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Stimulation Strategies for Selective Activation of Retinal Ganglion Cells

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Abstract

Retinal prosthetic implants have shown potential to restore partial vision to patients blinded by retinitis pigmentosa or dry age-related macular degeneration, via a camera-driven multielectrode array that electrically stimulates surviving retinal neurons. Commercial epi-retinal prostheses mostly use charge-balanced symmetric cathodic-first biphasic pulses to depolarize retinal ganglion cells (RGCs) and bipolar cells (BCs), resulting in the perception of light in blind patients. However, previous clinical study for patients with Argus II epiretinal implants reported most percepts evoked by single electrode stimulation were elongated and aligned with estimated axon path of retinal ganglion cells, suggesting the activation of axon bundles. In this project, using an established genetically encoded calcium indicator (GECI), we performed *in vitro* calcium imaging for different stimulation paradigms, focusing primarily on short duration pulse that can avoid axonal stimulation and selectively activate targeted RGC soma. The findings support the possibility to manipulate the responses of RGCs through varying the stimulation waveform, thus potentially forming more ideal shape perception with higher spatial resolution in future retinal prosthesis design.

Keywords

Retinal prosthesis; retinal degeneration; electrical stimulation; calcium imaging

I. Introduction

Retinitis pigmentosa (RP) and dry age-related macular degeneration (AMD) [1] are two prevalent degenerative diseases of retina that lead to significant visual impairment or blindness. Prior clinical testing demonstrated that applying electrical stimulation to a degenerated retina can elicit visual percepts [2]. Based on these findings, several retinal prostheses have been developed and two systems have regulatory approval with the best reported 20/1260 and 20/546 visual grating acuity respectively [3].

One of the most critical challenges of retinal implants is to achieve ideal shape perception. That is, each electrode should only activate nearby retinal cells, thus forming small round

visual percepts. Integrating multiple percepts generated from different electrodes, the complex perception of shape such as a letter can be created. However, clinical studies of patients with Argus II epiretinal implants reported most evoked percepts by single electrode were elongated and aligned with estimated axon path of retinal ganglion cells, suggesting the activation of axon bundles [4]. Spatial responses consistent with axonal stimulation have also been measured using calcium imaging techniques *in vitro* retina [5]. Stimulation strategies that avoid axonal stimulation may significantly improve prosthetic vision in terms of spatial resolution.

The final output of the retina is retinal ganglion cells (RGCs) that collectively transmit preprocessed visual information to the brain, and those neurons are a main targets for retinal prosthetic research since the visual percepts are mostly determined by their activation patterns. The RGC neurons can be activated directly by sufficient depolarization of the RGC membrane or indirectly via synaptic transmission from activated bipolar cells (BP) [6-9]. Direct stimulation has the advantages of precise control of retinal output, thus providing superior temporal resolution. Advantages of indirect stimulation include the retention of the neural processing that occurs at the inner plexiform layer and avoiding direct activation of axons of passage. Studies have showed the BPs tend to respond preferentially to longer pulse width (25 Hz sinusoidal) [10] and more localized responses of RGCs mainly resulted from indirect stimulation can be achieved with 25-ms duration pulses [11]. However, desensitization of the ganglion cell responses to continuous indirect stimulation argues against this approach. It is well documented that, depending on the stimulation frequency, sensitivity of the electrically-evoked ganglion cells progressively decreased with the repeated indirect stimulation [12]. This desensitization in the cellular response is observed from multiple animal models through electrical recording and is believed to be highly correlated with the percept fading [13,14] reported by patients. Therefore, the possibility for direct stimulation of RGC cell bodies, which are more easily excited by short duration pulse ($< 150 \mu\text{s}$) [8], is worth further investigation to alleviate phosphene fading in response to continuous stimulation.

To test different potential stimulation parameters, we developed a virus-transduced GECI GCaMP6f and performed calcium imaging to record the neural activity from RGCs at single cell resolution in wholemount retinas while applying short duration (less than $120 \mu\text{s}/\text{phase}$) pulse through a microelectrode array (MEA) with transparent indium tin oxide electrodes. The results suggest that the electrical stimulation thresholds and response patterns of RGCs can be manipulated through pulse duration and the use of conditioning pre-pulses.

II. Methods

A. Overview

Adult mice (C57BL6/J) receiving an intravitreal injection of an AAV vector encoding a GECI (AAV2-CAG-GCaMP6f) were used to perform the calcium imaging experiment. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) at the University of Southern California.

B. Virus-transduced Calcium Indicator

The pGFP plasmid was selected for constructing the GECI-containing vector where the original GFP sequence was removed and inserted with the GECIs GCaMP6f [15] to create pAAV-CAG-GCaMP6f-WPRE. Before GCaMP6f cDNA, the CMV enhancer, chicken β -actin promoter (CBA promoter), exon, and intron were collectively used to form ubiquitously strong CAG promoter. To enhance protein translation, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was placed downstream of the transgene. The entire cassette was flanked by AAV2 inverted terminal repeats. Recombinant AAV vectors were produced by the two-plasmid co-transfection method [5]. Final concentration of AAV2-CAG-GCaMP6f was 2.6×10^{12} vector genomes per milliliter. Viral stock was diluted to 1.04×10^{12} with balanced salt solution before injection.

C. Calcium Imaging

Virus-transduced mice were euthanized at 3–4 weeks post injection, which was determined to be the best time window for optimal GCaMP6f expression in RGCs [16]. After anesthesia with ketamine/xylazine, the mice were rapidly decapitated and the treated eye was enucleated and hemisected with Vannas spring scissors. To flatten the retina, 4 cuts were made, from periphery to center, to create quadrants with near equal size. Vitreous was gently peeled from the retina surface with fine forceps to allow for a tight interface between the retina and MEA. After removal from the eye cup, the retina was mounted on a porous membrane (cat. No. JVWP01300; Millipore) and placed on the transparent MEA chamber with ganglion cell side facing down. The retina was imaged using a customized up-down microscope (Olympus, Center Valley, PA). Fluorescence excitation was provided by a super bright cool white light-emitting diode (LED). Excitation and emission light were filtered through a commercial filter set (49002 - ET - EGFP (FITC/Cy2), Chroma Technology Corp, Bellows Falls, VT) for GCaMP6f. Images were viewed through an Olympus (Center Valley, PA) UPLFLN 0.3-numerical aperture (NA) x10 objective and captured by an electron-multiplied charge-coupled device (EMCCD) camera. (iXon 897, Andor Technology, Belfast, Northern Ireland). For superfusion, the bicarbonate-buffered Ames' Medium (Sigma-Aldrich, St. Louis, MO) was used in all procedures. 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 mOsm. During the course of each experiment, the retina was continuously superfused at a flow rate of 4–5 ml/min and a temperature of 33 °C.

D. Electrical Stimulation

Transparent MEAs were fabricated in a class 100 cleanroom. Arrays were patterned by selective etching of indium tin oxide (ITO) on no. 1 cover glass substrates (Vaculayer, Mississauga, Ontario, Canada). A dual-insulation layer, including SU-8 epoxy photoresist and silicon nitride, was formed atop the ITO. The insulation layer was removed to create 200 μ m diameter disk electrodes, in a 6×10 pattern with 500 μ m electrode pitch. These dimensions are within the range of present day retinal prostheses.

Electrical stimuli consisted of symmetric biphasic square current pulse waveform to ensure charge balance. Current stimuli were generated from a computer-controlled stimulus

generator (STG-2008, Multi Channel Systems, Reutlingen, Germany) and fed through a custom capacitive isolation circuitry for preventing leaking current. A customize interface circuit board was used to relay the signal to the designated electrode. 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 [5]. Two types of rectangular pulses, including symmetric cathodic-first and anodic-first pulse (anodic-first) were applied to the retina, with individual pulse durations from 40 μ s to 4 ms at 120 Hz depending on different experimental setting. Table 1 provides the pulse width selection of cathodic phase for each waveforms. For each waveform and duration, stimulation protocols were designed so that current amplitude progressively increase from subthreshold to suprathreshold. A total of 10 amplitudes were used, each lasting 5 secs with 20 secs interval in between to bring calcium transient back to baseline. No stimuli were delivered during the first and last 5 secs of each protocol. The overall stimulation protocol is shown in Fig. 3.

E. Spatial Threshold Maps

For each stimulation paradigm, the calcium imaging of RGCs nearby the working electrode is recorded at 10 fps. Based on the stimulation paradigm, the calcium images during stimulation (2-3 secs after stimulation initiation) were extracted and subtracted with the baseline selected from resting intervals (1 sec before stimulation initiation), so the calcium transient of responsive RGCs can be detected. Further normalization of obtained transient with respect to baseline will be used to calculate the percentage of fluorescent change (F/F). With proper threshold selection to remove noise ($> 15\%$), processed spatial and temporal information in the region of interest can form the spatial threshold maps. The 2-D mapping information can further be used to perform activated region analysis which directly corresponds to shape of visual percepts and spatial acuity. Moreover, experimental data with different pulse width of stimulation can be used to establish the strength duration or frequency curves for targeted RGCs or specific regions, thus offering the possibility for optimizing the stimulation parameters. All post-processing steps and analysis of calcium imaging were analyzed by MATLAB (The Mathworks, Natick, MA).

III. Results

A. Single Cell Calcium Transient

Nonmalized changes in fluorescence in response to stimulation for two randomly selected RGC expressing GCaMP6f are shown in Fig 4. The initiation of each stimulus is indicated by the red arrow. The result clearly show that increasing stimulus amplitude leads to larger calcium transient, which corresponds to stronger RGC activation. In addition, the chosen 20 secs resting interval between stimuli is sufficient for the calcium fluorescence to return back to baseline for next stimulus.

B. Duration Variation

Fig. 5 shows spatial threshold maps for pulse widths ranging from 0.04 to 4 ms with symmetric cathodic-first waveform at 120 Hz. The responses of somatic or axonal

activations were defined by inside the light blue contour (location of electrode) or outside the green contour (two times the radius of electrode) respectively. Results demonstrate that all pulses $\sim 150 \mu\text{s}$ duration do not significantly activate axon bundles and result in more focused response near the working electrode if proper stimulation current amplitude is selected. On the contrary, pulses $\sim 0.5 \text{ ms}$ cause more non-selective elongated responses extending away from the optic disc at threshold. The strength duration curves for somatic and axonal responses from multiple ROIs were also fitted using exponential model for all pulse durations of symmetric cathodic-first pulses in Table 1. We can observe that the largest percentage difference of average soma and axon thresholds occurred when short duration pulse ($40 \mu\text{s}$) was used, indicating that the selective activation of soma and axon can be more easily achieved due to larger manipulation ranges of current amplitude.

C. Symmetric Anodic-first Pulse

Responses of RGCs generated by symmetric cathodic-first pulses versus anodic-first pulses (reverse) with identical current amplitude are demonstrated in Fig. 6. The spatial patterns of threshold maps demonstrate the symmetric anodic-first produces more localized RGC responses compared with cathodic-pulse, especially with $40 \mu\text{s}$ pulses, though the required current amplitudes are universally higher. A similar phenomenon can also be observed for pulses with 0.5 ms duration, but less significant. Comparison of responsive area from multiple ROIs for different paradigms using the mean soma and axon threshold for stimulation shows the same tendency, indicating the anodic-first pulse with extremely short duration can effectively avoid axonal activation. A possible explanation for this finding is that the sharp anodic phase can effectively inhibit the following cathodic-driven depolarization of axonal bundles across the electrode, but do not have a strong inhibitory effect on RGC somas.

IV. Conclusion

We have presented a calcium imaging technique accompanied with transparent MEA platform that provides the unique ability to visualize spatial patterns of RGC activation to electrical stimulation in real time. The responses of RGC support the possibility to manipulate the activation of RGCs through varying the stimulation durations and phases. This proposed waveforms have great potential to improve the spatial resolution of retinal prosthetic implants through forming more ideal shape perception.

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Figure 1.
Map of pAAV2-CAG-GCaMP6f-WPRE.

Figure 2.

Calcium imaging experimental setup. (A) Recording chamber contains a transparent microelectrode array that delivers current-controlled electrical stimulation. An inverted microscope equipped with an EMCCD camera is juxtaposed below the recording chamber. (B) An inverted and upright microscope with the EMCCD camera installed at bottom left port. (C) MEA recording chamber and interface board, with glass pipes for superfusion. (D) Heat controller, MCS stimulator, and the other end of interface board.

Figure 3.

Stimulation protocol. Stimuli were delivered 10 times on 5-seconds intervals with monotonically increasing amplitudes and 20-seconds resting intervals in between. Each stimulus was a burst of rectangular pulses with symmetric cathodic-first or anodic-first pulse paradigm designed to evoke a burst of spikes and generate a detectable calcium transient.

Figure 4:

Normalized changes in fluorescence ($\Delta F/F$) for two RGCs expressing GCaMP6f in response to the 120 Hz 40 μ s duration biphasic symmetric pulse train stimuli with different amplitude of stimuli, from subthreshold to supra threshold. Each stimulus sustained for 5 secs with 20 secs inter-stimulus interval for calcium level returning back to normal. The red arrows indicate the onset of each stimulation pulse train.

Figure 5:

(A) Threshold maps for pulses with different duration in wild-type retina. The color bar shows thresholds in terms of current amplitude (μ A). The light blue and green contours represent the location of electrode and two times the radius of electrode respectively, defining the somatic activation region (inside the light blue circle) axonal activation region (outside the green circle). (B) The strength duration curve plotted in terms of current amplitude for somatic and axonal activations respectively. The blue and red curves are decaying exponentials that were fit to the data. Error bars indicate SD. The number listed near each error bar shows the percentage difference between mean soma and axon thresholds.

Figure 6:

(A) Threshold maps for symmetric cathodic-first and anodic-first in wild-type retina.

Identical setting as Figure 5. (B) The responsive area for different paradigms using the mean soma and axon threshold for stimulation. The red and blue bar demonstrate the percentage of somatic and axonal activated region.

Table 1.

Electrical stimulation parameters.

	40μs	80μs	120μs	0.5ms	1ms	4ms
Symmetric						
Cathodic-first						
Asymmetric						
Anodic-first						

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