

# Responses of Rabbit Retinal Ganglion Cells to Subretinal Electrical Stimulation Using a Silicon-Based Microphotodiode Array

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**PURPOSE.** With subretinal prostheses, retinal ganglion cells (RGCs) are activated by electrical stimulation of the retinal neural network. The aim of this study was to evaluate the efficacy of silicon-based solar cells in evoking RGC responses by electrically stimulating the photoreceptor side of an isolated retina.

**METHODS.** A light-bleached retina of an adult New Zealand White rabbit was placed with its photoreceptor side down onto a silicon chip that consisted of a 4 × 4 microphotodiode array (MPDA). The stimulating current was elicited by activating the solar cell with a 532-nm laser light source. Responses of the ON and OFF alpha RGCs on electrical stimulation were recorded extracellularly. Recorded RGCs were then injected with 4% N-(2-aminoethyl)-biotinamide hydrochloride to allow cell type identification.

**RESULTS.** Using a design that includes a circumvented ground electrode, the authors successfully evoked spiking responses by the ON and OFF alpha RGCs in an isolated rabbit retina using low light power to activate the MPDA (equivalent to 39  $\mu\text{C}/\text{cm}^2$ ). The charge density-dependent response and the frequency-dependent pair-pulse suppression were characterized. The spike latency of the RGC responses triggered by electrical stimulation was equivalent to the latency of its light response, which supports the hypothesis that the activation is mediated by the retinal neural network.

**CONCLUSIONS.** Reliable activation of RGCs by electrical stimulation in vitro using an MPDA demonstrates the feasibility of developing solar cell-based subretinal prostheses that potentially could be developed into a power-free device able to restore

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To restore the visual function of the blind, various retinal prostheses have been developed over a number of years. In general, there are two main approaches that are based on the site of electrical stimulation. These are epiretinal prosthesis<sup>1–8</sup> and subretinal prosthesis.<sup>8–12</sup> One of the most significant advantages in subretinal prosthesis is that the remaining retinal network can be directly stimulated by the electrodes, which are intended to substitute for photoreceptor function in retinitis pigmentosa (RP) and age-related macular degeneration (ARMD) patients. In past decades, various different techniques have been developed for implementing the concept of subretinal prosthesis, and numerous animal experiments have proved the principle of this prosthetic design. However, most studies have examined cell responses in animal models by directly applying voltage/current pulses from the subretinal side. For example, Zrenner's group<sup>13</sup> demonstrated that cortical activation can be elicited after subretinal stimulation using an electrode array in the rabbit. Similarly, Jensen and Rizzo<sup>14</sup> showed that the spiking activities of retinal ganglion cells (RGCs) depend on the spatiotemporal patterns of the injected charge from the microelectrode array. Other studies<sup>15–18</sup> also systematically characterized the responses of the RGC on electrical stimulation using single stimulating electrodes from the subretinal side in animals with and without photoreceptor degeneration. Whether an implantable solar cell-based subretinal microphotodiode array (MPDA) can elicit RGC responses on light activation has not really been examined in an animal model in vitro.

The MPDA, which is designed to convert light energy into electrical current without a power supply, has been developed to replace the photoreceptor function in subretinal prosthesis. Chow's group<sup>9,19,20</sup> was the first to report that a silicon chip with microphotodiodes, when implanted in the subretinal space of patients, can partially restore vision. Zrenner's group<sup>12</sup> tested a similar design in RCS rats and subsequently implanted MPDAs into patients with retinal degenerative diseases and successfully restored their vision,<sup>10,21</sup> although the MPDA they implanted is an active device that requires additional power.

However, given the successes of using the MPDA as a subretinal prosthesis, a detailed in vitro study of RGC responses on electrical stimulation by a light-activated MPDA is lacking. The aim of the present study was to characterize the elicited responses of ON and OFF alpha RGCs on activation by an implantable silicon-based MPDA from the subretinal side in rabbits. The upstream retinal network responsible for these RGC responses on the same subretinal electrical stimulation was also studied pharmacologically. By systematically examining the RGC responses under the MPDA in vitro, the present

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study provides insight into the synaptic mechanisms of the RGC responses on electrical stimulation from an implantable subretinal chip. These findings are crucial to the development of a power-free MPDA for future clinical use.

## MATERIALS AND METHODS

### Retinal Preparation

New Zealand White rabbits of either sex, weighing 2 to 3 kg, were anesthetized by intramuscular injection of ketamine (75–100 mg/kg; Imagine 1000; Merial, Lyon, France) and xylazine (15–20 mg/kg; Rompun 2%; Bayer HealthCare, Wuppertal, Germany). The local anesthetic proparacaine hydrochloride 0.5% ophthalmic solution (Alcaine; Alcon-Couvreur, Puurs, Belgium) was applied to the eye before the intraocular injection of 2  $\mu\text{L}$  of 4,6-diamidino-2-phenylindole (DAPI; 2  $\mu\text{g}/\mu\text{L}$ ; Sigma, St. Louis, MO). DAPI injection was used to aid visualization of the nucleus and thus identification of the cell type. The animal was allowed to recover. One day after DAPI injection, the animal was dark-adapted for 1 hour before dissection. The rabbit was enucleated under deep anesthesia by intramuscular (130–160 mg/kg ketamine, 26–32 mg/kg xylazine) and intravenous (50 mg/kg ketamine) injections. The eyes were also anesthetized locally with a few drops of 0.5% proparacaine hydrochloride ophthalmic solution before enucleation and hemisection. All surgery and electrophysiological experiments were accomplished under dim red illumination. The animal was then euthanized with  $\text{CO}_2$ . All procedures were approved by the institutional animal care and use committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The anterior segment of the eyeball and the vitreous humor were removed. The eyecup was immersed in oxygenated (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) modified Ames medium (120 mM NaCl, 3.1 mM KCl, 0.5 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 1.15 mM  $\text{CaCl}_2$ , 6.0 mM D-glucose, 23 mM  $\text{NaHCO}_3$ , pH 7.7), and the retina was carefully peeled from the retinal pigment epithelium and the sclera. A small piece of retina (7-mm diameter) was cut off and placed on a silicon chip, photoreceptor side down, with the aid of a large piece of membrane filter (0.45- $\mu\text{m}$  MF-Millipore; Millipore, Bedford, MA) attached under the silicon chip (Fig. 1A). This prepared unit was then transferred to a recording chamber mounted on the stage of a fluorescence microscope (Axioskop 2 FS Plus; Zeiss, Oberkochen, Germany)

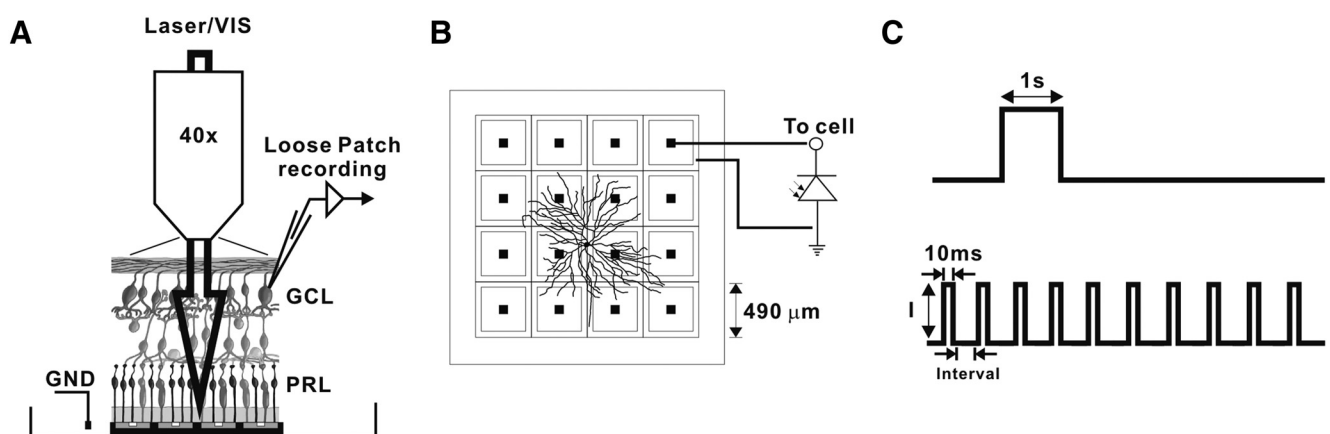
and was superfused at 1.5 to 2.5 mL/min with oxygenated modified Ames medium at 35°C to 37°C.

### Silicon-Based Microphotodiode Array

The MPDA in the subretinal prosthesis is based on an array of photodiodes formed by a PN junction structure that is able to convert light to electrical current. An array of photodiodes can be easily implemented in any semiconductor process with parasitic PN junctions. Our MPDA was designed using the parasitic P+/N Well junction as the photodiode, where the P+ region serves as the anode of the diode, and the N Well region serves as the cathode. When a photodiode is illuminated with light, free electron-hole pairs are generated in the depletion region of the PN junction, and the electrons/holes are swept to the cathode/anode by the built-in potential of the PN junction, which results in a photocurrent. The current strength is proportional to the light intensity, and a maximum voltage of 0.4 to 0.5 V across the cathode/anode can be produced by a single photodiode, depending on the loading impedance and the impinging light. Our MPDA ( $4 \times 4$  pixels) was fabricated by a standard CMOS process. The exposed electrode was made of aluminum, and the rest of the pixel was covered with a transparent passivation layer made of silicon nitride for isolation, protection, and antioxidation. A single pixel in the array is composed of a stimulating electrode ( $75 \times 75 \mu\text{m}$ ), photodiodes ( $490 \times 490 \mu\text{m}$ ), and a surrounding return electrode (Fig. 1B). In the present study, the cathode was used as the stimulating terminal and the anode as the return. The measured impedance of the electrodes (4294A Precision Impedance Analyzer; Agilent, Santa Clara, CA) was approximately 1 M $\Omega$  in this configuration.<sup>22</sup>

### Visual Stimulation

Visual stimuli generated by a software for visual psychophysics (VisionWork 4.0; Vision Research Graphics, Durham, NH) were displayed on a specialized microscopic mini-CRT monitor ( $800 \times 600$ ; Lucivid; MicroBrightField, Colchester, VT) with a 60-Hz refresh rate and driven by a personal computer. Images were projected onto the retina through the camera port of the microscope. A  $40\times$  water immersion objective (Achromplan, NA 0.8; Zeiss) was used to focus the visual stimuli onto the photoreceptor side of the retina and provide the visual stimulation (Fig. 1A).



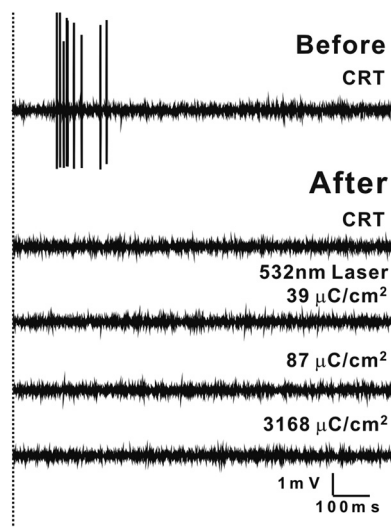
**FIGURE 1.** Schematic representation of the experimental design. (A) An isolated rabbit retina was placed with its photoreceptor side down on a silicon-based MPDA in a perfusion chamber. The visual stimulus (VIS) provided by a mini-CRT monitor or the electrical current elicited by activation of the MPDA by a 532-nm laser was used to stimulate the photoreceptors. The responses of ON and OFF alpha RGCs on light or electrical stimulation were extracellularly recorded by a loose-patch electrode. PRL, photoreceptor layer; GCL, ganglion cell layer; GND, ground electrode. (B) Camera lucida drawing of an alpha ganglion cell on the  $4 \times 4$  MPDA. The MPDA was designed with a parasitic P+/N Well junction as the photodiode, where the P+ region serves as the anode of the diode, and the N Well region serves as the cathode. A single pixel in the array is made up of a stimulating electrode ( $75 \times 75 \mu\text{m}$ ), photodiodes ( $490 \times 490 \mu\text{m}$ ), and a surrounding return electrode. (C) Paradigm of the visual and electrical stimulations. *Top*: flashing light for mapping the receptive fields of ganglion cells was set at 1 second. *Bottom*: repetitive electrical stimulation used to characterize the suppressive effect was generated by 10 laser pulses (10 ms) at various intervals. Laser intensity (I) was varied to determine the threshold of the ganglion cells with regard to electrical stimulation.

The ON and OFF alpha RGCs were identified initially by their transient ON and OFF spiking response to a circle of flashing light (150- $\mu\text{m}$  diameter, 1 second). A flashing square measuring 180  $\times$  180  $\mu\text{m}$  was then used to manually map the overall receptive field. The concentric center-surround receptive field was determined by varying the diameter of flashing circles (1 second; Fig. 1C). These responses to visual stimulation served as a control for comparison with those caused by electrical stimulation.

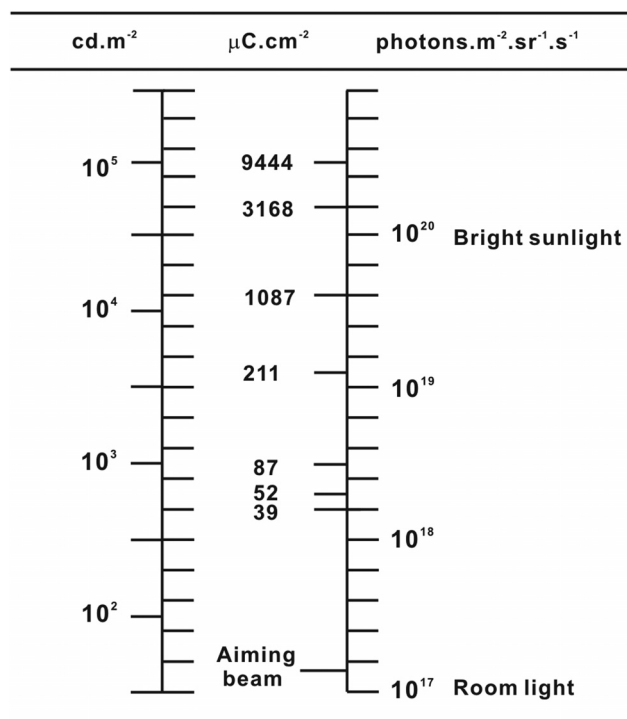
### Electrical Stimulation

The electrical current was generated by activating the MPDA with a 532-nm laser (LightLas 532 Green Laser; LightMed, Taipei, Taiwan) positioned at the second camera port of the microscope. The spot size of the laser measured 30  $\mu\text{m}$  in diameter under the same 40 $\times$  water immersion objective (Fig. 1A). Because the laser is within the visible spectral range, it can also readily stimulate the photoreceptors of the rabbit retina and activate the MPDA in our electrical stimulation paradigm. To remove this ambiguity, all photoreceptors were bleached completely to eliminate any light responses by exposing the retina to intense light from the mercury lamp of the fluorescence microscope for 4 to 6 minutes before the laser was used to activate the MPDA for electrical stimulation. After photoreceptor bleaching, neither visual stimulation with the maximum mini-CRT intensity nor laser stimulation at various powers was able to evoke spiking responses by the RGCs in the absence of the MPDA (Fig. 2). This result supports the hypothesis that our photoreceptor bleaching protocol is able to efficiently eliminate all light responses by the RGCs before electrical stimulation. This also ensures that the remaining responses of RGCs are generated exclusively by activating the MPDA with a 532-nm laser.

To determine the threshold of the RGCs on electrical stimulation, various power levels as a laser pulse (10 ms) were applied to activate the MPDA. The equivalent charge densities of the photocurrent generated by the MPDA were 39, 52, 87, 211, 1087, 3168, and 9444  $\mu\text{C}/\text{cm}^2$ . Note that the laser intensities were generally in the range of typical ambient illuminations (Fig. 3). To characterize the effect of repetitive electrical stimulation, 10 laser pulses at various intervals (1000, 500, 200, 100, and 50 ms; or, 1, 2, 5, 10, and 20 Hz in temporal frequency) were used to evoke RGC responses (Fig. 1C).



**FIGURE 2.** All light responses of the ganglion cells on mini-CRT and laser stimulation can be efficiently eliminated by the photoreceptor bleaching protocol. A burst of spikes of a recorded OFF alpha RGC was successfully elicited by mini-CRT visual stimulation before photoreceptor bleaching, which was performed by exposing the retina to intense light from the mercury lamp of a fluorescence microscope for 4 to 6 minutes. After photoreceptor bleaching, no light response could be evoked by either the mini-CRT stimulation or various strengths of laser stimulations used to activate the MPDA for electrical stimulation.



**FIGURE 3.** The strength of the photocurrent (*middle*) generated by activating the MPDA at various laser powers. The aiming beam is a spot of continuously visible red light used to point the laser beam at the stimulating area of the MPDA. Ambient light intensities equivalent to the laser powers in photometric (*left*) and radiometric (*right*) units are shown for comparison.

### Electrophysiological Recording

The tissue was viewed through the same 40 $\times$  water immersion objective. Under fluorescence illumination, numerous types of retinal neurons can be visualized by DAPI staining. Ganglion cells with large somata and round dim nuclei were considered potential alpha RGCs<sup>23</sup> and were subjected to loose-patch extracellular recording.<sup>24</sup> The microelectrodes were pulled from borosilicate glass capillaries (outer diameter, 1.0 mm; inner diameter, 0.5 mm; Sutter Instruments, Novato, CA) using a programmable puller (Flaming/Brown, P-97; Sutter Instruments) and were filled with normal HEPES pipette solution. An electrode holder (World Precision Instruments, Sarasota, FL) with a silver wire was used to connect the electrode to a differential amplifier (ISO-80; World Precision Instruments). The resistance of these electrodes ranged from 1 to 3 M $\Omega$ . A graphical program (LabVIEW; National Instruments, Austin, TX) was used to record the RGC responses elicited by either visual or electrical stimulation. Signals were sampled at 10 kHz, digitized using an analog-digital converter (NI 6040E; National Instruments), and stored in a separate personal computer for later offline analysis.

### Pharmacologic Experiments

To examine the upstream retinal network responsible for the RGC responses on subretinal electrical stimulation, various agonists or antagonists of neurotransmitter receptors were added into the perfusion chamber during the pharmacologic experiments. To block the AMPA/kainate glutamate receptors of the excitatory synapses, the potent antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50  $\mu\text{M}$ ; Tocris, Bristol, UK) was used. Similarly, to block the GABA<sub>A</sub> and GABA<sub>C</sub> receptors of the inhibitory synapses, picrotoxin (PTX; 100  $\mu\text{M}$ ; Tocris) was applied. Furthermore, the specific mGluR6 agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4, also called APB; 20  $\mu\text{M}$ ; Tocris) was used to distinguish the ON and OFF pathways in the retinal circuit by

selectively deactivating the ON bipolar cells. All reagents were freshly prepared for the present study.

### Morphologic Identification

To verify that the recorded ganglion cells were indeed alpha RGCs, microinjection was carried out after physiological recording. The recording electrode was replaced with the micropipette filled with 4% N-(2-aminoethyl)-biotinamide hydrochloride (Neurobiotin; Vector Laboratories, Burlingame, CA) and 2% lucifer yellow (Sigma) in 0.1 M Tris buffer, (pH 7.6). The borosilicate micropipettes (resistance range, 100–200 M $\Omega$ ) were first backfilled at their tips with the dye and then filled with 4 M potassium acetate. An intracellular amplifier (Neuroprobe Amplifier 1600; A-M Systems, Carlsborg, WA) was used to perform the iontophoresis. The lucifer yellow was injected first for several seconds with a continuous current of  $-2$  nA to check whether the primary dendrites were successfully filled. 4% N-(2-aminoethyl)-biotinamide hydrochloride (Neurobiotin; Vector Laboratories) was then injected continuously with a positive current modulating between  $+2$  and  $+2.5$  nA for 5 to 10 minutes.

After microinjection, the retinas were fixed immediately in 4% formaldehyde in 0.1 M PB (pH 7.4) for 1 hour. To obtain a better immunoreaction, all immunocytochemical processing was performed at 37°C, and 0.3% sodium azide was added to reagents to protect the tissue against bacterial infection. The retinas were incubated with the FITC-conjugated streptavidin (20  $\mu$ g/mL; Vector Laboratories) and 0.1% Triton X-100 in 0.1 M PB overnight. After rinsing, the retinas were mounted in mounting medium (Vectashield; Vector Laboratories) for confocal imaging.

All images were acquired using a confocal scanning module (LSM 5 Pascal; Zeiss) mounted on a fluorescence microscope (Axioskop 2 Plus Mot; Zeiss). A single image at low magnification was taken (10 $\times$ , Fluor, NA 0.5; 20 $\times$ , Plan-Neofluar, NA 0.5; Zeiss) to reveal the entire dendritic fields and to confirm the identity of the alpha RGCs.

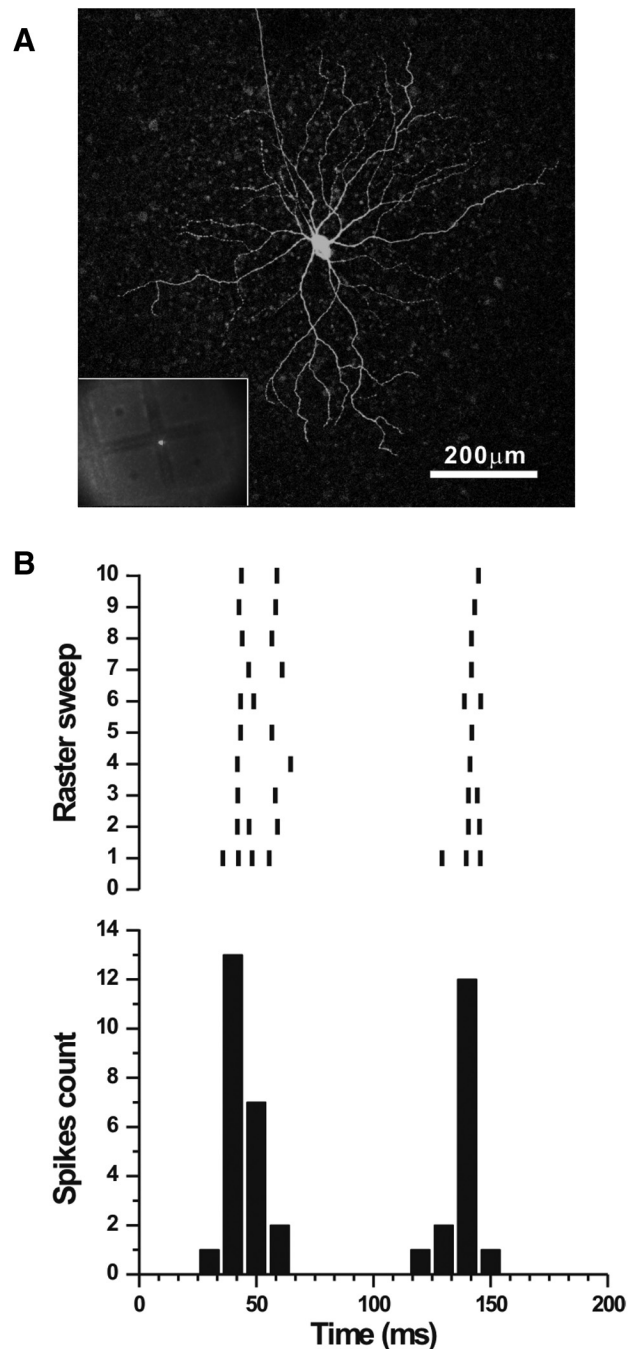
### Statistical Analysis

All spiking responses of the RGCs were analyzed offline (MatLab; The MathWorks Inc., Natick, MA), and the results were plotted (Origin; OriginLab Corporation, Northampton, MA). To characterize the first spike latency of the RGCs after either visual or electrical stimulation, the time between the stimulus onset and the first evoked spike was determined. Similarly, to estimate the threshold of the RGCs on electrical stimulation, the lowest electrical charge density that was able to activate  $>50\%$  of the RGC responses in 10 repeated trials was determined. To compare the differences in RGC response under different electrical stimulation conditions, a statistical program (SPSS, version 17.0; IBM, New York, NY) was used. For unbalanced data, the general linear model was applied.  $P < 0.05$  was considered significant.

## RESULTS

### Spiking Activity of alpha RGCs Can Be Electrically Evoked by Activating the MPDA

To characterize the responses of morphologically identified RGCs on electrical stimulation by a 532-nm laser-activated MPDA from the subretinal side, both ON and OFF alpha RGCs in the normal rabbit retina were recorded extracellularly. The typical response of an ON alpha RGC to 10 electrical stimulations showed two distinct bursts of spikes for each electrical pulse (Fig. 4). This result demonstrates that an RGC response can be successfully elicited using our silicon-based MPDA in vitro. This also suggests that the electrically evoked spiking pattern is apparently different from that of the visual response. A total of 17 alpha RGCs were recorded, and the majority (ON cells, 6/7; OFF cells, 8/10) showed multiple bursts of spikes when activated by the MPDA (Table 1). These bursts of spikes



**FIGURE 4.** Responses of a morphologically identified ON alpha RGC to electrical stimulation. (A) Confocal image of an alpha RGC injected with 4% N-(2-aminoethyl)-biotinamide hydrochloride after recording to show its dendritic morphology. *Inset:* cell soma on the MPDA was revealed by lucifer yellow injection. (B, *top*) Raster plots of the ON alpha RGC spiking response to 10 electrical stimulations. *Bottom:* post-stimulus time histogram (10-ms bins) generated by averaging ten stimulations. Note that two distinct bursts of spikes can be seen to be evoked by a single electrical pulse stimulation in this ganglion cell.

are indicative of oscillatory responses in the retinal network, although their origin is not known.

### Responses of alpha RGCs Evoked by Visual and Electrical Stimulations Are Different

In previous retinal prosthesis studies, it is rare that the responses of the RGCs to visual stimulation and to electrical

TABLE 1. Burst Patterns of alpha RGCs on Electrical Stimulation

	ON alpha GC				OFF alpha GC			
	Burst 1	Burst 2	Burst 3	Burst 4	Burst 1	Burst 2	Burst 3	Burst 4
Frequency of burst occurrence, %	100 (7/7)	86 (6/7)	29 (2/7)	14 (1/7)	100 (10/10)	80 (8/10)	50 (5/10)	20 (2/10)
First spike latency in each burst, ms	47.9 ± 1.5	163.9 ± 6.0	297.3 ± 10.15	360.9 ± 1.2	93.3 ± 4.6	153.8 ± 7	244.0 ± 21.5	345.3 ± 61.4
Spike/burst	2.1 ± 0.2	1.3 ± 0.1	1 ± 0.0	1 ± 0.0	3.8 ± 0.5	1.6 ± 0.2	1.8 ± 0.3	1.4 ± 0.4

Values are mean ± SEM.

stimulation were compared for the same cell. Using the normal rabbit retina, we attempted to examine the spiking responses of morphologically identified RGCs using visual stimulation before photoreceptor bleaching and using subretinal electrical stimulation after the light response was abolished. The first spike latencies of both the ON and OFF alpha RGCs on electrical stimulation were, on average, 8.8% and 13% shorter than those for visual stimulation, respectively (Table 2), although these differences were not statistically different (ON cells,  $P = 0.3$ ; OFF cells,  $P = 0.19$ ). The spike rates of the ON and OFF alpha RGCs on electrical stimulation were also less than when they were visually stimulated (ON cells,  $P = 0.54$ ; OFF cells,  $P = 0.34$ ). Furthermore, most alpha RGCs showed multiple bursts of spikes on electrical stimulation (Table 1) but only a single burst of spikes on visual stimulation (data not shown). Taken together, these results suggest that the response of the same alpha RGC cell on visual stimulation is different from the response on electrical stimulation.

### Characterization of the alpha RGC Responses to a Single Pulse of Electrical Stimulation

To determine the threshold of the alpha RGC response on electrical stimulation of the MPDA, single pulses at different stimulation intensities (Fig. 3) were applied. Even at the lowest charge density ( $39 \mu\text{C}/\text{cm}^2$ ) in our system, both the ON and OFF alpha RGCs still showed a  $>50\%$  response level (Fig. 5A). This indicates that the threshold of the alpha RGCs on electrical stimulation is much lower than in most previous studies (see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7808/-/DCSupplemental>) and also suggests that the silicon-based MPDA is capable of generating enough electrical current to stimulate the retina from the photoreceptor side. This result is consistent with previous findings that direct activation of the retina by a subretinal MPDA implant induces activity in the superior colliculus of RCS rats.<sup>25</sup>

To further characterize the alpha RGC response to a single pulse of electrical stimulation, the spiking patterns of the cells stimulated with different electrical currents were

TABLE 2. Average Response of alpha RGCs to Light and Electrical Stimulation

	ON alpha GC		OFF alpha GC	
	Light ( $n = 6$ )	Electrical ( $n = 7$ )	Light ( $n = 11$ )	Electrical ( $n = 10$ )
First spike latency, ms	52.5 ± 5.0	47.9 ± 1.5	107.3 ± 10.7	93.3 ± 4.6
Spike/burst	5.0 ± 2.1	2.1 ± 0.2	6.2 ± 1.4	3.8 ± 0.5

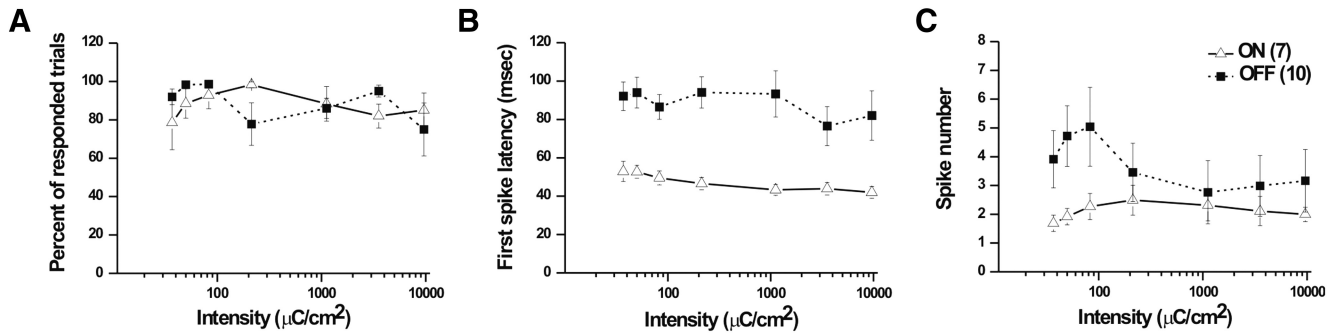
Values are mean ± SEM. Only spikes in the first burst were analyzed.

analyzed. The first spike latencies of both the ON and OFF alpha RGCs were slightly decreased as the electrical stimulation increased (Fig. 5B). In contrast, the spike rates of both the ON and OFF cells increased initially with moderate electrical current increases, but the response was reduced as the electrical stimulation increased further (Fig. 5C). These results indicate that the alpha RGC response is relatively insensitive to the strength of the suprathreshold electrical stimulation, which implies that a minimal electrical current that is just above the threshold may be the optimal stimulation strength for a subretinal prosthesis. However, this also suggests that the electrically evoked RGC response does not have a large dynamic range that would allow variation in stimulation strength to be encoded. This implies in turn that modulating the pulse frequency rather than the pulse intensity might be a key component in prosthetic design.

### Responses of alpha RGCs to a Series of Electrical Stimulations

To examine the effect of repetitive electrical stimulation on alpha RGCs, 10 repeated stimulus pulses at different intervals were used. The spiking responses of an alpha RGC to 5- and 20-Hz stimulation revealed that repetitive electrical stimulation had a suppressive effect (Fig. 6A). The normalized responses of an alpha RGC to 10 repetitive electrical stimulations at various temporal frequencies also showed that the suppressive effect increased on repetitive stimulations at increasing temporal frequencies (Fig. 6B). This result indicates that the suppressive response of an ON alpha RGC to repetitive electrical stimulations is temporal frequency dependent.

To further characterize the alpha RGC responses to trains of electrical stimulations, the spiking patterns of cells stimulated with repetitive electrical pulses at different temporal frequencies were analyzed. It is apparent that pair-pulse suppression of both ON and OFF alpha RGCs on repetitive stimulations was significantly increased when the temporal frequency increased (Fig. 7A). We also found that both ON and OFF cells evoked spiking response levels of  $<50\%$  when the temporal frequency was above 10 Hz (Fig. 7B). Furthermore, the normalized average response of the ON cells showed a lower level of frequency dependency than that of the OFF cells, in which the normalized average response was significantly decreased at temporal frequencies of the repetitive stimulation above 5 Hz (Fig. 7C). Taken together, these results suggest that the response of alpha RGCs to repetitive electrical stimulation is significantly reduced at high temporal frequencies. They also imply that modulating the pulse frequency in a subretinal prosthesis will be limited to low frequency. However, the fact that ON and OFF alpha RGCs showed distinct responses to repetitive electrical stimulation suggests that selectively activating the ON pathway in the retina with a higher temporal frequency may be possible.



**FIGURE 5.** The low threshold of the alpha RGCs on electrical stimulation using the silicon-based MPDA. (A) Average percentage response level of the ON and OFF alpha ganglion cells on stimulation with various electrical currents. Even using the lowest charge density ( $39 \mu\text{C}/\text{cm}^2$ ) available to our system, both the ON and OFF alpha RGCs still showed a better than 50% response, which makes the thresholds of alpha RGCs on electrical stimulation much lower than in previous studies. (B, C) Characteristic responses of the alpha RGCs stimulated by increasing electrical currents. The first spike latencies of both the ON and OFF alpha RGCs were slightly decreased as the electrical stimulation increased. In contrast, the spike rates of both the ON and OFF cells increased initially at moderate electrical current levels as the current was increased, but this response was muted by further increases in electrical stimulation at higher current levels. Numbers in parentheses indicate the number of cells examined. Error bars indicate mean  $\pm$  SEM.

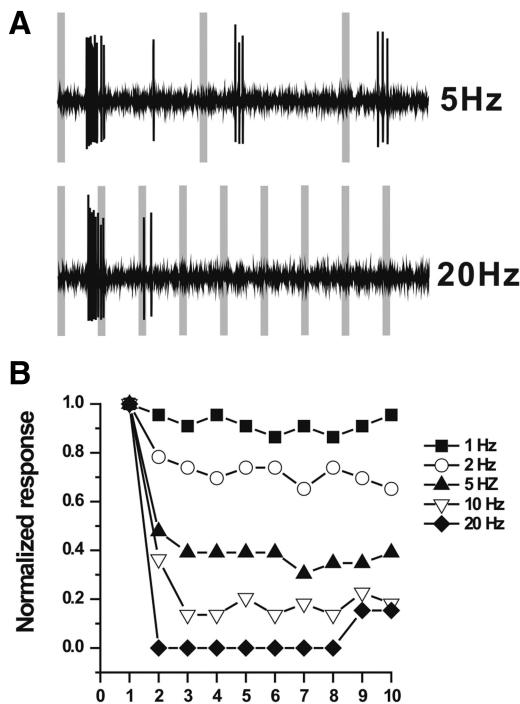
### Pharmacologic Studies of the Retinal Neural Network Responsible for the alpha RGC Response to Subretinal Electrical Stimulation

To determine whether the alpha RGC responses evoked by subretinal electrical stimulation by an MPDA did indeed result from activation of the retinal neural network that is presynaptic to ganglion cells, various pharmacologic blockers known to inactivate excitatory and inhibitory synapses were tested. The

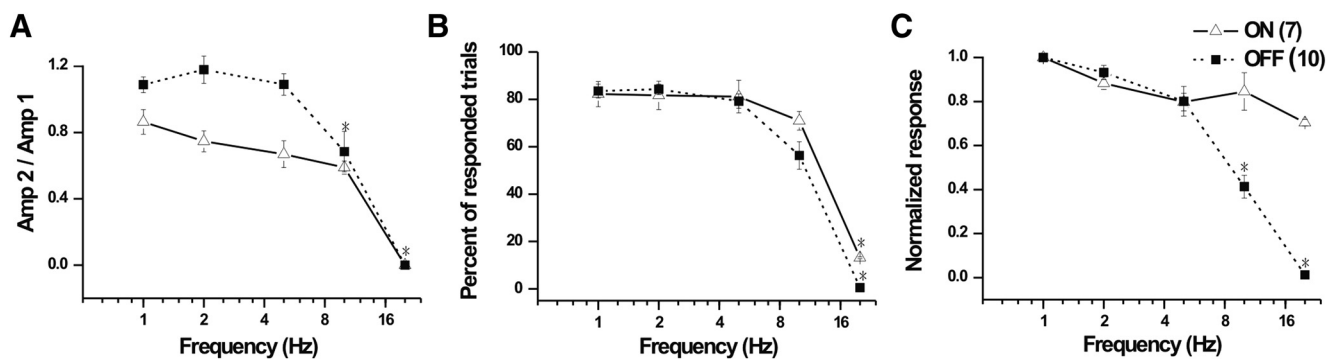
spiking response of an ON alpha RGC before treatment with the AMPA/kainate receptor antagonist CNQX ( $50 \mu\text{M}$ ) appeared normal (Fig. 8, top trace). However, no electrically evoked response was present after treatment with CNQX (Fig. 8, bottom trace), indicating that CNQX, which blocks the major excitatory inputs to ganglion cells, completely abolishes the electrically evoked responses of the alpha RGCs. It also suggests that the alpha RGC responses evoked by subretinal electrical stimulation by an MPDA are not the result of direct excitation of ganglion cells but, rather, involve the activation of upstream presynaptic components.

Similarly, the electrically evoked responses of alpha RGCs after treatment with PTX ( $100 \mu\text{M}$ ), an antagonist of the  $\text{GABA}_A$  and  $\text{GABA}_C$  receptors, were significantly increased, though the first spike latency was relatively independent of PTX treatment (Fig. 9). This indicates that PTX, which removes the major inhibitory inputs to ganglion cells, increases the spike rate of alpha RGCs evoked by electrical stimulation and suggests that the alpha RGC response evoked by subretinal electrical stimulation with an MPDA is subject to inhibitory circuit modulation, which is a property of the retinal neural network.

The fact that the first spike latency of alpha RGCs on subretinal stimulation was only slightly shorter than that of the same cells on visual stimulation (Table 2) suggests that the alpha RGC responses evoked by subretinal electrical stimulation by an MPDA may result from direct stimulation of the photoreceptor cells and does not occur by the stimulation of secondary retinal neurons. To verify this possibility, APB (a group III metabotropic glutamate receptor agonist) was used to selectively block synaptic transmission between the photoreceptors and the ON bipolar cells. The spike rate of the ON alpha RGCs was completely abolished after the application of APB ( $20 \mu\text{M}$ ), but that of the OFF alpha RGCs was not significantly affected (Fig. 10A). Furthermore, the average percentage level of response of the ON cells to 10 electrical stimulations was completely abolished after the application of APB, whereas that of the OFF cells was not significantly altered (Fig. 10B). This indicates that APB, which specifically inactivates the ON pathway in the mammalian retina, completely abolishes the electrically evoked response of the ON alpha RGCs without affecting the response of the OFF alpha RGCs. This result also suggests that bipolar cells are not excited by subretinal stimulation; rather, it seems likely that the photoreceptor cells are directly activated by subretinal electrical stimulation by the MPDA.



**FIGURE 6.** Suppressive responses of an ON alpha RGC to repetitive electrical stimulations depend on the temporal frequency. (A) Response of an alpha RGC to 5 and 20 Hz electrical stimulations. Gray bars: duration of the electrical stimulation. At both frequencies, the alpha RGC showed a suppressive response to repetitive electrical stimulations. (B) The normalized responses of an alpha RGC to 10 repetitive electrical stimulations at various temporal frequencies. Spike rates were normalized against the first response of the cell at each frequency. The alpha RGCs show an increase in suppression on repetitive stimulations at increasing temporal frequencies.



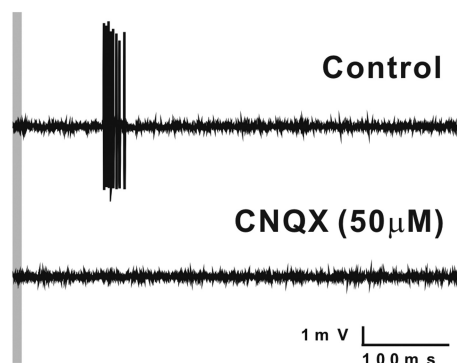
**FIGURE 7.** Responses of alpha RGCs to repetitive electrical stimulations are significantly reduced at high temporal frequencies. (A) Pair-pulse suppression of ON and OFF alpha RGCs on repetitive stimulations. The ratio of the second response amplitude to the first response amplitude (Amp2/Amp1) was significantly decreased when the temporal frequency was above 5 Hz. (B) Average percentage response of ON and OFF cells to 10 repetitive electrical stimulations at various temporal frequencies. Both types of cells gave rise to spiking responses in <50% of cases when the temporal frequency was above 10 Hz. (C) Normalized average responses of both ON and OFF cells to 10 repetitive electrical stimulations at various temporal frequencies. The ON cells showed lower frequency-dependent responses, whereas the response of OFF cell was significantly decreased when the temporal frequency of the repetitive stimulation was above 5 Hz. \**P* < 0.05. Numbers in parentheses are the number of cells examined. Error bars indicate mean ± SEM.

**DISCUSSION**

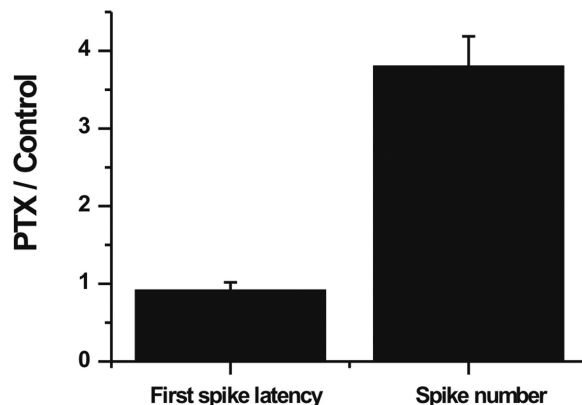
The present study demonstrates the feasibility of activating RGCs using a power-free MPDA to stimulate the retina from the subretinal side. Although this is not the first study to show the capacity of an MPDA to elicit a retinal network response in animal models, there are several features of our current approach that distinguish the present study from previous attempts. Our MPDA used a silicon-based solar-cell design that is passive and does not have to be powered. This means that it can be fabricated by a standard CMOS process and does not require additional power to drive the chip. To enhance the current density at the site of electrical stimulation, the surrounding return electrode of each pixel was designated as ground (Fig. 1B). This feature has been shown to significantly strengthen the density of the electrical current in simulation.<sup>22</sup> In addition, the responses of the RGCs to light and electrical stimulation were obtained from the same cell before and after photoreceptor bleaching, which allows direct comparison between visual and prosthetic stimulation. Furthermore, the use of pharmacologic interventions to examine the retinal network response on electrical stimulation by an MPDA allowed us to dissect the retinal circuitry that was responding to the subretinal prosthesis.

**Comparison between the Response of the alpha RGCs to Light and Electrical Stimulation**

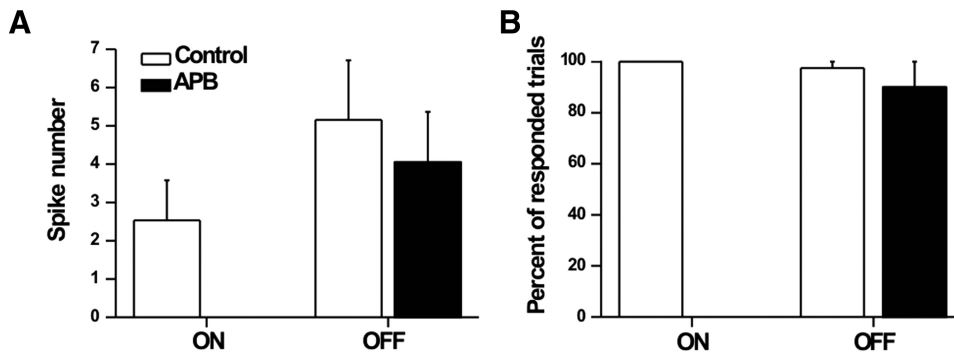
Consistent with a previous study<sup>14</sup> that showed good correspondence between electrical and visual receptive fields in RGCs, we also found that the first spike latencies of the alpha RGCs on light and electrical stimulation were similar (Table 2). This finding suggests that the observed electrically evoked responses by the RGCs are a result of activating the same retinal network as that involved in light stimulation. The fact that CNQX completely abolished the electrically activated response also strongly supports the presynaptic origin of the stimulation (Fig. 8). Furthermore, our observation that APB selectively eliminates the electrically evoked response of the ON alpha RGCs but does not eliminate that of the OFF alpha RGCs supports the hypothesis that the activated MPDA stimulates the upstream photoreceptors rather than bipolar cells (Fig. 10). However, the burst patterns of the alpha RGCs on light and electrical stimulation are distinct, namely there is only one burst of spikes on light stimulation but there are multiple bursts of spikes on electrical stimulation (Table 1, Fig. 4). This



**FIGURE 8.** CNQX completely abolishes the electrically evoked responses of an ON alpha RGC. The spiking response of the ganglion cell before application of AMPA/kainate receptor antagonist CNQX (50 μM) was normal (*top trace*). There was no electrically evoked response after application of CNQX (*bottom trace*). Gray bars: duration of electrical stimulation.



**FIGURE 9.** PTX increases the spike rate, but not the first spike latency, of alpha RGCs evoked by electrical stimulation. The electrically evoked responses of alpha RGCs (*n* = 3) after application of PTX (100 μM), an antagonist of the GABA<sub>A</sub> and GABA<sub>C</sub> receptors, were normalized against the control to reveal a difference. The first spike latency was relatively independent of PTX application, whereas the spike rate significantly increased after application of PTX. Error bars indicate mean ± SEM.



**FIGURE 10.** APB completely abolishes the electrically evoked responses of an ON alpha RGC but does not alter the responses of an OFF alpha RGC. (A) The spike rate of ON alpha RGCs ( $n = 3$ ) was completely abolished after the application of APB ( $20 \mu\text{M}$ ), an mGluR6 agonist, but there was no effect on the OFF alpha RGCs ( $n = 4$ ). (B) Average percentage response of ON cells to 10 electrical stimulations was completely abolished after treatment with APB, whereas that of OFF cells was not significantly altered. Error bars indicate mean  $\pm$  SEM.

is consistent with the findings of Jensen and Rizzo,<sup>15</sup> who showed that two bursts could be evoked by the anodal and cathodal currents for ON and OFF alpha RGCs, respectively. The spike numbers per burst on light and electrical stimulation were also different, with about twice as many spikes evoked by light activation (Table 2). These findings suggest that direct electrical stimulation from the photoreceptor side in an intact retina may induce an oscillation of excitation and inhibition in the outer retinal neurons<sup>16,26</sup> and that this phenomenon is absent during light stimulation. The fact that PTX increased the spike rate but not the first spike latency of the alpha RGCs on electrical stimulation partially supports the role of inhibitory neurons in modulating the responses of RGCs to the subretinal prosthesis (Fig. 9).

### Characteristics of the alpha RGCs on Electrical Stimulations by an MPDA

In the present study, the first spike latencies were  $47.9 \pm 1.5$  and  $93.3 \pm 4.6$  ms for ON and OFF alpha RGCs on electrical stimulation, respectively. This finding is consistent with previous studies, in which it was reported that the burst latency was around 50 ms.<sup>11</sup> Interestingly, although Stett et al.<sup>11</sup> showed that the burst latency of RGCs on electrical stimulation in a chicken retina was approximately 50 ms when activated by spot stimulation (similar to the one used in the present study), their response threshold was much higher than ours ( $500 \mu\text{C}/\text{cm}^2$  vs.  $39 \mu\text{C}/\text{cm}^2$ ). However, the burst latencies reported in other previous studies were shorter than what we found<sup>15-17</sup> (see Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7808/-DCSupplemental>). This discrepancy may result from the variation in the electrical stimulation strength that was applied in these studies. In contrast to earlier experiments, no artifact of initial electrical stimulation was seen in our recording traces (Figs. 2, 6A, 8). This implies that a lower current density was present in our experiments, which may also explain the observed longer first spike latency of the RGCs on electrical stimulation in the present study.

It is apparent that even at the lowest charge density ( $39 \mu\text{C}/\text{cm}^2$ ) in our system, both the ON and OFF alpha RGCs still showed a greater than 50% response level (Fig. 5A); thus, the threshold of alpha RGCs on electrical stimulation was much lower than that in previous studies (Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7808/-DCSupplemental>). For technical reasons, we were unable to test a charge density lower than  $39 \mu\text{C}/\text{cm}^2$  when activating the MPDA with our 532-nm laser. Nevertheless, it is likely, using the subretinal prosthetic design described here, that even smaller currents, when generated by a less intense light (i.e., indoor lighting), will be able to activate the MPDA (Fig. 3) and that the voltage produced will be capable of evoking an RGC response. In addition, several factors, such as duration of the laser pulse and materials of the stimulating elec-

trode, may also affect the response threshold. We have used a longer duration of laser pulse (100 ms) in a subset of tests and found that the threshold was not significantly altered (data not shown). Future studies may consider the capacitive effect of the stimulating electrode in affecting the response threshold.

It is known that the electrical field generated by the MPDA is dependent on the configuration and positions of the anodes and cathodes (Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7808/-DCSupplemental>). Stimulating currents that are perpendicular rather than inward or outward relative to the long axis of the photoreceptors change the response threshold of the recorded ganglion cells.<sup>6,27</sup> In the present MPDA configuration, the surrounding return electrode of each pixel was designated as ground (anode). As a result, the distance between stimulating and return electrodes was kept small, and thus the interface impedance was reduced. Reduced impedance can increase the output current and lower the response threshold.<sup>22</sup> Moreover, the efficiency of the MPDA is largely dependent on how closely the chip contacts the retina. The three-dimensional electrodes or in vivo implantation would form a tighter contact and thus a more efficient interface. This would probably result in even lower thresholds. However, the present study stimulated non-diseased retinas in rabbits, and it is known that diseased retinas in humans, such as in RP, have higher electrical thresholds than healthy retinas for producing phosphenes (Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7808/-DCSupplemental>). Thus the expected response threshold with this MPDA may still be higher in vivo than in vitro.

It has been shown that the response threshold of RGCs on epiretinal electrical stimulation is proportional to the distance of the cell soma location from the stimulating electrode.<sup>6,27,28</sup> Although we had no control over the cell soma location relative to the stimulating electrode, because the retina was placed on the MPDA before an alpha RGC was selected for recording, careful photographing of the location of each recorded cell soma after each experiment allowed the relationship between the response threshold and the cell soma location to be determined. It was found that the thresholds for the ON and OFF alpha RGCs on electrical stimulation did not correlate with their corresponding soma locations on the MPDA (see Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7808/-DCSupplemental>). The observation that the thresholds of the RGCs and their soma locations are independent further substantiates the idea that the present system of subretinal electrical stimulation by an MPDA is unlikely to directly activate ganglion cells. It seems likely that the recorded RGC responses must result from excitation of the upstream retinal network, probably the photoreceptor cells.

Although the first spike latencies of both the ON and OFF alpha RGCs were slightly decreased as electrical stimulation



increased, the spike rates of both cell types did not increase monotonically with an increased electrical current (Figs. 5B, 5C). This result differs from the findings of Gekeler et al.,<sup>13</sup> who showed that the electrically evoked cortical potentials were proportional to the strength of the subretinal electrical stimulation in the rabbit retina. Although the origin of this reduced responsiveness of RGCs on intense electrical stimulation is unknown, our finding highlights the importance of selecting an optimal electrical current strength for a subretinal prosthesis.

Furthermore, consistent with previous studies<sup>14,16</sup> that the responses of RGCs to repetitive subretinal electrical stimulations were gradually reduced, we also found that alpha RGCs on high-frequency electrical stimulation decreased their responsiveness significantly (Figs. 6, 7), although ON cells were less severely than OFF cells (Fig. 7C). A similar reduction in RGC response after repetitive epiretinal electrical stimulation was observed in other studies,<sup>27,29</sup> and this RGC desensitization probably involves multiple components.<sup>30</sup> It has been suggested that the inability of RGCs to respond to highly repetitive electrical stimulation may not be a limitation of the temporal resolution of the retinal prosthesis; this is because the response of RGCs to stroboscopic flash stimuli are also diminished at increased temporal frequency.<sup>16</sup> Nonetheless, this inherent temporal resolution limitation will make motion detection in a subretinal prosthesis more difficult than in an epiretinal prosthesis. However, the fact that the ON and OFF alpha RGCs responded to highly repetitive electrical stimulation differently (Fig. 7C) may provide a strategy that allows the selective activation by a retinal prosthesis of the ON pathway without affecting the OFF pathway. Although the present study used the rabbit retina with functional photoreceptors and demonstrated that electrical stimulation by an MPDA from the subretinal side can elicit RGC responses, future studies using animal models with progressive retinal atrophy such as RCS rats and Abyssinian cats would provide more substantial support in vitro.

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