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Shinya Sato[1], Igor V. Peshenko[2], Elena V. Olshevskaya[2], Vladimir J. Kefalov[1] and Alexander M. Dizhoor[2]

[1]*Department of Ophthalmology and Visual Sciences, Washington University in St. Louis, St. Louis, MO 63110*
[2]*Pennsylavania College of Optometry, Salus University, Elkins Park, PA 19027*

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Correspondence should be addressed to Co-corresponding authors: Alexander M. Dizhoor, Pennsylvania College of Optometry, Salus University, Elkins Park, PA 19027, adizhoor@salus.edu; Vladimir J. Kefalov, Department of Ophthalmology and Visual Sciences, Washington University in St. Louis, St. Louis, MO 63110, kefalov@wustl.edu

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GUCY2D Cone-Rod Dystrophy-6 is a 'Phototransduction Disease' Triggered by Abnormal Calcium Feedback on Retinal Membrane Guanylyl Cyclase 1

Shinya Sato[1,#], Igor V. Peshenko[2], Elena V. Olshevskaya[2], Vladimir J. Kefalov[1*], and Alexander M. Dizhoor[2*]

[1]*Department of Ophthalmology and Visual Sciences, Washington University in St. Louis, St. Louis, MO 63110;* [2]*Pennsylavania College of Optometry, Salus University, Elkins Park, PA 19027*

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* Co-corresponding authors:

Alexander M. Dizhoor

Pennsylvania College of Optometry,

Salus University, Elkins Park, PA 19027

adizhoor@salus.edu

Vladimir J. Kefalov

Department of Ophthalmology and Visual Sciences,

Washington University in St. Louis, St. Louis, MO 63110

kefalov@wustl.edu

[#]*Present address for Shinya Sato: Graduate School of Biostudies, Kyoto University, Yoshida-Konoecho, Sakyo-ku, Kyoto 606-8315, Japan; sato.shinya.7e@kyoto-u.ac.jp*

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ABSTRACT

The Arg838Ser mutation in retinal membrane guanylyl cyclase 1 (RetGC1) has been linked to autosomal dominant cone-rod dystrophy type 6 (CORD6). It is believed that photoreceptor degeneration is caused by the altered sensitivity of RetGC1 to calcium regulation via guanylyl cyclase activating proteins (GCAP). To determine the mechanism by which this mutation leads to degeneration, we investigated the structure and function of rod photoreceptors in two transgenic mouse lines, 362 and 379, expressing R838S RetGC1. In both lines, rod outer segments became shorter than in their non-transgenic siblings by 3-4 weeks of age, before the eventual photoreceptor degeneration. Despite the shortening of their outer segments, the dark current of transgenic rods was 1.5—2.2-fold higher than in non-transgenic controls. Similarly, the dim flash response amplitude in *R838S*$^{+}$ rods was larger, time to peak was delayed, and flash sensitivity was increased, all suggesting elevated dark-adapted free cGMP in transgenic rods. In rods expressing R838S RetGC1, dark current noise increased and the exchange current, detected after a saturating flash, became more pronounced. These results suggest disrupted Ca^{2+} phototransduction feedback and abnormally high free Ca^{2+} concentration in the outer segments. Notably, photoreceptor degeneration, which typically occurred after 3 months of age in R838S RetGC1 transgenic mice in *GCAP1,2*$^{+/+}$ or *GCAP1,2*$^{+/-}$ backgrounds, was completely prevented in *GCAP1,2*$^{-/-}$ mice lacking Ca^{2+} feedback to guanylyl cyclase. In summary, the dysregulation of guanylyl cyclase in RetGC1-linked CORD6 is a ‘phototransduction disease’, associated with increase in free cGMP and Ca^{2+} levels in photoreceptors.

SIGNIFICANCE STATEMENT

In a mouse model expressing human membrane guanylyl cyclase 1 (RetGC1, *GUCY2D*), a mutation associated with early progressing congenital blindness, cone-rod dystrophy type 6 (CORD6), deregulates calcium-sensitive feedback of phototransduction to the cyclase mediated by calcium-sensor proteins GCAPs. The abnormal calcium sensitivity of the cyclase increases cGMP-gated dark current in the rod outer segments, re-shapes rod photoresponses, and triggers photoreceptor death. This work is the first to demonstrate a direct physiological effect of *GUCY2D* CORD6-linked mutation on photoreceptor physiology *in vivo*. It also identifies the abnormal regulation of the cyclase by calcium-sensor proteins as the main trigger for the photoreceptor death.

INTRODUCTION

Retinal membrane guanylyl cyclase (RetGC), one of the essential enzymes in photoreceptor signaling, enables photosensitivity of rods and cones by opening cGMP-gated channels in the outer segment plasma membrane. The partial depolarization of photoreceptors caused by the inward current of Na^+ and Ca^{2+} through cGMP-gated channels in the dark becomes reversed when light activates the hydrolysis of cGMP by phosphodiesterase 6 (PDE6) and suppresses the influx of Na^+ and Ca^{2+} (Yau and Hardy, 2009; Arshavsky and Burns, 2012; Koch and Dell'Orco, 2015). While Ca^{2+} influx is suppressed upon light stimulation of rods, its extrusion via $Na^+/Ca^{2+},K^+$ exchanger 1 (NCKX1) (Reilander et al., 1992) still carries on to produce a decrease in rod outer segment Ca^{2+} concentration. This upregulates the negative Ca^{2+} feedback on RetGC (Koch and Stryer 1988; Pugh et al., 1999; Burns et al., 2002), which is mediated by the Ca^{2+}/Mg^{2+} sensors guanylyl cyclase activating proteins (GCAPs) (Palczewski et al., 1994; Dizhoor et al., 1995; Imanishi et al., 2004; Dizhoor et al., 2010; Makino et al., 2012). As a result of the light-induced decrease in Ca^{2+}, GCAPs convert to a Mg^{2+}-liganded state that stimulates RetGC activity. Upon the return of photoreceptors to darkness, PDE6 is inactivated, the influx of Ca^{2+} through the cGMP-gated channels resumes, and Ca^{2+} concentration returns to its dark-adapted level. Subsequently, GCAPs convert back to Ca^{2+}-liganded state and decelerate production of cGMP.

Two isozymes of RetGC are present in photoreceptors, RetGC1—the predominant isozyme in rods and cones— and RetGC2, an ancillary isozyme in rods (Lowe et al., 1995; Yang et al., 1995; Peshenko et al., 2011; Xu et al., 2013). Multiple mutations in the human *GUCY2D* gene, coding for the RetGC1 isozyme, have been linked to different types of congenital blindness. Some mutations cause a severe recessive blindness—Leber's Congenital Amaurosis (LCA), mostly a non-degenerative

disease resulting from loss of RetGC1 activity and/or activation that renders rods and cones non-functional from birth (Stone, 2007; Jacobson et al., 2013). Unlike *GUCY2D* LCA, autosomal dominant *GUCY2D* cone-rod dystrophy 6 (CORD6) is an early-onset progressing degeneration of photoresponsive cones and rods, frequently linked to substitutions, Cys, Ser, Pro, His or Gly, replacing Arg838 in the dimerization domain of RetGC1 (Ramamurthy et al., 2001; Hunt et al., 2010; Ito et al., 2004; Garcia-Hoyos et al., 2011), a part of the GCAP-regulated interface on the enzyme (Peshenko et al., 2015a,b). A number of previous *in vitro* studies using recombinant RetGC1 expressed in cultured non-photoreceptor cells (Tucker et al., 1999; Ramamurthy et al., 2001; Peshenko et al., 2004) have shown that the Arg838 substitutions cause dysregulation of the recombinant cyclase via Ca^{2+} feedback *in vitro* such that the mutant RetGC1 complex with GCAP not only becomes activated at the low Ca^{2+} concentrations typical for light-adapted photoreceptors, but also retains elevated activity at Ca^{2+} levels typical for dark-adapted rods and cones. We recently demonstrated that mouse rods expressing R838S RetGC1 in a transgenic model rapidly degenerate, causing early-onset progressive blindness (Dizhoor et al., 2016). We now present physiological *in vivo* evidence that *GUCY2D* CORD6 is a 'phototransduction disease' triggered specifically by abnormal Ca^{2+} feedback to the cyclase via GCAPs.

MATERIALS AND METHODS

Animals — All experiments involving animals were conducted in accordance with the Public Health Service guidelines and approved by the Institutional Animal Care and Use Committees. Transgenic mice expressing R838S RetGC1 under control of rod opsin promoter [RRID: currently available from the Dizhoor lab; a request has been also submitted to JAX laboratories and assigned interim accession numbers MGI_5908882 and MGI_5908875] were produced as previously described (Dizhoor et al.,

2016); *GCAP1,2*$^{-/-}$ mouse line (Mendez et al., 2001) originally provided by Dr. Jeannie Chen and *RetGC1*$^{-/-}$ mice (GC-E null, Yang et al., 1999) originated from Dr. David Garbers laboratory were outcrossed for ten generations to C57B6J mouse strain purchased from JAX Research (Jackson Laboratory) before breeding the knockout alleles into a homozygous state. The *R838S*$^{+}$ mouse lines were propagated by breeding lines 362 and 379 (Dizhoor et al., 2016) to wild type C57B6J, and the transgene-negative progeny from the breeding, both males and females, were used in the study as a littermate control.

Immunofluorescence and immunoblotting — Mice anesthetized by intraperitoneal injection of ketamine/xylazine/urethane (80/32/800 mg/kg) cocktail were perfused through the heart with phosphate buffered saline (PBS), followed by 10% paraformaldehyde in PBS. The enucleated eyes were placed in 10% formaldehyde/PBS for 5 min at room temperature, then the cornea and the lens were removed and the eye cup was fixed in 5% formaldehyde/PBS for 1 hour, cryoprotected by immersing in 30% sucrose/PBS overnight at 4 °C twice, frozen in OCT medium (Electron Microscopy Science) and sectioned using a Hacker-Bright cryomicrotome. Sections were probed with a rabbit polyclonal antibody raised against the catalytic domain of RetGC1 (Laura et al., 1996) and developed using goat anti-rabbit antibody (Molecular Probes/Fisher) and fluorescein-labeled peanut agglutinin (Vector Laboratories) as described (Dizhoor et al., 2016). Confocal images were acquired using an Olympus FV1000 Spectral instrument controlled by FluoView FV10-ASW software, collecting in a sequential mode. No changes were made to the original images except for minor gamma correction applied to the whole image for more clear presentation in print. For Western immunoblotting, proteins extracted from the retinas with a RIPA buffer (Abcam) were mixed with equal volumes of 2x Laemmli SDS sample buffer and subjected to electrophoresis in 7% SDS-PAAG, then transferred to PVDF membrane and probed with anti-

NCKX1 or anti-CNG1 alpha mouse monoclonal antibodies (both were generously provided by Dr. Robert Molday, University of British Columbia) or anti-actin antibody (Abcam) and developed using anti-mouse IgG peroxidase-conjugated secondary antibody and a Femto SuperSignal chemiluminescence substrate (Pierce/Thermo Fisher). Chemiluminescence image was acquired using a Fotodyne Luminous FX instrument.

Retinal Morphology — Mice were perfused through the heart as above with PBS and then with 2.5% glutaraldehyde in PBS. The eyes were surgically removed and fixed overnight in 2.5% glutaraldehyde/2.5% formaldehyde at 4 °C. The fixed eyes were washed three times for 15 min each in PBS, soaked in PBS overnight, processed for paraffin embedding, sectioned, and stained with hematoxylin/eosin (AML Laboratories, Saint Augustine, FL). The outer segment length was measured from confocal differential interference contrast image. The retina morphology was analyzed using images taken by an Olympus Magnafire camera mounted on an Olympus BX21 microscope. The photoreceptor nuclei in the outer nuclear layer of the retina were counted from a 425-μm fragment of the retina, mid-way between the optic nerve and the periphery of the retina, and the densities of the nuclei per 100 μm distance were averaged from several frames for each retina.

Guanylyl cyclase assay — Mice were dark-adapted overnight and their retinas were dissected and assayed under infrared illumination as previously described (Peshenko et al., 2011; 2016). Briefly, the assay contained in 25 μL 30 mM MOPS-KOH (pH 7.2), 60 mM KCl, 4 mM NaCl, 1 mM DTT, 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, 10 mM creatine phosphate, 0.5 unit of creatine phosphokinase (Sigma-Aldrich), 1 μCi of [α-^{32}P]GTP, 0.1 μCi of [8-^{3}H]cGMP (Perkin Elmer), phosphodiesterase inhibitors zaprinast and dipyridamole, and 2 mM Ca^{2+}/EGTA buffer, with $MgCl_2$ added to maintain variable [Ca^{2}

163 $^{+}]_{free}$ at 0.9 mM free Mg^{2+} (Peshenko and Dizhoor, 2006). Where indicated, myristoylated recombinant
164 mouse GCAP1 expressed in *E. coli* and purified as previously described (Peshenko and Dizhoor, 2006;
165 Peshenko et al., 2015) was added to the assay.
166
167 *Electrophysiology* — Single-cell suction recordings were done as previously described (Wang et al.,
168 2014). Mice were dark-adapted overnight, euthanized by 5 min CO_2 inhalation, and their eyes were
169 enucleated under dim red light using forceps. Retinas were then isolated into Locke's solution (112.5
170 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM HEPES, 20 mM $NaHCO_3$, 3 mM
171 sodium succinate, 0.5 mM sodium glutamate, 0.02 mM EDTA, 10 mM glucose, 0.1% minimum
172 essential media vitamins and 0.2% minimum essential media amino acids, pH 7.4) using microscissors
173 and fine forceps under a stereomicroscope fit with infrared image converters. A retina was chopped by a
174 razor blade into small pieces and transferred into the recording chamber perfused with 33-37 °C Locke's
175 solution equilibrated with 95%O_2/5%CO_2. Single rod outer segment was then drawn into micro glass
176 pipette under microscope (IX51, Olympus Corporation, Tokyo, Japan) with infrared illumination and
177 camera. The pipette was filled with electrode solution (140 mM NaCl; 3.6 mM KCl; 2.4 mM $MgCl_2$; 1.2
178 mM $CaCl_2$; 3 mM HEPES; 0.02 mM EDTA; 10 mM glucose, pH 7.4 with NaOH) and controlled by
179 micro manipulator (MP-225, Sutter instrument, Novato, CA, USA). Test flashes of 500 or 505 nm were
180 generated in a calibrated LED light stimulus system and delivered to the recording chamber through a
181 custom-made optical system. Flash intensity and duration were controlled by an LED driver (LDC210,
182 Thorlabs Inc., Newton, NJ, USA) connected to a computer and operated by pClamp9 software
183 (Molecular Devices, Sunnyvale, CA, USA). Signals from individual rods were amplified by an amplifier
184 (Axopatch 200B, Axon Instruments, Inc., Union City, CA, USA) and tunable active filter (Model 3382,
185 Krohn-Hite Corporation, Brockton, MA, USA), low-pass filtered at 30 Hz (8-pole Bessel, Model 3382,

Krohn-Hite Corporation), digitized at 1 kHz (Digidata 1322A, Axon Instruments, Inc.), stored and analyzed on a computer using pClamp9.

Experimental design and statistical analysis — All animal experiments indiscriminately utilized male and female mice. Since the magnitude of the expected effects, by the nature of the experiments, was not feasible to predict a priori, the size of the animal groups was not explicitly planned in advance and the statistical analysis was applied to the data collected upon the completion of the experiments. Unless noted otherwise, two-tailed unpaired Student's t-test (Origin8, OriginLab Corporation, Northampton, MA) was used to test for the significance of differences in the mean values of two sample groups. P-values of < 0.05 were considered to be statistically significant. Where indicated, one-way ANOVA/Bonferroni test at alpha = 0.01 (Synergy KaleidaGraph) was applied for comparison of photoreceptor loss between several animal groups.

RESULTS

The R838S RetGC1 expression dysregulates cyclase modulation by Ca^{2+}.

Two mouse lines expressing the R838S RetGC1 under control of rod opsin promoter, lines 362 and 379, have been shown to undergo rapid early-onset loss of rod ERG concomitant with the degeneration of rods, more severe in line 362 (Dizhoor et al., 2016). To determine whether the R838S RetGC1 transgene in the fast-degenerating line 362 is expressed in rod photoreceptor outer segments, the line was outcrossed to *RetGC1*$^{-/-}$ background, which eliminates interference from the endogenous mouse RetGC1 ortholog (Yang et al., 1999). Immunolabeling of retinal sections demonstrated the robust and uniform expression of RetGC in rod outer segments of *R838S*$^{+}$*RetGC1*$^{-/-}$ mice (**Fig. 1**). Thus, R838S RetGC1 was

specifically expressed in line 362 transgenic rods. This result, together with the previous finding that the mutant cyclase is expressed in the rods of line 379 (Dizhoor et al., 2016), allowed investigation of the function of individual rods expressing R838S RetGC1 in the two transgenic lines. The experiments described further were conducted using the original *R838S*$^{+}$ mouse lines, propagated in RetGC1$^{+/+}$ background.

The guanylyl cyclase activity measured in the retina using our standard assay (Peshenko et al., 2011) directly assesses Ca^{2+} sensitivity of the photoreceptor-specific RetGC activity (Olshevskaya et al., 2004, 2012; Woodruff et al., 2007; Makino et al., 2008, 2012). Despite the fast degeneration of *R838S*$^{+}$ rods in line 362 (Dizhoor et al., 2016), their cyclase activity was still measurable at 3 weeks of age, allowing for a comparison of RetGC Ca^{2+} sensitivity between transgene-positive and transgene-negative littermates (**Fig. 2A**). The CORD6-linked R838S mutation in RetGC1 has dual effect on the cyclase regulatory properties in the retina. It increases EC_{50} for the inhibitory effect of Ca^{2+} (from 76 nM in wild type littermates to 123 nM in the transgene-positive mice of line 362) and causes nearly two-fold reduction of the Hill coefficient (from 1.73 to 0.99). As a result, the cyclase activity remained markedly elevated at the free Ca^{2+} concentrations typical for the normal dark-adapted mouse photoreceptors (Woodruff et al., 2007; Olshevskaya et al., 2004) (**Fig. 2A**). We hence reasoned, that if the altered Ca^{2+} sensitivity of the cyclase measured in retinal homogenate *in vitro* translates into a corresponding increase of free cGMP in living *R838S*$^{+}$ rods, then one would expect a larger fraction of the channels to remain open in dark-adapted *R838S*$^{+}$ rods, producing a larger inward dark current. In order to adequately interpret any potential changes in the dark current, we also verified that dysregulation of the cyclase does not result in a compensatory upregulation of the CNG1 channel in the transgenic retinas (**Fig. 2B**).

The presence of a CORD6 RetGC1 in rods elevates their dark current and delays their response kinetics.

The outer segment of degenerating *R838S*$^{+}$ rods were still present at young age for both lines 362 and 379, despite showing nearly 2-fold and 1.5-fold reduction in length at 3.5 weeks of age (**Fig. 3A**) and 4.5 weeks (**Fig. 3B**) respectively, which preceded their pronounced degeneration at later stages (Dizhoor et al., 2016; also see **Fig. 9**). This made possible the functional analysis of *R838S*$^{+}$ rods prior to their degeneration by recordings with suction pipet electrode (**Figs. 4-8**, **Tables 1 and 2**). Notably, despite the reduction of their outer segment length, *R838S*$^{+}$ rods from both mouse lines exhibited significantly elevated dark current compared to their respective non-transgenic littermates. The increase in dark current was 2-fold in line 362 (**Fig. 4; Table 1**) and 1.5-fold in line 379 (**Fig. 5; Table 2**) at 4 weeks of age. Taking into account the reduced length of the outer segment (**Fig. 3**), such increase in the dark current indicates an even more drastic increase in the cGMP-gated current density in *R838S*$^{+}$ rods. This result is consistent with the inefficient suppression of the RetGC1 activity at dark-adapted Ca^{2+} levels in *R838S*$^{+}$ (**Fig. 2**). The larger fraction of open cGMP-gated channels in the dark and the slower activation of RetGC due to reduced cooperativity for Ca^{2+} (**Fig. 2A**) can explain the increased flash sensitivity of mutant rods, such that a larger response amplitude was produced by a given flash in lines 362 and 379 in comparison with their respective wild type littermates (**Fig. 4C** and **Table 1**; **Fig. 5C** and **Table 2**, respectively). The half-saturation flash strength, $I_{1/2}$, which is independent of the size of the dark current, was less affected in the mutant rods than their dim flash sensitivity (**Fig. 4D and 5D, Tables 1 and 2**).

Unexpectedly, some of the parameters for the wild type animals differed slightly between Tables 1 and 2. These differences are likely attributable to differences in the genetic background of the wild type animals used as controls in each case (derived from independent crosses of *R838S*$^{+}$ mice of each transgenic line with C57B6J), slight variability in age, and possibly stress from the shipment of the

255 animals from Pennsylvania to Missouri the day prior to the recordings. However, as in each case we
256 used littermates as controls, hence, the control wild type animals had the same genetic background, were
257 of the same age, and were shipped together with their respective *R838S*$^{+}$ siblings. As a result, the
258 differences between wild type and *R838S*$^{+}$ transgenic rods in each case would be independent of other
259 factors and reflect the change in rod function caused by the expression of R838S RetGC.

260 The shape of dim flash responses in *R838S*$^{+}$ rods also changed in a manner consistent with
261 elevated free cGMP content – the amplitude of the flash response rose and reached its peak later in both
262 line 362 (**Fig. 6A, B**) and line 379 (**Fig. 6C, D**). However, the rod dim flash recovery time constant,
263 dominated by the inactivation of transducin (Krispel et al., 2006), was not significantly different
264 between transgenic and wild type littermate controls (**Fig. 6, Tables 1 and 2**), indicating normal
265 transducin/PDE6 complex inactivation. Similarly, the rising phase of the fractional response was
266 unaffected by the expression of R838S RetGC1 in both line 362 (**Fig. 6B**) and line 379 (**Fig. 6D**). Thus,
267 the activation of the phototransduction cascade remained unaltered in the mutant rods.

268 The current carried through the NCKX1 exchanger can be observed following the rapid
269 closing of all cGMP-gated channels by a saturating flash (Yau and Nakatani, 1984). Notably, this
270 exchange current was more pronounced in *R838S*$^{+}$ rods than in wild type littermates (**Fig. 7A**). Direct
271 comparison of the exchange currents, measured from the point of saturation of the cGMP-gated channels
272 revealed that it is ~3-fold larger in *R838S*$^{+}$ rods compared to wild type controls (**Fig. 7B**). We found no
273 indications of the NCKX1 content being upregulated in *R838S*$^{+}$ rods by Western immunoblotting (**Fig.**
274 **2B**), therefore, the more pronounced exchange current in *R838S*$^{+}$ rods is consistent with their larger dark
275 current and argues that the free Ca^{2+} concentration in their outer segments becomes elevated.
276
277

The presence of a CORD6 RetGC1 in rods increases their dark noise.

During the recordings from *R838S*$^{+}$ rods, we noticed that the baseline of the recordings in darkness had frequent 'bumps'. Such increase in dark noise was observed in rods from both 362 and 379 lines but not in control wild type rods (**Fig.8A** and **Fig. 8B**). This observation is reminiscent of the increased dark noise in *GCAPs*$^{-/-}$ mouse rods (Burns et al., 2002). In that case, the lack of proper phototransduction feedback and slow Ca^{2+}-mediated inactivation renders the discrete events caused by spontaneous activation of visual pigment 4-5 times larger than normal, making them readily observable in the dark. To test if the increased dark noise in *R838S*$^{+}$ rods was caused by abnormal Ca^{2+}-mediated feedback on rod phototransduction, we first evaluated the instrumental noise in *R838S*$^{+}$ rods under saturating background light. In this condition, the cGMP-gated channels are closed so that phototransduction signals, including those from the spontaneous activation of the pigment, become undetectable. Notably, the discrete bumps were not observed in bright light recordings (**Fig. 8A** and **Fig. 8B**; bottom traces), indicating that they were not derived from the instrumental noise, but rather, were generated by rod phototransduction. We next analyzed the power spectrum of the dark noise (**Fig. 8C** and **Fig. 8D**). Its phototransduction component was extracted by subtracting the instrumental (bright light) spectrum from the total dark noise spectrum. The so obtained difference spectra were well-fitted with the scaled power spectrum of the averaged dim flash response for both line 362 (**Fig. 8E**) and line 379 (**Fig. 8F**), demonstrating that the origin of the discrete dark noise in *R838S*$^{+}$ rods is the spontaneous activation of visual pigments. Thus, similar to the case in *GCAPs*$^{-/-}$ rods (Burns et al., 2002), the abnormal Ca^{2+}-mediated feedback on the cyclase resulted in increased dark noise in both lines expressing the mutant R838S RetGC.

R838S^{+} rod degeneration is triggered by dysregulation of the Ca^{2+} feedback via GCAPs.

Mouse photoreceptors express only two homologs of GCAPs – GCAP1 and GCAP2 (Howes et al., 1998). To test if the abnormal regulation of the RetGC1 harboring CORD6 mutation was the main event triggering the downstream processes eventually leading to the photoreceptor death, we compared the rate at which *R838S*$^{+}$ rods degenerated in mice having normal (*GCAP1,2*$^{+/+}$) or partially reduced (*GCAP1,2*$^{+/-}$) content of GCAPs *versus* mice completely lacking both GCAPs (*GCAP1,2*$^{-/-}$) (Mendez et al., 2001). The respective genotypes were bred by repetitively crossing line 362 to the GCAP1,2$^{-/-}$ double knockout mice. In contrast to the normal (*GCAP1,2*$^{+/+}$) and hemizygous (*GCAP1,2*$^{+/-}$) backgrounds, a complete lack of GCAPs to mediate Ca^{2+} feedback on the CORD6 cyclase preserved normal retinal morphology in *R838S*$^{+}$ mice (**Fig. 9A, B**). Even after 5 months, when the photoreceptor layer in line 362 was obliterated, the histology of *R838S*$^{+}$*GCAP1,2*$^{-/-}$ retinas remained indistinguishable from that of wild type (**Fig. 9A**). While the line 362 *R838S*$^{+}$ rod nuclei count in *GCAP1,2*$^{+/+}$ or *GCAP1,2*$^{+/-}$ mice fell by more than 80% after 3 months of age, the thickness of the outer nuclear layer in *R838S*$^{+}$*GCAP1,2*$^{-/-}$ retinas did not differ from wild type controls and remained normal even after 6 months (**Fig. 9B, C**).

To further verify that the CORD6 cyclase mutant expressed in the *R838S*$^{+}$*GCAP1,2*$^{+/-}$ rods still retained its propensity for abnormal regulation by GCAPs, we compared the Ca^{2+} sensitivity of the cyclase in *GCAP1,2*$^{-/-}$ mice harboring normal complement of RetGC1 and RetGC2 (Mendez et al., 2001; Peshenko et al., 2011) and in *R838S*$^{+}$*GCAP1,2*$^{-/-}$ mice, by reconstituting the homogenates of their dark-adapted retinas with purified recombinant mouse GCAP1. The Ca^{2+} sensitivity of the cyclase in *R838S*$^{+}$*GCAP1,2*$^{-/-}$ retinas in the presence of the added GCAP1 was shifted toward higher Ca^{2+} concentrations in a manner similar to the original line 362 (**Fig. 9D**). We also bred four *R838S*$^{+}$*GCAP1,2*$^{-/-}$ mice (two males and two females), subsequently verified as having normal, similar

to the C57B6J wild type, retinal histology at 4 months and confirmed that the retinas of their *R838S*$^{+}$*GCAP1,2*$^{+/-}$ progeny underwent rapid degeneration after two months of age (not shown). Hence, only the lack of abnormal Ca^{2+} feedback mediation via GCAPs prevented the CORD6 RetGC1 mutant present in the *R838S*$^{+}$*GCAP1,2*$^{-/-}$ rods from triggering the photoreceptor death.

DISCUSSION

Among the R838 substitutions in RetGC1 linked to the CORD6 (Kelsell et al., 1998; Gregory-Evans et al., 2000; Wegell-Weber et al., 2000; Downes et al., 2001; Payne et al., 2001; Ramamurthy et al., 2001; Kitiratschky et al., 2008), the R838S mutation, usually present in combinations with other substitutions not directly related to the phenotype, causes one of the most severe forms of *GUCY2D* CORD6 (Ramamurthy et al., 2001). Expression of R838S RetGC1 in mouse rods (Dizhoor et al., 2016), unlike overexpression of normal RetGC1 (Boye et al., 2013, 2015), results in a rapid decline of rod electroretinography (ERG) responses due to progressive degeneration of rod photoreceptors. Notably, photopic ERG responses from cones, which by design of the model do not express the CORD6 cyclase mutant (**Fig. 1**), remain strong in *R838S*$^{+}$ mice, even after the loss of rod ERG (Dizhoor et al., 2016). The clinical symptoms of CORD6 (Kelsell et al., 1998; Gregory-Evans et al., 2000; Hunt et al., 2010) include early onset and decline of vision in childhood due to progressive degeneration of functional photoreceptors. Consistently with that, individual *R838S*$^{+}$ rods in the mouse model remain highly functional at the early stage preceding the massive retinal degeneration albeit with markedly changed shape of photoresponses (**Fig. 4-7**).

Variability of the photoresponse between different *R838S*$^{+}$ rods (**Tables 1 and 2**) likely originates from a non-uniform cell-to-cell transgene expression, and the larger variability in line 379

would be consistent with a larger variation in *R838S*$^{+}$ ROS length for this line (**Fig. 3**). The variability of expression between different rods in these two lines could also explain why, after the initial rapid decline, both rod ERG and rod nuclei count in line 379 reach a plateau after 3 months of age and then the remaining rods survive for much longer (Dizhoor et al., 2016), while in line 362 *R838S*$^{+}$ all rods die out after 3 months of age (**Fig. 9**).

Photoreceptor degeneration in the CORD6 R838S RetGC1 model resembles the phenotype caused by mutations in GCAP1 associated with clinically distinct from the CORD6 dominant cone and cone-rod degenerations (Olshevskaya et al., 2004; Woodruff et al., 2007). Mutations such as Y99C or E155G render GCAP1 less sensitive to Ca^{2+} and hence shift Ca^{2+} sensitivity of the cyclase in a manner similar to the R838S RetGC1 mutation. Also, similarly to the *R838S*$^{+}$ mice, cGMP-gated channel activity and Ca^{2+} influx in rods expressing mutant GCAP1 increase and the abnormal Ca^{2+} feedback results in photoreceptor death (Olshevskaya et al., 2004; Woodruff et al., 2007). Nonetheless, the molecular events underlying the pathology are different between the GCAP1 and RetGC1 mutations. While the mutations in GCAP1 directly affect its Ca^{2+} sensor function by reducing GCAP1 affinity for Ca^{2+}, the Ca^{2+} sensor properties of GCAPs per se remain unaffected in CORD6. Instead, the R838S mutation in RetGC1 biases its binding to Mg^{2+}-liganded *versus* Ca^{2+}-liganded GCAP, even in the presence of a larger Ca^{2+}GCAP pool (Ramamurthy et al., 2001: Peshenko et al., 2004). As a result, the R838S mutant RetGC1 remains in its activated state at higher than normal Ca^{2+}.

The longer time to peak and the increased amplitude of dim flash response (**Fig. 6**) as well as the more prominent dark noise in the *R838S*$^{+}$ rods (**Fig. 8**) all indicate that the dynamic regulation of the cyclase by Ca^{2+} becomes altered, suppressing its activation in response to the reduction in free Ca^{2+} after illumination. On the other hand, the increased exchange current in *R838S*$^{+}$ rods demonstrates more robust efflux of Ca^{2+} in dark-adapted conditions, indicative of higher intracellular Ca^{2+} concentrations

(Iwamoto et al., 2000). However, the increased dark current also suggests that the elevation of Ca^{2+} levels in these rods does not reach the levels sufficient for complete inhibition of the mutant cyclase in the dark. If the cyclase activity is not fully inhibited in the dark and merely stabilizes a higher RetGC/PDE6 activities equilibrium, then a less cooperative (**Fig. 2A**) response of the cyclase to the decrease of free Ca^{2+} concentrations could make its light-driven acceleration slower than normal.

Conforming with the hypothesis, that the CORD6 mutation of cyclase leads to elevation of free cGMP and Ca^{2+} in the outer segments (see also Tucker et al., 1999; Ramamurthy et al., 2001; Peshenko et al., 2004), the abnormalities in *R838S*$^{+}$ rod photoresponses can be attributed to the change in Ca^{2+} sensitivity of the cyclase regulation. Indeed, the larger dark current carried by Na^{+} and Ca^{2+} through the cGMP-gated channels in *R838S*$^{+}$ rods indicates elevated free cGMP concentrations in outer segments (**Fig. 4-5**), consistent with the right-shift in the Ca^{2+} sensitivity of the mutant cyclase and the reduced cooperativity of its Ca^{2+}-dependent deceleration (**Fig. 2A**). The more prominent exchange current produced by Ca^{2+} efflux through the NCKX1 (**Fig. 7**), under the conditions of the experiment limited by the NCKX1 affinity for Ca^{2+} (Iwamoto et al., 2000), indicates that the free Ca^{2+} concentration is also increased. Hence, both elevated cGMP and/or Ca^{2+} in the diseased photoreceptors, both being considered likely apoptotic agents (Olshevskaya et al., 2004; Xu et al., 2013), could trigger the photoreceptor death.

Our results directly demonstrate that the abnormal Ca^{2+} feedback on the cyclase via GCAPs is the principal initiating event leading to CORD6 – this is strongly argued for by preservation of *R838S*$^{+}$ rods in GCAP-deficient mice (**Fig. 9**). The dramatic rescue of degeneration in the absence of GCAPs also suggests that processes other than abnormal Ca^{2+} feedback, such as, for example, potential unfolded protein response directly provoked by the mutant cyclase, are unlikely to create the apoptotic trigger in this case. However, the exact pathway(s) through which the apoptotic progression builds up

remains to be established. The elevated free cGMP content detectable by the increased *R838S*$^{+}$ rod photocurrent could conceivably lead to apoptosis via activation of cGMP-dependent protein kinase (PKG, or cGK) in photoreceptors (Xu et al., 2013; Paquet-Durand et al., 2009). Both isozymes of the kinase (PKG1 and 2, or cGKI and II) are present in photoreceptors (Feil et al., 2005; Gamm et al., 2000). Deletion of a single isozyme, PKG1, does not affect the severity of rod degeneration in *rd1* mouse model, in which cGMP rises due to the lack of PDE6 activity (Wang et al., 2017). However, PKG2 could not be deleted in that study, because *rd1* and the *Prkg2* gene coding for PKG2 are located on the same chromosome. This leaves open a possibility that PKG2 can specifically mediate the apoptotic pathway or does so in a compensatory fashion in the absence of PKG1. Therefore, any future studies of the degeneration mechanism in *R838S*$^{+}$ rods will have to address the possible effects of PKG1 and PKG2 individual and double gene knockouts in order to delineate or exclude a possible PKG-activated apoptotic pathway in CORD6.

Increase in Ca^{2+} flux could provide another strong possibility for triggering apoptosis, similar to that in *rd1*, where deletion of the rod CNG1 channel slows the pace of degeneration (Wang et al., 2017). A larger cGMP-gated current in the outer segments would cause stronger depolarization of *R838S*$^{+}$ rods in the dark. Therefore, the increased Ca^{2+} influx through cGMP-gated channels in the outer segment and/or the voltage-gated Ca^{2+} channels in the synaptic terminals of the *R838S*$^{+}$ rods could overload the Ca^{2+} scavenging capacity of the mitochondria (Giarmarco et al., 2017) and set the apoptosis in motion. One could also speculate that the increased Ca^{2+} flux elevates tonic release of glutamate from the synapse in the dark and thus affects the synaptic transmission. However, the early onset of morphological changes and rod degeneration in *R838S*$^{+}$ mice reduce scotopic ERG b-wave even at young age (Dizhoor et al., 2016), making this issue difficult to address. We reason that studies of the degenerative processes in *R838S*$^{+}$ rods should help better understand general mechanisms of CORD6

pathology and, in a long term, could facilitate development of gene therapy approaches to treat the disease.

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FIGURE LEGENDS

Fig. 1. Rod-specific expression of R838S RetGC1 in mouse line 362. The R838S RetGC1 expressing line 362 (Dizhoor et al., 2016) was outcrossed to *RetGC1*$^{-/-}$ background (Yang et al., 1999) to eliminate interference from the endogenous mouse RetGC1 immunoreactivity. Cryosections from *RetGC1*$^{-/-}$ (*top row*) and *R838S*$^{+}$*RetGC1*$^{-/-}$ (*bottom row*) retinas were probed by rabbit polyclonal anti-RetGC1 antibody

(Laura et al., 1996) and developed using AlexaFluor 568 goat anti-rabbit (*red*) secondary antibody. Cone sheaths were labeled using FITC-conjugated peanut agglutinin (PAg, *green*). The fluorescence images were superimposed on differential contrast image (DIC) in the rightmost panels. *RPE* – retinal pigment epithelium, *ROS* - rod outer segments, *COS* – cone outer segments, *ONL* – outer nuclear layer.

Fig. 2. Expected increase in guanylyl cyclase activity in *R838S*$^{+}$ rods due to the change in its Ca^{2+} sensitivity. (A) Guanylyl cyclase activity, mean ± S.D., in dark-adapted wild type (closed triangles ▲) and *R838S*$^{+}$ littermates (open circles ○) retinal homogenates was assayed as described in Materials and Methods. The activities were normalized as the percent of maximal catalytic activity of the cyclase in each preparation and fitted with the Hill function, $A = (A_{max} - A_{min})/(1 + ([Ca] / K_{1/2\ Ca})^{h}) + A_{min}$, where A_{max} and A_{min} are the respective maximal and minimal activity, *[Ca]* – free Ca^{2+} concentration, $K_{1/2\ Ca}$ – free Ca^{2+} concentration causing two-fold inhibition of the cyclase activity, and *h* – Hill coefficient. The *arrows* indicate the approximate range of free Ca^{2+} change in mouse rods between light- and dark-adapted state (Woodruff et al., 2002; Olshevskaya et al., 2004). (B) Altered Ca^{2+} feedback on the cyclase predicted to increase cGMP production and Ca^{2+} influx in the dark does not upregulate CNG1 and NCKX1 expression in *R838S*$^{+}$ retinas. Protein fractions extracted from *R838S*$^{+}$ and wild type littermates at 3 weeks of age were equalized by total protein concentration and 10, 20 and 30 µl of the samples were separated using 7% SDS-PAAG. Western immunoblot was probed with anti-NCKX1 (*top*), anti-CNG1 alpha (*middle*), and beta actin (*bottom*) antibodies. Chemiluminescence for CNG1 was proportional to the sample load (*right panel*) and was quantified from the immunoblot in *R838S*$^{+}$ retinas as 1.1 ± 0.2 (SD), N = 3, of the wild type level. Signal-to-background ratio on immunoblots with NCKX1 did not allow for a reliable quantification, but the observed band intensities gave no indication of upregulation.

686 **Fig. 3**. Shortening of the outer segment in *R838S*$^{+}$ rods occurs at early ages in lines 362 (A) and 379 (B).
687 Top panels – DIC images of sections from wild type and *R838S*$^{+}$ littermates perfused and fixed with
688 glutaraldehyde/paraformaldehyde solution at 3.5 and 4.5 weeks, respectively. Bottom panels – length
689 distribution for the outer segments of wild type and identifiable *R838S*$^{+}$ rods. The horizontal bars
690 represent the mean average for each group. *RPE* – retinal pigment epithelium, *ROS* - rod outer segments,
691 *RIS* - rod inner segments, *ONL* – outer nuclear layer.
692
693 **Fig. 4.** Increased dark current in line 362 of *R838S*$^{+}$ rods. (A, B) Representative response families to
694 flash stimuli recorded by suction electrode from a wild type (A) and line 362 *R838S*$^{+}$ rods (B). Data
695 were obtained at 4-4.5 weeks of age. Flash strength ranged from 1 to 3200 photons·μm^{-2} with 0.5 log
696 unit steps and flash duration was 2 or 20 ms. Bold traces represent the responses of the two rods to a
697 flash of 32 photons·μm^{-2}. (C, D) Intensity-response curves of wild type (filled circles ●) and line 362
698 *R838S*$^{+}$ (open diamonds ◇) rods shown in absolute (C) or normalized (D) values. Data are shown as
699 mean ± SEM, N = 6 (wild type) and 16 (*R838S*$^{+}$). Data in (D) were also fitted with the Naka-Rushton
700 equation (the fitting curves are not shown in the panel), $r = I^n / (I^n + I_0^n)$, where r is a normalized
701 photoresponse amplitude, I - flash intensity, I_0 - half-saturating flash intensity and n - a constant that
702 controls the slope; there was no significant difference in slope between the wild type (n = 1.42 ± 0.06)
703 and the *R838S*$^{+}$ rods (1.25 ± 0.06); P-value for 2-tailed unpaired test was 0.15.
704
705 **Fig. 5.** Increased dark current in line 379 of *R838S*$^{+}$ rods. (A, B) Representative response families to
706 flash stimuli recorded by suction electrode from a wild type (A) and line 379 *R838S*$^{+}$ rods (B). Data
707 were obtained at 4-4.5 weeks of age. Flash strength ranged from 1 to 3200 photons·μm^{-2} with 0.5 log
708 unit steps and flash duration was 2 or 20 ms. Bold traces represent the responses of the two rods to a

flash of 32 photons·μm^{-2}. (C, D) Intensity-response curves of wild type (filled circles ●) and line 379 *R838S*$^{+}$ (open diamonds ◇) rods shown in absolute (C) or normalized (D) values. Data are shown as mean ± SEM, N = 11 (wild type) and 24 (*R838S*$^{+}$). In (D), the slope parameter *n* from the Naka-Rushton equation (fitting curves not shown) was not significantly different between wild type (1.20 ± 0.07) and *R838S*$^{+}$ (1.23 ± 0.04); P-value for 2-tailed unpaired test was 0.74.

Fig. 6. Delayed onset of dim flash response recovery in *R838S*$^{+}$ rods. Averaged normalized (A, C) and fractional (B, D) dim flash responses from wild type (black traces) and *R838S*$^{+}$ (red traces) rods from line 362 (A, B) and line 379 (C, D) mice. Fractional responses (photons^{-1}·μm^{2}) were obtained by dividing dim flash responses (pA) by dark current (pA) and flash intensity (photons·μm^{-2}). Flash strength in panels A and B were 10 photons·μm^{-2} for wild type and 3 or 10 photons·μm^{-2} for *R838S*$^{+}$. Flash strength in panels C and D were 3 or 10 photons·μm^{-2} for wild type and 1, 3 or 10 photons·μm^{-2} for *R838S*$^{+}$, all with a flash duration of 2 or 20 ms. The specific flash intensity was selected depending on the sensitivity of each rod. Data are shown as mean ± SEM, N = 6 (wild type in line 362 panel), 17 (*R838S*$^{+}$ line 362), 11 (wild type in line 379 panel) and 24 (*R838S*$^{+}$ line 379).

Fig. 7. Increased NCKX1 exchange current in *R838S*$^{+}$ rods. (A) Averaged saturating flash responses of wild type (black) and *R838S*$^{+}$ (red) rods from line 362. Each trace was set to 0 pA at 0.08 sec after the 3200 photons·μm^{-2} flash stimulus when the cGMP-gated current becomes fully blocked. (B) Magnified view of the peaks of the responses from (A). Data are shown as mean ± SEM, N = 10 (wild type) and 16 (*R838S*$^{+}$).

Fig. 8. Increased dark noise in *R838S*$^{+}$ rods. (A, B) Representative traces demonstrating noise in darkness (*Dark*) and in bright light (*Instrumental*) recorded from wild type (left) and *R838S*$^{+}$ (right) rods of line 362 (A) or line 379 (B) mice. Data were low-pass filtered at 10 Hz. (C, D) Power spectra of dark noise (Dark, filled circles ●) and instrumental noise (Instrument, open circles ○) from wild type (left) and *R838S*$^{+}$ (right) rods of line 362 (C) or line 379 (D) mice. Data are shown as mean ± SEM, the N values are shown in the labels. (E, F) Phototransduction component of the noise power spectrum of *R838S*$^{+}$ rods (filled circles ●), fitted with a scaled power spectrum of dim flash responses. Plots were obtained by subtracting instrumental power spectrum from dark noise power spectrum. Curves were fitted with least-square method. Data are shown as mean ± SEM.

Fig. 9. Retinal degeneration in *R838S*$^{+}$ mice is triggered by abnormal Ca^{2+} feedback on R838S RetGC1 via GCAPs. (A) Representative microphotographs of retinal histology at 5 months of age. Top to bottom - wild type, *R838S*$^{+}$*GCAP1,2*$^{+/+}$, *R838S*$^{+}$*GCAP1,2*$^{+/-}$, and *R838S*$^{+}$*GCAP1,2*$^{-/-}$ mice. The retinas from aldehyde-perfused mice were embedded and cross-sectioned as described in Methods; *RPE* – retinal pigment epithelium, *ROS* - rod outer segments, *ONL* – outer nuclear layer, *OPL* – outer plexiform layer, *INL* – inner nuclear layer, *IPL* – inner plexiform layer, *GCL* – ganglion cell layer; *bar* – 100 μm. (B) Photoreceptor degeneration in wild type (open diamonds ◇), *R838S*$^{+}$*GCAP1,2*$^{+/+}$ (open circles ○), *R838S*$^{+}$*GCAP1,2*$^{+/-}$ (open triangles △), and *R838S*$^{+}$*GCAP1,2*$^{-/-}$ (closed circles ●) mice. The ONL nuclei count per 100 μm retina section length between the optic nerve and the periphery of the retina was sampled at different ages. Each data point represents separate animal. (C) The reduction of ONL thickness at 3 months of age in wild type, *R838S*$^{+}$*GCAP1,2*$^{+/+}$, *R838S*$^{+}$*GCAP1,2*$^{+/-}$, and *R838S*$^{+}$*GCAP1,2*$^{-/-}$ mice was tested by one-way ANOVA with a post-hoc Bonferroni all-pairs comparison (alpha 0.01) using a Synergy Kaleidagraph software; ***P < 0.0001; no difference was

found in wild type vs. *R838S*$^{+}$*GCAP1,2*$^{-/-}$. (D) In *R838S*$^{+}$*GCAP1,2*$^{-/-}$, the R838S RetGC1 activity, masked by the lack of GCAPs, retains the propensity for abnormal Ca^{2+} sensitivity. Guanylyl cyclase activity, mean ± S.D., in dark-adapted *GCAP1,2*$^{-/-}$ (open diamonds ◇) and *R838S*$^{+}$*GCAP1,2*$^{-/-}$ (closed circles ●) mouse retinal homogenates was assayed as described in Fig. 2A, except that the assay was supplemented with 5 μM purified recombinant mouse GCAP1 to enable activation and Ca^{2+} sensitivity of the endogenous RetGC. The data were fitted with the Hill function.

760 Table 1. Parameters of single-cell suction recordings for *R838S*$^{+}$ rods from Line 362

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Parameter	*Wild type*	*R838S*$^{+}$	P-value*
Dark current (pA)	7.9 ± 1.3	19 ± 2	**0.014**
Exchange current (pA)**	NA	1.2 ± 0.3	-
Flash sensitivity (pA·photons^{-1}·μm^{2})	0.13 ± 0.03	0.77 ± 0.15	**0.021**
Fractional sensitivity (photons^{-1}·μm^{2})	0.016 ± 0.002	0.039 ± 0.006	**0.033**
Time to peak (msec)	142 ± 7	232 ± 15	**0.003**
Recovery time constant (msec)	180 ± 27	219 ± 33	0.503
Integration time (msec)	281 ± 48	329 ± 37	0.494
Half-saturation flash sensitivity (photons·μm^{-2})	34 ± 3	24 ± 4	0.129
Number of cells	6	17	-

762 *Two-tailed unpaired t-test. Statistically significant differences are highlighted in bold.
763 ** Observed in 1 of 6 WT rods and in 10 of 17 R838S^{+} rods
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765 Table 2. Parameters of single-cell suction recordings for *R838S*$^{+}$ rods from Line 379

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Parameter	*Wild type*	*R838S*$^{+}$	P-value*
Dark current (pA)	9.9 ± 1.1	14.1 ± 1.1	**0.031**
Flash sensitivity (pA·photons^{-1}·μm^{2})	0.29 ± 0.06	0.80 ± 0.14	**0.023**
Fractional sensitivity (photons^{-1}·μm^{2})	0.028 ± 0.003	0.056 ± 0.009	**0.037**
Time to peak (msec)	150 ± 6	198 ± 9	**0.02**
Recovery time constant (msec)	182 ± 24	188 ± 15	0.824
Integration time (msec)	215 ± 25	321 ± 19	**0.02**
Half-saturation flash sensitivity (photons·μm^{-2})	24 ± 3	15 ± 2	**0.005**
Number of cells	11	24	-

767 * Two-tailed unpaired t-test

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