Axonal Sodium-Channel Bands Shape the Response to Electric Stimulation in Retinal Ganglion Cells

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Fried SI, Lasker ACW, Desai NJ, Eddington DK, Rizzo JF 3rd. Axonal sodium-channel bands shape the response to electric stimulation in retinal ganglion cells. J Neurophysiol 101: 1972–1987, 2009. First published February 4, 2009; doi:10.1152/jn.91081.2008. Electric stimulation of the retina reliably elicits light percepts in patients blinded by outer retinal diseases. However, individual percepts are highly variable and do not readily assemble into more complex visual images. As a result, the quality of visual information conveyed to patients has been quite limited. To develop more effective stimulation methods that will lead to improved psychophysical outcomes, we are studying how retinal neurons respond to electric stimulation. The situation in the retina is analogous to other neural prosthetic applications in which a better understanding of the underlying neural response may lead to improved clinical outcomes. Here, we determined which element in retinal ganglion cells has the lowest threshold for initiating action potentials. Previous studies suggest multiple possibilities, although all were within the soma/proximal axon region. To determine the actual site, we measured thresholds in a dense two-dimensional grid around the soma/proximal axon region of rabbit ganglion cells in the flat mount preparation. In directionally selective (DS) ganglion cells, the lowest thresholds were found along a small section of the axon, about 40 μm from the soma. Immunochemical staining revealed a dense band of voltage-gated sodium channels centered at the same location, suggesting that thresholds are lowest when the stimulating electrode is closest to the sodium-channel band. The size and location of the low-threshold region was consistent within DS cells, but varied for other ganglion cell types. Analogously, the length and location of sodium channel bands also varied by cell type. Consistent with the differences in band properties, we found that the absolute (lowest) thresholds were also different for different cell types. Taken together, our results suggest that the sodium-channel band is the site that is most responsive to electric stimulation and that differences in the bands underlie the threshold differences we observed.

INTRODUCTION

Several research groups are actively developing retinal prosthetics—devices designed to restore vision to patients blinded by retinal degenerative diseases (Chow et al. 2004; Gekeler et al. 2006; Hornig et al. 2005; Humayun et al. 1996, 2003; Rizzo 3rd et al. 2003a,b). These devices work by electrically stimulating inner retinal neurons, large numbers of which have been shown to survive the disease process (Humayun et al. 1999b; Santos et al. 1997; Stone et al. 1992; but see also Jones and Marc 2005; Marc et al. 2003). The ability of this approach to elicit light percepts, called phosphenes, has been successfully demonstrated by several independent groups (Humayun et al. 1996; Rizzo 3rd et al. 2003b).

Despite the initial success of these efforts, several considerable obstacles must be overcome before a meaningful level of vision can be restored to patients. For example, the appearance of individual phosphenes is variable (Humayun et al. 1996, 2003; Rizzo 3rd et al. 2003b; Weiland et al. 1999) and single phosphenes are not predicatably “assembled” into more complex percepts (Rizzo 3rd et al. 2003b). This greatly reduces the spatial information that can be conveyed with these devices. In addition, the thresholds required to elicit phosphenes in visually impaired humans are quite high—several orders of magnitude larger than those required to elicit single-neuron action potentials in normally sighted animals (summarized in Sekirnjak et al. 2006). Elevated thresholds raise safety concerns (Margalit et al. 2002; Merrill et al. 2005) and necessitate the use of relatively large stimulating electrodes, further reducing the ability to convey spatial detail.

The mechanism(s) by which phosphenes are created is not well understood. Presumably, the highest level of vision would arise if the patterns of neural activity elicited by the prosthetic match the patterns created normally by light. However, there are at least two considerable obstacles that impede the prosthetic creation of such patterns. First, the degenerate retina must remain viable, e.g., capable of generating and transmitting a high level of neural activity. The evidence for this is mixed, with some studies suggesting that nearly all layers of the retina are subject to degeneration (Jones and Marc 2005; Marc et al. 2003), whereas others suggest that significant portions of the inner retina, including ganglion cells (retinal output cells), remain intact (Humayun et al. 1999b; Santos et al. 1997; Stone et al. 1992). Along similar lines, the synaptic pathways that connect the retina to higher visual centers must also remain intact. If rewiring occurs (Baker et al. 2005; Sabel 1999), the retinal signal may not reach the appropriate sites in the visual cortex and the quality of elicited vision will be reduced. A second factor impeding the creation of physiological-like patterns arises from the stimulation methods used by existing implants. These devices typically use a small number of widely spaced and relatively large diameter stimulating...
electrodes (Humayun et al. 1999a; Mahadevappa et al. 2005; Rizzo 3rd et al. 2003a); the resulting patterns are presumably crude and thus significantly different from the complex patterns created normally. This suggests that even if the underlying retinal structure and synaptic pathways remain intact, the neural activity that arrives at the visual cortex may not support complex visual properties. It is likely that each factor contributes to both the high-threshold level and the limited quality of elicited percepts (Rizzo 3rd et al. 2003b), but further study is needed to assess the individual contribution of each. Here, we are studying the mechanism(s) by which retinal neurons respond to electric stimulation as a first step toward developing methods that create more complex patterns of neural activity.

Many basic questions about the retinal response to electric stimulation remain unanswered. For example, the specific part of the ganglion cell in which spikes are initiated has not yet been determined. The few studies that have explored this suggest that the site of lowest threshold—presumably the site in which spikes are initiated—is in the soma/proximal axon region. Jensen (2003) measured threshold in and around the receptive field center (presumably the soma) of rabbit ganglion cells and found that thresholds were lowest for locations of the stimulating electrode that were slightly offset from the soma, although their methods did not allow correlation to a specific anatomical site. Sekirnjak et al. (2008) inferred the location of the site of lowest threshold were found at the soma (Greenberg et al. 1999), the axon bend (Schiefer and Grill 2006), and in the initial segment (Rattay 1999). Each study also predicted that the site of spike initiation during synaptic input (intracellular activation).

A dense band of voltage-gated sodium channels, immunohistochemically localized to the proximal axon in many different types of neurons (Boiko et al. 2003; Inda et al. 2006; Kole et al. 2008; Meeks and Mennerick 2007; Van Walt et al. 2007; Wollner and Catterall 1986), seems a likely possibility for the site of spike initiation. In response to synaptic input, the axon initial segment has long been thought to be the site of spike initiation (Carras et al. 1992; Colbert and Johnston 1996; Coombs et al. 1957a; Meeks and Mennerick 2007; Rattay 1999; Stuart et al. 1997). However, there is conflicting evidence as to whether the band is (Jenkins and Bennett 2001; Kole et al. 2008; Komada and Soriano 2002; Wollner and Catterall 1986) or is not (Clark et al. 2005; Colbert and Johnston 1996; Colbert and Pan 2002) the site of spike initiation in nonretinal neurons. Synaptic input studies in the retina (Carras et al. 1992) suggest that spikes are initiated in the proximal axon of salamander ganglion cells, possibly within a small-diameter (thin) segment that starts about 40 μm from the soma (Carras et al. 1992; Fohlmeister and Miller 1997). However, computational models derived from these physiological studies require a high density of sodium channels in the thin section to replicate physiological findings (Carras et al. 1992; Sheasby and Fohlmeister 1999). This requirement is seemingly in conflict with more recent immunochemical findings that suggest the dense region of sodium channels lies proximal to the thin section (Van Walt et al. 2007). Regardless, it is not clear whether the site of spike initiation during electric stimulation (extracellular activation) is the same as the site of spike initiation during synaptic input (intracellular activation).

At least 12 different types of retinal ganglion cells have been identified in all mammalian species (DeVries and Baylor 1997; O’Brien et al. 2002; Rockhill et al. 2002), including primate (Dacey et al. 2003). Different types are thought to detect different features of the visual world and each uses different patterns of spiking to transmit information to higher visual centers (DeVries and Baylor 1997; O’Brien et al. 2002; Roska and Werblin 2001). Many of the underlying anatomical and biophysical properties are different for each type, raising the question of whether the sites of spike initiation and/or the dense bands of sodium channels might also be different in different types. This has not yet been explored.

In this study, we identified the site at which threshold for spike initiation in response to electric stimulation was lowest by mapping threshold in and around the soma and proximal axon of retinal ganglion cells. Measurements within a single ganglion cell type (directionally selective [DS]) were consistent; each had a single low-threshold region centered about 40 μm from the soma. Immunochemical staining for voltage-gated sodium channels revealed that the dense band of sodium channels within the proximal axon was closely aligned with the low-threshold region, suggesting that the band may somehow underlie the low thresholds. Immunochemical staining of additional ganglion cell types revealed that the properties of sodium-channel bands (length, location) were different in different types. We found that both absolute threshold levels and threshold maps were also different for different ganglion cell types, perhaps as a result of the differences in band properties. The implications of our findings for creating more physiological patterns of neural activity using electric stimulation are discussed.

**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 Subcommittee 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 killed 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, the vitreous was eliminated, and the eye cup was dissected so that the retina could be flattened. Care was taken to minimize handling of the visual streak and ventral regions. For electrophysiology experiments, three rectangular pieces from the region just ventral to the streak (~5 mm), each about 5 × 7 mm, were extracted and stored in oxygenated Ames medium. For immunochemistry experiments, three larger pieces, each around 7 × 15 mm, were similarly processed. Storage times prior to use ranged from 15 min to 6 h.
Measuring light responses

Just before use, the retina was separated from the retinal pigment epithelium and mounted, photoreceptor side down, to a 10-mm-square piece of Millipore filter paper (0.45-μm HA Membrane Filter) that was mounted with vacuum grease to the recording chamber (~1 ml volume). A 4-mm-square hole in the center of the Millipore paper allowed light from below to be projected onto the photoreceptors. Retinas were superfused continuously at 7–10 ml/min with Ames medium (Sigma; pH 7.4, 36°C), equilibrated with 95% O₂-5% CO₂.

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 loose, cell-attached patch electrode (5–6 MΩ), filled with Ames medium. Two silver-chloride–coated silver wires served as the ground and were positioned at opposite edges of the recording chamber each around 15 mm from the targeted cell.

The stimulus presentation and data acquisition software were written by G. Spor, T. Muench, and D. Balya. Light stimuli were projected onto the retina from below through a liquid crystal display projector (Dell) and focused onto the outer segments of the photoreceptor. A photopic background intensity was maintained throughout the experiment (~4 nW/m²) (Roska and Werblin 2001). Contrast levels, defined as [L – Lmean]/Lmean × 100, were fixed at 200%. Light stimuli consisted of stationary flashed squares (size range: 100–1,000 μm), 1-s duration, centered at the soma, moving bars (300 × 1,800 μm moving at 600 μm/s in four orthogonal directions), and large flashed squares (1,000 μm) presented at temporal frequencies of 15 and 30 Hz.

Cells were classified as directionally selective (DS) if their response to the flashed 200-μm square was ON–OFF and if their response to back-and-forth motion of the bars was directional, e.g., spiking levels were considerably higher in one direction versus the other (Barlow and Levick 1965). These cells are more accurately classified as ON–OFF DS cells to distinguish them from a different type of DS cell that generates responses only at light ON (ON DS cells). All cells used in this study were of the ON–OFF variety and the term DS throughout this study refers exclusively to the ON–OFF population. Cells were classified as local edge detectors (LEDs) based on physiological responses described in previous studies (Roska and Werblin 2001; van Wyk et al. 2006); that is, that the cell gave ON and OFF responses to either light or dark stimuli and that the responses were largest for small squares (100 μm). In addition, the light response was significantly reduced for squares of increasing size. Cells were classified as brisk transient/alpha cells (BT) if they responded with high frequency and transient bursts of spiking to stimuli centered in their receptive field (Cleland and Levick 1974; DeVries and Baylor 1997; Roska and Werblin 2001). Consistent with previous reports, responses were largest for larger squares and were typically small or nonexistent for small squares (≤100 μm). Similar to previous studies, we found both ON and OFF varieties of these cells.

Construction of threshold maps

The use of cell-attached patch-clamp recordings allowed direct visualization of the elicited action potentials with no confusion from the stimulus artifact (Fried et al. 2006). Short-duration pulses were used exclusively (100–200 μs) to ensure that responses were the result of direct ganglion cell activation only (no presynaptic responses) (Fried et al. 2006; Jensen et al. 2005a; Sckirnjak et al. 2006). Under these conditions, only one spike is elicited per pulse and the responses to consecutive short pulses are not altered, even at stimulus rates as high as 300 Hz, suggesting that the response to each pulse is independent (no suppression of response by the previous pulse). Electric stimulation was delivered via a 100-kΩ platinum–iridium electrode (MicroProbes); the exposed area was conical with an approximate height of 35 μm and base diameter of 30 μm. Two silver-chloride–coated silver wires served as the return; each was positioned about 8 mm from the targeted cell and about 12 mm from each other.

At a given location of the stimulating electrode, a series of 10 biphasic pulses was delivered at 10 Hz; amplitude increased by 2 μA with each consecutive pulse. Each biphasic pulse consisted of equal and opposite square waves (MultiChannel Systems hardware and software); cathodic pulses were delivered first and intervals between phases were typically 10 ms, which was large enough for the neural response to the cathodic pulse to be completed before the onset of the anodic phase. The series of 10 biphasic pulses was repeated three times at each location.

The lowest amplitude level at which two of three pulses (cathodic phase) elicited a spike was considered threshold (Fig. 1). Because the percentage of successful trials did not always increase monotonically with increasing stimulus amplitude (i.e., Fig. 1B), we fit the raw data with a sigmoid curve (Fig. 1B, dashed line) and defined threshold as the amplitude level at which the percentage of successful trials line between 0.67 or two of three pulse–elicited spikes. For consistency, the threshold level as determined with the sigmoid curve was used for all data (monotonic and nonmonotonic increasing).

After determining threshold at one spatial location, we moved the stimulating electrode to a new location and repeated the process. The position of the stimulating electrode was controlled with a precision micromanipulator (Sutter). The distance between adjacent measurements was typically 10 μm and the height of the stimulating electrode was fixed at 25 μm above the surface of the inner limiting membrane. This process allowed us to construct two-dimensional maps of threshold as a function of the position of the stimulating electrode.

Defining the proximal axon’s low-threshold region

For all cells in which we obtained threshold maps, we defined a low-threshold region along the proximal axon. However, we did not have anatomical data for five of the six cells in which threshold maps were obtained and, since the area of the low-threshold region was relatively large, it was difficult to determine the exact location of the proximal axon. Therefore we estimated its location by connecting a line between the soma and the start of the distal axon. The start of the distal axon was estimated by measuring threshold measurements just beyond the proximal axon region (e.g., Fig. 6A). For each series of measurements (e.g., each column in Fig. 6A), the axon position was estimated as the point corresponding to the minimum value of a second-order curve that was fit to the raw data. This method allowed us to fairly accurately determine the path of the distal axon and to trace it back to the soma/proximal axon region. Support for this method of approximation is derived from the one cell in which we were able to obtain both physiology and anatomy—the estimate trajectory of the proximal axon is nearly identical to the actual trajectory (compare Fig. 6, A and C). Once its trajectory was determined, threshold values along the proximal axon were fit with a second-order curve. The low-threshold region was defined as the segment of the curve within 2 μA of the curve minimum. The location of the center of the low-threshold region corresponded to the location of the curve minimum.

Abbreviated mapping routine

In all cases for which the complete threshold map was obtained, the lowest observed threshold was at or near the center of the low-threshold region. Movement of the stimulating electrode toward the center resulted in decreasing thresholds, whereas movement away from the center resulted in increasing thresholds. This consistency allowed us to quickly approximate the center of the low-threshold region by moving the stimulating electrode only in the direction in which thresholds decreased. Using an ad hoc iterative process, we were able to find the approximate center and lowest threshold for
targeted cells with many fewer measurements than otherwise needed to obtain the complete map.

**Imaging ganglion cells: plasmid insertion (gene transfer) and incubation**

Pieces of rabbit retina about 1 cm² in size were placed ganglion cell side up on a 0.4-μm Millicell tissue culture insert (Millipore). The quality of the tissue after incubation depended on smooth attachment to the membrane. Filter stands (2-cm diameter, 1 cm high) were cut from the caps of 1-ml Monoject Tuberculin syringe jackets, so that the Millicell filter rested on three “stands” when it was placed into a 60 × 20 cell-culture dish (Nunc). Approximately 25 ml Ames medium (Sigma) containing 1% horse serum, 1% N2 supplement, and 100 U/ml penicillin, 100 U/ml streptomycin, 0.3 mg/ml l-glutamine (Invitrogen) were added to the dish, so that the retina was in contact with the medium via the Millicell filter over the photoreceptor side and with the incubator atmosphere (5% CO₂, 35°C, humidified) over the ganglion cell side. All further manipulations, including gene gunning, were carried out with the retina attached to the Millicell filter.

Transformation of the plasmid pEGFP (GenBank accession number 6084; Clontech, Palo Alto, CA) into Escherichia coli (DH5) and bacterial cell culture was performed according to standard procedures (Sambrook et al. 1989). For large-scale preparations of purified plasmid, the Qiagen (Valencia, CA) Maxi Prep kit was used. The plasmid was delivered to cells in the ganglion cell layer via particle-mediated transfection (Koizumi et al. 2007; Lo et al. 1994). Gold particles were coated with the green fluorescent protein (GFP) plasmid according to the manufacturer’s instructions. In brief, for each preparation, 12.5 μg of plasmid, 100 μl of 1 M CaCl₂, and 100 μl of 0.05 M spermidine stock solution were added to 12.5 mg of gold particles (1.6 μm; Bio-Rad, Hercules, CA) under continuous, slow vortex. Plasmid was allowed to attach to the gold particles for 10 min before recentrifugation and resuspension in absolute ethanol. The gold-plasmid suspension was then applied to the inner-surface diameter of plastic tubing using a tube prep station (Bio-Rad) and dried in place with nitrogen. The gene gun (Bio-Rad) was loaded with the gold plasmid-coated tubing. The gun was primed to 115 psi and the retina was flattened out on the Millicell filter. The nozzle was placed over the entire retina and fired. The retina was then placed on a rocker inside an incubated chamber (30°C) for 2 days with daily medium changes. This time is sufficient to allow expression of GFP, leading to bright labeling of individual ganglion cells of various types, and other cells in the ganglion cell layer (mainly displaced amacrine cells). With this method, 10–20 brightly labeled ganglion cells were typically visible. Morphological identification of DS cells was performed by identifying several characteristic features (Amthor et al. 1989; Rockhill et al. 2002) including a large soma (~25 μm), a bistratified dendritic tree, and recursive dendritic processes (e.g., see Fig. 4A).

**Imaging ganglion cells: whole cell patch**

Whole cell patch-clamp electrodes were used in two sets of experiments. First, they were used in the one DS cell in which we were able to first obtain a threshold map and then complete immunocytochemistry. The second set of experiments was used to capture the sodium channel...
bands in BT cells. Unlike classification of DS cells, we were not able to unequivocally classify a cell as BT purely by morphological features. Therefore it was necessary to first measure light responses and, once identified, fill the cell via whole cell patch. Whole cell patch electrodes were 6–7 MΩ and filled with (in mM) 112.5 CsMeSO4, 1 MgSO4, 0.0078 CaCl2, 10 HEPES, 4 ATP-Na2, 0.5 GTPNa3, 5 lidocaine N-ethyl bromide (QX314-Br), and 7.5 neurobiotin chloride (pH 7.2). Alexa 488 (Molecular Probes) was added to the intracellular solution to visualize the dendritic morphology of recorded cells after the electrical recordings were completed. In these experiments, Streptavidin with Alexa Fluor 488 2 mg/ml (Invitrogen/Molecular Probes) was conjugated to the neurobiotin that was delivered to the cell via the whole cell patch. Tissue was fixed for 30 min, washed in phosphate buffer (PB) three times for 5 min each (3 × 5), and then placed in 500 μl blocking solution overnight. Additional immunohistochemistry (e.g., for sodium channels) was typically performed in parallel with the streptavidin.

Immunohistochemistry

All antibodies used have been previously characterized. Mouse monoclonal anti-ankyrin-G antibody, described by Jenkins and Bennett (2001), was purchased (Catalog no. 12719; Santa Cruz Biotechnology, Santa Cruz, CA). K58/35, mouse monoclonal pan-specific anti-Nav referred to as PAN, described in detail in Rasband et al. (1999) was purchased (Catalog no. S8809; Sigma–Aldrich, St. Louis, MO). Rhodamine mouse IgG secondary antibodies were used for visualization of primary antibodies (Jackson ImmunoResearch, West Grove, PA).

After gene transfer and incubation for 2 days, the retinas were fixed with 4% paraformaldehyde for 25–30 min at room temperature (RT) and washed three times for 5 min each in phosphate-buffered saline (PBS), then placed in blocking solution consisting of 4% donkey serum in PBST (PBS, then placed in blocking solution consisting of 4% donkey serum in PBST (PBS + 0.3% Triton-X). Primary antibodies were diluted in blocking solution and applied to the tissue for 3–4 days at RT. They were then washed three times for 5 min each in PB. Secondary antibodies were diluted in blocking solution and applied at RT in the dark for 1 day. After washing three times for 5 min each in PB, the retinas were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Image acquisition and analysis

Individual cells were imaged using a confocal microscope (Radiance, BioRad) with water-immersion objectives (×25/0.8 Plan Apochromat, ×40/1.2 C-Neofluar, or ×63/1.4 C-Neofluar, all from Carl Zeiss Microimaging). Through-focus series of images were taken of labeled cells in the ganglion cell layer. Optical sections were taken at thicknesses of 0.25 and 0.5 μm. The resulting images were adjusted for brightness and contrast with volume-reconstruction software (VolView2, Kitware). The images were median filtered and collapsed into a maximum-intensity projection in ImageJ software (National Institutes of Health, Bethesda, MD) and VolView. The confocal images in Figs. 4 and 6 show planar projections of a series of successive confocal images.

To estimate the length and location of sodium-channel bands, lines were drawn at the proximal and distal edges of the high-density region using ImageJ software. Contrast differences (between band and background) were at least twofold and often larger than threefold, thus facilitating the visual detection of the band edges. Use of a ×63 objective in this region further enhanced our estimates of edge locations. The length of the high-density sodium-channel band was taken as the distance between the two edges. The distance between the soma and the high-density region was taken as the distance between the proximal edge of the soma and the proximal edge of the high-density region. Images were formatted in Photoshop CS2 (Adobe).

RESULTS

Our general approach was to map thresholds in and around the soma/proximal axon region and then determine the specific anatomical site aligned with the low-threshold region. To eliminate potential sources of variability that might arise from differences between ganglion cell types, we restricted our initial analysis to a single type, known as the directionally selective (DS) ganglion cell.

Threshold maps

We refer to the plot of threshold versus position of the stimulating electrode as a threshold map. A typical map is shown in Fig. 2A. The color of each square represents the threshold for eliciting action potentials when the stimulating electrode was positioned at that spatial location (see METHODS). The location and approximate size of the soma are depicted by the black circle.

The overall appearance of the threshold map shows a central region of low threshold surrounded by concentric “rings” of increasing threshold. This arrangement suggests a single region
of the neuron is associated with low thresholds—placement of
the stimulating electrode near this region results in the lowest
thresholds.

Threshold maps vary for different ganglion cell types

To explore differences across different ganglion cell types,
we measured threshold maps in two additional cell types: local
dge detectors (LEDs) (Rockhill et al. 2002; Roska and
Werblin 2001; van Wyk et al. 2006) and brisk transient/alpha
(BT) cells (Caldwell and Daw 1978; Peichl et al. 1987; Rock-
hill et al. 2002). Threshold maps from these other ganglion cell
types were qualitatively similar to those of DS cells in that they
both consisted of a single low-threshold region that was offset
from the soma. However, both the center and size of the
low-threshold region varied by type. For example, in the maps
from LEDs, the center of the low-threshold region was closer
to the soma than those from DS cells (Fig. 2B, n = 2, compare
with Fig. 2A). Also, the size of the low-threshold region
appeared smaller than that of DS cells. A threshold map for one
BT cell was also qualitatively similar, although the size of the
low-threshold region was larger than that of either LEDs or DS
cells (n = 1, data not shown). The center of the low-threshold
region in the BT cell was at a similar distance from the soma
as that of DS cells.

Shape of equal threshold contours

The contours of equal threshold (i.e., the “rings” of similar
colored squares) appeared circular in some cases (i.e., Fig. 2A),
whereas in others they appeared more elliptical (i.e., Fig. 3A).
To estimate the circularity of these contours, we measured their
diameter in two orthogonal directions. The first measurement
was along a line that was coextensive with the proximal axon.
The second diameter was orthogonal to the first and passed
through the center of the low-threshold region. The ratio of the
two diameters provided a measure of eccentricity: ratios close
to 1.0 indicated contours that were approximately circular,
whereas ratios above or below 1.0 indicated contours that were
more elliptical. The mean ratio was 1.09 ± 0.07 (n = 6);
values ranged from 0.99 to 1.18. In nearly all cases (n = 5/6),
the diameter measured along the proximal axon was longer

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**FIG. 3.** Low-threshold regions are spatially distinct from the soma and axon bend. A, top: the trajectory of the proximal axon was estimated by drawing a
line from the soma to the start of the distal axon (single column of threshold measurements at left; see also Figs. 2B and 6A). The scale correlating color to
amplitude is identical to that of Fig. 2A. Bottom: measured thresholds along this line (“X”) were fit with a second-order curve (solid line); the minimum point
along the fit curve was set as the center of the low-threshold region. The length of the axon for which thresholds were within 2 μA of minimum determined the
length of the low-threshold region. The length of the axon bend region, to the left of the soma, represents a 6-μm-wide region adjacent to the soma (see text). B: each thin line corresponds to the low-threshold region from a threshold map for a different DS cell. The length of each line is proportional to the length of the low-threshold region and the distance between the closest point of the region and the closest edge of the soma is proportional to the distance between the region and the soma. The thick horizontal line represents the mean (size and location) of all low-threshold regions. The
axon bend region, to the left of the soma, represents a 6-μm-wide region adjacent to the soma (see text). C: 2 adjacent ganglion cells (green) extend dendritic
processes down into the inner plexiform layer and axons up to the nerve fiber layer (NFL, arrowhead). For the cell on the right (DS), the axon emerges from
the vitreal end of the soma and ascends directly to the NFL; the bend (arrow) occurs within the lateral boundaries of the soma. For the cell on the left (non-DS),
the axon emerges from the approximate midline of the soma and ascends slowly to the NFL; the bend is about 45 μm from the soma (arrow). Scale bars in A
and C are both 50 μm.
than the corresponding orthogonal measurement. In the one case for which it was not, the two diameters were approximately equal (ratio = 0.99).

Absolute thresholds vary across ganglion cell types

Casual comparison of the maps from different ganglion cell types suggested that the absolute thresholds (lowest threshold across the map) were different for each type. To explore this further, we determined the absolute threshold in BT, DS, and LED cells using an abbreviated mapping routine (METHODS). This method allowed us to determine the approximate lowest threshold in individual cells without having to complete lengthy maps for each cell. A summary of the thresholds for each of the three types is shown in Fig. 2C. The difference in threshold for each of the three possible pairs of cell types was found to be highly significant ($t > 4$, $P < 0.0007$ for all pairs). A one-way ANOVA also showed a significant difference in the distribution of threshold across the three cell types [$F(2,19) = 42$, $P < 0.0001$]. The difference in absolute thresholds between types suggests that the part(s) of the neuron underlying the response to electric stimulation is different for these three types.

Region of minimum threshold is offset from the soma

The region of lowest threshold identified in threshold maps was always offset from the soma (DS cells: $n = 6/6$; LED cells: $n = 2/2$; BT cells: $n = 1/1$). During preparation of our slides, the optic disk was placed on the left side (left/right orientation is as seen in Fig. 2) so that axons generally coursed to the left. With this arrangement, the region of low threshold was always offset to the left side of the soma. This orientation is maintained in all subsequent figures, unless specified. To quantify the offset, we fit the raw threshold data along the estimated trajectory of the proximal axon (METHODS) with a second-order curve (Fig. 3A, bottom); the center of the low-threshold region was taken as the spatial location corresponding to the minimum value of the curve. The length of the low-threshold region was defined by the projected portion of the curve (thick horizontal bar in Fig. 3A, bottom) within 2 $\mu$A of the minimum.

A summary of low-threshold regions for all measured DS cells is shown in Fig. 3B. Each horizontal thin line represents a single low-threshold region: the length and position represent the length and position of the corresponding low-threshold region. The mean center of all low-threshold regions was 36.8 $\pm$ 10 $\mu$m from the soma. This relatively large distance suggests that the soma is not the site of lowest threshold. Thus our results do not support the conclusions of a previous computational simulation (Greenberg et al. 1999) (see DISCUSSION). The mean length of all low-threshold regions was 36.8 $\pm$ 5.6 $\mu$m. This value is somewhat arbitrary, however, because it is determined by the offset from minimum used with the second-order curve; e.g., use of a 3-$\mu$A offset would yield a longer low-threshold region. Other factors, such as the distance of the stimulating electrode from the retinal surface, would also affect the length of this region. Therefore we used only the center of the low-threshold region to analyze the spatial correlation between regions.

The offset of the low-threshold region was always toward the optic disk, suggesting that one or more anatomical features of the proximal axon (axon bend, axon hillock, sodium-channel band, initial segment, etc.) might align with the low-threshold region.

Axon bend is not aligned with the region of low threshold

One site reported to have the lowest threshold is the axon bend (Schiefer and Grill 2006)—that portion of the proximal axon that curves to orient the emerging axon with other fibers of the nerve fiber layer (NFL). We examined this proposition by visualizing individual cells following the methods of Koizumi et al. (2007), which resulted in a few brightly filled ganglion cells per retina (METHODS). Once the filled ganglion cell was identified as DS (METHODS), a three-dimensional confocal reconstruction was used to directly visualize the position of the axon bend (Fig. 3C).

In DS cells, we found that the bend typically occurred within 6 $\mu$m of the soma (Fig. 3C, cell on right, $n = 16/18$). In some cases, (e.g., the DS cell in Fig. 3C), the bend in the axon occurred even before the axon extended beyond the lateral edge of the soma. In the two cases for which the bend was located >6 $\mu$m from the soma, it appeared as if the emergent axon fiber was “trapped”—caught below a bundle of axon fibers that prevented the lone axon from ascending to the NFL. As a result, the single axon ran parallel to, and below, the NFL for as much as 125 $\mu$m before finally curving up to enter the NFL (not shown).

The “axon bend region” in Fig. 3B depicts a 6-$\mu$m-wide region that is adjacent to the soma. Most axon bends (16/18) occurred within, or to the right of, this region. Therefore the bend was typically >30 $\mu$m from the center of the low-threshold region, suggesting that it is not associated with the region of low thresholds. This is also true for the other two cases (2/18) since in these instances the bend location was >100 $\mu$m from the soma, far beyond the distal edge of all measured low-threshold regions. Therefore in all examined DS cells ($n = 18$), the axon bend was not aligned with the region of low threshold. Thus our results do not support the conclusion of the above-mentioned computer simulation (Schiefer and Grill 2006) (DISCUSSION).

We noticed that the nature and location of the axon bend varied across ganglion cell types (for example, compare the two cells in Fig. 3C). However, we did not attempt to quantify the number of different bend types that existed or to characterize their properties along different cell types.

Imaging Na$^+$ channel distribution

Electric stimulation is thought to initiate spiking by triggering voltage-gated sodium channels (for a review see Ratnav 1999); high-density regions of such channels are therefore likely to be associated with low thresholds. Dense bands of voltage-gated sodium channels have been localized within the proximal axons of retinal (Boiko et al. 2003; Van Wart et al. 2007; Wollner and Catterall 1986) and several different types of nonretinal neurons (Inda et al. 2006; Kole et al. 2008; Meeks and Mennerick 2007), making the bands a candidate for alignment to the region of low thresholds. However, the precise location and extent of sodium-channel bands in retinal ganglion cells have not been previously reported (but see Boiko et al. 2003; Van Wart et al. 2007; Wollner and Catterall 1986).
In addition, bands from specific ganglion cell types have not been studied and therefore it is not known whether differences exist between types. Therefore we characterize the properties of ganglion cell sodium-channel bands here.

Following methods similar to those of previous studies (Boiko et al. 2003; Inda et al. 2006; Kole et al. 2008; Meeks and Mennerick 2007; Van Wart et al. 2007), we immunohistochemically stained for one of two antibodies associated with voltage-gated sodium channels of the axon: 1) Ankyrin G, a structural protein associated with high-density sodium-channel bands found at the initial segment (Jenkins and Bennett 2001) and at nodes of Ranvier (Caldwell et al. 2000) or 2) pan sodium antibody (PAN), which binds to all known subtypes of voltage-gated sodium channels (Boiko et al. 2003; Van Wart et al. 2007). Once a cell was anatomically confirmed as DS (Methods) (Fig. 4A), high magnification was used to examine the proximal axon, an approximately 150-μm length, as it emerged from the soma (Fig. 4B). Overlay of the Ankyrin G staining and the filled ganglion cell axon (Fig. 4D) revealed that one of the long thin bands of dense Ankyrin G staining corresponded with a portion of the ganglion cell axon (region between the vertical lines in Fig. 4C). It is likely that the other long thin bands in Fig. 4C (arrows) are associated with axons of other ganglion cells that were not filled with GFP and thus are not visible (Boiko et al. 2003; Van Wart et al. 2007; Wollner and Catterall 1986). We also observed highly variable punctate staining across preparations (Fig. 4C) that was likely a result of nonspecific binding of the Ankyrin G antibodies. We did not investigate the source of this “noise” since it did not interfere with our subsequent analysis of the lengths and locations of the dense bands. Consistent with previous reports (Boiko et al. 2003), we found similar patterns of staining when PAN was used (data not shown).

We quantified the physical properties of the region of high-density staining in a total of 21 DS ganglion cells: 12 stained with Ankyrin G and 9 with PAN. Using Ankyrin G, the mean length of the high-density region was 28.0 ± 4.0 μm and the mean distance from the soma was 24.4 ± 6.6 μm. Using PAN, the mean length was 29.4 ± 5.8 μm and the mean distance from the soma was 21.7 ± 5.8 μm. There was no statistical difference between the length or distance-to-soma measures (P = 0.522 and 0.190, respectively, unpaired t-test) when values from Ankyrin G and PAN were compared. Therefore we grouped results for Ankyrin G and PAN together in the following analyses. A previous study (Koizumi et al. 2007) suggests that the methods we used to investigate DS ganglion cells and their sodium-channel bands (gene-gun of the retinal extract followed by 48-h incubation) did not alter the properties of the sodium-channel bands. For additional confirmation, we compared the length and location of DS cells that were fixed within 2 h of enucleation (n = 2) to those that were processed as described earlier (n = 19): the average length and distance from the soma were 30.7 ± 10.9 and 30.5 ± 2.2 μm, respectively. There was therefore no significant change in band properties as a result of these methods (P = 0.5948 for length and 0.1161 for distance from the soma).

We found that the position of the sodium-channel band completely filled the tapered region of the axon, the part in which the axon diameter transitioned from the relatively large hillock to the relatively thin diameter section originally described by Carras et al. (1992) (Fig. 4, E and F; n = 16/18). The alignment of the sodium-channel band to the taper persisted over the range of band lengths (23–40 μm) and locations (proximal edge: 14–36 μm from the soma). In many cases, sodium-channel staining extended slightly beyond the taper into the thick, thin, or both portions of the axon but did not extend along the majority of the thin section as suggested by previous computer models (Carras et al. 1992; Fohlmeister and Miller 1997). The alignment of the sodium-channel band to the tapered region is consistent with findings from Van Wart et al. (2007).

High-density sodium channels are aligned with the low-threshold region

Since the region of low threshold and the region of high sodium-channel density were both localized to a similar portion of the proximal axon, we explored the correlation between the physiologically and anatomically identified regions. We started by plotting representations of all low-threshold and all high-density sodium-channel regions with respect to their respective somas (Fig. 5). The representation of low-threshold regions (thin solid lines) is identical to that shown in Fig. 3B. Thin dashed lines were used to similarly represent sodium-channel bands. The location of the “average” region of low threshold (thick solid line) and the location of the “average” sodium-channel band (thick dotted line) were similar: the centers of the two “average” regions were offset by <1 μm. This suggests that the lowest thresholds arise when the stimulating electrode is closest to the sodium channel band.

In one of the six cells for which we obtained a threshold map (one of the six cells described in Fig. 3B), we were also able to complete immunochemistry (Fig. 6, n = 1). For this cell, we imaged the dendrites by filling the cell with neurobiotin via whole cell patch clamp (Methods). The cell was subsequently labeled via a GFP-streptavidin conjugate to capture the cell’s morphology. Simultaneous immunostaining for sodium channels was done with Ankyrin G as described previously. Each of the physiological and immunochemical procedures for this one experiment required a different mounting of the retina; therefore it was necessary to align the threshold map (Fig. 6A) obtained in the first procedure, to the Ankyrin G immunostain obtained in the second procedure. This was accomplished by aligning the two-dimensional trajectory of the distal axon from both plots (Fig. 6B). Each column of threshold measurements along the distal axon was fit with a second-order curve; the axon location was assigned as the location corresponding to the curve minimum (“X”) (Jensen et al. 2003). This allowed corresponding points along the distal axon from both the threshold map and the filled cell to be aligned. In addition, the first threshold measurement (of the map) was always centered over the soma, providing an additional point of alignment. This method fixed the relative positioning of the two images and allowed the locations of the physiological and anatomical regions to be accurately compared. A higher-magnification view of the overlaid images (Fig. 6C) confirms that the region of low threshold is coextensive with the region of high-density sodium channels. This close alignment is also seen in Fig. 5: the low-threshold region and sodium-channel band from this one cell are indicated by arrows. The close alignment between the two regions, in both population and
FIG. 4. Dense sodium-channel staining is limited to a small region of the axon. A: confocal image stack of a green fluorescent protein (GFP)-filled DS ganglion cell. The characteristic morphological features include a relatively large soma as well as bistratified and recursive dendritic processes. B: higher-magnification view of the soma and initial portion of the axon. C: immunocchemical staining for Ankyrin G (red) reveals several long, thin segments (arrows). The small, punctuate staining is an artifact associated with nonspecific binding of one of the antibodies. D: overlay of B and C reveals that one of the long Ankyrin G segments is coextensive with a portion of the axon. Vertical lines from C help clarify the edges of the Ankyrin G staining. E: the dense sodium-channel region is colocalized to the region where the axon diameter decreases. F: close-up view of the soma and axon from a DS ganglion cell (green) with pan sodium antibody (PAN) immunostaining (red). The solid vertical lines indicate the approximate extent of the dense sodium-channel region and reveal that the dense sodium-channel region fills the tapered portion of the axon (compare the axon diameter at the right and left solid lines in E). The dashed vertical line indicates the edge of the soma. The distance between the 2 solid lines was used to determine the length of the band; the distance between the dashed line and the closest solid line was used to determine the distance between the soma and the band. Scale bar in A: 25 μm. Scale bar in B: 25 μm also applies to C and D. Scale bar in E: 20 μm, also applies to F.
single-cell results, provides strong support for a spatial association between the band of dense sodium channels and the region of low threshold.

**Differences across ganglion cell types**

The differences in size and location of the low-threshold region for different ganglion cell types (Fig. 2), coupled with spatial alignment of the sodium-channel band and region of low thresholds, suggest that sodium-channel-band properties may vary between cell types. To examine this, we measured band properties in additional cell types (non-DS). The average length of all measured sodium-channel bands in BT cells ($n = 13$) was $40.6 \pm 5.39 \, \mu m$ and the average distance between the proximal edge of the band and the proximal edge of the soma was $26.9 \pm 5.5 \, \mu m$. Therefore sodium-channel bands in brisk transient cells were longer than those from DS cells ($28.6 \pm 4.8 \, \mu m$ for DS vs. $40.6 \pm 5.39 \, \mu m$ for BT, $P \ll 0.001$), although there was no statistical difference between their location—e.g., distances from the soma were similar (DS: $22.8 \pm 6.4 \, \mu m$; BT $26.9 \pm 5.5 \, \mu m$, $P = 0.065$). To better visualize the difference in band properties, we plotted length versus location (distance from the soma) for measured bands of each type (Fig. 7A). The scatterplot reveals that there was minimal overlap between bands from the two types. Interestingly, bands from each type appear to be separated by a line that is a function of both length and distance. In other words, bands from both DS and BT cells can be the same length (e.g., $35 \, \mu m$), but if so, the bands from DS cells tend to be closer to the soma than those from BT cells. The negative slope of the line separating the two populations raises the possibility that for these two cell types the length and location of sodium channel bands are not independent variables and, in fact, may be inversely correlated. This is similar to a recent finding in chick auditory neurons, which showed a similar inverse correlation between band length and distance from the soma (Kuba et al. 2006).

**Fig. 5.** High-density sodium-channel and low-threshold regions are spatially coextensive. Each thin dashed line represents a measured sodium-channel band. The length of the line corresponds to the length of the band and the distance between the line and the soma corresponds to the distance between the band and the soma. The thick dashed line represents the arithmetic mean of all high-density regions. The solid lines are identical to those in Fig. 3B: each thin line corresponds to the length and position of each region of low threshold. The thick line represents the mean size and location of all low-threshold regions. The axon bend region is identical to that of Fig. 3B and represents a distance of 6 \( \mu m \) from the soma. The top and bottom arrows indicate the low-threshold region and the sodium-channel band, respectively, from the same cell (see Fig. 6).
To further explore the differences in sodium-channel bands between types, we measured the bands in 19 additional non-DS, non-BT ganglion cells. We found additional diversity across the population (Fig. 7, B and C). For example, some bands were much closer to the soma than either DS or BT cells (Fig. 7B, left), whereas other bands were much shorter than those of DS (and BT) cells (right panel). The bands from unidentified ganglion cell types were added to the length versus location scatterplot (Fig. 7C), revealing that not all cell types have unique band properties. For example, many non-DS, non-BT cells (triangles) had sodium-channel bands with properties that were similar to those of DS cells. Across the population, band lengths and locations were bounded: lengths ranged from 10 to 50 μm, whereas distance from soma ranged from 0 to 40 μm.

**DISCUSSION**

**Sodium-channel bands are the source of low thresholds**

Our study is the first to precisely map threshold in response to electric stimulation of retinal ganglion cells and then correlate the physiologically identified low-threshold region to a specific anatomical site. We showed a correlation between the low-threshold region and the band of sodium channels located in the tapered segment of the proximal axon. In DS cells, the low-threshold region and sodium-channel band were both centered nearly 40 μm from the soma. The locations varied in other ganglion cell types (Figs. 2 and 7); however, the location of the low-threshold region presumably corresponds to the sodium-channel band for all cell types. The close association of the low-threshold region with the dense band of sodium channels leads us to speculate that this region is the same site in which spikes are initiated in response to electric stimulation.

**Comparison with previous studies**

Previous physiological studies in both retinal and nonretinal neurons support our finding that the location of the sodium channel band is coextensive with the location of the region of lowest thresholds. For example, Gustaffson and Jankowska (1976) concluded that thresholds in response to electric stimulation were lowest in the initial segment (proximal axon) of cat spinal motoneurons, although they were not able to identify a more specific location within the initial segment. In response to electric stimulation of retinal ganglion cells, Jensen et al. (2003) found that thresholds were lowest when the stimulating electrode was positioned close to (but offset from) the soma. The appearance of the “map” obtained by Jensen et al. is not consistent with the maps presented here. For example, their low-threshold region was not radially symmetric around the lowest point, nor was the site of lowest threshold offset toward the optic disk. The reasons for the discrepancies are not clear—perhaps the finer grid spacing used here resulted in a more uniform appearance in our plots. The difference in offset is harder to reconcile. Sekirnjak et al. (2008) similarly found that the region of lowest threshold was offset from the soma. Estimations of soma and axon positions derived from multielectrode array recordings allowed them to generate a map of

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**FIG. 6.** The sodium-channel band is coextensive with the region of low threshold. A: threshold map of a DS ganglion cell. The map includes threshold measurements over the soma/proximal axon region (right) as well as along more distal sections of the axon (columns in the middle and the left). The threshold values given by each color are identical to those in Fig. 2A. The circle indicates the position of the soma. B: overlay of the threshold map with the dye-filled ganglion cell. “X”s indicate the position of lowest threshold in each column and were aligned to the corresponding portion of the distal axon in the dye-filled cell. C: a higher-magnification view of the soma/proximal axon region from B (indicated by the rectangular box in B). The position of the sodium-channel band, indicated by the arrows, is in the approximate center of the low-threshold region. Scale bar in A, B, and C: 50 μm.
thresholds for the population of parasol cells; the general appearance of the map derived from their population results is consistent with our findings that thresholds are lowest along the proximal axon. They conclude that the center of the low-threshold region was probably along the proximal axon, in line with our findings. The estimated center of their low-threshold region was about 13 μm from the soma, leading us to predict that the center of the sodium-channel band in parasol cells is at this same location.

The site of lowest threshold identified here is different from those predicted by each of two previous computational models. Greenberg et al. (1999) found that thresholds were lowest when the stimulating electrode (point source or disk) was positioned over the soma. Surprisingly, the predicted site of

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**FIG. 7.** Different ganglion cell types have different sodium-channel bands. A: each point represents a single sodium-channel band from either a DS (filled) or BT (open) cell and plots the length vs. distance from the soma for the band. B: examples of sodium-channel bands from 2 unidentified ganglion cell types. Note the variability in length and location of the bands. C: sodium-channel bands from additional, unidentified ganglion cell types are shown alongside the bands from DS and BT cells. Scale bar in B: 25 μm.
spike initiation was the proximal axon, regardless of electrode type. This is easier to understand for disk electrodes: when the electrode is centered over the soma, the largest current densities and electric fields (found in the disk periphery) are centered over the proximal axon. It is harder to interpret the results from the point source electrode since in this case the maximum “activating force” is centered over the soma—it seems that threshold should be lowest when centered over the proximal axon. In our experiments, the stimulating electrode was conical with a height and base diameter that were each about 30 μm. Although this is not quite a point source at the distances we used (25 μm), both voltage measurements and modeling data (not shown) indicate that the electric field was largest near the tip of our electrode. With this configuration, we found thresholds were lowest when the stimulating electrode was positioned over the sodium-channel band in the proximal axon for all ganglion cell types tested (i.e., not over the soma). Thus we conclude that the soma is not the site of lowest threshold in retinal ganglion cells. We cannot reconcile the discrepancy with the Greenberg et al. model, although we note that the modeled neuron from their simulation does not contain a dense band of sodium channels in the proximal axon, perhaps contributing to the different findings.

Our results are also inconsistent with those from another computational model that predicted the lowest thresholds to electric stimulation occurred when the stimulating electrode was positioned over the axon bend (Schiefer and Grill 2006). We examined three-dimensional confocal reconstructions of DS cells, which allowed us to directly visualize the bend (Fig. 3C), revealing that it occurred within 6 μm of the soma edge in DS cells. The bend was therefore typically offset from the center of the low-threshold region by >30 μm. If the bend contributed to low thresholds in other cell types, we might have expected to see an inflection in threshold amplitudes as the stimulating electrode was moved across the bend, but this was not the case (data not shown). Therefore we conclude that the bend is not associated with the region of low thresholds in the retinal ganglion cell types we examined. Like the Greenberg et al. study, the Schiefer and Grill model did not include a dense sodium-channel region in the proximal axon, perhaps contributing to the discrepancy in results.

Comparison with synaptic input studies

With the site of low threshold in response to electric stimulation identified, we can now compare this to the site of spike initiation in response to synaptic stimulation. Similar to earlier synaptic input studies in nonretinal neurons (Coombs et al. 1957a,b), the site of spike initiation in retinal ganglion cells was determined to be within the proximal axon (Carras et al. 1992). Although this is consistent with the low-threshold region that we describe, a more precise site within the proximal axon was not specified, making more detailed comparison impossible. Most synaptic input studies in nonretinal neurons indicate that the sodium-channel band is the site of spike initiation, although others indicate that spiking is initiated in other portions of the proximal axon. Several of the studies that found spiking initiated in nonband locations also did not find clear evidence of a high-density region in the proximal axon. This raises the possibility that, when present, the band is the site of spike initiation for both electric stimulation and synaptic input.

Should the contours of equal threshold appear circular?

Given that the sodium-channel bands are thin and elongated, it seems reasonable to assume that the contours of equal threshold from the threshold maps might be elliptical. Although the contours were somewhat elliptical in a few cases (i.e., Fig. 3A), in others they appeared more circular (Fig. 2). This visual observation is supported by the ratios of orthogonal diameters that ranged from 0.99 to 1.18. In nearly all cases (n = 5/6), the major diameter was along the line containing the sodium-channel band, suggesting that the long, thin nature of the band may contribute, at least in some cases, to the elliptical appearance of the map. If this is the case, however, it is not clear why the contours of equal threshold appear circular in other cases.

The circularity of the contours may be influenced by several factors besides the shape of the sodium-channel band. For example, although we originally believed that the electric field arising from the stimulating electrode should be circularly symmetric (similar to that of a point source) (McIntyre and Grill 2001), it is possible that this was not the case in our setup. Examination of the tips of the stimulating electrode under high magnification often revealed imperfections in the shape and axis (of the cone). In addition, the angle of insertion of the stimulating electrode was 30° (the angle between the long axis of the stimulating electrode and the plane of the retina) and the long axis of the stimulating electrode was generally orthogonal to the long axis of the band. Any or all of these factors could make the electric field asymmetric. If the field was elliptical, with its long axis perpendicular to the long axis of the sodium-channel band, it is possible that this might serve to “round out” the contours. Another possibility is that the density of sodium channels within the band is not uniform. If the density was such that sodium channels were highly concentrated within a small region (i.e., much smaller than the band), the expected contours could be circular.

Still another potential factor that might influence the shape of the contours arises from the population of voltage-gated sodium channels within the band. There are many different subtypes of voltage-gated sodium channels (Trimmer and Rhodes 2004); each type has different kinetics and different sensitivity to voltage changes. Recently, it has been shown that the sodium-channel band in rat ganglion cells is comprised of at least two different subtypes (Van Wart et al. 2007) (the immunostaining methods that we used do not distinguish between types). If one of the subtypes is more sensitive to extracellular electric stimulation, it is possible that a smaller portion of the band underlies the response. This arrangement would similarly lead to contours that were more circular. Our methods did not allow us to quantify these different factors and therefore it is difficult to speculate as to the contour shape that should be expected. Further studies are needed to quantify each of these potential influences and determine how each modulates the response to electric stimulation.
SODIUM-CHANNEL BANDS SHAPE THE RESPONSE TO ELECTRIC STIMULATION

Sodium-channel band properties vary across ganglion cell types

Our results suggest that the spatial properties of the dense bands of voltage-gated sodium channels located within the proximal axon of retinal ganglion cells can be different for different ganglion cell types. Initially, we measured the length and location of bands in two ganglion cell types—DS and BT—and found that BT bands were significantly longer, although the distance from the soma to the proximal edge of the bands was comparable (Fig. 7A). Additional measurements of sodium-channel bands in unspecified ganglion cell types indicate that additional variations in both length and location are present (Fig. 7C). In some cases, we found band length and location were distinct for different ganglion cell types—e.g., DS versus BT (Fig. 7A), whereas in others, the lengths and/or locations overlapped (Fig. 7C). Since band properties were consistent for each of the two ganglion cell types we studied in detail (DS, BT), it is likely that band properties are also consistent within other ganglion cell types, although further study is needed to confirm this.

Why do the properties of sodium-channel bands vary in different ganglion cell types? It is tempting to speculate that the differences in band properties contribute to some aspect of spike generation. For example, the sodium-channel band may be optimized to reduce threshold for spike initiation in response to synaptic input or it may somehow modulate one or more properties of the spike train that are known to be different in different ganglion cell types (DeVries and Baylor 1997; O’Brien et al. 2002; Roska and Werblin 2001). This hypothesis is supported by findings outside the retina (Kuba et al. 2006): each auditory neuron that responds to different characteristic frequencies (CFs) has different band properties (length, location). Within neurons of a given CF, the band was positioned at the site that maximized the spiking difference between inputs that were in phase versus those that were out of phase. Adjusting band position in either direction reduced the difference, making the cell less sensitive for that CF. In ganglion cells, band locations may be similarly optimized for some aspect of spike timing as well, perhaps underlying the precise spike timing that occurs between individual spikes of nearby ganglion cells (Mastronarde 1989; Meister et al. 1995). Differences in band properties across other types of CNS neurons (Inda et al. 2006; Meeks and Mennerick 2007) support the notion that bands for each type are optimized for one or more aspects of normal neural function.

Our results also allow us to speculate that other properties of the band contribute to the spiking response. For example, since BT cells have bands longer than those of DS cells (Fig. 7A) and since they also respond with higher spike frequencies (Caldwell and Duw 1978; DeVries and Baylor 1997), it is possible that longer bands underlie higher spike frequencies. Our results also suggest that individual properties (e.g., length, location) of the sodium-channel band may not vary independently (Fig. 7A). For example, the length and location of a given band may be optimized to reduce spike frequencies, similar to bands in neurons of the nucleus laminaris (Kuba et al. 2006). It is likely that other properties of the sodium-channel band (e.g., density of sodium channels), not quantified in this study, also contribute to the spiking response. Further studies are needed to determine how each property of the sodium-channel band affects the spiking properties of retinal ganglion cells.

Implications for the mechanism of retinal ganglion cell activation

Our findings have three important implications for the development of a retinal prosthesis. First, knowledge of the region in which spikes are initiated allows the mechanism of activation to be studied. The mechanism underlying the activation of axons of the peripheral nervous system has been well studied and has led to improvements in stimulation efficiency and to more selective stimulation methods, such as methods that allow selective activation of specific axons within a fiber bundle (Grill and Mortimer 1997). The mechanism underlying activation of CNS neurons is not well understood; our identification of the site of lowest threshold is a step toward resolving the underlying mechanism. In addition, our findings can be incorporated into new computer models with which improvements in stimulation efficiency and methods of selective activation can be studied more accurately. A better understanding of the mechanism of activation for the proximal axon region may also help to reduce activation of distal axons, previously shown to have thresholds only slightly higher than those of the soma/proximal axon region (Greenberg et al. 1999; Jensen et al. 2003, 2005b; Schiefer and Grill 2006).

Second, our results may help to better understand responses in the degenerate retina. Several studies suggest that thresholds for activating ganglion cells in degenerate retina are larger than those in healthy retina (Chen et al. 2006; Humayun et al. 1994; O’Hearn et al. 2006). Synaptic reorganization of the retina (Jones and Marc 2005; Marc and Jones 2003; Marc et al. 2003) may contribute to these changes—e.g., if the amount of synaptic input to ganglion cells is reduced, the cell may hyperpolarize with a corresponding increase in threshold. Our findings suggest an alternative, or possibly complementary, explanation. Both the function and distribution of voltage-gated sodium channels are altered as the result of some disease processes (Chahine et al. 2008; Dib-Hajj et al. 2007; Gold 2008), raising the possibility that sodium-channel bands in ganglion cells of degenerate retina may be altered as well. The effect of changes to the sodium-channel bands is not addressed directly by our results, although the threshold differences that we found (Fig. 2B) suggest that changes to the band may alter thresholds. The impact of band variations on threshold should be assessed relative to the effects from other possible causes, such as a change in synaptic input.

Finally, the differences in sodium-channel bands and other anatomical/physiological properties between ganglion cell types raise the possibility that each type may be preferentially activated by a different stimulus (i.e., a different electric field). For example, a given stimulus may activate one type of ganglion cell strongly while minimally activating a different type. This is supported by our finding that different ganglion cell types had different thresholds (Fig. 2B). Since much of primate vision is thought to be mediated by only two ganglion cell types (midget and parasol), methods that allow selective activation of even one of these types might dramatically improve control of elicited percepts. At the very least, our threshold results suggest that not all ganglion cell types are activated equally. Knowledge of these differences is essential.
for a better understanding of the retinal response—and ultimately the psychophysical response—to electric stimulation.

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