Selective Activation of Neuronal Targets With Sinusoidal Electric Stimulation

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¹Center for Innovative Visual Rehabilitation, Boston Veterans Administration Healthcare System, Boston; ²Department of Neurosurgery, Massachusetts General Hospital and Harvard Medical School, Boston; ³Department of Otology and Laryngology, Harvard Medical School, Boston; ⁴Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge; and ⁵Cochlear Implant Research Laboratory and ⁶Department of NeuroOphthalmology, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts

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Freeman DK, Eddington DK, Rizzo JF, Fried SI. Selective activation of neuronal targets with sinusoidal electric stimulation. J Neurophysiol 104: 2778-2791, 2010. First published September 1, 2010; doi:10.1152/jn.00551.2010. Electric stimulation of the CNS is being evaluated as a treatment modality for a variety of neurological, psychiatric, and sensory disorders. Despite considerable success in some applications, existing stimulation techniques offer little control over which cell types or neuronal substructures are activated by stimulation. The ability to more precisely control neuronal activation would likely improve the clinical outcomes associated with these applications. Here, we show that specific frequencies of sinusoidal stimulation can be used to preferentially activate certain retinal cell types: photoreceptors are activated at 5 Hz, bipolar cells at 25 Hz, and ganglion cells at 100 Hz. In addition, low-frequency stimulation (≤ 25 Hz) did not activate passing axons but still elicited robust synaptically mediated responses in ganglion cells; therefore, elicited neural activity is confined to within a focal region around the stimulating electrode. Our results suggest that sinusoidal stimulation provides significantly improved control over elicited neural activity relative to conventional pulsatile stimulation.

INTRODUCTION

The remarkable successes of cochlear implants (Wilson and Dorman 2008) and deep brain stimulation (DBS) for the treatment of Parkinson's disease (Gale et al. 2008) suggest a wide range of neurological disorders could also be treated with electric stimulation from a neural prosthetic. Clinical trials are underway targeting epilepsy (Loddenkemper et al. 2001), cluster headaches (Sillay et al. 2009), depression (Stefurak et al. 2003), certain types of blindness (Rizzo et al. 2003), and other diseases of the CNS. Despite considerable effort, however, the outcomes of many of these applications remain limited. Improved stimulation methods that selectively activate individual classes of neurons or target specific neuronal substructures would be a significant benefit to neural prostheses.

Retinal prostheses aim to restore vision to those blinded by outer retinal diseases by electrically stimulating the surviving neurons in the inner retina (Winter et al. 2007; Zrenner 2002). Although electric stimulation of the retina in blind subjects typically elicits a visual percept (Humayun et al. 2003; Rizzo et al. 2003), the ability to elicit more complex pattern vision with multielectrode stimulation has not yielded consistent results (Caspi 2009; Lowenstein 2004; Rizzo et al. 2003; Weiland et al. 2004). Although several factors are thought to limit the quality of elicited vision, the inability to control the pattern of elicited neural activity is thought to play a critical role. Presumably, stimulation methods that could replicate one or more aspects of normal retinal signaling would lead to the highest quality of elicited vision.

Efforts to control the spatial pattern of neural activation in retinal explants have had only limited success (Behrend et al. 2009; Greenberg 1998; Jensen et al. 2003). This is thought to arise from two factors: 1) the ganglion cell bodies that are the target of stimulation are overlaid by axons that arise from distant cell bodies and 2) the threshold for activation of these passing axons is higher than that of the soma region, but only by a factor of two (Rizzo et al. 2003). Incidental stimulation of these passing axons will be perceived by the brain as coming from ganglion cells with distant cell bodies, thereby reducing the spatial control over the elicited visual percept. Given that the activation threshold varies for different types of ganglion cells (e.g., brisk-transient versus local edge detectors) (Fried et al. 2009), the ability to activate a large number of ganglion cells while avoiding the activation of passing axons may not be possible with existing stimulation methods. A similar challenge exists in many other CNS-based neural prosthetic applications because targeted cell bodies often lie in close proximity to passing axons that arise from distant regions of the brain (Behrend et al. 2009; Histed et al. 2009; Jensen et al. 2003; Ranck 1975; Schiefer and Grill 2006). For example, in DBS treatment of Parkinson's disease, the activation of passing axons in the limbic system is thought to underlie a number of adverse side effects, such as cognitive and mood changes (Wichmann and Delong 2006).

The ability to selectively target individual classes of neurons would be another significant benefit to many neural prostheses. In the retina, selective activation of bipolar cells would use circuits in the inner retina, creating spiking patterns in ganglion cells that better resemble those that arise under physiological conditions. Bipolar cells can be activated by long-duration pulses (>1 ms) (Fried et al. 2006; Greenberg 1998; Jensen et al. 2003, 2005); however, such pulses also activate ganglion cells, both at the soma and the distal axon. This results in spiking patterns that are highly complex and do not resemble those that arise under physiological conditions. The ability to selectively activate particular classes of neurons could also be useful to many other neural prostheses (McIntyre and Grill 2002) because stimulating electrodes are typically surrounded by heterogeneous populations of neurons.

The use of alternative stimulation waveforms (i.e., nonpulsatile) for electric stimulation has not been well explored (but see also Cantrell and Troy 2009; Langille et al. 2008). This may be caused in part by the early successes of pulsatile

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stimulation in cochlear implants and DBS for Parkinson's disease. Given that the membrane properties of different neuronal substructures (e.g., soma vs. axon) vary considerably in terms of the types and densities of voltage-gated ion channels, input resistance, capacitance, and synaptic contacts (Carras et al. 1992; Fried et al. 2009; O'Brien et al. 2002), it is likely that such variability will lead to different frequency-dependent response properties for each substructure. This raises the possibility that the use of narrowband waveforms, such as sinusoids, may provide selective control over the targets of neuronal activation. Conversely, pulses contain broad spectral energy that may limit the ability to preferentially activate neuronal targets even if they exhibit different frequency-dependent properties.

We measured spike trains from rabbit retinal ganglion cells in response to sinusoidal electric stimulation of various frequencies (5–100 Hz) and compared the responses to that of conventional pulse trains. Because of the well-defined organization of the retina, we were able to deliver stimulation near the soma as well as over the distal axon (\sim 1 mm from the soma) of the same cell and thus directly compare the response for each location. Also, using pharmacological blockers, we were able to elucidate which components of the response were caused by direct activation of the ganglion cell and which were caused by activation of presynaptic neurons.

METHODS

Animal preparation and retina isolation

The care and use of animals followed all federal and institutional guidelines, and all protocols were approved by the Institutional Animal Care and Use Committees of the Boston VA Healthcare System and/or the Subcommittsee of Research Animal Care of the Massachusetts General Hospital. New Zealand White rabbits (~2.5 kg) were anesthetized with injections of xylazine/ketamine and subsequently euthanized with an intracardial injection of pentobarbital sodium. Immediately after death, the eyes were removed. All procedures following eye removal were performed under dim red illumination. The front of the eye was removed, and the vitreous was eliminated. The retina was separated from the retinal pigment epithelium and mounted, photoreceptor side down, to a 10-mm² piece of Millipore filter paper (0.45 μ m HA membrane filter) that was mounted with vacuum grease to the recording chamber (~1.0-ml volume). A 2-mm circle in the center of the Millipore paper allowed light from below to be projected on to the photoreceptors.

Electrophysiology and light responses

Patch pipettes were used to make small holes in the inner limiting membrane, and ganglion cells with large somata were targeted under visual control. Spiking was recorded with a cell-attached patch electrode (4–8 MΩ) filled with superfusate. For whole cell recordings, the patch electrode was filled with (in mM) 113 CsMeSO₄, 1 MgSO₄, 7.8 × 10⁻³ CaCl₂, 0.1 BABTA, 10 HEPES, 4 ATP-Na₂, 0.5 GTP-Na₃, 5 lidocaine *N*-ethyl bromide (QX314-BR), and 7.5 neurobiotin chloride, pH 7.2. Excitatory currents were shown by clamping at –60 mV ($E_{\rm Cl}$). Two silver chloride–coated silver wires served as the ground and were positioned at opposite edges of the recording chamber each ~15 mm from the targeted cell. The retina was continuously perfused at 4 ml/min with Ames (pH 7.4) at 36°C, equilibrated with 95% O₂-5% CO₂. Pharmacological agents were applied to the bath by switching a three-way stopcock to a 200-ml reservoir of Ames containing one or more of the following blockers:

50 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 100 μ M CdCl₂.

The light stimulus was controlled by VisionWorks software, and data acquisition and stimulus triggering were controlled by custom software written in LabView (National Instruments) and Matlab (Mathworks). Light stimuli were projected on to the retina from below through an LCD projector (InFocus) and focused onto the photoreceptor outer segments with a steady, photopic background. Light stimuli consisted of stationary flashed squares (size range: 100–1,000 μ m), 1-s duration, centered at the soma. Stimulus intensity was 50–75% above background light level. Other than noting whether targeted ganglion cells were ON or OFF, they were not further classified.

Electric stimulation

Electric stimulation was delivered via a 10-k Ω platinum-iridium electrode (MicroProbes); the exposed area was conical with an approximate height of 125 μ m and base diameter of 15 μ m, giving a surface area of \sim 5,900 μ m², comparable to a 40- μ m diameter disk electrode. Pulse and sinusoidal stimuli were controlled by Multi-Channel Systems STG2004 hardware and software. Two silver chloride–coated silver wires served as the return; each was positioned ~ 8 mm from the targeted cell and ~ 12 mm from each other. The height of the stimulating electrode remained fixed at 25 μ m above the inner limiting membrane. The stimulating electrode was placed either directly over the sodium channel band on the proximal axon or $\sim 1 \text{ mm}$ lateral to the soma directly over the distal axon. Because of the use of patch clamp, spikes were clearly visible through the stimulus artifact. The efficacy of various stimulation waveforms (0.2-ms pulses and 5to 100-Hz sinusoids) was tested for the two different electrode positions.

Location of the sodium channel band

In response to short-duration pulses, the location of the sodium channel band has been shown to correspond to the center of the region with the lowest threshold and is generally centered between 20 and 60 μ m from the soma along the proximal axon (Fried et al. 2009). Using an iterative process, we were able to quickly find the center of the low-threshold region: movement of the stimulating electrode toward the center of the low-threshold region resulted in decreasing thresholds, whereas movement away from the center resulted in increasing thresholds. We used this location as the approximate center of the sodium channel band. Preliminary testing indicated that thresholds for sinusoidal stimulation were also lowest over the sodium-channel band (Fig. 1).

Location of the distal axon

The trajectory of the distal axon was ascertained by studying the pattern of thresholds in response to rectangular pulses of electric stimulation. During the dissection of the retina, the location of the optic disk was noted, and the tissue was oriented so that axons generally coursed in a constant direction (from right to left in our preparation). Electric pulse stimulation was used to more precisely define the axon location. A typical search algorithm placed the stimulating electrode 100 μ m left of the soma and then delivered a series of 10 increasing-amplitude pulses. If the pulses elicited spikes, the stimulating electrode was moved perpendicular to the presumed axon trajectory in 10- μ m steps to find the location at which the lowest pulse amplitudes could elicit spikes. This was considered to be the axon location. The stimulating electrode was moved an additional 100 μ m to the left, and the process was repeated until the axon position was determined at a distance of ~1,000 μ m from the soma.

Rectangular pulses

Pulsatile stimuli were biphasic pulses (equal and opposite rectangular phases) delivered at 10 pulses/s (phase duration: 200 μ s;



interphase delay: 10 ms; cathodic phase 1st). The interphase delay was long enough for the neural response to the cathodic pulse to be completed before the onset of the anodic phase. For each stimulus amplitude, 15–30 pulses were delivered, and there was a delay of >5 s between stimulation epochs. We found that pulses of this duration and over this range of stimulus amplitudes produced either a single spike or no spike. If a spike was elicited, it immediately followed the cathodal pulse. Therefore the number of pulses that elicited a spike was normalized to the total number of pulses delivered to give the fraction of pulses that elicited spikes.

Sinusoidal waveforms

Sinusoidal waveforms were delivered at frequencies of 5, 10, 25, and 100 Hz. Sinusoidal stimuli were delivered for 1 s, using a linear onset and offset ramp of 40 ms to reduce the spectral splatter induced by sudden stimulus onset/offset. Because a typical cell was held for <30 min and there were several stimulus conditions to be tested on a given cell, time constrains limited the number of stimulus presentations; each stimulus amplitude was delivered once, with a delay of ≥ 5 s between consecutive stimuli. An array of stimulus amplitudes was delivered in steps of 1–2 μ A, where the amplitudes were chosen with the goal of covering the full dynamic range of the neuron. For each cell, the order of presentation for the various stimulus waveforms was randomized. The maximum amplitude for which the charge density of the stimulating electrode remained below safe limits was estimated using a method described previously (Brummer and Turner 1977): the stimulus amplitude was increased until microscopic bubbles were seen to form on the electrode tip. Based on these results, the maximum stimulation levels were set at 4, 9, 18, and 36 μ A for 5, 10, 25, and 100 Hz, respectively. For pulses, the stimulus level that exceeded charge density limits was not estimated because a threshold response was always achieved below this stimulus level. Because sinusoidal stimulation typically elicited multiple spikes per stimulus period, we plotted the number of spikes elicited by the 1-s stimulus as a function of stimulus amplitude. This is a different measurement than the probability curves used for pulsatile stimulation, and this should be taken into account when comparing data from pulsatile and sinusoidal stimulation. Stimulus amplitude was reported in terms of current levels (μA) instead of charge per phase (nanocoulombs/phase) to facilitate comparison across stimulus frequencies (charge/phase varies considerably across the frequencies tested).

Stimulus threshold and statistical tests

The cells used in this study did not exhibit spontaneous firing, and therefore all recorded spikes were assumed to be stimulus induced. The number of spikes (R) was measured for a range of stimulus amplitudes (S) in steps of 1–2 μ A, and sigmoidal curves were found to fit the data well ($\langle r^2 \rangle = 0.913 \pm 0.097$) using the following equation: $R = A * S^n / (S^n + \sigma^n)$, where A is the saturation level, σ is the input current required to reach one half of saturation, and *n* is the order of the sigmoid. Stimulus threshold was therefore defined as the stimulus amplitude necessary to produce the number of spikes equal to one half the number of stimulus periods (e.g., for a 100-Hz, 1-s sinusoidal stimulus, the stimulus level required to elicit 50 spikes is defined as threshold). Because of the limits on stimulus levels for sinusoidal stimulation, saturation level could not be reached in many cells, and σ could not be used to define threshold. If a cell did not elicit a threshold number of spikes for the highest stimulus amplitude tested (as determined by the amplitude levels at which micro-bubbles were produced), the highest stimulus amplitude tested was taken to be threshold. For pulses, threshold was defined as the stimulus level necessary to elicit a spike on one half the number of pulses delivered, as estimated by the best-fit sigmoidal curve. All tests for statistical significance are paired *t*-test using a significance level of 5% (α = 0.05).

FIG. 1. Schematic of the experimental

setup. A: spikes were recorded from a single

ganglion cell (gray) using cell-attached patch

clamping (patch electrode is orange). Epi-

retinal stimulation is applied for 2 different

positions of the stimulating electrode (blue):

the Soma Position is centered over the so-

dium channel band (green, $\sim 40 \ \mu m$ lateral

from the soma) and the Distal Axon Position

is ~1 mm lateral from the soma. Both loca-

tions are 25 μ m above the inner limiting

membrane. *B*: sample of a response to 10-Hz stimulation where large spikes (arrows) are

easily discerned from the sinusoidal stimulus artifact. *C* and *D*: the number of spikes elic-

ited in response to a 1-s sinusoidal stimulus

of 25 (C) and 100 Hz (D) as a function of

stimulating electrode position for 2 different

cells. The soma is defined as 0 on the x-axis,

and the negative positions are distance from

the center of the soma along the axon. Stim-

ulus amplitudes used were 8, 11, 14, and 17μ A for 25 Hz and 9, 12, and 15 μ A for

100 Hz, with increasing amplitudes indicated

by circle, square, triangle, and diamond.

Computational modeling

Models of a voltage-gated sodium channel and an L-type calcium channel were developed from previous physiology and modeling studies of retinal ganglion cells (Benison 2001; Huang and Robinson 1998). T-type calcium channels in retinal neurons have been characterized physiologically, but an explicit model of these channels in the retina has not been developed. Therefore model equations were based on work from cerebellar Purkinje neurons (Schutter and Bower 1994), which have similar physiological properties as the T-type calcium channels in retinal bipolar cells (Hu et al. 2009). The voltage across the channels was varied sinusoidally or stepwise, and the resulting sodium and calcium currents were calculated. Currents took on the general form of

$$I_{\text{Na}} = g_{\text{Na}} \ m^3 \ h \ (V - E_{\text{Na}})$$
$$I_{\text{CaL}} = g_{\text{Ca}} \ n^2 \ (V - E_{\text{Ca}})$$

25Hz

15

80

where $g_{\text{Na}} = 150 \text{ nS}$, $g_{\text{CaL}} = 2.0 \text{ nS}$, $g_{\text{CaT}} = 1.0 \text{ nS}$, $E_{\text{Na}} = 75 \text{ mV}$, and $E_{\text{Ca}} = 45 \text{ mV}$. The gating parameters were calculated with the equation

$$dp/dt = \alpha_p(V)(1-p) - \beta_p(V)p$$

where p = m, h, n, k, and q. The gating parameters m, k and n are activating (open in response to depolarization), and the parameters hand q are inactivating (open in response to hyperpolarization). The functions $\alpha_{p}(V)$ and $\beta_{p}(V)$ can be found in Benison et al. (2001) for I_{Na} and I_{CaL} and Schutter and Bower (1994) for I_{CaT} . Differential equations were solved in Matlab using Euler's method with a time step of 0.01 ms.

RESULTS

Α

30

25

SomaDistal Axor

We used cell-attached patch-clamp recordings to measure spiking from retinal ganglion cells in response to electric stimulation with sinusoidal and pulsatile waveforms. Stimuli were delivered either in the soma region or over the distal axon (Fig. 1A). A typical response to one period of a 10-Hz stimulus delivered near the soma is shown in Fig. 1B—the use of patch-clamp recordings allowed individual spikes (arrows) to be visualized without obstruction by the stimulus artifact. Previous work has shown that there is a dense band of sodium channels in the proximal axon ($\sim 40 \ \mu m$ lateral from soma), and in response to pulses, this region has the highest sensitivity to stimulation (Fried et al. 2009). We extended this result to

В

80

10Hz

include sinusoidal stimulation, where the maximal response to 25 and 100 Hz was found to occur \sim 40–50 μ m from the soma (Fig. 1, C and D) (n = 3). For these preliminary experiments, synaptic input to the ganglion cell was blocked with application of CdCl₂ (100 μ M) to confirm that the response was mediated by direct activation of the ganglion cell and not activation of presynaptic neurons. All of the stimulation delivered near the soma in this study was approximately centered over the cell's sodium channel band (see METHODS).

Avoiding axonal activation with sinusoidal stimuli

To determine whether sinusoidal stimulation can be used to activate retinal ganglion cells without simultaneously activating passing axons (Behrend et al. 2009; Histed et al. 2009; Jensen et al. 2003; Schiefer and Grill 2006), we compared responses from electric stimuli delivered near the soma to responses from electric stimuli delivered over the distal axon, typically ~ 1 mm from the soma (Fig. 1A). Stimulation waveforms consisted of 1) low-frequency sinusoidal stimuli (LFSS) of 10 and 25 Hz, 2) high-frequency sinusoidal stimuli (HFSS) of 100 Hz, and 3) brief cathodic pulses of 0.2 ms delivered at 10 pulses/s. Sinusoidal stimulation of 10 Hz elicited a strong response when the stimulating electrode was positioned near the soma (Fig. 2A, \bullet), but spiking could not be elicited when the electrode was moved to a position over the distal axon (Fig. $2A, \bigcirc; n = 10/10$ cells). Even the highest amplitude levels we

20 FIG. 2. Avoiding axonal activation with low-frequency sinusoidal stimulation. A: the number of spikes elicited in response to a 1-s, 10-Hz sinusoidal stimulus is plotted as a function of stimulus amplitude (peak-to-peak μA) for stimuli delivered near the soma (filled circles) and the distal axon (open circles). B and C: similar plots for stimulus frequencies of 25 and 100 Hz. D: the probability of eliciting a spike in response to 0.2-ms cathodal pulses (15-30 repeats at each stimulus level). All data in A–D are from a single cell. E: summary data showing the mean ratio of distal axon threshold to soma threshold for each stimulus waveform (n = 10 cells per wave-)form). The upward-pointing arrows indicate values presented are lower bounds. Error bars indicate SE.



10Hz 25Hz 100Hz Pulses Stimulus

could safely deliver (see METHODS) failed to elicit spiking at the distal axon position using 10-Hz stimulation. Similar results were obtained for stimulation at 25 Hz (Fig. 2*B*): cells were highly sensitive to stimulation near the soma, whereas stimulation over the distal axon elicited no spiking in most cases (n = 9/10 cells) and elicited a response above threshold in only one cell (n = 1/10).

Increasing the stimulus frequency to 100 Hz resulted in strong spiking responses, both when the stimulating electrode was positioned near the soma and also when it was positioned over the distal axon (Fig. 2C). Unlike LFSS, responses to 100 Hz typically consisted of a single spike per stimulus period, resulting in a maximum of ~ 100 spikes for a 1-s stimulus. This maximum response level was reached for stimulation at both locations, although larger stimulus amplitudes were required when the stimulating electrode was over the axon (P < 0.001, paired *t*-test). When short-duration (0.2 ms) cathodal pulses were applied, no more than a single spike per pulse was elicited (Fried et al. 2006; Sekirnjak et al. 2006). For each stimulus amplitude, 15-30 pulses were delivered, and the number of pulses that elicited a spike was normalized to the total number of pulses delivered to give the fraction of pulses that elicited spikes (Fig. 2D). Similar to the 100-Hz responses, pulses also elicited reliable spiking at each of the two stimulating electrode locations (Fig. 2D).

These findings suggest that LFSS elicits a spiking response when the stimulating electrode is positioned near the soma but typically elicits no response when the stimulating electrode is positioned over the distal axon. HFSS and pulses elicit responses for both electrode positions. To quantify these results, we computed the stimulus amplitude that was needed to elicit a given response level (threshold, see METHODS) at each of the two locations. The threshold ratios (distal axon/soma region) measured for HFSS and pulses were 2.29 ± 0.07 and 3.22 ± 0.08 (SE), respectively (Fig. 2*E*). The threshold ratios for LFSS were 10.0 ± 0.66 for 10 Hz and 7.08 ± 0.43 for 25 Hz. Because LFSS stimulation near the axon did not elicit spiking at the maximum level tested for 18/20 cells, we could not accurately determine the average threshold ratios for LFSS. Therefore we used the maximum amplitude tested as a lower bound of threshold and a lower bound on the distal axon-to-soma threshold ratios for 10- and 25-Hz stimulation (indicated by the arrows in Fig. 2*E*). The HFSS and pulse threshold ratios are each significantly smaller than each of the LFSS threshold ratios (maximum value of all comparisons, P < 0.015). The relatively high-threshold ratios for LFSS suggest that ganglion cells whose somas are close to the stimulating electrode will respond, whereas nearby passing axons will not. Thus LFSS may be useful in confining elicited activity to a small, "focal" region around the electrode.

Responses to LFSS are synaptic in origin

To determine whether presynaptic activation played a significant role in the high sensitivity of the soma region to LFSS, we measured the response to stimulation near the soma while pharmacologically blocking synaptic transmission. The primary source of excitatory input to ganglion cells arises via glutamatergic release from the axon terminals of bipolar cells and is mediated through AMPA/kainate receptors on the ganglion cell dendrites. In the presence of 50 μ M CNQX, an antagonist of AMPA/kainate receptors, the response to 10-Hz stimulation was greatly reduced (n = 3/6; Fig. 3A, red trace) or completely eliminated (n = 3/6; data not shown). To determine whether the CNQX-insensitive portion of the response was mediated by one or more additional synaptic components, we added 100 µM CdCl₂ to block all synaptic transmission (Margalit and Thoreson 2006) and found that, now, the response was mostly eliminated (Fig. 3A, green trace; n = 2/2). Similar findings were obtained in CdCl₂ alone: the response to 10-Hz stimulation at the soma was completely eliminated (n = 3/4) or greatly reduced (n = 1/4). Taken together, these results suggest that the response to 10 Hz is primarily mediated through synaptic activity.

The amount of presynaptic activation was similarly determined for sinusoidal stimulation at 25 and 100 Hz and with



FIG. 3. Input from presynaptic neurons underlies the response to low-frequency sinusoidal stimuli (LFSS). *A*–*D*: the response to sinusoidal stimulation (10, 25, 100 Hz) and 0.2-ms cathodal pulses delivered near the soma for control (blue circles), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (red squares), and CNQX + CdCl₂ (green triangles). For sinusoidal stimulation, the number of spikes elicited in response to a 1-s stimulus is plotted as a function of stimulus amplitude (peak-to-peak μ A). In response to pulses, the probability of eliciting a spike is plotted against stimulus amplitude (15–30 repeats at each stimulus level). All data in *A*–*D* are from a single cell. Legend in *C* applies to *A*, *B*, and *D*.

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FIG. 4. Whole cell patch clamp reveals synaptic currents. Whole cell voltage clamping an OFF-cell at -60 mV shows excitatory currents in response to 5-Hz sinusoidal stimulation for 1 s. By convention, inward currents are depicted as negative deflections. Note that the sinusoidal stimulus artifact is also embedded in the response. However, the artifact is a 0-mean signal, and the fact that the current is skewed negatively is indicative of a stimulus-induced inward current.

0.2-ms pulses (Fig. 3, B-D). The response to 25-Hz stimulation was greatly reduced in the presence of CNQX (n = 6) or CdCl₂ (n = 4, data not shown) or both (n = 2). Because the response to 25 Hz was not completely eliminated by synaptic blockers, our results suggest that the response consists of two components: a portion arising from direct activation of the ganglion cell and a portion that is synaptically mediated. The responses to 100 Hz (Fig. 3*C*) and to pulses (Fig. 3*D*) were affected very little by synaptic blockers, suggesting the response to these waveforms arose predominantly from direct activation of the ganglion cell.

To further confirm that the spiking responses to LFSS resulted from modulation of synaptic input to the ganglion cell, we used whole cell patch clamp to record ganglion cell input currents. This allowed us to eliminate the possibility that the application of synaptic blockers simply reduced the level of tonic glutamate release from bipolar cells, thus decreasing the sensitivity of ganglion cells to electric stimulation. Voltage clamping at E_{Cl} and stimulating at 5 Hz gave a response that consisted of both the stimulus artifact and any inward (excita-

tory) currents (Fig. 4). Because the stimulus artifact is zeromean, and the response shifted toward negative currents, we can infer that stimulation at 5 Hz elicited inward currents. This further supports the view that the spiking response of ganglion cells to LFSS results from activation of presynaptic neurons. We did not attempt to separate the whole cell currents from the stimulus artifact and therefore did not quantify the level of input currents.

We quantified the effect of synaptic blockers in two ways. First, we compared the response threshold in control conditions to the response threshold in CNQX or CdCl₂. The ratio of thresholds before and after the application of synaptic blockers for each stimulus waveform is shown in Fig. 5, A and B, indicating that responses to 10- and 25-Hz stimulation were more strongly affected by the blockers than responses to 100-Hz and pulsatile stimulation. These results are consistent with the view that the response to 10- and 25-Hz sinusoidal stimulation activate neurons presynaptic to the ganglion cell, whereas the response to 100-Hz sinusoids and 0.2-ms pulses are mediated by direct activation of the ganglion cell. Thresholds increased significantly in the presence of each blocker for stimulation at 10 (P < 0.001 for CdCl₂ and P < 0.05 for CNQX) and 25 Hz (P < 0.001 for CdCl₂ and P < 0.02 for CNQX). The effect of the blockers was not significant for 100-Hz stimulation (P >0.07 for CdCl₂ and P > 0.6 for CNQX) but was statistically significant for 0.2-ms pulses (P < 0.001 for CdCl₂ and P < 0.01for CNOX).

The second method used to quantify the level of synaptic input was to compare the maximum number of elicited spikes in control conditions versus the maximum number of spikes elicited with synaptic blockers (Fig. 5, *C* and *D*). The data for 10-Hz stimulation in either CNQX (Fig. 5*C*) or CdCl₂ (Fig. 5*D*) were largely clustered around the *x*-axis, again suggesting that synaptic input underlies most of this response. In contrast, the data from 100-Hz stimulation were largely clustered around the line of unity slope, confirming that synaptic input had little effect. The results for 25 Hz were mostly scattered between the



FIG. 5. Effects of pharmacological blockers on threshold and maximal response. The ratio of stimulus thresholds measured across all cells in CNQX (*A*) (n = 6) or CdCl₂ (*B*) (n =4) relative to control for 10, 25, and 100 Hz and 0.2-ms cathodal pulses (error bars indicate SE; arrows indicate bar is lower bound). The maximum number of spikes elicited in response to 1 s of sinusoidal stimulation in control conditions (no drugs) is plotted vs. the maximum number of spikes in CNQX (*C*) and CdCl₂ (*D*) for all cells. Circles: 10 Hz; triangles: 25 Hz; squares: 100 Hz.

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line of unity slope and the *x*-axis, consistent with the response to 25 Hz arising from both presynaptic and direct activation.

Preferential activation of individual neuronal classes

Surprisingly, we found that the class of presynaptic neurons activated by LFSS could be altered by changes in the stimulus frequency. In response to 5-Hz stimulation delivered near the soma, spikes occurred near the peak of the cathodal phase for OFF ganglion cells (n = 6; Fig. 6B) as expected (Ranck 1975; Tehovnik et al. 2006), but spikes occurred near the peak of the anodal phase for on-type ganglion cells (Fig. 6A; n = 6). Because ON and OFF ganglion cells are thought to have similar intrinsic properties (O'Brien et al. 2002), we hypothesized that the mechanism responsible for this ON and OFF difference originates at a site presynaptic to ganglion cells. A likely site is the photoreceptor-to-bipolar cell synapse in the outer retina, where the ON and OFF pathways diverge. Thus if the cathodal phase of the 5-Hz sinusoidal stimulus depolarizes photoreceptors, it would lead to a depolarization of OFF-bipolar cells and subsequent increased spiking in OFF ganglion cells. The same depolarization of photoreceptors would lead to hyperpolarization of ON bipolar cells because of sign inverting metabotropic glutamate receptors (mGluR) found in their dendrites and a corresponding reduction in spiking for ON ganglion cells. Analogously, the anodal phase of 5-Hz stimulation would hyperpolarize photoreceptors, which would decrease glutamatergic input to, and depolarize, ON bipolar cells, thereby causing increased spiking in ON ganglion cells.

Increasing the stimulus frequency to 25 Hz resulted in spikes that occurred exclusively near the cathodal peak for both on and oFF cells (Fig. 6, *C* and *D*). This suggests that the mechanism of excitation at 25 Hz shifts to a location downstream of photoreceptors, most likely in bipolar cells because CNQX blocks much of this response. A summary of the average phase at which spiking occurs in on and oFF cells for each stimulus frequency tested (Fig. 6*E*) shows that the on and oFF phase differences occur for 5 and 10 Hz, but for frequencies of 25 Hz and above, the response phase remains cathodal for both cell types.

Do ion channel properties underlie the frequency-dependent responses?

The frequency-dependent response properties of retinal neurons to electric stimulation are likely to be influenced by the properties of voltage-gated ion channels. It is well established that different types of ion channels are distributed heterogeneously across different classes of retinal neurons, as well as between different subregions of a given neuron. Because the kinetics by which different types of ion channels respond to changes in membrane voltage can also vary considerably, the possibility exists that differences in the frequency sensitivity that we observed experimentally may arise from differences in



FIG. 6. Stimulus frequency alters the response phase. Portion of the response to 1-s sinusoidal stimulus delivered near the soma at 5 (*A* and *B*) and 25 Hz (*C* and *D*). At 5 Hz, spikes occurred during the peak of the sinewave (cathodal phase) for OFF cells and the trough of the sinewave (anodal phase) for oN cells. At 25 Hz, spikes occurred during cathodal phase for both cell types. *E*: summary plot of stimulus frequency vs. the average phase at which the peak spiking response occurs for ON cells (squares, n = 6) and OFF cells (circles, n = 6). Note that the ON and OFF phase difference occurs at low but not high frequencies. Error bars indicate SE.

the distribution and/or kinetics of the ion channels inherent within the different classes of retinal neurons.

To explore this possibility, we constructed a computational model to examine the response of ion channels to different frequencies of sinusoidal stimulation. The channels tested were those thought to underlie the physiological responses we observed: *I*) voltage-gated sodium channels that underlie the spiking response in ganglion cells and are found in high densities in the proximal axon and *2*) both L- and T-type calcium channels that have been shown to modulate synaptic release in bipolar cells and photoreceptors (only the L-type calcium channel has been identified in photoreceptors). These model ion channels were examined individually so that the frequency-dependent response properties of each could be isolated. The voltage across the channel was modulated, and the resulting current was calculated according to equations that were based on previous studies (Benison et al. 2001; Schutter and Bower 1994).

To test the kinetics and activation/inactivation properties of the individual ion channels within our model, steps of voltage were applied, and the resulting current through each ion channel was calculated (Fig. 7, A-C). Consistent with previous studies, the L-type calcium channel activated slowly with a time course of several milliseconds (Fig. 7*B*) (Protti and Llano 1998), whereas the sodium channel activated rapidly (Fig. 7*C*), opening in less than a millisecond (Fohlmeister and Miller 1997). The inactivating mechanism of the sodium channel also acts fairly quickly (\sim 1 ms), causing the sodium channel to close in response to sustained depolarization and reducing the sodium current back to baseline. Because the model L-type calcium channel does not inactivate, the L-type calcium current persists for the duration of the voltage step. Similar to the response of the sodium channel, the activation and inactivation mechanisms of the T-type channel combine to cause a transient increase in current in response to a step depolarization (data not shown). However, because the activation and inactivation kinetics of T-type channel are both slower than that of the sodium channel, the current increase starts after and persists longer than that of the sodium current.

To determine whether the response kinetics and activation/ inactivation properties of the ion channel might contribute to the frequency dependence observed experimentally, voltage was varied sinusoidally, and the resulting sodium and calcium currents were calculated. Example response currents elicited by low (10 Hz) and high (200 Hz) frequencies are shown in Fig. 7, D–F. The response of the L-type calcium channel was significantly stronger in response to low-frequency stimulation



FIG. 7. A model sodium channel and two calcium channels respond optimally to different stimulus frequencies. A-C: ten voltage steps were made starting from -80 mV in steps of 10 mV and the resulting L-type calcium and sodium currents were computed. D-F: also, voltage was sinusoidally varied around -80 mV at 10 Hz (black) and 200 Hz (gray) (peak-to-peak amplitude: 100 mV), and the resulting currents were calculated. *G*: the peak current was computed and normalized for all three channels (L- and T-type calcium and sodium) for stimulus frequencies ranging from 1 to 1,000 Hz.

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than to high-frequency stimulation. The slow activation kinetics of the L-type channel was responsible for the weaker response at the high stimulus frequency. In contrast, the rapid activation kinetics associated with the sodium channel enabled the channel to open and close in response to the relatively rapid fluctuations in voltage associated with the high stimulus frequency. The weak response to the low-frequency stimulus was caused by the relatively fast inactivation mechanism of the sodium channel.

To determine the optimal range of stimulus frequencies for each model channel, the peak-to-peak current was calculated as a function of stimulus frequency (Fig. 7G). As suggested by the results at 10 and 200 Hz (Fig. 7, D-F), the L-type calcium channel elicited strong responses at low frequencies but responded only weakly to higher stimulus frequencies. In contrast, the strongest response of the sodium channel was observed for a range of relatively high stimulus frequencies (centered around 200 Hz), consistent with its relatively fast activation kinetics. As with the step depolarization, the inactivation mechanism closes the sodium channel during slow depolarizations, thus suppressing the response to low-frequency stimulation. Similar to the sodium channel, the T-type channel also exhibited a band-pass response. However, the relatively slow activation kinetics of the T-type channel resulted in an optimum frequency of ~ 10 Hz, which is much lower than that of the sodium channel. The presence of an inactivation mechanism in the T-type channel limits the responsiveness at very low frequencies, similar to that of the sodium channel. The general shape of the frequency response was similar across a wide range of initial membrane voltages and stimulus amplitudes (data not shown).

Interestingly, the model T-type channel maintained a moderate response level even at the highest frequency simulated (1,000 Hz; Fig. 7*G*). Although the fluctuations in voltage were too rapid to cause changes to the activation or inactivation state of the channel, the steady-state conductance in response to high-frequency stimulation was nonzero (i.e., the activation and inactivation variables k and q were significantly different from 0, see METHODS). Therefore current flowed through even for rapid fluctuations in voltage. The same was not true for sodium and L-type calcium channels, where the channels were closed in response to high-frequency stimulation, preventing any current flowing through the channel.

These computational results are highly consistent with the physiological data. For example, the ganglion cell responses that were mediated by activation of presynaptic neurons (Fig. 3) were strongest at low frequencies. This is consistent with the model results in which calcium channels, known to mediate synaptic release, responded optimally to low stimulus frequencies (Fig. 7G). Additional correspondence between the model and experimental results arise from comparison of the direct (nonsynaptic) activation of ganglion cells to the frequencydependent characteristics of the model sodium channel. Previous physiological data have suggested that the direct activation of ganglion cells is mediated by sodium channels, and the model predicted that sodium channels respond optimally to high stimulus frequencies. This is consistent with our experimental findings in which direct (nonsynaptic) activation of ganglion cells is strongest for high-frequency stimulation. The correspondence between the physiological and modeling results suggests that the activation and/or inactivation kinetics of voltage-gated ion channels is likely to contribute to the frequency-dependent response of neurons in response to electric stimulation. Therefore customizing stimuli based on the frequency-dependent characteristics of voltage-gated ion channels endogenous to the target neuron may optimally activate the target neuron(s) and allow selective activation of individual classes of neurons or neuronal substructures.

DISCUSSION

Our results suggest that electric stimulation with sinusoidal waveforms provides a level of control over neuronal activation that has not been possible with more conventional pulsatile stimulation. We showed that LFSS avoids the activation of axons while still eliciting robust responses in the target neuron. In addition, we showed that the specific class of neuron being activated depends on the frequency of sinusoidal stimulation: photoreceptors are activated at 5 Hz, bipolar cells at 10-25 Hz, and ganglion cells at 100 Hz. The ability to target specific classes of neurons has important implications for the retinal prosthetic as well as for a wide range of other neural prostheses.

LFSS avoids the activation of axons

One of the principal findings of this study is that LFSS is much more effective than short-duration pulses at avoiding the activation of passing axons. Previous physiological studies found that, for short-duration pulses, the threshold for activation of the distal axon was only two times greater than the threshold for activation for the soma region (Jensen et al. 2003). This is consistent with our results, which found that the threshold ratio with short-duration pulses was \sim 3 (Fig. 2). The slight difference between our findings and the previous study was likely because of the difference in stimulation parameters (0.2- vs. 0.1-ms pulses, 10- vs. 1-M Ω impedance of the stimulating electrode). The threshold ratios were significantly higher with LFSS: at 25 Hz, the threshold ratio was >7 and for 10 Hz the ratio was >10. The ratios for LFSS are lower bounds because we could not elicit responses from the distal axon, even at the highest stimulus amplitudes that could be safely delivered (METHODS). The higher ratios associated with LFSS suggest that it is a significant improvement for avoiding the activation of passing axons.

The ability to avoid the activation of passing axons in retinal prostheses will reduce the spatial spread of activation, potentially improving the control over the spatial pattern of the elicited percept. For example, in human trials, blind patients often report a percept that is oval in shape, and this is potentially because of incidental activation of passing axons (Horsager et al. 2010). There are also other factors that influence the spatial pattern of elicited activity. Previous work has shown that increased stimulus amplitude for pulsatile stimuli activates cells further from the stimulating electrode, thus spreading the area of elicited activity (Jensen et al. 2003). Whether sinusoidal stimulation shows a similar dependence was not studied here and remains an open question.

We do not believe variations in pulse rate would have a significant effect on the results. The responses to short-duration pulses arises predominantly from direct activation of the ganglion cell and not activation of presynaptic neurons (Fig. 3). Previous work has shown that the ability to elicit spikes through direct activation delivered near the soma varies little for pulse rates ≤ 100 Hz (Sekirnjak et al. 2006). Therefore we do not expect changes in pulse rate to have a significant effect on the relative threshold of the distal axon versus the soma region.

Different frequencies activate different neuronal classes

Another principal finding is that changes to the frequency of sinusoidal stimulation altered the class of retinal neuron that was activated. We were able to infer this by observing the frequency-dependent change in the phase during which the responses were elicited. For example, OFF-ganglion cells tended to respond during the cathodal phase of the stimulus for both 5- and 25-Hz stimulation. ON-ganglion cells, however, responded during the cathodal phase for 25-Hz stimulation, but responded during the anodal phase for 5-Hz stimulation (Fig. 6). Given that the traditional view of electric stimulation is that neurons are depolarized in response to cathodal stimulation, it was surprising that ON-ganglion cells elicited a response during the anodal phase. One explanation for the response differences between ON- and OFF-cells is that photoreceptors are activated by 5-Hz stimulation; because the photoreceptor output is inverted at the on-bipolar synapse (but not the off-bipolar synapse), depolarization of photoreceptors (during the cathodal phase) would elicit spiking in OFF-ganglion cells, whereas hyperpolarization of photoreceptors (during the anodal phase) would elicit spiking in ON-ganglion cells. For 10-Hz stimulation, the spikes elicited in on-ganglion cells occurred during the transition between the anodal and cathodal phase. The phase shift that occurs as the stimulus frequency increases from 5 to 25 Hz suggests that the neural class activated shifts from photoreceptors to bipolar cells.

We cannot rule out the possibility that the ON-OFF phase difference for 5-Hz stimulation arises from the activation of horizontal cells and not photoreceptors. However, we do not believe that this is likely because the anticipated response polarity from horizontal cell activation is inconsistent with the data. For example, if the cathodal phase of the stimulus depolarizes horizontal cells, photoreceptors would be inhibited and there would be a reduction in glutamate release on to the bipolar cell dendrites. Because ON-bipolar cells depolarize in response to reduced glutamate input, ON-ganglion cells should exhibit increased spiking during the cathodal phase. However, this is inconsistent with the observed data (Fig. 6), suggesting that the response at 5 Hz is most likely the result of photoreceptor activation.

In addition to activating photoreceptors and bipolar cells with stimulation at 5 and 25 Hz, respectively, our data suggest that ganglion cells can also be directly activated by increasing the stimulus frequency. The response of ganglion cells to 100-Hz stimulation was not significantly affected by the application of synaptic blockers (Fig. 5), consistent with the response arising primarily from direct excitation of the ganglion cell. Thus our results suggest that different classes of retinal neurons can be targeted with the appropriate tuning of stimulus frequency; photoreceptors at 5 Hz, bipolar cells at 25 Hz, and ganglion cells at 100 Hz. Although the ability to target photoreceptors is of limited use for retinal prostheses because these cells have degenerated, the ability to preferentially target

specific classes of neurons has important implications. For example, in the retinal prosthetic, the ability to activate bipolar cells (e.g., at frequencies of 10-25 Hz) may be advantageous if it allows the inner retinal circuitry to be used and results in neural activity in ganglion cells that more closely resembles physiological signaling patterns.

The synaptically mediated response of ganglion cells to stimulation at 5-25 Hz was greatly reduced following application of CNQX. However, the additional application of cadmium was necessary to completely abolish the response. There are several possible sources for this CNQX-insensitive response component (difference between the red and green traces in Fig. 3A), including acetylcholine from activation of starburst amacrine cells (Famiglietti 1983), glutamatergic activity mediated by N-methyl-D-aspartate (NMDA) receptors (Kalloniatis et al. 2004), or reduced inhibitory input to ganglion cells mediated via activation of a serial inhibitory pathway (Roska et al. 1998). It is also possible the presence of cadmium reduced the response by blocking voltage-gated calcium channels that are intrinsic to the ganglion cell (Benison et al. 2001). However, we do not believe these channels play a major role, because direct activation of the ganglion cell is thought to be largely mediated by the dense band of sodium channels in the initial segment (Fried et al. 2009). Our data do not allow us to determine unequivocally the origin of the CNQX-insensitive response. However, because most of the synaptic response was eliminated in CNQX, it is likely that increased bipolar cell output is the primary source of the synaptic response.

Although HFSS was effective at exciting the ganglion cell directly and LFSS was not, it should be noted that higher stimulus amplitudes were delivered with HFSS compared with LFSS because of the charge-density limitations imposed (see METHODS). Therefore it was not possible to precisely measure the relative sensitivity of HFSS and LFSS for direct excitation of the ganglion cell. Nevertheless, our results suggest that *1*) LFSS is much more effective at eliciting a synaptically mediated response than a response from direct activation of the ganglion cell, and 2) the response to HFSS is primarily through direct excitation of the ganglion cell and not through synaptic activation.

Do ion channel properties underlie the frequency-dependent responses?

In general, the neuronal response to direct electric stimulation (i.e., nonsynaptic component) is thought to be governed by at least two factors: first, the membrane potential of the target neuron is modulated by the electric field of the stimulus with a time course determined by the resistive and capacitive properties of the membrane and any cells or tissue between the stimulating electrode and the target neuron (Tehovnik et al. 2006). Second, the change in membrane potential will open or close voltage-gated ion channels that will, in turn, further influence the membrane potential. The expression of ion channels is heterogeneous across cell classes, cell types, and across individual neuronal substructures. In addition, the kinetics and/or activation/inactivation properties of each channel type can be different as well. This suggests that knowledge of both ion channel distributions and their corresponding response properties may be necessary to understand the neuronal response to electric stimulation.

We used a computational model to explore the possible contribution of specific types of ion channels to the frequencydependent responses that were observed experimentally. Previous studies have shown that voltage-gated sodium channels underlie the response of ganglion cells (and axons) to direct activation, whereas both T- and L-type calcium channels underlie the release of neurotransmitter from the presynaptic neurons (bipolar cells and photoreceptors) that lead to indirect (synaptic) activation (Thoreson 2007). Therefore we used a model to determine how each of these three channels respond to the range of sinusoidal frequencies delivered experimentally.

In the model, we found that current through L- and T-type calcium channels was maximal at low stimulus frequencies (Fig. 7*G*). This is consistent with the results from our physiological experiments that found that presynaptic neurons (photoreceptors and bipolar cells) were maximally activated with low-frequency sinusoidal stimulation (Fig. 3). At higher stimulation frequencies, the model showed that an L-type calcium channel responds weakly, consistent with the lack of synaptically mediated activity in ganglion cells found experimentally.

The moderate level of activity in response to high-frequency stimulation of the modeled T-type calcium channel was somewhat surprising. It is possible that the small amount of synaptic activity seen experimentally in response to high-frequency stimulation was mediated by T-type channels. However, this synaptic response was relatively weak, and therefore the ability of T-type channels to respond to high stimulus frequencies may be an artifact of the specific T-type channel that we modeled.

The model showed that the sodium channel responded optimally to relatively high stimulus frequencies, consistent with the results from our physiological experiments that showed that direct activation of the ganglion cell can be achieved with high-frequency stimulation (Fig. 3). The ability of ganglion cells to respond to such high frequencies is likely the result of the rapid activation kinetics of sodium channels. At low frequencies, the modeled sodium channel responded poorly (weak responses up to \sim 40 Hz; Fig. 7G), consistent with experimental results in which low-frequency stimulation did not elicit responses via direct activation of the ganglion cell. The relatively weak response of the model sodium channel to low-frequency stimulation is a result of the inactivation mechanism, causing the channel to close during the depolarizing phase of stimulus. Thus at stimulus frequencies around 10-25 Hz, the sodium channels that underlie the direct activation of ganglion cells (and their axons) may be inactivated, whereas the calcium channels that underlie the response of presynaptic bipolar cells and photoreceptors are strongly activated. Clearly, not all sodium channels are inactivated as low frequencies; otherwise, the cell would not spike in response to increased excitatory input. Although mechanisms to explain this discrepancy can be postulated (i.e., inactivation of a subset of sodium channels), alternatives to sodium channel inactivation at low frequencies must also be considered.

Much previous work on neural prostheses has studied the ability of electric stimulation to elicit action potentials (Nowak and Bullier 1998; Tehovnik et al. 2006). As a result, such studies have largely focused on the role of voltage-gated sodium channels in the neural response to electric stimulation. Importantly, our work here suggests that voltage-gated sodium channels are not a necessary component for a neuron to respond to electric stimulation. In the physiological experiments, bipolar cells and photoreceptors were highly sensitive to LFSS, despite the fact that they are nonspiking, do not exhibit voltage-gated sodium currents (Kawai et al. 2001, 2002), and do not express dense regions of sodium channels (Cui and Pan 2008). This strongly suggests that other types of voltage gated ion channels underlie the response to electric stimulation in these cells; results from the computer simulation implicate voltage-gated calcium channels as a likely candidate. It is likely that other types of voltage-gated ion channels, not explored in this study, will also influence the response to electric stimulation.

Our experimental results suggested that bipolar cells and photoreceptors are optimally activated at different stimulation frequencies (Fig. 6). Although our model results do not offer a definitive mechanism responsible for this difference, we can draw some inferences about factors that may contribute. First, both Land T-type calcium channels are known to mediate release from bipolar cells (Hu et al. 2009; Protti 1998), whereas only L-type channels mediate release in photoreceptors (Thoreson 2007). The high sensitivity of L-type channels to low frequencies in the model is consistent with our experimental finding that photoreceptors were activated by the lowest stimulation frequencies we tested (5 Hz). Second, the synaptic terminals of several bipolar cells subtypes are thought to contain T-type channels exclusively (Pan et al. 2001). The model suggests that T-type channels respond optimally to low-to-mid frequencies but respond weakly to very low frequencies. This is consistent with the experimental observation that bipolar cell activation was stronger at 25 Hz than at 5 Hz. However, for bipolar cell terminals that do express both L- and T- type channels, it is not clear why their frequency sensitivities are different from photoreceptors. It may be that photoreceptors and some bipolar cells respond to 5-Hz stimulation but that the photoreceptor response is much stronger and overwhelms the bipolar cell response. Third, each class of ion channel contains multiple subtypes, each of which can have different kinetics. For example, three subtypes of T-type channels have been identified, and each activate and inactivate with different kinetics (Hu et al. 2009). The model contained only a single type each of L- and T-type channels, and it is possible therefore that differences in the kinetics between the channels in our model and the actual channels present in the retina may account for the observed differences between photoreceptor and bipolar cell responses.

It is important to note that many other mechanisms may contribute to the frequency-dependent responses that we observed experimentally. For example, the resistive and capacitive properties of the tissue between the stimulating electrode and the target neuron will likely influence the frequency dependence of the response (e.g., the bipolar cells and the stimulating electrode are separated by a layer of ganglion cells). Also, the membrane properties of the target neuron (e.g., its time constant) are likely to influence the frequency response. In addition, the differential response of each class of retinal neuron to different frequencies of stimulation could arise, at least in part, from several other factors associated with synaptic release and neuronal signaling. These include the temporal relationship between internal calcium concentration and subsequent release of transmitter vesicles, desensitization of ligand-gated channels, and transmitter depletion and uptake kinetics. Further effort will be needed to determine the extent to which these factors influence the frequency dependence we observed. Because our model did not include all of the elements that could potentially modulate the frequency response,

it is likely that the specific frequency predictions for a given ion channel will not match the physiological response exactly. However, the key result from the model is that the different kinetics and distribution of ion channels influence the response sensitivity to different frequencies of electric stimulation.

Implications for use of sinusoidal stimulation in a retinal prosthetic

Interestingly, our results also suggest that the use of LFSS in retinal prostheses may reduce the need to position the stimulating electrode close to the targeted neurons. Using conventional pulsatile stimulation, stimulating electrodes must be positioned relatively close to the ganglion cell layer to reduce the thresholds required to elicit percepts (Jensen et al. 2003; Sekirnjak et al. 2006, 2008). Using LFSS, however, we found presynaptic neurons were highly sensitive to stimulation even at relatively large distances from the stimulating electrode (Figs. 3 and 6). In our experimental setup, photoreceptors were \sim 4 times farther from the stimulating electrode than ganglion cells (125 vs. 30 μ m) and bipolar cells were ~2 times farther (75 vs. 30 μ m). It is somewhat surprising therefore that photoreceptors were preferentially activated by 5-Hz stimulation and bipolar cells by 25-Hz stimulation because much previous work indicates that activation thresholds are inversely proportional to the square of distance from the stimulating electrode (Tehovnik et al. 2006). This suggests that the challenge of positioning the stimulating electrode extremely close to the ganglion cell layer may be less critical for success with LFSS. In our experimental setup, the stimulating electrode was positioned on the vitreal side of the retina (epiretinal). Positioning the stimulating electrode closer to bipolar cells (e.g., subretinally or with penetrating electrodes) may further reduce the thresholds we observed.

Before sinusoidal stimulation techniques are implemented in a retinal prosthetic, several important considerations must be evaluated. First, because this study was performed on healthy retina, it will be necessary to confirm that similar results are obtained when LFSS is applied to the degenerate retina. The activation of photoreceptors at very low stimulus frequencies (5–10 Hz) will not be useful in retinal prostheses because these cells have degenerated as a result of outer retinal diseases. Also, because LFSS targets presynaptic neurons, it will be necessary that bipolar cells remain viable and that they maintain synaptic connections with ganglion cells. These are both likely to be the case; anatomical studies have shown that bipolar cells remain largely intact (Gargini et al. 2007), and physiological studies suggest that synaptic connections to ganglion cells remain functional, although the nature of these connections may vary from normal (Margolis et al. 2008; Stasheff 2008). Another consideration is that there are many subtypes of bipolar and ganglion cells (Masland 2001). This raises the possibility that a particular frequency of sinusoidal stimulation may preferentially activate only a subset of bipolar or ganglion cells. The particular subtypes of neurons that are activated will likely have a corresponding effect on the elicited visual percept (e.g., activation of the magnocellular vs. parvocellular pathways).

Charge density limits are another important consideration before the implementation of sinusoidal stimulation in a neural prosthetic. A previous study using pulsatile stimulation found that the charge density at threshold was 0.093 mC/cm² for direct activation of the ganglion cell and 0.219 mC/cm² for activation of presynaptic neurons (Fried et al. 2006). In this study, we also found that the charge density at threshold was relatively low for short-duration pulses (0.046 mC/cm²). However, for sinusoidal stimulation, the charge density levels at threshold were relatively high, both for HFSS (0.35 mC/cm^2) and LFSS $(0.49-0.51 \text{ mC/cm}^2)$. These values are slightly higher than the safe limit of charge density of 0.3 mC/cm² widely used in similar types of studies (Brummer and Turner 1977; Sekirnjak et al. 2006). There are several factors that will determine whether sinusoidal waveforms can be safely implemented in a neural prosthetic. First, although the charge densities used here were relatively high, new electrode materials are being developed that allow higher charge densities to be safely delivered (Cogan 2008). Second, our study involved epi-retinal stimulation where the stimulating electrode is 25 μ m above the tissue, allowing a significant amount of current spread through the bathing solution. Other electrode configurations, such as subretinal or penetrating electrodes, may reduce the stimulus levels necessary to produce the desired response, thereby reducing the charge density levels. Finally, the appropriate charge density safety limits for sinusoidal stimulation are not known and may be different from the estimated charge density limits for pulsatile stimulation (Mc-Creery et al. 1990).

Implications for use of sinusoidal stimulation in other types of neural prosthetics

Our results have important implications for DBS as well as for other types of neural prostheses. For example, DBS of the subthalamic nucleus (STN) for the treatment of Parkinson's disease (Bejjani 1999; Parsons et al. 2006; Stefurak et al. 2003) often results in side effects, such as cognitive and mood changes, that are thought to arise from incidental activation of passing axons from nearby limbic circuits. LFSS may reduce these side effects by avoiding activation of passing axons that arise from these nearby circuits. However, for LFSS to be implemented, it will be necessary to evaluate whether the elicited neural activity achieves similar clinical outcomes. Previous work has shown that the activation of afferent fibers projecting to the STN underlies the effectiveness of DBS for Parkinson's disease (Gradinaru et al. 2009). This raises the possibility that LFSS-mediated activation of presynaptic neurons in the STN could reproduce similar patterns of neural activity to those elicited by DBS for Parkinson's disease. Further support for the use of LFSS in other neural prosthetic applications comes from a recent study that used sinusoidal modulation of an electric field across the hippocampus to reduce seizures in an epileptic model of rat (Sunderam et al. 2009). The mechanisms of neuronal activation were not elucidated in that study; it will be interesting to learn whether mechanisms similar to the ones we describe here underlie the reported effectiveness.

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DISCLOSURES

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