Imaging the response of the retina to electrical stimulation with genetically encoded calcium indicators


Department of Biomedical Engineering, University of Southern California, Los Angeles, California; Department of Electrical Engineering, University of Southern California, Los Angeles, California; Department of Physiology & Biophysics, University of Southern California, Los Angeles, California; Department of Pharmacology, Toxicology, and Neuroscience, Louisiana State University Health Sciences Center, Shreveport, Louisiana; Department of Ophthalmology, University of Florida, Gainesville, Florida; and Department of Ophthalmology, University of Southern California, Los Angeles, California

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Weitz AC, Behrend MR, Lee NS, Klein RL, Chiodo VA, Hauswirth WW, Humayun MS, Weiland JD, Chow RH. Imaging the response of the retina to electrical stimulation with genetically encoded calcium indicators. J Neurophysiol 109: 1979–1988, 2013. First published January 23, 2013; doi:10.1152/jn.00852.2012.—Epiretinal implants for the blind are designed to stimulate surviving retinal neurons, thus bypassing the diseased photoreceptor layer. Single-unit or multielectrode recordings from isolated animal retina are commonly used to inform the design of these implants. However, such electrical recordings provide limited information about the spatial patterns of retinal activation. Calcium imaging overcomes this limitation, as imaging enables high spatial resolution mapping of retinal ganglion cell (RGC) activity as well as simultaneous recording from hundreds of RGCs. Prior experiments in amphibian retina have demonstrated proof of principle, yet experiments in mammalian retina have been hindered by the inability to load calcium indicators into mature mammalian RGCs. Here, we report a method for labeling the majority of ganglion cells in adult rat retina with genetically encoded calcium indicators, specifically GCaMP3 and GCaMP5G. Intravitreal injection of an adeno-associated viral vector targets ~85% of ganglion cells with high specificity. Because of the large fluorescence signals provided by the GCaMP sensors, we can now for the first time visualize the response of the retina to electrical stimulation in real-time. Imaging transduced retinas mounted on multielectrode arrays reveals how stimulus pulse shape can dramatically affect the spatial extent of RGC activation, which has clear implications in prosthetic applications. Our method can be easily adapted to work with other fluorescent indicator proteins in both wild-type and transgenic mammals.

Address for reprint requests and other correspondence: R. H. Chow, Univ. of Southern California, 1501 San Pablo St., #323, Los Angeles, CA 90089 (e-mail: rchow@usc.edu).
and blocks solvent access to the cpGFP chromophore, causing it to become brighter (Akerboom et al. 2009).

Until recently, GECIs have produced signals inferior to those of synthetic calcium dyes (Hendel et al. 2008; Palmer and Tsien 2006). However, recent advances in GECI biotechnology have led to sensors with signal-to-noise ratios (SNRs) that rival those of synthetic probes (Akerboom et al. 2012; Tian et al. 2009; Zhao et al. 2011). GECIs are also more photostable than their synthetic counterparts (Borghuis et al. 2011). They can be delivered to cells via electroporation, viral vectors, or generation of transgenic animals (Zariwala et al. 2012). Of these three methods, viral vectors have shown the greatest cellular specificity in the retina (Borghuis et al. 2011; Hellström et al. 2008).

Recombinant adeno-associated viral (AAV) vectors are most commonly used for retinal gene transfer due to their lack of pathogenicity and toxicity as well as their superior ability to target specific retinal cell types (Grimm and Kay 2003). AAV tropism is dictated by the viral capsid, which differs among serotypes (Hellström et al. 2008). By using various combinations of serotypes and promoters, Borghuis et al. (2011) delivered the GECI GCaMP3 (Tian et al. 2009) to the five major neuron classes in mouse retina. Intravitreal injection of AAV2/1-SYN1-GCaMP3 targeted RGCs with high specificity, although some horizontal cells were also labeled. In general, labeling was described as patchy, with >70% of RGCs in a patch showing GCaMP3 expression (Borghuis et al. 2011).

Of the many AAV serotypes that have been identified (Schmidt et al. 2008), AAV2 has been reported to be best for labeling RGCs (Auricchio et al. 2001; Hellström et al. 2008). AAV2 also transduces the greatest number of cells following intravitreal injection (Hellström et al. 2008). When coupled with the CAG promoter, AAV2 vectors can transduce ~85% of RGCs in adult rat retina (Martin et al. 2002).

Based on these findings, we designed AAV2-CAG vectors incorporating the GECIs GCaMP3 (Tian et al. 2009) and GCaMP5G (Akerboom et al. 2012). We found these vectors to label the majority of RGCs in adult rat retina with high specificity. Subsequent electrical stimulation with MEAs evoked large increases in fluorescence intensity, much larger than we observed in amphibian retina with synthetic calcium dye. Subsequent electrical stimulation with MEAs evoked large increases in fluorescence intensity, much larger than we observed in amphibian retina with synthetic calcium dye. Subsequent electrical stimulation with MEAs evoked large increases in fluorescence intensity, much larger than we observed in amphibian retina with synthetic calcium dye.

Recombinant AAV vectors were produced by the two-plasmid cotransfection method (Zolotukhin et al. 1999). Briefly, one CellSTACK (Corning, Corning, NY) supplemented with 5% fetal bovine serum and antibiotics (cDMEM). A calcium phosphate precipitation transfection was set up by mixing a 1:1 molar ratio of vector plasmid DNA and a serotype-2-specific rep-cap helper plasmid, pDG. This precipitate was added to 1,100 μl of cDMEM, and the mixture was applied to the cell monolayer. The transfection was allowed to incubate at 37°C, 7% CO2, for 60 h. The cells were then harvested and lysed by three freeze/thaw cycles. The crude lysate was clarified by centrifugation, and the resulting vector-containing supernatant was divided among four discontinuous iodixanol step gradients. The gradients were centrifuged at 350,000 g for 1 h. Five milliliters of the AAV containing 40–60% interface was removed from each gradient and combined. This combined iodixanol fraction was further purified and concentrated by column chromatography on a -ml HiTrap Q Sepharose (anion exchange) column using an AKTA FPLC system (Pharmacia, Piscataway, NJ). The vector was eluted from the column using 215 mM NaCl, pH 8.0, and the AAV peak was collected. The AAV-containing fraction was then concentrated and buffer exchanged in Alcon Balanced Salt Solution (BSS) with 0.014% Polysorbate 20 using a Bio-max 100K concentrator (Millipore, Billerica, MA). The AAV was titered for DNase-resistant vector genomes by real-time PCR relative to a standard. Purity of the final vectors was assayed by polyacrylamide gel electrophoresis to determine the fraction of total protein that was AAV viral capsids VP1, VP2, and VP3 (>95%). Final concentrations of AAV2-CAG-GCaMP3 and AAV2-CAG-GCaMP5G were 4.0 × 1012 and 3.2 × 1012 vector genomes per milliliter, respectively. AAV2/1-SYN1-GCaMP3 (University of Pennsylvania Vector Core, Philadelphia, PA), which was used in some experiments, had a concentration of 1.7 × 1013 vector genomes per milliliter. Viral stock was diluted in BSS before injection.

Intravitreal injections. Adult female Long-Evans (Harlan Laboratories, Indianapolis, IN) rats, aged postnatal day 60-256, were anesthetized via intraperitoneal injection of ketamine (60 mg/kg) and xylazine (5 mg/kg). Phenylephrine (2.5%) and tropicamide (1%) were dropped into one eye to induce pupil dilation. Tetracaine (0.5%) was applied to the same eye as a local anesthetic. A 30-gauge needle was used to make a pilot hole through the sclera, choroid, and retina, 1–2 mm posterior to the corneal limbus. A microliter syringe (Hamilton, Reno, NV) attached to a blunt 32-gauge needle was used to inject 4–5 μl of virus into the vitreous. Care was taken to avoid injury of the lens. Injections were given slowly over a period of 30 s to allow diffusion of the virus. The needle was left in place for 30 s after the
injection and withdrawn slowly to prevent leakage. The injection site was visualized with an ophthalmic microscope to confirm absence of cataract, intraocular bleeding, and air bubbles. Antibiotic eye ointment (neomycin and polymyxin B sulfates and bacitracin zinc) was applied to prevent infection.

Fluorescent dye loading. Rats were deeply anesthetized with ketamine/xylazine and rapidly decapitated. The treated eye was enucleated and hemisectioned with dissection scissors. Vitreous was removed with a custom extractor (Sekirnjak et al. 2006) to allow for a tight interface between the retina and MEA. The red tracer dye Alexa Fluor 594 hydrazide, sodium salt (30 mM, 1.5 μl; Life Technologies, Grand Island, NY) was retrogradely loaded into RGCs via the cut optic nerve, as described previously (Behrend et al. 2009). This enabled us to determine whether GECI-expressing cells were RGCs or other cell types. Dye loading was performed at 30°C for 1 h while superfusing the eyecup at 4–5 ml/min.

Bicarbonate-buffered Ames’ Medium (Sigma-Aldrich, St. Louis, MO) was used as superfusate in all experiments. Media was supplemented with penicillin-streptomycin to prevent bacterial growth, equilibrated with 5% CO₂-95% O₂ gas, and adjusted to pH 7.4 and 280 mM. Ames’ Medium used before dye loading contained 2.5 mM MgCl₂ and 5 mM EGTA to chelate calcium (Baldridge 1996) and prevent Ca²⁺-dependent resealing of the optic nerve (Yawo and Kuno 1985).

In experiments separate from those involving GECIs, we attempted retrograde loading of synthetic calcium dyes (Life Technologies) via the optic nerve. These experiments were performed in adult Long-Evans rats (Harlan Laboratories) and C57BL/6j mice (The Jackson Laboratory, Bar Harbor, ME). All dyes were dextran-conjugated potassium salts: 10-kDa fluo-4-dextran (20 mM), 10-kDa fura-dextran (20 mM), and 70-kDa Calcium Green-1-dextran (5 mM). An equimolar mixture (10 mM) of 10-kDa Oregon Green 488 BAPTA-1 (OGB-1)-dextran and 10-kDa Alexa Fluor 594-dextran was also applied in some experiments. Required loading times were predicted by a finite element model based off of the diffusion equation (Behrend et al. 2009). All 10-kDa dyes were loaded for 4–14 h. Calcium Green-1-dextran (70 kDa) was loaded for 15 h. Probenecid (2.5 mM) and sulfinpyrazone (250 mM) were added to the superfusate to inhibit organic anion transporter activity (Di Virgilio et al. 1988).

Imaging and electrophysiology. After dye loading, the retina was removed from the eyecup and mounted on a porous membrane (cat. no. JWVP01300; Millipore). The retina was placed ganglion cell-side down on a transparent MEA and imaged with an inverted epifluorescence microscope. Fluorescence excitation was provided by a super bright white light-emitting diode (LED) except for fura-dextran, which was excited by an ultraviolet LED. Excitation and emission light were filtered through Semrock (Rochester, NY) filter sets: cat. no. GFP-4050A for GCaMP3/GCaMP5G, cat. no. TXRED-4040B for Alexa Fluor 594, and cat. no. FITC-3540B for the remaining calcium dyes. Images were viewed through a Nikon (Tokyo, Japan) Plan Apo 10 objective and captured with a high-speed camera (Pulnix, Mississauga, Ontario, Canada). A dual-insulation layer, consisting of silicon nitride and SU-8 epoxy photore sist (Gholzien et al. 2006), was deposited atop the ITO. Vias were opened over the electrodes and contact pads on the perimeter of the substrate. Some electrodes were electroplated with platinum/iridium by cyclic voltammetry to increase charge injection limits.

Electrical stimuli consisted of a train of charge-balanced, biphasic current pulses or sinusoids. Voltage stimuli were output from a computer-controlled stimulator generate (Multi Channel Systems, Chagaltingen, Germany) and fed through a custom voltage-to-current converter. Signals were relayed to MEA electrodes through a custom circuit board that interfaced with the electrode array. A platinum wire encircling the top of the recording chamber served as the return electrode.

All stimuli were repetitive to evoke a burst of spikes and generate a detectable calcium transient (Akerboom et al. 2012; Behrend et al. 2009; Borghuis et al. 2011; Tian et al. 2009). Rectangular pulse trains (167 or 333 Hz, 120- or 360-ms duration, cathodic-first) and sine waves (20 Hz, 200-ms duration, anodic-first) were applied in different experiments. Rectangular pulses consisted of a 60- or 400-μs cathodic phase followed by an anodic phase of twice the duration and half of the amplitude. (All stated amplitude and pulse width values are for the cathodic phase. For sinusoidal stimulation, current is specified in zero-to-peak amplitude.) Images were acquired at 5 or 10 Hz and synchronized with the onset of each stimulus. Kynurenate acid (1 mM), a broad-spectrum glutamate receptor antagonist, was applied in one experiment to isolate RGCs from bipolar cell input pharmacologically (Massey and Miller 1988).

Electrical thresholds of RGCs were measured as described previously (Behrend et al. 2009, 2011), with slight modifications. Stimuli were delivered 14 times on 3.6-s intervals and repeated over 10 monotonically increasing amplitudes. Electrically evoked responses were detected by convolving the fluorescence intensity of each ganglion cell body with a difference filter and identifying rapid changes in fluorescence temporally correlated with the stimuli. A dose-response curve was generated for every RGC by plotting the fraction of the 14 stimuli that elicited a response at each amplitude. A sigmoidal function was fit to this curve, and threshold was defined as the stimulation amplitude that yielded a 50% response. Threshold maps (Fig. 7) were generated by binning cells in a grid according to their spatial location relative to the stimulating electrode. Maps from separate retinas were rotated and shifted into the same reference frame (relative to the optic disc position). Thresholds of cells in each grid bin were averaged. Data processing was performed in ImageJ 1.43u (National Institutes of Health, Bethesda, MD) and MATLAB R2009b (The MathWorks, Natick, MA). Except for the identification of ganglion cell bodies, all processing steps were automated and identical for each data set. Statistical significance was determined with unpaired t-tests using a significance level of 5%.

Histology. Eyes treated with AAV were enucleated, and their anterior segments were carefully removed. Posterior eyecups were fixed in 4% paraformaldehyde in 1× PBS for 1 h. Following fixation, eyecups were washed three times in 1× PBS for 10 min each. Samples were transferred to 30% sucrose in 1× PBS and kept overnight at 4°C. Eyecups were then embedded in O.C.T. compound (Tissue-Tek; Sakura Finetek, Torrance, CA) and sectioned to a thickness of 8 μm. Sections were collected on slides and stored at −80°C until imaging. Before imaging, slides were thawed at room temperature and immersed in 1× PBS for 10 min. Nuclei were stained with ProLong Gold Antibody Reagent with 4',6'-diamidino-2-phenylindole (DAPI; Life Technologies). Imaging was performed at the Doheny Eye Institute Specialized Microscopy Core on a Zeiss (Thornwood, NY) LSM 510 confocal laser scanning microscope equipped with a Plan-Neofluor 1.3-NA ×10 objective. GCaMP fluorescence was excited with a 488-nm argon laser and collected through a 560- to 530-nm band-pass filter. DAPI was excited at 800 nm by a Ti:sapphire laser and collected through a 390- to 465-nm band-pass filter.

RESULTS

Following intravitreal injection of AAV2-CAG vectors encoding for GECIs, ganglion cells began to show green fluorescence after 1 wk. Expression levels became strong after 2 wk, at which point retinas were harvested for experiments. Transduced retinas were dissected and mounted on a transparen-
**GCaMP expression profiles.** Retinal whole mounts showed widespread GCaMP expression with anywhere from one-quarter to the entire whole mount exhibiting green fluorescence (Fig. 2). Ganglion cell bodies were generally brighter than their axons, which permitted imaging of somata through the superficial nerve fiber layer (Fig. 3A). When experiments were performed <4 wk postinjection, fluorescence remained predominantly localized to RGC cytoplasms (Fig. 3C, inset). As time progressed, baseline fluorescence increased and became apparent in ganglion cell nuclei, indicating GCaMP overexpression and cytomorbidity (Akerboom et al. 2012; Borghuis et al. 2011; Tian et al. 2009). Indeed, we found that very bright cells and/or ones with filled nuclei did not fire spikes or exhibit GCaMP fluorescence transients during electrical stimulation. To limit overexpression, viral stock was diluted in BSS before administration. Optimal dose was roughly $1 - 5 \times 10^9$ vector genomes per injection.

Double-labeling RGCs with the red tracer dye Alexa Fluor 594 enabled us to determine whether transduced cells were RGCs or other cell types (Fig. 3). We compared expression profiles induced by our AAV2-CAG-GCaMP3 vector with those of AAV2/1-SYN1-GCaMP3, which labels mouse RGCs with high efficiency (Borghuis et al. 2011). Cell counting revealed that both vectors had a high specificity for RGCs. Although differences were not statistically significant, AAV2-CAG-GCaMP3 labeled more RGCs and less non-RGCs than AAV2/1-SYN1-GCaMP3 (Table 1). Roughly 83% of ganglion cells were transduced by AAV2-CAG-GCaMP3, which achieved a labeling density of $1,813 \pm 343$ cells/mm$^2$. Similarly, Martin et al. (2002) found that AAV2-CAG-GFP targeted 84.5% of adult rat RGCs and labeled $1,828 \pm 299$ cells/mm$^2$. Given that 85.3% of all AAV2-CAG-GCaMP3-labeled cells (i.e., ~1,550 cells/mm$^2$) were RGCs (Table 1), our counts are consistent with counts of approximately 1,500–1,600 RGCs per square millimeter obtained by FluoroGold backfilling through rat optic nerve and superior colliculus (Salinas-Navarro et al. 2009).

To determine which types of non-RGCs were targeted by AAV2-CAG-GCaMP3 and AAV2/1-SYN1-GCaMP3, we examined cross-sections of retina transduced by each vector. With rare exception, we found AAV2-CAG-GCaMP3 labeling to be confined to the ganglion cell layer (GCL), implying transduction of RGCs and displaced amacrine cells (Fig. 4A). In contrast, AAV2/1-SYN1-GCaMP3 expression often extended into the inner nuclear layer, where it labeled amacrine cells (Fig. 4B). Coupled with our double-labeling and cell counting results, these data indicate that the 14.7% of non-RGCs transduced by AAV2-CAG-GCaMP3 (Table 1) were displaced amacrine cells. Similarly, Harvey et al. (2002) found that 12–13% of all cells transduced by intravitreal injection of AAV2-CMV-GFP in adult rat were amacrine cells.
Table 1.  Percentage of RGCs and other retinal cells labeled with GCaMP3 following intravitreal injection

<table>
<thead>
<tr>
<th></th>
<th>AAV2-CAG</th>
<th>AAV2/1-SYN1</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>RGCs labeled, %</td>
<td>82.9 ± 9.2</td>
<td>75.4 ± 7.5</td>
<td>0.14</td>
</tr>
<tr>
<td>Labeled cells that were not RGCs, %</td>
<td>14.7 ± 7.6</td>
<td>17.6 ± 3.9</td>
<td>0.39</td>
</tr>
<tr>
<td>Labeled cell density, cells per square millimeter</td>
<td>1,813 ± 343</td>
<td>1,553 ± 367</td>
<td>0.24</td>
</tr>
<tr>
<td>No. counted cells</td>
<td>2,714</td>
<td>1,492</td>
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</table>

Percentages indicate means ± SD. Cells were counted in regions with dense genetically encoded calcium indicator GCaMP3 labeling. We counted 8 400-× 400-μm regions from 2 retinas transduced with AAV2-CAG-GCaMP3 and 5 400-× 400-μm regions from 2 retinas transduced with AAV2/1-SYN1-GCaMP3. Retinal ganglion cells (RGCs) were identified after retrograde loading with Alexa Fluor 594. Values imply no significant differences between the labeling profile of each vector.

GCaMP responses to electrical stimulation. Electrical stimulation evoked large calcium transients that were clearly visible when viewed through the microscope. As shown in Fig. 5, burst stimulation with a 20-μm-diameter electrode elicited strong fluorescence responses in GCaMP3-expressing RGCs. A rapid train of 120 pulses (1.2 μA, 400-μs pulse width, 333 Hz) stimulated a bundle of passing axons, antidromically activating a streaklike pattern of RGC somata (see Supplemental Video S1 available in the data supplement online at the Journal of Neurophysiology web site). Normalized change in fluorescence (ΔF/F) for responding cells was 75.9 ± 21.5% (n = 34). Application of 1 mM CdCl2 to the superfusate abolished all fluorescence signals within minutes.

We compared calcium transients from GCaMP3-, GCaMP5G-, and Oregon Green-labeled RGCs by delivering bursts of 40 suprathreshold pulses (60-μs pulse width, 333 Hz) through a 200-μm-diameter electrode (Fig. 6). GCaMP3 fluorescence responses in rat RGCs were similar in amplitude to Oregon Green responses in salamander RGCs (Behrend et al. 2009) but were generally less noisy. GCaMP5G signals in rat were roughly 3–4 times larger than those of GCaMP3. After rising to full magnitude, GCaMP3 and GCaMP5G fluorescence decayed with time constants of 0.91 ± 0.10 SE (n = 24) and 0.50 ± 0.03 SE (n = 66), respectively. Single stimulus pulses never elevated GCaMP5G fluorescence above the baseline noise level; a rapid burst of 5–10 suprathreshold pulses was needed. For RGCs expressing GCaMP3, 20–30 pulses were needed to generate a detectable calcium transient. Because of the short pulse width (60 μs), it is likely that each stimulus pulse elicited one or two action potentials per RGC (Ahuja et al. 2008; Freeman et al. 2011; Jensen et al. 2005; Sekirnjak et al. 2006).

We gathered statistics about the properties of electrically responsive cells across seven GCaMP5G-labeled retinas. Retinas were stimulated with a transparent 200-μm-diameter electrode, and only RGCs lying directly above the electrode were used for analysis (n = 418). In response to high-amplitude stimulation, 82.3 ± 10.5% of cells responded with detectable calcium transients (stimulus amplitude was 2–3 times greater than the mean threshold of responding RGCs). For responding cells, there was little to no correlation between baseline fluorescence, F0, and response magnitude, ΔF = Fstim - F0 (correlation coefficient = 0.19 ± 0.24). Within each retina, baseline fluorescence of RGC somata was highly uniform (mean coefficient of variation = 12.4 ± 4.9%).

To investigate whether GCaMP overexpression (i.e., very bright baseline fluorescence) was the cause of nonresponsive-ness, we compared F0 of responding cells with that of nonresponsive ones. Baseline fluorescence between these two populations varied by only 1.0 ± 5.9% (P = 0.67), indicating that...
cytomorbidity was not the primary cause. Indeed, cells that were bright ($F_0 > 1$ SD from the mean) and not responsive made up only 3.1% of the total population.

Stimulus threshold mapping. Previous studies have suggested that pulse shape affects the target of epiretinal electrical stimulation: short pulses excite RGCs directly, whereas longer pulses target bipolar cells (Freeman et al. 2010; Greenberg 1998; Jensen et al. 2005; Margalit and Thoreson 2006; Shah et al. 2006). By avoiding RGC axon bundles, long pulses can potentially confine the response area to the site of stimulation. This would have implications for epiretinal prostheses, which currently face the potential problem of stimulating RGC axons (Nanduri et al. 2011).

Because prior studies have relied on single-unit recordings, they were unable to quantify the spatial extent of RGC activation. To investigate this, we mapped RGC thresholds in response to stimulation with two markedly different pulse shapes: 400-µs rectangular pulses and 20-Hz sine waves. As shown in Fig. 7A, 400-µs pulses activated a streak of RGC somata, the path of which followed the trajectory of their axons. (Although not shown in the figure, this streak extended to the edge of the retina.) Mean RGC threshold directly under the electrode was $2.6 \pm 0.5 \mu$C/cm². The shape of the response map resembles those of prior calcium imaging studies by our group in salamander retina (Behrend et al. 2009, 2011).

In contrast to short, rectangular pulses, 20-Hz sine waves produced focal activation of RGC somata (Fig. 7B). Responding cells were largely confined to the vicinity of the electrode. Mean threshold charge density was $49.3 \pm 18.5 \mu$C/cm², much higher than for 400-µs pulses. Sine-wave thresholds increased by $32.7 \pm 4.1\%$ SE ($n = 31$; $P < 0.001$) in the presence of kynurenic acid, suggesting that responses were mediated in part by inner retinal stimulation. Comparatively, Freeman et al. (2010) found that low-frequency sinusoids (10–25 Hz) preferentially activated bipolar cells and avoided passing axons.

Effects of rat strain and temperature. All data reported in this study were collected from Long-Evans rats, an outbred strain. Injecting AAV2-CAG-GCaMP into the vitreous of inbred Copenhagen rats also led to widespread RGC transduction. However, GCaMP-induced cytomorbidity (Borghuis et al. 2011; Tian et al. 2009) was much more pronounced in Copenhagen rats. Injecting synthetic bipolar cells and avoided passing axons.

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Synthetic dye loading. Before employing GECIs, we attempted to load synthetic calcium dyes down the cut optic nerve. Previous attempts with 10-kDa OGB-1-dextran in adult
rat were unsuccessful, presumably owing to dye extrusion by organic anion transporters (Behrend et al. 2009). In this study, we tried loading other dextran-conjugated calcium dye variants in both adult rats and mice. Experiments were performed in the presence of the organic anion transporter blockers probenecid and sulfinpyrazone.

As a control experiment, we applied an equimolar mixture of 10-kDa Alexa Fluor 594-dextran and 10-kDa OGB-1-dextran to the cut optic nerve. The weakly charged Alexa tracer was retained by RGC somata (Fig. 8A), but the highly charged Oregon Green was not (Fig. 8B), further supporting the hypothesis that organic anion transporters are responsible for calcium dye extrusion. Attempts to load 10-kDa fluo-4-dextran (Fig. 8C) and 10-kDa fura-dextran (Fig. 8D) were also unsuccessful. Reasoning that a heavier dye might be more resistant to externalization, we tried loading 70-kDa Calcium Green-1-dextran; however, this dye was not retained (Fig. 8E).

**DISCUSSION**

We have described an optical technique to record from populations of mammalian RGCs during electrical stimulation. By relying on calcium imaging, rather than traditional electrical recording approaches, we can identify the precise location of every activated neuron, including those distant from the stimulation site. Furthermore, patterns of electrical activity can be visualized in real-time due to the large fluorescence transients produced by GCaMP3 and GCaMP5G.

*Comparison with other calcium indicator loading techniques.* A number of studies have focused on bulk-labeling RGCs with fluorescent calcium dyes. Early protocols utilized membrane-permeant dye loading or biolistic delivery (via gene gun), although these methods have significant drawbacks: membrane-permeant dyes do not load well in adult retina and lack cell-type specificity (Morgan et al. 2005). Biolistic delivery is also not cell-type specific and results in sparse labeling (Kettenun et al. 2002; Roizenblatt et al. 2006). In search of a new method, our group found that dextran-conjugated calcium dyes could be loaded into ganglion cell populations through optic nerve backfilling (Behrend et al. 2009). This technique labeled nearly all RGCs with complete specificity yet was ineffective in mature mammalian retina. We have since tested other calcium dye variants, including heavier dextran conjugates, in

![Fig. 7. Left: threshold maps of RGCs in response to 167-Hz pulsatile stimulation with 400-μs pulses (A) and 20-Hz sinusoidal stimulation (B). Stimuli were delivered by a transparent 200-μm-diameter electrode (black circle). RGCs are drawn as colored circles according to their stimulus thresholds. Small, medium, and large circles indicate 1–2, 3–4, and 5+ cells, respectively. Data in A were generated from 2 retinas (682 somata), and data in B were generated from 3 retinas (686 somata). Maps from each retina were rotated and shifted into the same reference frame such that the optic disc lies to the left of each image. Cells were binned in a grid according to spatial location relative to the electrode, and thresholds in each bin were averaged. Right: background-subtracted fluorescence responses to the highest stimulus amplitude. Images from each retina were transformed to the same reference frame and averaged. Brightness and contrast were adjusted to accentuate the responses. The electrode perimeter is outlined in gray.

![Fig. 8. Attempts to load dextran-conjugated calcium dyes via the cut optic nerve were unsuccessful in adult rat. Retrograde loading with an equimolar mixture of 10-kDa Alexa Fluor 594-dextran and 10-kDa OGB-1-dextran led to retention of the Alexa dye (A) but not Oregon Green (B). Very weak OGB-1 staining can be seen in some RGC somata, but we could not evoke calcium transients in these cells. Ten-kilodalton fluo-4-dextran (C), 10-kDa fura-dextran (D), and 70-kDa Calcium Green-1-dextran (E) were also not retained by adult rat RGCs even in the presence of probenecid and sulfinpyrazone. Results in adult mice were similar. Scale bars are 100 μm.](http://jn.physiology.org/doi/10.220.33.3.onJune11,2017)
both adult rat and mouse (Fig. 8). None were retained to a level sufficient for detecting fluorescence transients.

To date, only one group has reported a method for bulk-loading mature mammalian RGCs with calcium dye (Briggman and Euler 2011). Their approach relies on in vitro electroporation and is able to stain the entire GCL. However, displaced amacrine cells, which constitute roughly 50–60% of cells in the rodent GCL (Jeon et al. 1998; Perry 1981), are labeled indiscriminately. Because we are interested in recording RGC responses to electrical stimulation (since RGC activity defines retinal output), we wanted a method for labeling populations of RGCs preferentially over other cell types. This led us to investigate GECIs, which have become an attractive alternative to synthetic dyes. Recent advancements in GECI engineering have led to novel proteins (Akerboom et al. 2012; Zhao et al. 2011) that exceed small molecule dyes in terms of SNR and photostability (Borghuis et al. 2011).

Various approaches have been developed for loading GECI genetic material into RGCs. In vivo (Dezawa et al. 2002; Mo et al. 2002) and in utero (Garcia-Frigola et al. 2007) electroporation can transfect RGC populations, but the extent of labeling is limited and is not RGC-specific. Transgenic mouse lines offer an alternative means to express GECIs in neuronal populations. The Pvalb-2A-Cre:Ai38 line, for example, expresses GCaMP3 in RGCs, horizontal cells, and Müller glia (Zariwala et al. 2012). Because expression levels remain stable without increases over time, GCaMP3-induced cytomorbidity does not occur in these mice, even after several months. However, the lack of a pan-ganglion-cell-specific promoter makes it difficult to generate a mouse line that expresses GCaMP exclusively in RGCs (Feng et al. 2000). Furthermore, establishing stable transgenic lines is costly and takes many months, making it impractical to incorporate new GECIs as they become available. Viral vectors overcome these limitations. They can be designed to target specific neuronal classes and produced within a matter of weeks. We chose to use AAV because it is easily administered (via intravitreal injection), is not pathogenic, and can be designed to target the overwhelming majority of RGCs in mammalian retina (Martin et al. 2002).

The AAV2-CAG-GCaMP vectors we generated in this study labeled ~83% of adult rat RGCs while causing minimal expression in other cell types (Table 1). We found that AAV2-CAG-GCaMP3 transduced more RGCs and fewer non-RGCs than AAV2/1-SYN1-GCaMP3, which targets mouse RGCs with high specificity (Borghuis et al. 2011). This was not unexpected, given that prior studies have reported superior RGC labeling with AAV2 vs. AAV2/1 (Auricchio et al. 2001; Hellström et al. 2008). Although both vectors we tested contained different promoters, CAG and synapsin-1 are both strong promoters that produce similar amounts of transgene expression (Shevtsova et al. 2005). Furthermore, the extent of retinal transduction is limited by the ability of the AAV particles to penetrate the retina, which is determined by the capsid serotype, not the promoter (Dalkara et al. 2009; Petrs-Silva et al. 2010).

Effects of rat strain and temperature. We were surprised to find that GCaMP expression led to different phenotypes in different rat strains. GCaMP-induced cytomorbidity was much more pronounced in Copenhagen RGCs than in Long-Evans. We believe this behavior may be attributed to differences in genetic background: Long-Evans is an outbred strain, whereas Copenhageners are inbred. Prior studies have reported problems with inbred lines that do not occur with outbreds, possibly due to deleterious homozygous recessive alleles that result from inbreeding. For example, Carlson et al. (1997) found that overexpressing Alzheimer amyloid precursor protein (Kang et al. 1987) was lethal to inbred mice but not to outbreds. Similarly, Kinney and Sidman (1986) found that a mutation causing spongiform encephalopathy killed inbred mice within 3 mo but was well-tolerated by outbred strains. Although the focus of these studies is unrelated, their findings demonstrate how phenotype can be affected by genetic background. We also observed that higher temperatures exacerbated the effects of cytomorbidity, especially in Copenhagen rats. This resembles a finding by Newman (2003) in which temperatures above 24°C altered calcium fluorescence in rat retina and caused deterioration of retinal function.

Limitations. Despite the relative ease of AAV vector production and delivery to the eye, GCaMP-induced cytomorbidity limits the types of experiments that can be performed. In agreement with another study, we found that retinas harvested after ~4 wk exhibited abnormal cellular physiology (Borghuis et al. 2011). This has been suggested to arise from GCaMP overexpression and interaction of the sensor CaM and/or M13 motifs with endogenous proteins (Hasan et al. 2004). It may be possible to limit overexpression by systematically administering hyperosmotic mannitol before AAV injection (Burger et al. 2005; Kuhn et al. 2012), although this might also cause GCaMP expression to extend beyond the GCL.

Because the degree of cytomorbidity varies with rat strain, investigators wishing to use a different mammalian species or rat strain (other than Long-Evans) should test for cytomorbidity before proceeding with experiments. Since GCaMP expression will eventually reach a level that is toxic to cells (after a few weeks), our method does not permit chronic in vivo imaging. Chronic experiments would require the use of transgenic GECI knockins, as expression levels in these animals remain stable over time (Zariwala et al. 2012).

Future applications. Although we were unable to detect single action potentials in GECI-expressing RGCs, future generations of calcium indicators may overcome this limitation. GECIs still lag behind synthetic dyes in terms of detecting sparse neural activity (Akerboom et al. 2012). However, given the rate at which calcium indicator technology is progressing (Looger and Griesbeck 2011), measurement of single spikes may soon become possible. This can already be accomplished with genetically encoded voltage sensors (Kralj et al. 2011), although the fast signals of those sensors make it difficult to image simultaneous activity from neuronal populations.

In this study, we confirmed that changing the stimulus pulse shape can dramatically alter the pattern of electrically activated RGCs. Importantly, 20-Hz sine waves avoid axon bundles and stimulate cells only near the electrode. This may have implications for epiretinal prostheses, which currently face the problem of stimulating RGC axons (Nanduri et al. 2011). Since these prostheses operate on diseased retina, it would be helpful to understand how retinal degeneration affects the spatial patterns of RGC activation. This could be investigated in transgenic rodent models (Chader 2002; Marc et al. 2003) such as the S334ter-line-3 rat model of retinitis pigmentosa (Steinberg et al. 1996). Despite extensive retinal remodeling that
occurs during degeneration (Jones and Marx 2005), AA2 transduction profiles in S334ter-line-3 retina are similar to those of wild-type rats (Kolstad et al. 2010).

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DISCLOSURES

M. S. Humayun has a financial interest in Second Sight Medical Products, Inc. Although no Second Sight products were used in the study, we did perform basic research on electrical stimulation of the retina.

W. W. Hauswirth and the University of Florida have a financial interest in the use of AAV therapies and own equity in a company (AGTC, Inc.) that might, in the future, commercialize some aspects of this work.

AUTHOR CONTRIBUTIONS

A.C.W. performed the experiments, analyzed the data, and wrote the manuscript; M.R.B. developed many of the experimental procedures; N.S.L. and P.R.H. contributed with construction of the viral plasmid; V.A.C. and W.W.H. produced the AAV vectors; M.S.H., J.D.W., and R.H.C. oversaw the research and provided experimental guidance.

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