Foveal RGCs develop abnormal calcium dynamics weeks after photoreceptor ablation

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Abstract

Objective or purpose

Physiological changes in retinal ganglion cells (RGCs) have been reported in rodent models of photoreceptor (PR) loss but this has not been investigated in primates. By expressing both a calcium indicator (GCaMP6s) and an optogenetic actuator (ChrimsonR) in foveal RGCs of the macaque, we reactivated RGCs *in vivo* and assessed their response in the weeks and years following PR loss.

Design

We used an *in vivo* calcium imaging approach to record optogenetically evoked activity in deafferented RGCs in primate fovea. Cellular scale recordings were made longitudinally over a 10 week period following photoreceptor ablation and compared to RGC responses from RGCs that had lost photoreceptor input more than two years prior.

Participants

Three eyes received photoreceptor ablation, OD of a male *Macacus mulatta* (M1), OS of a female *Macacus fascicularis* (M2) and OD of a male *Macacus fascicularis* (M3). Two animals were used for *in vivo* recording, one for histological assessment.

Methods

Cones were ablated with an ultrafast laser delivered through an adaptive optics scanning light ophthalmoscope (AOSLO). A 0.5 s pulse of 25Hz 660nm light was delivered to optogenetically stimulate the deafferented RGCs, and the resulting GCaMP fluorescence signal was recorded from RGCs with an AOSLO. These measurements were repeated over the 10 weeks immediately after photoreceptor ablation and at 2 years.

Main Outcome measures

The rise time, decay constant and response magnitude of the deafferented RGCs responding to optogenetic stimulation was derived from GCaMP fluorescence recordings from 221 RGCs (Animal M1) and 218 RGCs (Animal M2) *in vivo.*

Results

The mean time to the peak calcium response was stable in deafferented RGCs over the 10-week post ablation observation period, while the mean decay constant of the calcium response decreased by 1.5 fold 1.6±0.5 s to 0.6±0.3 s within 10 weeks in subject 1 and 2.1 fold (2.5±0.5 s to 1.2±0.2 s SD) within 8 weeks in subject 2.

Conclusions

We observe abnormal calcium dynamics developing in primate foveal RGCs in the weeks after photoreceptor ablation. The mean decay constant of the optogenetic mediated calcium response decreased 1.5 - 2-fold. This is the first report of this phenomenon in primate retina and further work is required to understand the role these changes play in cell survival and activity.

Nevertheless, the presence of optogenetic mediated responses 2 years after PR loss and the stable rise time remain promising for vision restoration therapies.

Introduction

In diseases such as age-related macular degeneration and retinitis pigmentosa, photoreceptor death leads to irreversible vision loss. A range of vision restoration therapies are currently under development that aim to restore light sensitivity to the retina including optogenetic and optoelectronic prostheses, and regenerative stem cell transplantation. However, all current vision restoration approaches that act at the retinal level require reactivation of the remaining retinal architecture to restore interpretable signals to the brain. These approaches therefore require not only structural preservation of the inner retina, but high-quality functional preservation following extended periods of vision loss.

Previous studies have demonstrated RGC survival following long-term photoreceptor loss both in mice[1,2] and primates[3–5]. While RGCs may survive for many years in cases of advanced retinal degeneration, there is evidence of cytopathologies and ultrastructural 'whorls' suggestive of organelle stress[6]. Physiological studies of RGCs in murine models of retinal degeneration report increased spontaneous activity, including higher a oscillatory firing frequency and an increased sustained firing rate[7–10]. In primates, structural rewiring of the midget pathway has been reported following acute photoreceptor loss[11], but physiological changes in primate RGCs has yet to be investigated. If altered physiology does develop in primate RGCs in the absence of photoreceptor input, this may compromise the efficacy of vision restoration therapies. In this study, we investigated the *functional* preservation of RGCs after photoreceptor ablation with the aim of understanding how deafferented RGCs in the fovea respond to restored activity with increasing time after photoreceptor loss.

In humans, high acuity vision is mediated by the specialized anatomy and physiology of the fovea. Previous attempts to restore vision in patients with retinal prostheses have been most successful

when the fovea is stimulated[12], and optimizing therapies at this location may provide a pathway toward improved visual outcomes[13,14]. Due to the degree of similarity in foveal anatomy, physiology, and downstream processing, the non-human primate model is the gold standard animal model for preclinical development of vision restoration therapies. While naturally occurring retinal degeneration models have recently been identified in NHPs[15,16], they are not widely available at present. Here we adopt a photoreceptor ablation approach using a femtosecond laser exposure delivered through an adaptive optics scanning laser ophthalmoscope (AOSLO). This results in acute photoreceptor loss that is laterally and axially confined[17].

In the absence of photoreceptors, investigating the functional status of deafferented RGCs requires an alternative stimulation approach. In retinal explants electrical stimulation of RGCs is typical, however longitudinal monitoring of responses in these preparations is impossible. In this study, we take an *in vivo* retinal imaging approach, optically stimulating and recording from deafferented RGCs for weeks, months, and years after photoreceptor ablation by combining AOSLO with the optogenetic actuator ChrimsonR and the calcium indicator GCaMP6s expressed in the foveal RGCs of the NHP. Calcium imaging with AOSLO enables a longitudinal readout of the intracellular calcium response to stimulation at the cellular scale *in vivo*[18]. In this study, we presented a brief pulsed optogenetic stimulus focused onto deafferented RGCs, and recorded the optogenetic mediated calcium rise and decay in the weeks and years following photoreceptor loss. This approach allows us to observe the impact of localized photoreceptor loss on the calcium dynamics of the same group of RGCs over time and in doing so make observations that have previously been experimentally inaccessible.

Methods

Animal Care and housing

The primates in this study were housed in an AAALAC-approved facility, where the animals were provided with free access to water and nutritious chow together with various treats and vegetables. Primates were provided with daily enrichment including 2-4 pieces of manipulata such as mirrors and puzzle feeders rotating between animals, daily movies and music, and rotating access to larger free roaming space with swings and elevated perches. A team of 4 full-time veterinarians, 5 veterinary technicians, and an animal behavior specialist monitored animal health, body condition and checked for any signs of discomfort daily. Subjects were housed in diffuse ambient illumination during the daytime (equivalent to 620 nm 0.05mW at the pupil plane), significantly lower than the ChrimsonR activation threshold[19], thus deafferented RGCs were only stimulated during the optogenetic trials. This study was performed in strict accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals, and the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the University of Rochester Committee in animal resources (PHS Assurance No. 1160743209A1).

Co-expression of GCaMP6s and td-Tomato labeled ChrimsonR

AAV2-CAG-tdTomato-ChrimsonR and AAV2-CAG-GCaMP6s were synthesized by the University of Pennsylvania vector core and intravitreally injected into the left eye of one female *Macaca fascicularis* (M1) 65 weeks before recording, the right eye of one male (M2) 11 weeks before recording, and the right eye of a male *Macaca fascicularis* (M3) 164 weeks before euthanasia. Ocular tissues were sterilized with 50% diluted Betadine prior to the injection and a lid speculum was placed. The viral vector was injected into the middle of the vitreous from a location ~3 mm behind the limbus using a 30-gauge needle with a tuberculin syringe. Neutralizing antibodies to

AAV2 were measured in serum prior to purchase of NHPs and only animals which showed an antibody response to a serum dilution of 1:25 or less were included in the study. Animals received daily subcutaneous cyclosporine A injections to suppress the immune system 1-4 weeks prior to and after injection of the viral vectors. A starting dose of 6 mg/kg was titrated into a therapeutic range of 150–200 ng/mL by monitoring blood trough levels. Once the desired blood trough level was achieved, that dose was maintained throughout the experiment. Signs of intraocular inflammation were monitored by slit lamp examination, fundus photography, and observation of the pupillary response.

Photoreceptor Ablation by ultrafast laser exposure

A patch of photoreceptors was ablated in the central fovea by 106 ms exposure of a 0.8 x 0.7-degree field to an ultrafast pulsed, 730 nm laser focused onto the photoreceptor layer (PRL) using the AOSLO (average power of 4.48 Wcm^{-2} and a repetition rate of 80 MHz). Dhakal *et al.* have previously shown that this approach creates damage to the PRL that is both axially and laterally confined[17]. Furthermore, as the RGCs in the fovea are laterally displaced from the photoreceptors that drive them, the deafferented RGCs under study here were never directly exposed to the high intensity laser during PRL ablation. This femtosecond laser ablation approach has previously been used as a model of vision loss in photoreceptor transplantation studies[20] and to demonstrate optogenetic restoration of RGC activity [5,18].

Fundus imaging/OCT/cSLO Imaging

Imaging was performed with a conventional scanning light ophthalmoscope (Heidelberg specialist), OCT, and a fundus camera before and weekly following PRL ablation. A fundus camera (Topcon TRC 50ex) with customized fluorescence filters for GCaMP imaging (excitation 466/40 nm, emission 520/28) and tdTomato imaging (excitation 549/25 nm and emission 586/20

nm) was used to assess the fluorescence of GCaMP6s and tdTomato-ChrimsonR independently. The axial impact of laser exposure was monitored with OCT.

Adaptive optics calcium imaging

Foveal RGCs were stimulated and recorded through an AOSLO system described in Gray *et al*[21]. To summarize, a Shack-Hartmann wavefront sensor and a deformable mirror in the subject pupil plane detected and compensated ocular aberrations in a closed-loop correction system using 847 nm laser diode (Thorlabs) in an SLO. A 796 nm superluminescent-diode laser source (Superlum) was focused on the photoreceptor mosaic layer, and reflectance images collected through the 25.6 Hz adaptive optics SLO with a 2-airy disk, confocal pinhole. GCaMP6s was excited by focusing a 488 nm laser source (Qioptics) at the RGC layer over an area of 398x398 um^2 and emitted photons detected in 517/20 nm band using an 8-airy disk pinhole to maximize signal collection.

Visual stimulation

Both optogenetic and photoreceptor stimulation were delivered through the 25 Hz AOSLO system. Both types of trials began with a period of adaptation to the imaging light (67.5 sec for the optogenetic trials and 60 sec for the photoreceptor trials). To drive the optogenetic actuator ChrimsonR, a 0.5 sec pulse of 25Hz, 640 nm light was focused onto the RGC layer, (mean luminance 21.37 mw/cm^2, 205x205 um^2). The calcium responses were recorded for 22.5 s following stimulation to capture both the calcium rise and decay in response to the optogenetic stimulus. To evoke photoreceptor mediated responses and confirm deafferentation, a 561 nm, 0.025 Hz square wave uniform flicker stimulus with a mean luminance of 0.25 mw/cm^2 was delivered to central photoreceptors for 80 seconds while GCaMP recordings were made from the laterally displaced foveal RGCs they drive.

Experimental Design

Trials were performed prior to PRL ablation to ensure that we were able to generate robust and reproducible photoreceptor and optogenetic responses. In the 1-5 weeks post ablation photoreceptor stimulation trials were performed to ensure complete deafferentation of the RGCs under study. To assess changes in the calcium response of RGCs in the weeks following deafferentation optogenetic stimulation were performed both in the recently deafferented area and an eccentricity matched region of RGCs that had been deafferented for over 2 years on the opposite side of the RGC ring. These experiments were performed 5 and 6 times over the 10 week period following PRL ablation in subject M1 and M2 respectively. Control experiments were also performed including only presenting 488 nm stimulation to confirm the response was optogenetically driven, and a ChrimsonR stimulus only trial to ensure no bleed through of the 640 nm stimulus into the detection channel.

Data analysis

To correct for eye motion, *in vivo* fluorescence videos containing the calcium imaging data from the ganglion cell layer were co-registered to simultaneously acquired, higher signal to noise IR reflectance images of the photoreceptor mosaic. For each imaging location, a reference frame was used to perform a strip-based cross-correlation[22–24] to remove translation and rotation. Motion corrected reflectance and fluorescence videos were summed over time to generate higher signal to noise reflectance images of the photoreceptor ablation site and fluorescence images of intact and deafferented RGCs for manual segmentation. To ensure the same group of cells were tracked longitudinally, the fluorescence GCaMP images of RGCs from each time point were registered to week 1 post ablation, prior to RGC segmentation.

To extract GCaMP recordings for each RGC, the segmentation mask was applied to the motion-corrected fluorescence videos and the initial 67.5s adaptation period was removed. The mean

signal within each individual cell mask was computed for a single frame (subject M1 96 RGCs, subject M2 109 RGCs).

In optogenetic stimulation trials, each trace was separated into a rising phase and a decay phase based on the frame containing the maximum fluorescence intensity. Rise time was calculated as the time between the stimulus onset and the frame containing the maximum fluorescence intensity. To determine the decay constant for single traces the data was first normalized by subtracting the average baseline fluorescence in the 100 frames prior to stimulus onset and dividing by the maximum fluorescence intensity. The resulting traces were fitted with an exponential function using a least-square approach. The mean responses presented in figures 2 and 3 were created by taking the mean of the normalized single cell traces from all segmented RGCs for each location.

The response magnitude was quantified in the following ways:

Absolute response magnitude To report the response magnitude excluding changes in signal baseline, F_{LT}-$F0_{LT}$ & F_{ST}-$F0_{ST}$ indexes are the difference between the maximum fluorescence signal (F) in a single cell and the pre-stimulus baseline (F0).

Normalized response magnitude Then the normalized response magnitude is calculated by dividing single RGC F_{ST}-$F0_{ST}$ indexes by the mean of F_{LT}-$F0_{LT}$ recorded in the same imaging session to exclude changes in eye quality or imaging conditions over weeks, and the sequence of experiments between sessions was not varied over the sessions.

Normalized pre-stimulus baseline The normalized F0 value was acquired by dividing single short-term deafferented RGC calcium floor level by the mean of long-term deafferented RGC

calcium floor level recorded within the same imaging session; the sequence of experiments between sessions is not varied over the sessions.

The sensitivity index ((F-F0) / std(F0)) was measured by dividing individual short-term deafferented RGC's F-F0 index by the standard deviation F0 value, F-F0 & F0 were calculated with the same method described above.

For photoreceptor stimulation trials, the 60-s adaptation period is removed from the registered video, and the mean signal within each cell mask is computed for each frame of the recording period. The resulting fluorescence traces were multiplied by a Hann windowing function to remove ringing artifacts and Fourier transformed into the frequency domain as described previously[18].

Light Safety

The total light exposure at each retinal location was calculated for each recording session, with all light sources taken into account (488 nm at 0.71 mw cm^{-2}, 796 nm at 1.30 mw cm^{-2}, and 843 nm at 0.19 mw cm^{-2}), optogenetic stimulation (640 nm at 21.37 mw cm^{-2}), and photoreceptor stimulation (561 nm at 0.25 mw cm^{-2}). Overall light exposure at a single retinal location did not exceed the American National Standard Institute (American National Standard for the Safe Use of Lasers ANSI Z136.1–2007) maximum permissible exposure of the human retina scaled by the ratio of numerical aperture difference between human and macaque (Photoreceptor ablation session excluded). The power of each light source was measured before the imaging session at the pupil plane, and accumulation of laser exposure between sessions is not considered since all sessions were separated by at least six days.

Histology

Subject 3 was euthanized with an intravenous injection of Euthasol and perfused with 1 L of heparinized saline for 23 min and 1 L of 4% paraformaldehyde for 7 min. After enucleation, the anterior segment was removed and the posterior segment was immersion-fixed in 4% paraformaldehyde for 90 min. After fixation, the posterior segment was washed for 5 min in PBS and cryoprotected in 10%, 20%, and 30% sucrose. Retinal pieces were embedded in Cryogel embedding compound (Leica) and frozen in isopentane cooled in liquid nitrogen. 20-μm thick cryosections were collected and dried for 60 min at 35°C on a slide warmer before storage at −80°C. To assess the cytoplasmic to nuclear ratio of GCamP6s expression in RGCs, nuclei were counterstained with Hoescht 33342. Samples were imaged on a laser scanning confocal microscope (Zeiss LSM 880) with the 405- and 561-laser lines using a Zeiss Plan-Apochromat 60×/1.4 N.A. oil immersion objective. Tiled image stacks were stitched in Image J software. To measure the cytoplasmic to nuclear ratio for GCaMP6s, line profiles were drawn to transect the center of each RGC in the image stack. Intensity profile plots of GCaMP6s and Hoescht fluorescence were exported to Igor Pro 9 (Wavemetrics) for further analysis. Intensity measurements were background subtracted using areas of the image without GCaMP6s fluorescence.

Results

Localized photoreceptor ablation in primate fovea deafferents RGCs allowing characterization of optogenetic mediated calcium dynamics

Intravitreal co-injection of the viral vectors AAV2-CAG-GCaMP6s and AAV2-CAG-tdTomato:ChrimsonR resulted in the foveal ring of RGCs expressing the calcium indicator

GCaMP6s (Figure 1A and Supp. Figure 3A-C) and the optogenetic actuator tdTomato-ChrimsonR in all 3 animals (Supp. Figure 3D-F) as observed in previous studies[5,18].

AOSLO NIR reflectance imaging of the cone photoreceptor mosaic *in vivo* confirmed photoreceptor ablation following laser exposure (Figure 1C). Optical coherence tomography (OCT) showed the absence of the foveal photoreceptor layer (PRL) post exposure (Supp. Figure 4A-C). Figure 1D shows GCaMP fluorescence from foveal RGCs one week after PRL ablation (9 weeks after the initial injection of AAV2-CAG-GCaMP6s and AAV2-CAG-ChrimsonR-tdTomato). As previously reported[5,18] fluorescence fundus imaging shows a reduction in activity dependent fluorescence from the RGC ring that is spatially consistent with the ablation of photoreceptors in the temporal hemifield of the central fovea. The loss of photoreceptor response was confirmed by presenting a 0.025 Hz square wave flicker stimulus to the central fovea. Figure 1E shows 0.025Hz GCaMP responses from RGCs prior to laser exposure and the absence of these signals from the same RGCs post exposure as anticipated.

To assess RGC calcium dynamics in these deafferented RGCs we presented a 0.5s, 640 nm, 0.9 mW stimulus focused at the ganglion cell layer through AOSLO to optogenetically stimulate RGCs. The optogenetic mediated calcium response leads to an increase of GCaMP fluorescence and then gradual decay. Figure 1F shows the calcium responses recorded from deafferented RGCs 1 week after PRL ablation including an inset showing an *in vivo* fluorescence image of the RGCs under study.

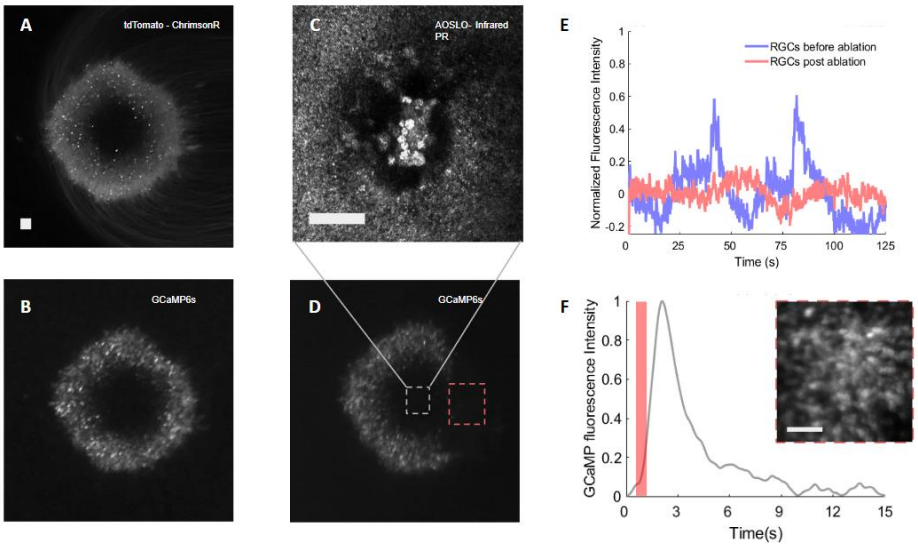

Fig. 1: Optogenetic mediated calcium response triggered in deafferented primate foveal RGCs. (A) Fundus fluorescence image of td-Tomato ChrimsonR expression 7 weeks after injection of the viral vector and 1 week prior to photoreceptor ablation. (B) AOSLO NIR reflectance image of photoreceptor ablation site 1-week following ablation. (C) Fundus fluorescence image of GCaMP6s expression 7 weeks after injection of viral vector and 1 week prior to photoreceptor ablation. (D) Fundus fluorescence image of GCaMP nine weeks after injection of viral vector and 1-week post photoreceptor ablation. The dark region of the RGC ring has photoreceptor inputs ablated and the activity dependent fluorescence is correspondingly reduced. (E) Clear 0.025Hz GCaMP response is observed from RGCs on the right side of the RGC ring before photoreceptor ablation, while no GCaMP response is observed from the same group of RGCs following photoreceptor ablation, supporting loss of photoreceptor inputs. (F) Optogenetic mediated GCaMP response to a 0.5 s 640 nm stimulation 1 week following laser ablation. Stimulus duration is shown in the red bar, AOSLO deafferented RGC image with low fluorescence expression in top right. All scale bars are 100 μm, and all data were acquired from subject M1.

Calcium responses to optogenetic stimulation decay more rapidly in RGCs within weeks following PRL ablation

The same laser ablation, optogenetic stimulation and GCaMP recording paradigm was also used to interrogate the calcium rise and decay in a second animal (M2). In this animal, recording were made from both a patch of RGCs that had been deafferented for 1 week (nasal: short-term deafferented), and also from an eccentricity-matched control patch of RGCs deafferented 2 years previously (temporal: long-term deafferented). By recording from these two regions during each session we were able to use long-term deafferented RGC responses as a control group to assess the stability of the imaging platform.

The NIR reflectance AOLSO image of the photoreceptor mosaic and OCT data show the two regions of photoreceptor ablation (Figure 2 A-B) in subject M2. The decreased activity dependent RGC GCaMP fluorescence in Figure 2D reflects the loss of photoreceptor input, consistent with the previous study[5]. Deafferentation was confirmed at the 1-week timepoint post ablation on the nasal side of the RGC ring, with no response to a 0.45 Hz photoreceptor stimulation detected (Figure 2D). Figure 2G-H shows the mean decay constant of the calcium response decreased 2.1-fold (from 2.5±0.5 s to 1.2±0.2 s SD) in the 8 weeks post PR ablation ($p<0.001$, paired t-test, n=109), with 87% of this decrease occurring within the first 5 weeks. The decay constant did not decrease further from week 8 to 10, and the calcium rise and decay speed stabilized from 101-122 weeks following photoreceptor loss (Figure 2E-F).

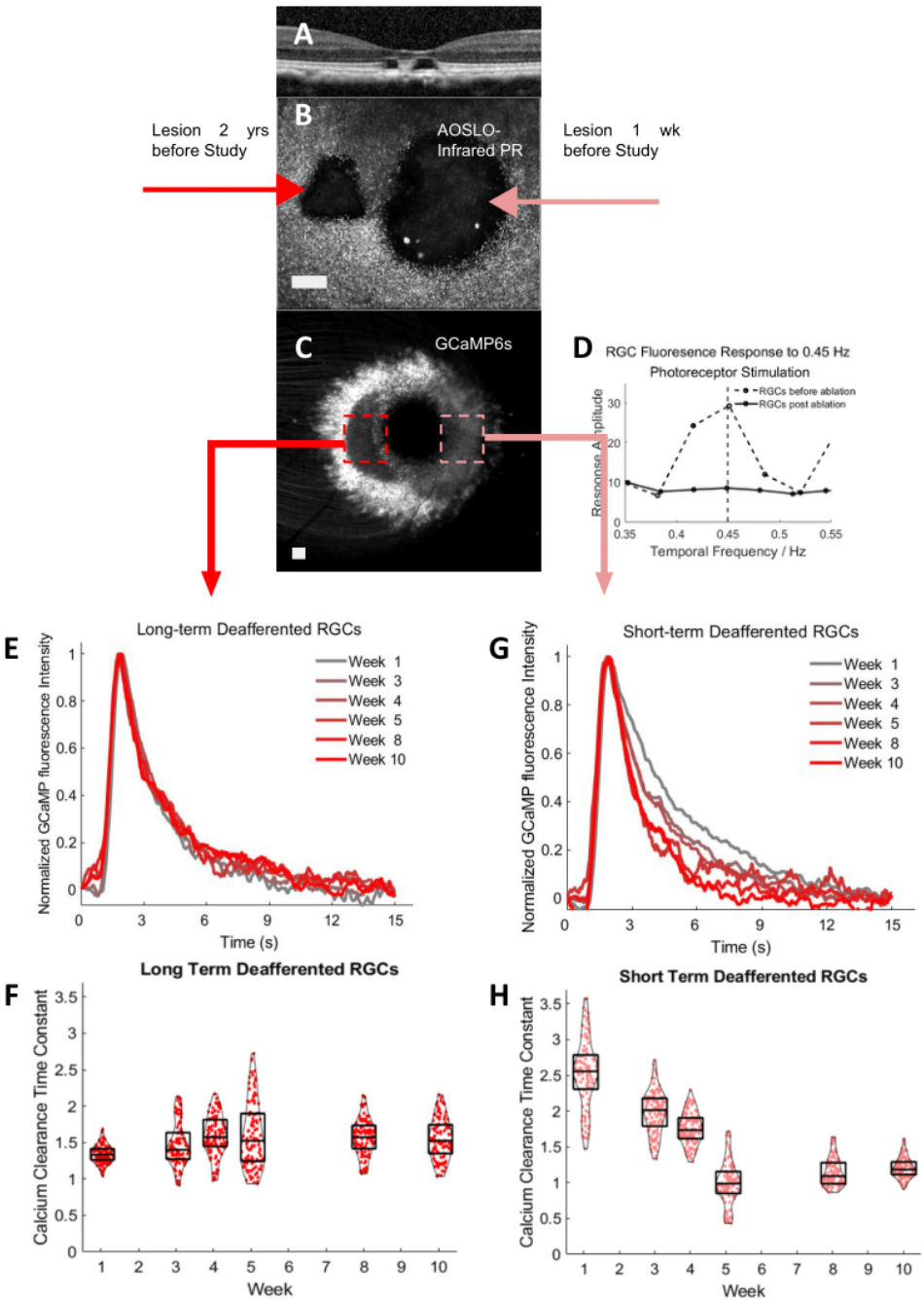

Fig. 2: Foveal RGCs show faster calcium decay in the five weeks following PRL ablation in subject M2. (A) OCT scan through the foveal region shows two localized regions of photoreceptor loss. (B) AOSLO NIR reflectance image of two photoreceptor ablation sites 122 weeks following the first ablation (left) and 10

weeks following the second ablation (right). (C) Fundus fluorescence image of GCaMP6s fluorescence 1 week following the second PRL ablation. (C) Fundus fluorescence image of GCaMP6s expression 7 weeks after injection of viral vector and 1 week prior to photoreceptor ablation. Two dark regions of the RGC ring have photoreceptor inputs ablated on the nasal and the temporal side of the RGC ring, reflecting the laser PRL ablation performed 2 years and 1 week before the study. The nasal side corresponds to the short-term deafferented RGCs, and the temporal side corresponds to the long-term deafferented RGCs. (D) A 0.45 Hz GCaMP response is observed in RGCs before photoreceptor ablation, while no GCaMP response is observed from the same group of RGCs following photoreceptor ablation. (E) Mean GCaMP response to optogenetic stimulation from 109 long-term deafferented RGCs from weeks 1-10. (F) GCaMP fluorescence decay constants from 109 individual long-term deafferented RGCs from weeks 1-10. (G) Mean GCaMP response to optogenetic stimulation from 109 short-term deafferented RGCs from weeks 1-10. (H) GCaMP fluorescence decay constants from 109 individual short-term deafferented RGCs from weeks 1-10.

The rate of calcium decay is stable in RGCs with intact photoreceptors and in RGCs prior to PRL ablation

Longitudinal recording from deafferented RGCs was repeated in a second subject (M1) this time comparing the optogenetic response in deafferented RGCs to the optogenetic response in RGCs with intact photoreceptors. Photoreceptor-mediated calcium responses to a 0.1 Hz flickering stimulus incident on foveal photoreceptors were recorded 1 week prior to, and 5 weeks post PRL ablation in a single field of view (Figure 3A-B). Laser ablation in subject M1 removed photoreceptor input from half of the RGCs in the recording area, leaving those at higher eccentricity with inputs intact. This scenario is shown in Figure 3C. After ablation, periodic RGC

responses to photoreceptor stimulation were absent in the deafferented zone (Figure 3D-E). Similar to animal M2, the mean decay constant of the optogenetically-evoked calcium responses in the deafferented RGCs decreased 1.5-fold (from 1.6±0.5 s to 0.6±0.3 s SD) in the 10 weeks post PR ablation (p<0.001, paired t-test, Figure 3F). In comparison, RGCs with photoreceptors intact in the same field of view showed relatively stable decay kinetics over the 10-week period. Use of a spatially limited optogenetic stimulus in the fovea ensured that the calcium responses from these non-deafferented cells were not photoreceptor mediated (Figure 3G, Appendix 1). Optogenetic stimulation of RGCs with intact photoreceptors in this region and in the deafferented region 1 week prior to photoreceptor ablation produced a stable decay constant (2.2±0.5 s), confirming that these results are not explained by a temporary increase in decay constant relating to e.g. inflammation post ablation.

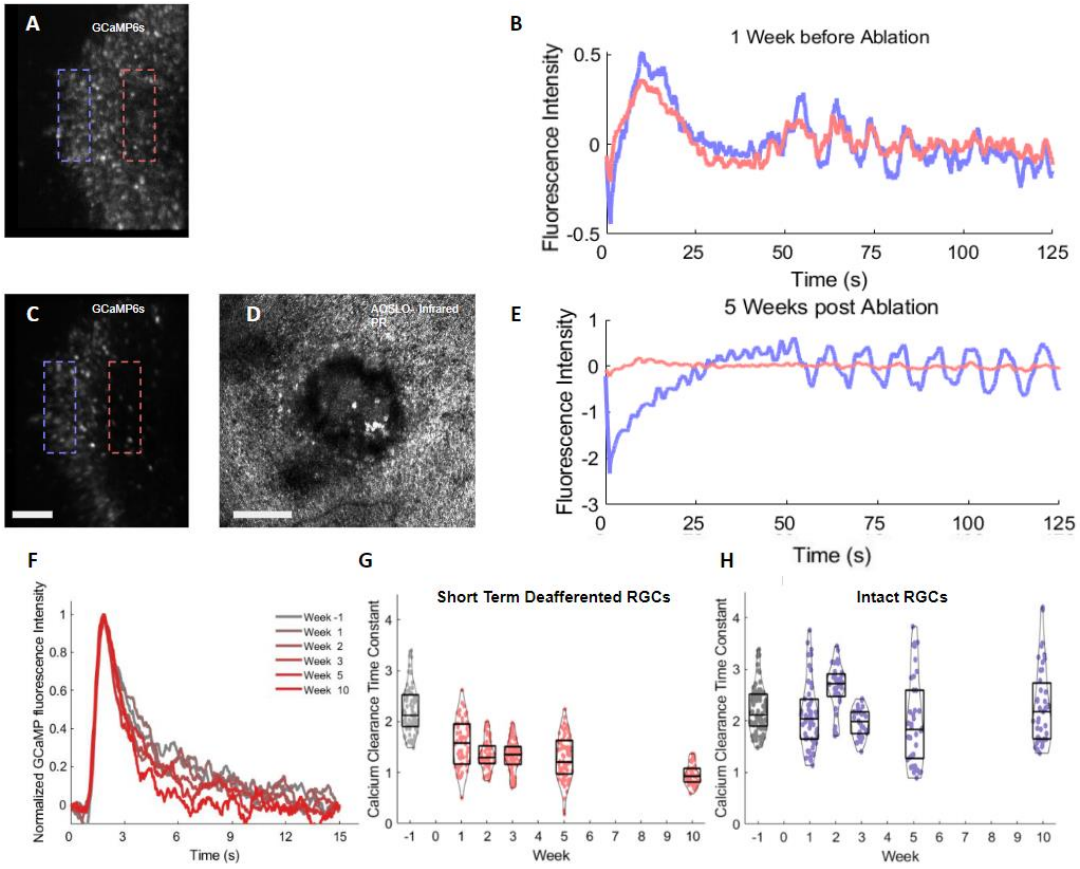

Fig. 3: Calcium response to PR stimulation and optogenetic decay constant in RGCs with photoreceptor loss and intact photoreceptor in a second primate. (A) SLO GCaMP fluorescence image showing the GCaMP fluorescence from the two test regions of foveal RGCs prior to photoreceptor ablation. (B) RGC photoreceptor mediated GCaMP fluorescence responses to 0.1 Hz flicker stimulation from the purple and pink regions 1 week prior to photoreceptor ablation (C) Same region as shown in (A) 5 weeks post PRL ablation deafferenting the RGCs in the pink region. (D) AOSLO NIR reflectance image of the photoreceptor ablation site 5 weeks after PRL ablation. (E) Photoreceptor mediated GCaMP responses to 0.1 Hz flicker stimulation in deafferented (purple) and intact (pink) areas 5 weeks post photoreceptor ablation. (F) The mean normalized optogenetic mediated GCaMP response recorded from 129 short-term deafferented RGCs over the observation period from 1 week before PRL ablation to 10 weeks post-ablation. (G) Calcium decay constants recorded from 129 single RGCs over the observation period from 1 week before PRL ablation to 10 weeks post-ablation. (H) Simultaneously acquired calcium decay constants recorded from 92 RGCs over the observation period from 1 week before PRL ablation to 10 weeks post-ablation. All scale bars are 100 μm, and all data were acquired from subject M1.

The optogenetic mediated calcium rise time is unaffected by deafferentation

The mean time to peak calcium response reflects the speed of the optogenetic mediated calcium influx. In animal M1, the mean rise time did not change significantly prior to and post PRL ablation (Week -1, 1.47±0.33s SD) & (Week 5, 1.44±0.14s SD). In animal M2, the mean rise time also did not differ significantly between the long-term (1.43±0.17s SD) and the short-term (1.45±0.30s SD) deafferented RGCs during the 10-week period (unpaired *t*-test, p=0.05, n=654) (Figure 4A-B).

These data suggest that photoreceptor ablation does not alter the rise time of calcium responses in downstream RGCs.

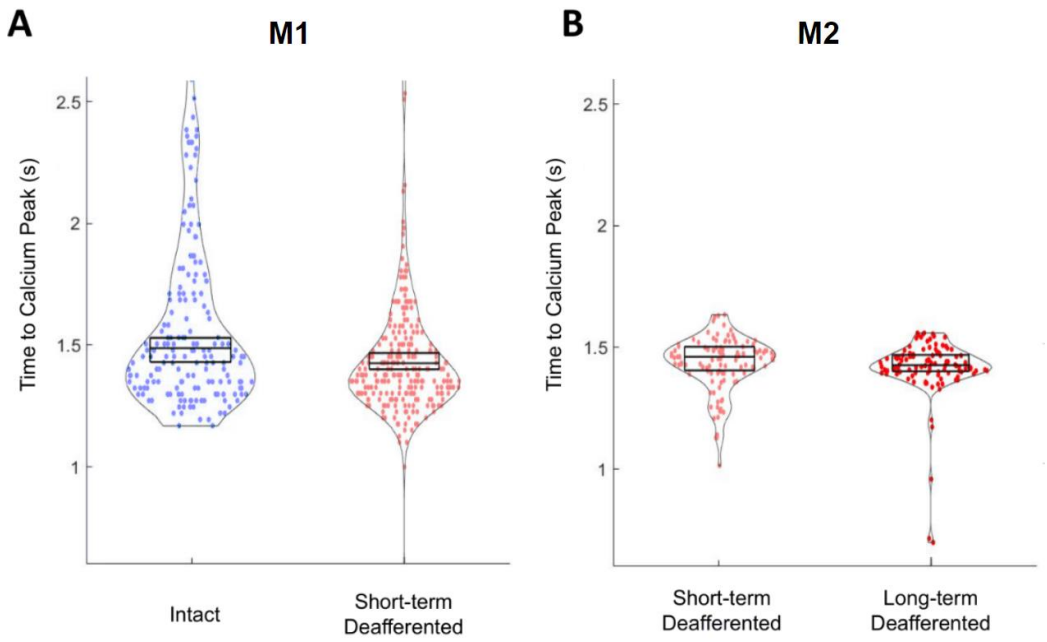

Fig. 4: Optogenetic mediated calcium rise time in deafferented and intact RGCs. (A) Optogenetic mediated calcium rise time in response to a 0.5 s, 25 Hz 640 nm stimulus delivered to RGCs with PR input intact (n=182) and RGCs with PR inputs ablated 1-10 weeks previously (short term deafferented) RGCs (n=257) in subject M1. Standard deviations of the RGC rise time are labeled with the upper and lower edge of the box plot, and the mean of RGC rise times are labeled with middle line in the box plot. Each data point shows the rise time recorded from a single RGC within an imaging session. (B) Optogenetic mediated calcium rise time for short term deafferented RGCs (n=106) compared to RGCs deafferented for 2 years (long-term, n=107), in animal M2. Each data point shows the mean RGC rise time collected from a single cell over the six imaging sessions within the 10-week observation period.

An optogenetic mediated calcium response can be evoked in deafferented RGCs 2.8 years following viral transduction and 2.3 years after photoreceptor ablation

In subject M2, robust optogenetic mediated responses were recorded 2.8 years post-injection of the viral vector carrying the ChrimsonR transgene. The RGCs in this area were deafferented for over 2.3 years at the time of recording. The decay constant in long-term deafferented RGCs was 1.6 ± 0.2s S.D.. The calcium rise time in these long-term deafferented RGCs (1.43 ± 0.10s) is not significantly different from RGCs with intact photoreceptors, indicating the speed of the optogenetic mediated calcium response is not impacted by long-term photoreceptor loss.

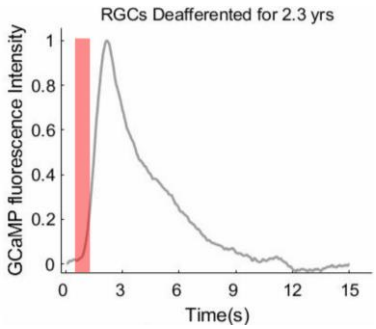

Fig. 5: Mean optogenetic mediated calcium response recorded from 109 RGCs deafferented over 2.3 years. (A) Optogenetic mediated GCaMP response to a 0.5 second 25 Hz 640 nm stimulus 2.3 years following photoreceptor ablation and 2.8 years following initial vector injection, the stimulation duration is shown by the red bar.

The decay constant is independent of response amplitude, stimulus power, and chronic expression of GCaMP6s

To assess the impact of potentially varying optical parameters, response magnitude and protein expression on the calcium decay constant, we calculated the sensitivity index of responses and the normalized response magnitude to control for these parameters. In addition, we performed

control experiments using a range of optogenetic stimulus powers and conducted a histological investigation of intact and deafferented RGCs in subject M3 with chronic GCaMP6s expression.

The sensitivity index scales the absolute response magnitude by the standard deviation of the baseline fluorescence, providing a discriminability metric for the response amplitude independent of fluorescence intensity. The division controls for parameters that may potentially vary between sessions such as the clarity of the anterior optics, system alignment, AO performance, pinhole position and the level of GCaMP expression. The sensitivity index of responses in deafferented RGCs was stable during the 10-week recording period, suggesting changes in the magnitude of the response do not account for the reported reduction in the decay constant (Fig. 6A).

Figure 6B shows the decay constant from deafferented RGCs under optogenetic stimulation powers ranging from 0.8 - 1 mW. The decay constants in deafferented RGCs show no linear trend with stimulus power, suggesting the changes we observed in the speed of the calcium decay are not linked to the power of the stimulus or to changes in the level of optogenetic actuator expression over time.

Between week 2 & 3 post PRL ablation, the normalized pre-stimulus GCaMP fluorescence baseline decreased 1.7-fold, consistent with the reduction observed in the fluorescence fundus images (Fig. 2C, Figure S2B). This decrease in baseline fluorescence has been observed and reported in previously work[5]. The normalized response magnitude in the short-term deafferented RGCs also decreased significantly between weeks 1 & 2 following photoreceptor loss (0.11±0.02s to 0.04±0.01s, Figure S2B). The trend of both pre-stimulus baseline and normalized response magnitude change did not follow the gradual decrease in decay constant decrease observed over 5 weeks.

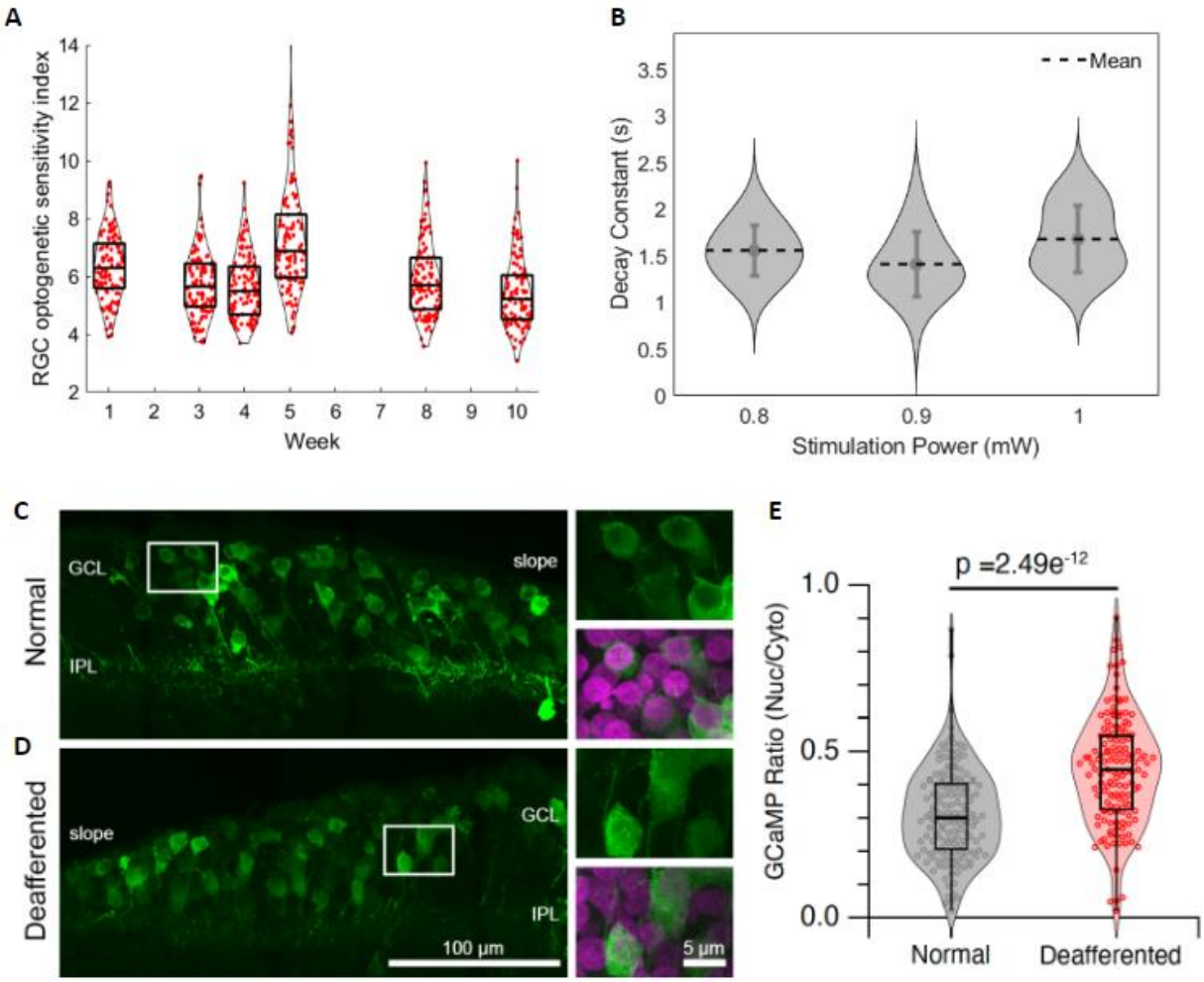

Fig. 6: Control experiments showed that the abnormal calcium dynamics are not explained by changes in response magnitude, stimulus power or nuclear expression of GcaMP6s. (A) Optogenetic sensitivity index of responses to optogenetic stimulation in short-term deafferented RGCs in the 10 weeks following photoreceptor ablation. (B) Calcium decay constant in deafferented RGCs following optogenetic stimulation by three different light intensities. Data collected from 109 short-term deafferented RGCs and 109 long-term deafferented RGCs in subject M2. (C-D), Confocal image (maximal z-projection) showing GCaMP6s expression (green) in a non-deafferented (normal, C) and deafferented (D) region

of the same retina. The foveal slope region is indicated. ROIs are shown enlarged on the right with and without a nuclear counterstain (Hoescht 33342). E, Violin/bar plots showing the nuclear:cytosolic ratio of GCaMP6s for RGCs in normal (n=152 cells) and deafferented (n=140 cells) retinal regions from subject 3. The horizontal line shows median, whiskers show min/max. Dots show data from individual cells. Data is pooled from two different sections of the same retina. Statistical comparison was with an unpaired, two-tailed t-test.

Previous studies in mouse cortical neurons have shown that long-term expression of GCaMP3 can lead to nuclear accumulation and prolongation of the decay of calcium signals [25]. However, recent longitudinal studies with GCaMP7s in rodents showed no abnormal light-evoked activity in RGCs following long-term expression of GCaMP7s[26]. To investigate whether changes in GCaMP6s localization could account for the observed changes in calcium dynamics, we quantified the nuclear to cytosolic (N/C) ratio of GCaMP6s in deafferented and intact RGCs in subject M3 (Figure 6E-F). In prior studies, a higher GCaMP6s N/C ratio was linked to slower decay of calcium signals. We found a significantly higher N/C ratio in deafferented compared to intact RGCs (0.44 ± 0.16 vs 0.31 ± 0.14 S.D.). These findings suggest that the faster decay time constant of calcium signals in deafferented RGCs cannot be explained by nuclear accumulation in intact RGCs associated with GCaMP toxicity.

Discussion

By co-expressing the calcium indicator GCaMP6s and optogenetic actuator ChrimsonR *in vivo*, we have demonstrated the development of abnormal calcium dynamics in adult primate foveal RGCs just weeks after photoreceptor loss. The timescale over which these changes were observed is similar to that associated with the development of retinal hyperactivity in rodent

models[7–10]. A 1.5-2-fold increase in the speed of calcium clearance was observed within 5 weeks in subjects M1 and M2 respectively. The decay constant in short-term deafferented RGCs recorded from subject M2 also approached the long-term deafferented RGC responses after week 8. Data from both subjects suggest that physiological changes occurred within weeks of photoreceptor loss. While there was an initial difference in calcium decay constant between the two subjects, it is important to note that both deafferented RGC groups approach a similar decay constant of 1.3 s and 1 s by 10 weeks after photoreceptor loss. The initial difference between the two subjects may be related to the substantial age difference between these animals (M1 7 years old, M2 17 years old, M3 9 years old). Data from post-mortem human retina has suggested that exposure to age-related stressors may generate differences in physiology[27].

The stable decay constant recorded from intact RGCs in subject M1 indicates the abnormal calcium dynamics observed in both subjects following deafferentation was not a result of optogenetic stimulation per se or a transient response to laser injury. We also demonstrated that with adaptive optics with active closed-loop tracking, the optogenetic stimulation of displaced RGCs will not trigger responses from nearby cells with photoreceptor crosstalk, confirming the initial longer decay constant prior to PRL ablation. Consistent with the reduction of GCaMP6s and tdTomato-ChimsonR fluorescence intensity in deafferented RGCs in the weeks of PRL ablation reported by McGregor *et al.*, the normalized response magnitude shows a significant reduction within 1 week following photoreceptor ablation. However, considering the sensitivity index of optogenetic responses, stimulus power control experiments, and histological evaluation of GCaMP6s expression, the gradual changes in the decay constant cannot be solely attributed to changes in absolute response magnitude or the level of GCaMP expression.

While this is the first report of abnormal calcium dynamics in primate retina, studies in a murine model of glaucoma have also revealed abnormal calcium dynamics developing in RGCs following

axon crush, although interestingly the decay constant increases after injury[28]. This is different from the more rapid decay observed in this study, but there are a number of important models and species differences to note. In the glaucoma model, lengthening of the decay constant appears to precede rapid cell death[28], but in the photoreceptor ablation model the inner retina remains structurally intact despite many years of photoreceptor loss. It is possible that the shorter decay constant we observe is the consequence of a protective adaptation to higher levels of intracellular calcium generated by hyperactivity. Studies in other parts of the primate visual system have observed abnormal calcium homeostasis following ablation[29]. It is also possible that the altered calcium clearance develops following photoreceptor loss to maintain calcium homeostasis. Additionally, primate foveal RGCs are largely midget ganglion cells, which are absent in mice. This study represents the first observations of this phenomenon and more work is required to understand the mechanism giving rise to this effect, and whether RGC types are differentially affected. To fully investigate the impact of physiological changes on restored vision, psychophysical tests are necessary.

Although physiological changes are observed in RGC calcium dynamics following photoreceptor loss, it is important to note that the optogenetic mediated calcium rise time was stable in intact and deafferented RGCs in both subjects and across all time points. Furthermore, robust optogenetic mediated responses are triggered in RGCs even 2.3 years following photoreceptor loss, and 2.8 years after viral transduction of the optogenetic actuator. This is a promising result with current clinical trials having reported optogenetic mediated light sensitivity remaining 1.6 years after administration of the therapy in patients[30].

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Conflict of Interest

The authors declare no competing financial interests.

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