Effects of hereditary retinal degeneration due to a *CEP290* mutation on the feline pupillary light reflex

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Abstract

Objective To understand how progressive rod cone degeneration due to a mutation in *CEP290* affects the pupillary light reflex (PLR) in domestic cats.

Animals studied Domestic cats identified as either normal wildtype (WT; *n* = 6), or homozygous for the rdAc mutation in *CEP290* and having early stage retinal degeneration (stage 2, S2; *n* = 4), or advanced retinal degeneration (S4; *n* = 6).

Methods The effect of light on pupil size was measured over a series of 10-s pulses of white and chromatic light in cats lightly sedated with medetomidine.

Results In WT cats, the PLR was characterized by a pronounced initial constriction that rapidly re-dilated during the stimulus (pupil escape), to a stable or sustained constriction. There was then a marked constriction at stimulus offset. Each component of the PLR was retained in affected cats, but with progressively reduced irradiance sensitivity from early to advanced retinal disease.

Conclusions The PLR of cats had multiple phases, with a remarkably high-amplitude 'paradoxical' off-constriction even in the absence of retinal disease. In rdAc cats, reduced irradiance sensitivity was consistent with progressive loss of rod and cone function. Based on previously characterized retinal pathology, this suggests the visual streak of the retina has a proportionally large contribution to PLR input. These findings support the hypothesis that the efficacy of planned therapeutic trials can be determined by careful evaluation of the PLR in cats.

Key Words: cat, CEP290, melanopsin, photoreceptor, pupillary light reflex, retinal degeneration

INTRODUCTION

The promising clinical trial phase of gene therapy for RPE65 deficiency has stimulated therapy development efforts in additional challenging inherited eye diseases.[1–3] *CEP290* mutations cause early onset blindness in human patients, and these individuals could in principle benefit from gene therapy intervention.[4] Pre-clinical therapy development for inherited retinal disorders is critically reliant on available gene- and/or mutation-specific animal models of the disease. A recessively inherited mutation in CEP290 causes retinal degeneration in otherwise healthy Abyssinian cats (rdAc).[5] Early functional and morphological deficits progress to more severe photoreceptor degeneration by 18–24 months, with end-stage photoreceptor degeneration usually at the age of

3–5 years.[6–9] However, objective *in vivo* evaluation of retinal function in domestic cats is currently limited to the electro-retinogram (ERG). Although undoubtedly useful as a measure of retinal function, an unrecordable ERG does not exclude a degree of meaningful rod/cone photoreceptor function.[10,11] Therefore, alternative objective measures of retinal function will be important for understanding the pathology and defining the efficacy of treatment.

The size of the pupil is regulated by light to optimize image-forming capacity for a given light condition, a response called the pupillary light reflex (PLR).[12] The PLR originates with circuits of the retina that integrate input from rod and cone photoreceptors with intrinsic photosensitivity of melanopsin ganglion cells.[13,14] The PLR has reduced sensitivity to light in humans with rod/cone

photoreceptor degenerations, and has been used as a measure of functional gain in animal models where direct measures of vision are difficult.[15–17]

To date, no studies characterize the effect of retinal degeneration on the PLR of cats. In addition, studies in cats have been limited to the 'sustained' pupillary response, with a considerable contribution from the intrinsic photosensitivity of melanopsin ganglion cells.[18–21] The initial pupil constriction to stimulus from the dark-adapted state is likely to provide a more useful measure of the rod and cone function.[21] However, dark-adaptation for measurement of initial responses in non-sedated cats is impractical because changing arousal state with restraint and changing point of focus will affect pupil size. Further, the movement of a restless animal affects the spatial relationship to the stimulus, causing variability in applied light. An alternative approach is to sedate animals during recording, but again there are limitations. The PLR is sensitive to pharmacological sedatives, and some otherwise useful agents are inappropriate because of negative health effects.[22–25]

Therefore, the utility of the PLR as a measure of efficacy in cat models of disease will depend on understanding the relationship between stimuli and response under a particular protocol, and how retinal pathology affects pupillary responses. The aim of this study in domestic cats was two-fold: to make a first assessment of the relationship between retinal clinical pathology on the PLR of the cat, and to determine the suitability of medetomidine sedation in recoding PLRs.

METHODS

All experiments were performed in accordance with the University of Missouri-Columbia Animal Care and Use Review. Animals were normally entrained to a 14-h light, 10-h dark daily light cycle with food available according to dietary requirements and water available *ad libitum*.

Clinical evaluation of disease stage in individual cats

Previously established methods were used to genotype and categorize the pathologic findings of individual rdAc cats used for this study. Cats were genotyped by bi-directional sequence analysis.[5] To determine the disease stage, cats were examined by indirect ophthalmoscopy after dilation of the pupils and fundus images were recorded with a digital fundus camera (Model 1000; Nidek, Fremont, CA, USA).[8] Early disease stage (S2) is identified by slight degenerative changes: a grayish discoloration in the mid-peripheral and peripheral fundus with a more normal appearing horizontal area along the visual streak and mainly normal retinal vasculature. Advanced disease stage (S4) is identified by signs of severe degeneration with retinal atrophy: hyper-reflective mid-peripheral and peripheral parts of the fundus with severely attenuated retinal vasculature and signs of moderate degeneration in the visual streak.

To illustrate the previously described relationship between indirect ophthalmoscopic findings and retinal function, bilateral full-field flash ERGs were recorded in selected cats. Our methods have been previously described.[26,27] In short, cats were dark-adapted overnight, fully anesthetized, temperature controlled, and prepared for ERGs under dim red light. Scotopic and photopic light intensity series were utilized with a standardized protocol.

Pupillometry

Animals entrained to the daily light–dark cycle were tested during the mid-light phase (ZT3–ZT9). Animals were sedated with medetomidine (0.09 mg/kg IM of Domitor; Novarits, Pfizer Animal Health. Exton, PA, USA) and placed onto a heated and padded test platform facing into a Ganzfeld illuminator. Pupil measurements were made from digital video equipment fitted to the Ganzfeld bowl, and pupil width automatically calculated based on ellipse fitting at 0.03-s intervals using ViewPoint Eye Tracker (Arrington Research Inc., Scottsdale, AZ, USA). Pupil width is almost linear to pupil area in the cat.[23] The light source was mounted in a light-tight chamber onto the Ganzfeld bowl. A Capsylite® 75PAR16 halogen spotlight (Osram Sylvania Inc., Danvers, MA, USA) provided broad band white light but intensity that increased with wavelength, particularly having high thermal output. To counter this, a pair of heat filters were used between the light source and shutter. Light was then filtered through neutral density and/or band-pass chromatic filters (half-bandwidth 10 nm) as required (Andover Corporation, Salem, NH, USA). A manual shutter controlled the timing of light applied into the Ganzfeld bowl. Both eyes were illuminated by the stimulus and only responses of the right eye recorded.

Light measurements throughout were made using a PM103 power meter (Macam Photometrics Ltd., Livingston, UK). Measurements are shown in either power for white light stimuli ($\mu W/cm^2$) or in photons for monochromatic stimuli (cm^2/s). Trolands can correct measures of applied light for the constriction of the pupil under a given stimulus, but are inappropriate in this study for three reasons: (i) the pupillary response is dynamic so the "troland" measurement would change over the course of a stimulus; (ii) trolands are calculated based on the photopic or scotopic sensitivity in a species but the relative contribution of the photoreceptors to the response is not known, and again is probably dynamic; (iii) the multifocal lens and elliptical pupil of the domestic cat mean that the spectral transmission into the eye will change with pupil constriction.

Under a standardized protocol, animals were dark-adapted for 5 min before testing. Then, the response to a series of light stimuli was tested at 60-s intervals. Light pulse duration was 10 s and stimuli were ordered to minimize light-adapting effects of preceding stimulus. Monochromatic stimuli were applied first alternating between 480 and 560 nm, and increasing in irradiance from −4 to 0 neutral densities. Then, after a 2-min dark adaptation, white light

was applied increasing in irradiance from −4 to 0 neutral densities.

In all cases, the effect of stimulus on pupil size was determined relative to the mean dark-adapted pupil size during the 5 s immediately preceding a treatment. Because shutter timing and data acquisition were not integrated, responses were calculated relative to the onset of response (time zero) and latency was not measured. For the dimmest stimuli where no response was apparent, responses were calculated relative to the recorded timing of stimulus application. The initial pupillary response was defined as the mean constriction over 1 s centered on the maximal constriction during the first 2 s of the response to stimulus. The sustained response was defined as the mean pupil size during the final 2 s of the response/stimulus (8.0–9.9 s). The off-constriction was defined as the mean pupil size during the first second after termination of the stimulus (10.0–10.9 s).

Only animals where PLRs were reliably recorded through the entire protocol were included (wildtype [WT] = 4 cats, S2 = 3 cats, S4 = 5 cats). Failed recordings (WT = 2, S2 = 1, S4 = 1) were due to technical difficulties in automated pupil width measurement; typically where cats had slightly pronounced upper eyelids that obscured the upper portion of the dilated pupil.

Statistical comparison of irradiance response ranges was by F-test comparison of fitted sigmoid dose–response curves made with Prism (GraphPad Software, La Jolla, CA, USA). Bonferroni corrections were applied to a P-value of 0.05 in determining significance.

RESULTS

Temporal dynamics of the PLR in normal cats

The PLR to white light in normal cats sedated with medetomidine was characterized by a pronounced initial constriction becoming maximally constricted within the first second of the stimulus (Fig. 1). The pupil then rapidly re-dilated (pupil escape) during the stimulus to a stable or sustained level of constriction. There was then a marked constriction of the pupil (decrease in pupil size) immediately following termination of the stimulus. The pupil then returned to the dark-adapted dilated state (full time course not shown). Each component of the PLR appeared light-dose dependent but a maximal ~25% constriction was seen within the irradiance range tested.

Clinical evaluation of disease stage in individual cats

Progressive retinal degeneration was observed ophthalmoscopically in all cats identified as homozygous for the *CEP290* mutation (Fig. 2). As expected, ERG changes corroborated fundus findings.[9] In unaffected control cats, the fundus appearance and ERG were normal. In cats identified with an early disease stage, mid-peripheral and peripheral parts of the fundus showed generalized grayish discoloration with a more normal appearing horizontal area along the visual streak and mostly normal retinal vasculature. In
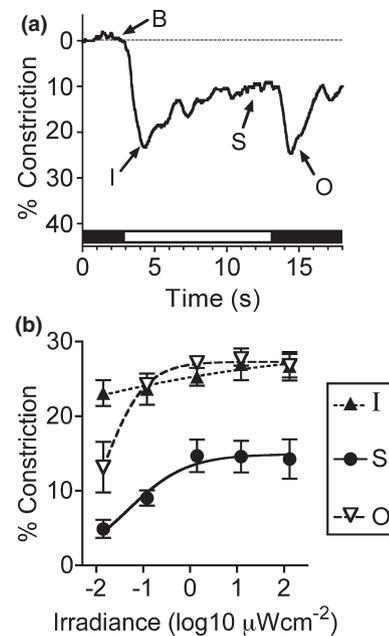
Figure 1. The pupillary light reflex in normal cats. Pupillary responses are shown as % of dark-adapted horizontal diameter of the pupil. Timings of 10-s light stimuli are indicated by black and white bars. (a) An example pupillary response for a normal cat shows the phases or components of the pupillary response at 1.4 µW/cm^2 identified by arrows (B, baseline; I, initial constriction; S, sustained constriction; O, off-response). (b) The irradiance response relationship of the initial, sustained and off-constriction are shown (mean and SEM) fitted with variable slope sigmoid dose–response functions.

early stage affected cats (Fig. 2b), ERG amplitudes were reduced, particularly for the photoreceptor-generated a-wave. At the advanced disease stage (S4, $n = 6$; Fig. 2c), there were severe atrophic changes in the mid-peripheral and peripheral fundus observed as tapetal hyper-reflectivity and severe vascular attenuation. However, in some cats, the area along the visual streak appeared less affected by disease. In advanced disease stage cats, the ERG was markedly reduced but still recordable, consistent with the opthalmoscopic observation of a relatively well-preserved visual streak in these cats.[28]

The PLR in rdAc cats

Initial, sustained, and off-constriction components of the PLR were all retained in rdAc cats but progressive effects of disease were apparent (Fig. 3). The initial response was not significantly reduced at S2 ($P = 0.023$; Bonferroni significance at 0.020), but was severely reduced at S4 ($P < 0.0001$). Similarly, the sustained response was not reduced at S2 ($P = 0.81$), but was severely reduced at S4 ($P < 0.0001$). In contrast, the off-constriction responses were severely reduced even at S2 ($P < 0.0001$).

Chromatic sensitivity of the PLR

Responses to chromatic stimuli again showed progressive effects of disease on sensitivity of specific components of the
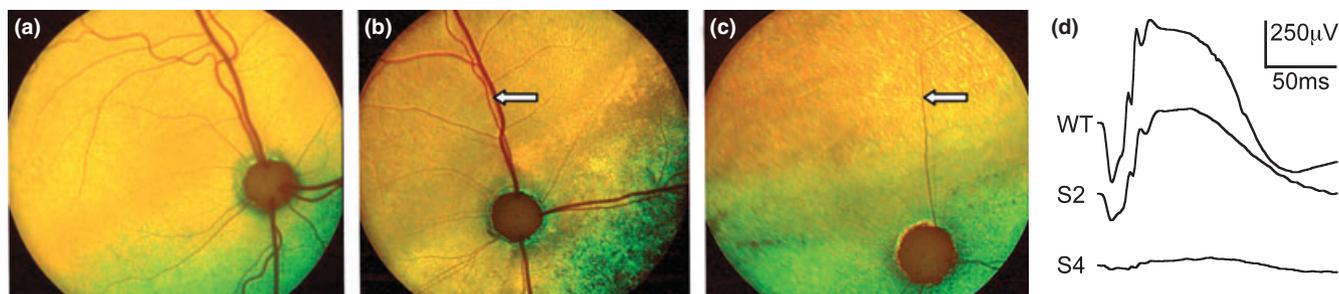
Figure 2. Fundus appearance and electroretinogram of rdAc cats. Representative fundus photographs are shown for (a) a 1-year-old unaffected Abyssinian cat (wildtype, WT), (b) a 2-year-old affected Abyssinian cat early in disease progression (S2), and (c) a 6-year-old affected Abyssinian cat with an advanced disease stage (S4). Arrows in (b) and (c) indicate retinal vasculature that is slightly attenuated in (b) and severely attenuated in (c). (d) For the same example WT, S2, and S4 cats, the waveform of the dark-adapted bright flash electroretinogram is shown using 4 cd.s/m² of white light stimulation for each. Amplitude and implicit time calibrations are indicated in the figure, vertically and horizontally, respectively.

PLR, and identified stage of disease differences in the sensitivity to 480- and 560-nm light.

In normal cats, the initial response to 480- and 560-nm stimuli was similar (comparison of fitted curves by *F*-test, $P = 0.903$; Bonferroni significance at 0.0045; Fig. 4). The sustained response had limited sensitivity to these irradiances and was also not significantly different at 480 and 560 nm ($P = 0.045$). The off-constriction was much less potently induced by 560-nm light than by 480-nm light ($P = 0.002$).

In rdAc cats the, initial response to 480-nm stimuli was progressively reduced with disease but not significantly with the Bonferroni corrected alpha value (S2: $P = 0.10$; S4: $P = 0.0137$). In contrast, the initial response to 560-nm stimuli was reduced sharply between early and advanced disease (S2: $P = 0.99$; S4: $P = 0.0002$). As in normal cats, the sustained component of the PLR had limited sensitivity to these irradiances at either wavelength. The off-constriction was again reduced in early disease stage (S2: 480 nm $P = 0.032$; 560 nm $P = 0.80$), but showed a marked loss of sensitivity in late-stage disease, particularly to 480-nm stimuli (S4: 480 nm $P < 0.0001$; 560 nm $P = 0.0042$).

DISCUSSION

The aim of this study in domestic cats was twofold: to make a first assessment of the relationship between retinal clinical pathology on the PLR of the cat, and to determine the suitability of medetomidine sedation in recording PLRs. This was directed to establishing additional tests of retinal function and treatment efficacy in cats.

Domestic cats had expected not only initial and sustained components of the PLR, but also a remarkable off-constriction under a range of irradiances. When compared with previous studies, the maximal constriction of the pupil appeared to be constrained under this protocol of medetomidine sedation. Despite this, irradiance deficits in the PLR of rdAc cats showed broadly reduced sensitivity with disease progression, consistent with progressive rod/cone degeneration. Of further interest, based on the previous extensive characterization of the retinal pathology of rdAc cats, findings suggest

that the area centralis and visual streak has a proportionally large input to the PLR.[8,28,29]

Initial constriction

Under this protocol, all aspects of the pupillary response were irradiance dependent. The initial PLR characterized by rapid constriction and subsequent partial re-dilation during the stimulus (pupil escape) was consistent with major input from a similarly rapidly activated and partially light-adapting input. Particularly at lower irradiances, based on other studies, this can be reasonably assumed to be generated largely by synaptically mediated rod/cone input.[21] In rdAc cats, the initial constriction was severely affected by disease. However, the initial response with an escape to the sustained response was not absent even in S4 rdAc cats, suggesting that functional loss of rods and cones is not absolute. The initial response to 560 nm also deteriorates in advanced disease much more than the response to a 480-nm stimulus. When compared with the similar amplitude of initial responses at 480 and 560 nm in normal cats, this suggests a late-stage loss of MWS-cones as has been identified in morphological studies.[29] Furthermore, the marked loss of sensitivity between S2 and S4 rdAc cats coincides with the pathology extending to the visual streak area.[6] This raises the possibility that as in people and rhesus monkeys, the central retinal field contributes more to the PLR than the peripheral retina.[30]

Sustained constriction

The stable 'sustained' constriction seen postescape was also consistent with predicted sustained input from rods and melanopsin ganglion cells. Although an effect of disease on the intrinsic photosensitivity of the latter cells cannot be discounted, loss of sensitivity tracks with previously described rod/cone degeneration. In addition, the cellular function of CEP290 in cilia vesicle-trafficking suggests disease effects in the eye would affect the ciliated rods and cones, but not the non-ciliated melanopsin ganglion cells.[31] Therefore, this finding would most parsimoniously suggest that the loss of rods and cones directly affects the sensitivity of the sustained
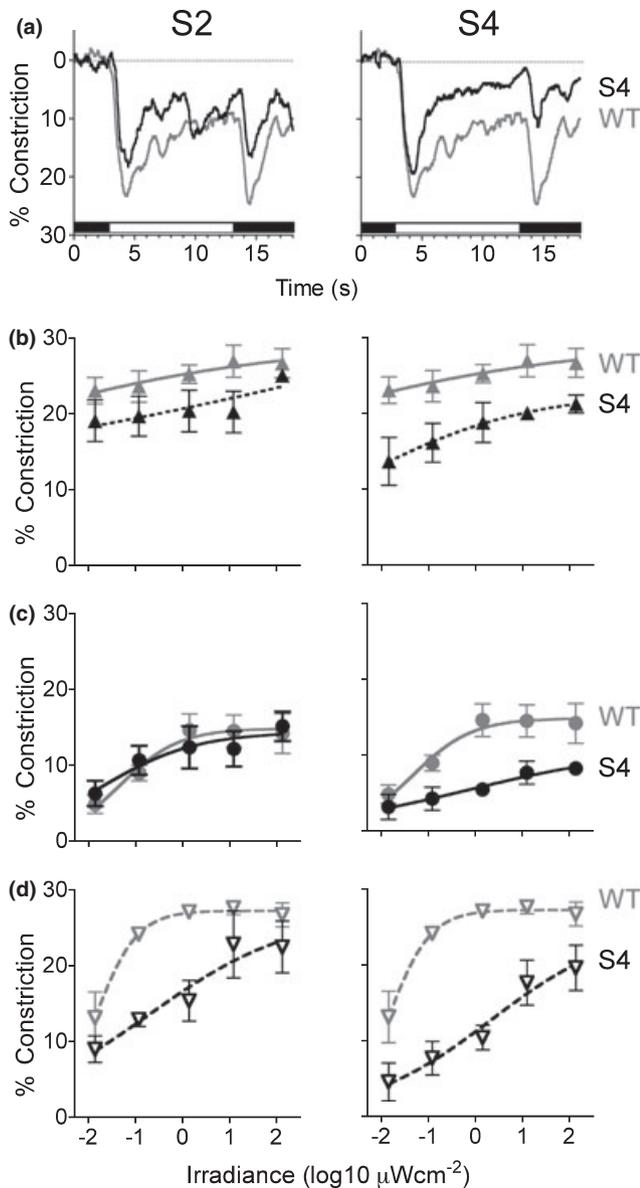
Figure 3. Effect of disease on pupillary light reflex responses to white light. Pupillary responses are shown as % of dark-adapted baseline horizontal diameter. Timings of 10-s light stimuli are indicated by black and white bars. Data for early disease rdAc (S2) and advanced disease rdAc cats (S4) are arranged in columns. Responses from unaffected (wildtype, WT) animals are shown on panels in gray for reference. (a) The dynamics of pupillary responses to 1.4 μW/cm^2 are shown. Derived irradiance response curves (mean and SEM) fitted with a variable slope sigmoid dose–response function are shown for (b) initial, (c) sustained, and (d) off-responses.

PLR, supporting other studies identifying a role of rod photoreceptors in generating the sustained PLR.[21]

Off constriction

A 'paradoxical' pupillary response is typically only seen in disorders of afferent pathways, congenital disorders of the eye, or very specific stimulus conditions.[32–34] In contrast, in cats, the off-constriction was clear even in the absence of ret-

inal disease, and under different stimuli. The basis for this off-constriction remains speculative but two observations suggest that the response is generated at the level of the retina: the unexpected off-constriction was irradiance dependent, and the effect of disease was pronounced. The similar dynamics of the initial and off-constriction would suggest a rod/cone basis, and the presumed effect of stimulus termination on melanopsin would be to allow dilation. Therefore, one plausible mechanism is that sudden absence of rod/cone input through the off bipolar cell pathway might dis-inhibit constriction, particularly under the non-physiological square wave stimulus. However, an effect of medetomidine cannot be discounted as contributing to this unusual pupillary response.

Protocol suitability for pathology characterization and efficacy testing

A major consideration in this study was a protocol that allowed measurement of the initial constriction of the pupil from a dark-adapted state, where the contribution of rods/cones to the response would predominate. The reversible sedative medetomine has been used with some success for recording PLRs in dogs, but its utility in cats has not been tested.[17] Alpha-2-adrenergic agonists such as medetomidine are known to cause mydriasis (dilation) of the pupil, but the extent to which this would interfere with the use of the PLR as a measure of ocular pathology was not known.[35] The lower irradiance range at which we begin to see pupil constriction is consistent with previous studies of the irradiance sensitivity of the sustained PLR in normal cats.[19,20,23] However, there was a divergence in response magnitude at higher irradiances with previous studies reporting an approximately 75% constriction at the highest light level we applied. Critically, despite this interference of medetomidine, sedation did allow quantification of changes in irradiance sensitivity of the initial PLR response with disease.

The additional use of chromatic stimuli was intended to provide some insight into the contribution of the different photoreceptors to specific components of the PLR.[21,36–38] Rods, cones, and melanopsin ganglion cells each have characteristic sensitivities to light depending on the irradiance (light level), duration, and wavelength (color) of the stimulus. The 480-nm stimulus should not only strongly activate rods and melanopsin (>90% of maximal absorption or quantum efficiency), but would also have activation of SWS (>68%) and MWS-cones (>40%). In contrast, the 560-nm stimulus should potently activate MWS-cones (~98%), but less potently activate rods (~28%), melanopsin (~8%), and SWS-cones (<1%). These properties mean that despite the overlap in spectral sensitivities of the photoreceptors, proportional changes in response to these chromatic stimuli were able to suggest the effect of pathology on particular photoreceptor populations.

Change in the sensitivity of the PLR was able to quantify the loss of photoreceptive function in a way suited to evaluation of treatment efficacy in rescue of rods and cones. Nor-
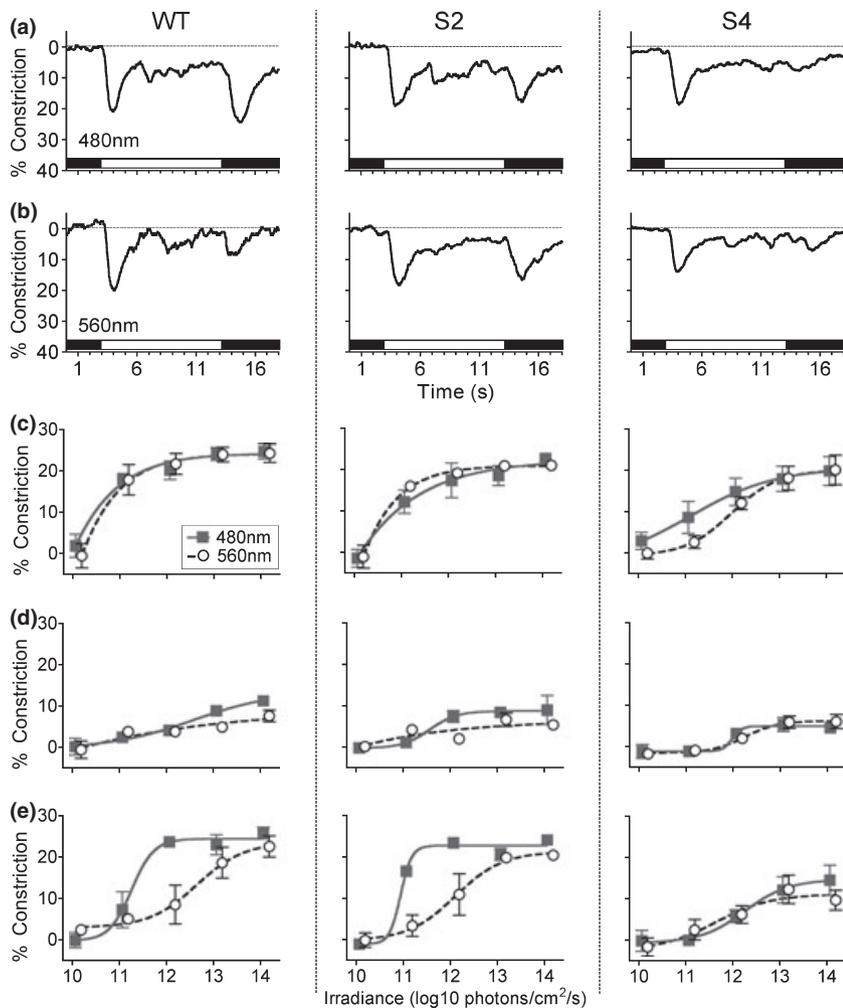
Figure 4. Pupillary light reflex responses to chromatic stimuli. Pupillary responses are shown as % of dark-adapted baseline horizontal diameter. Timing of the 10-s stimuli are indicated by black and white bars. Data for unaffected (wildtype, WT), early disease rdAc (S2), and advanced disease rdAc cats (S4) are arranged in columns. The dynamics of pupillary responses to a mid irradiance range stimulus ($\sim$0.5 $\mu W/cm^2$: 480 nm = 1.16×10^{12} photons; 560 nm = 1.55×10^{12} photons) are shown for (a) 480 nm and (b) 560 nm. Derived irradiance response curves (mean and SEM) are shown for (c) initial, (d) sustained, and (e) off-responses at 480 and 560 nm fitted with a variable slope sigmoid dose–response function.

malization of the initial response to white light would indicate general restored rod and cone function, and sensitivity to 560-nm stimuli could be used to indicate the rescue of MWS-cones. However, it remains the case that extensive testing of stimulus–response relationships in normal cats under different sedative agents and to several additional chromatic stimuli would be required to fully define effects of sedative and photoreceptor contribution to specific features of the PLR.

Understanding the pathology of CEP290 mutations
Pupillary light reflex irradiance sensitivity and residual ERGs in previous studies both suggest that some rod/cone function is retained late in disease progression.[6] This pathology is in contrast to *rd16* mice with severe photoreceptor degeneration within 10 days of eye opening.[39] One explanation for this divergence of retinal pathology is that the CEP290 protein in rdAc cats retains some function that results in less severe disease. Indeed, *rd16* mice have a large deletion in the CEP290 gene, but in rdAc cats there is truncation of only the last 4-exons of the 54-exon CEP290 protein. This mutation severity hypothesis would conveniently explain the spectrum of disease observed in patients who harbor different *CEP290* mutations.

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