Both electrical stimulation thresholds and SMI-32-immunoreactive retinal ganglion cell density correlate with age in S334ter line 3 rat retina

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Chan LL, Lee EJ, Humayun MS, Weiland JD. Both electrical stimulation thresholds and SMI-32-immunoreactive retinal ganglion cell density correlate with age in S334ter line 3 rat retina. J Neurophysiol 105: 2687–2697, 2011. First published March 16, 2011; doi:10.1152/jn.00619.2010.—Electrical stimulation threshold and retinal ganglion cell density were measured in a rat model of retinal degeneration. We performed in vivo electrophysiology and morphometric analysis on normal and S334ter line 3 (RD) rats (ages 84–782 days). We stimulated the retina in anesthetized animals and recorded evoked responses in the superior colliculus. Current pulses were delivered with a platinum-iridium (Pt-Ir) electrode of 75-μm diameter positioned on the epiretinal surface. In the same animals used for electrophysiology, SMI-32 immunolabeling of the retina enabled ganglion cell counting. An increase in threshold currents positively correlated with age of RD rats. SMI-32-labeled retinal ganglion cell density negatively correlated with age of RD rats. ANOVA shows that RD postnatal day (P)100 and P300 rats have threshold and density similar to normal rats, but RD P500 and P700 rats have threshold and density statistically different from normal rats (P < 0.05). Threshold charge densities were within the safety limits of Pt for all groups and pulse configurations, except at RD P600 and RD P700, where pulses were only safe up to 1- and 0.2-ms duration, respectively. Preservation of ganglion cells may enhance the efficiency and safety of electronic retinal implants.

Retinal remodeling may influence the treatments described above. Subsequent to the loss of photoreceptor synaptic inputs of the inner nuclear layers, bipolar and horizontal cells undergo remodeling and secondary death in both human and animal RP models (Marc and Jones 2003). In late-stage RP, severe rewiring of the retinal circuitry due to cellular translocation and neurite sprouting alters the normal neural pathway. Despite the loss of cells in the retinal layers reported in morphometric studies of late-stage human RP retinas (Humayun et al. 1999), the remaining cells are viable and blind humans perceive light when these cells are electrically stimulated, a finding that has motivated several major efforts toward bioelectronic ocular implants (retinal prostheses). Several groups worldwide have conducted clinical trials in which test subjects with implants could detect light and perform visually guided tasks (Besch et al. 2008; DeMarco et al. 2007; Gerdig et al. 2007; Rizzo et al. 2003b; Yanai et al. 2007; Zrenner et al. 2010).

Human studies support the finding that healthier retinas have lower electrical stimulation thresholds compared with diseased retinas (Rizzo et al. 2003a; Yanai et al. 2003). Morphometric analysis of enucleated eyes from late-stage human RP has demonstrated that retinal ganglion cells (RGCs) are reduced by as much as 60% (Humayun et al. 1999; Santos et al. 1997; Stone et al. 1992). Therefore, it is reasonable to speculate that a reduced number of cells may play a role in elevating the threshold values under electrical stimulation at a late stage of RP. However, obtaining simultaneous electrophysiology and histology in clinical trials of retinal prosthesis is almost impossible. Animal studies have investigated sensitivity to electrical stimulation in normal and degenerate retinas. Several studies have shown an increase of stimulus threshold in degenerate retina (Chan et al. 2008; O’Hearn et al. 2006; Suzuki et al. 2004; Ye and Goo 2007), including our previous report. One study reported a constant threshold increase at multiple ages of rd1 mouse (Jensen and Rizzo 2008). In contrast, P23H rats were shown to have no threshold changes even after 2 yr of degeneration (Sekirnjak et al. 2009). None of these studies included detailed anatomic analysis. Two studies examined thickness of degenerated retina (O’Hearn et al. 2006, Sekirnjak et al. 2009). Discrepancies exist among the morphological studies of degenerate animal retina, possibly due to differences in the strains and elapsed time of retinal degeneration. Most groups have reported a decline in RGC count in degenerate retina (Caley et al. 1972; Grafstein et al. 1972; Farber et al. 1994; Kolomiets et al. 2010; Ward 1982), while a few recent studies have shown near-normal ganglion cell layers (Mazzoni et al. 2008; Streit et al. 2002). Thus no clear explanation exists for the increased threshold consistently noted in human
studies. In this study, we measured both electrophysiological and morphometric properties over an extensive age range in a rodent model of retinal degeneration.

**MATERIALS AND METHODS**

**Animals**

Normal Copenhagen [postnatal day (P)90–P700, n = 11] and heterozygous S334ter line 3 (P84–P782, n = 25) rats were used in these studies; heterozygous S334ter line 3 rats are referred to here as RD rats. Animals were housed in covered cages and fed with a standard rodent diet ad libitum while kept on a 12:12-h light-dark cycle in the Doheny Eye Institute animal facility. Homozygous S334ter-line-3 rats were mated on site with Copenhagen rats (Charles River, Hollister, CA) to produce the heterozygous pigmented offspring. Homozygous breeding pairs of S334ter-line-3 rats were produced by Xenogen Biosciences (formerly Chrysalis DNX Transgenic Sciences, Princeton, NJ), developed and supplied with the support of the National Eye Institute by the courtesy of Dr. Matthew LaVail, University of California San Francisco. All experimental procedures were done according to protocols for animal care and use of animals reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California.

**Surgical Procedures**

All surgeries were performed under general anesthesia induced by intraperitoneal injection of a cocktail of ketamine (100 mg/kg; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (20 mg/kg; X-Ject SA, Butler, Dublin, OH) and maintained by sevoflurane (1% in 100% O2) throughout the entire experiment. Deeply anesthetized rats were positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), and the skin overlying the skull was cut open and retracted. Gas inhalant anesthetic was administered through an anesthetic mask. The animal’s temperature and pulse were monitored during the surgical procedures. Body temperature was monitored with a rectal thermometer and maintained at 37°C with a self-regulating electric heating blanket (model 50-7053-F; Harvard Apparatus, Holliston, MA). An effective anesthetic state was ensured throughout the experiment by monitoring the heart rate and blood pressure (Heska, Loveland, CO). Heart rate was monitored as feedback for adjustment of anesthetic level. After the experiments, all animals were euthanized by an intraperitoneal injection of pentobarbital (30 mg/kg; Butler).

**Insertion of Stimulating Electrode**

The surgical procedures of implantation of the stimulating electrode were similar to those reported in previous work (Colodetti et al. 2007; Ray et al. 2009). The pupil was dilated with 1% tropicamide (Tropicacyl, Akorn, Buffalo Grove, IL) and 2.5% phenylephrine (AK-Dilate, Akorn). The left eye was proposted under anesthesia. The fundus was viewed through an operating microscope. Focus was achieved by slightly flattening the cornea with a glass coverslip. With the eye gently proposted, an incision was made by a 25-gauge needle just behind the limbus. The needle was inserted at an angle of 45–60° with respect to the contact surface to avoid damaging the lens, which occupies roughly two-thirds of the globe in rodents. The stimulating electrode was the inner pole of a concentric bipolar Pt-Ir electrode (model CBDFG74, FHC, Bowdoin, ME) with a flat tip (inner pole diameter 75 μm, surface area 4.42 × 10⁻⁵ cm²). The stimulating electrode was mounted in a 1-ml syringe for handling and attached to a single-axis linear translational stage with 10-μm graduations (model NT33-475, Edmund Optics, Barrington, NJ) on a magnetic based articulating arm. The stimulating electrode was inserted through the insertion site along the same path as the needle and positioned in the ventral temporal quadrant without contacting the retina. Final positioning to ensure close proximity utilized impedance feedback as described below.

**Impedance Sensing for Positioning**

Electrode-tissue proximity can be indicated by an electrochemical impedance measurement (Duan et al. 2003; Zheng et al. 2000). Clinical and bench studies have shown that electrodes with increased impedance are closer to the retina (de Balthasar et al. 2008; Madevapampa et al. 2005; Shah et al. 2007). In our study, the proximity of the electrode to the retina was indirectly indicated by measurement of the electrochemical impedance with a commercial potentiostat (FAS1, Gamry Instruments, Warminster, PA). The Pt-Ir inner pole electrode was connected to the working electrode input, and two needle electrodes served as the counter and return electrodes, which were placed under the skin of the nose and tail, respectively. All impedances were measured at open-circuit potential with a 10-mV (r.m.s.) AC sinusoidal signal. The frequency chosen was 100 Khz. The impedance measurement was monitored before each stimulus current delivery. A custom circuit board was built to switch the recording between the impedance measurement and the stimulus.

**Recording Electrode Positioning**

To position the recording electrodes, the skull was exposed and a craniootomy of the right skull was made (caudal-medial corner: ~4 mm caudal and ~3 mm lateral to lambda) with a bit driven by a drill hand piece (Dremel, Robert Bosch Tool, Mount Prospect, IL). The overlying cortex was aspirated through the craniootomy until the surface of the superior colliculus (SC) was exposed (~depth of 4 mm from the dura mater). Epoxy-coated tungsten microelectrodes (10 MΩ, FHC) were positioned within the superficial gray or stratum griseum superficiale (SGS), the upper 100–200 μm after SC surface penetration. A characteristic noise produced by “juxtazonal potentials,” first described in experiments conducted on cats (Mcllwain 1978), was heard immediately after penetration of the pia over the SC from the speakers connected to the recording channel. This noise represents massive extracellular potentials from retinal axon spikes terminating in SGS layer, which exclusively processes visual information. Retinal input to SC is most dense in this layer. The SC was chosen because the majority of RGC axons terminate topographically onto SC surface in rodents (O’Leary et al. 1986; Simon and O’Leary 1992). The cells within this layer had no or very low spontaneous activity (Girman and Lund 2007). Spikes were recorded in 40% of experiments (example shown in Fig. 1A). Juxtazonal potentials were recorded in 60% of experiments (example shown in Fig. 1B). Both spikes and juxtazonal potentials are known as retina-driven SC responses. This recording technique is similar to other work that has used the SC recording to evaluate experimental treatments for retinal blindness (DeMarco et al. 2007; Girman et al. 2005; Kanda et al. 2004; Sagdullaev et al. 2003; Thomas et al. 2004).

The experiment required simultaneous positioning of two electrodes: one on the retinal surface and another on the SC surface. Above we described each positioning individually. Through trial and error, the following sequence was established to minimize animal movement during critical times of electrode positioning: 1) cranietomy, 2) stimulating electrode insertion into vitreous cavity, 3) SC exposure and recording electrode positioning, 4) stimulating electrode positioning on the epiretinal surface with impedance sensing.

**Electrical Stimulation**

Charge-balanced, cathodic first, biphasic currents (1–500 μA) were applied to the epiretinal surface across seven pulse durations (0.1, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0 ms) through a flat concentric bipolar stimulating Pt-Ir electrode (described above). The current pulses were generated

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by a voltage-to-current converter (model 2200, A-M Systems) driven by a voltage pulse from a programmable analog output card (DataWave Technologies, Berthoud, CO). The interphase interval was 100 μs. Blocking capacitors were used to minimize DC current. The electrode voltage was monitored after each delivery of a current pulse to ensure electrode integrity during the stimulation experiment and maintenance of the voltage within the compliance limits of the stimulator box. Electrode voltage was recorded and displayed by DataWave after signal conditioning with a commercial differential amplifier (model P55, Grass Technologies, West Warwick, RI).

Light Stimulation

A full-field strobe flash (1,000 cd/m², duration of 10 μs) was delivered to the test eye with a photostimulator (model PS 22 Photic stimulator; Grass Technologies) positioned ~30 cm in front of the rat’s eye. An interstimulus interval of 5 s was used. Light stimulation was performed to examine the condition of the retinas.

Measurement of Stimulus Threshold

The threshold current was determined as follows. The recording electrode was positioned in several locations within the medial-caudal SC region, where ganglion cell axons terminate from the ventral-temporal quadrant of the retina. With an X-Y micromanipulator with gradations, the coordinates of each location were recorded relative to the starting position to allow the electrode to be returned to the location with the lowest threshold. A series of stimulus pulses of increasing amplitude (1, 2, 5, 10, 20 μA; 1 ms/phase) was applied to the retina after repositioning the recording electrode. The low threshold location was selected based on visual inspection of the responses and the electrode positioned at the location where these responses were obtained. A more extensive series of stimulus pulses were applied. At each pulse duration (listed above), responses were recorded for several current levels. The starting stimulus level was based on the series of test pulses used to find the low threshold location. For example, if the 5-μA test pulse yielded a robust response and the 2-μA test pulse yielded no response, then the extensive threshold measurement began at 2 μA. Stimulus was increased in 1-μA steps, and 50 responses were recorded at each level. A custom algorithm processed this data immediately to determine whether threshold was reached. A response was defined as neural activity correlated with stimulus that exceeded 5 times the baseline noise level. Electrical activation threshold was defined as the amount of current required to evoke a response in 75% of all trials (n = 50) for the short-latency response (response window 0–10 ms). Others have noted that short-latency responses indicate the direct activation of the ganglion cell while long-latency responses indicate presynaptic activation of ganglion cells (Fried et al. 2006). This procedure was repeated across all pulse durations (100 μs to 2 ms). If stimulus amplitude reached 50 μA, the increment was increased to 10-μA steps.

Tissue Preparation

Upon completion of the electrophysiology, the animals were euthanized. After rapid enucleation, a small incision was made with a razor blade to indicate the superior pole of the globe. The corneas were slit, and the lens was removed. Eyecups were immersed in 4% paraformaldehyde in 0.4 M phosphate buffer (PB) for 2 h at 4°C. After fixation, eyecups were cryoprotected in 30% sucrose in 0.1 M PB. After 5 days, the eyecups were frozen in liquid nitrogen and stored at −70°C. For vertical sections, the eyecups were embedded in Tissue-Tek OCT compound (Tissue-Tek, Hatfield, PA) and fast frozen in liquid nitrogen. Vertical cryostat sections of 25-μm thickness were cut perpendicular to a plane tangent to the corneal surface from nasal to temporal order and collected onto Superfrost/Plus microscope slides (Fisherbrand, Fisher Scientific, Pittsburgh, PA).

Immunocytochemistry

For fluorescence SMI-32 immunocytochemistry, 25-μm-thick cryostat sections were incubated in 10% normal goat serum (NGS) and 1% Triton X-100 in 0.01 M PBS for 1 h at room temperature. This incubation was to block nonspecific binding sites. Sections were then incubated overnight with a mouse monoclonal antibody directed against SMI-32. The antiserum was used at a dilution of 1:1,500 in PBS containing 0.5% Triton X-100 at 4°C. Retinas were kept at room temperature for 30 min and then washed in PBS for 15 min (3 × 5 min). Afterwards, retinas were incubated for 2 h in Alexa Fluor 488 goat anti-mouse IgG (1:300 dilution; Invitrogen, Carlsbad, CA) at room temperature. Retinal sections were washed for 15 min with 0.1 M PBS and coverslipped with a Vectashield mounting medium (Vector Labs). For retinal whole-mount immunostaining, the same immunocytochemical procedures described above were used, but with longer incubation times (4 days in SMI-32 and 3 days in Alexa Fluor 488 goat anti-mouse IgG). Whole-mount retinas were also counterstained with TOPRO-3 iodide (1:1,000; Invitrogen). TOPRO-3 labels nucleic acid staining (cell bodies). Since the retinal condition may affect the protein level of an antibody such as SMI-32, we used TOPRO-3 as a control to confirm that any cell reduction labeled by SMI-32 is due to cell degeneration. SMI-32 has been reported to label several types of ganglion cells in mammalian retinas (Coombs et al. 2006; Lin et al. 2004; Straznicky et al. 1992). To verify SMI-32 activity in rat, we performed immunocytochemistry in a vertical section of a P21 normal rat retina (Fig. 2A); SMI-32 immunoreactivity was observed only in cells in the ganglion cell layer and the processes in the inner plexiform layer. In addition, the nerve fiber layer was labeled, providing strong evidence that SMI-32 labeled only RGCs (Fig. 2, B and C).

Immunofluorescence images were processed with Zeiss LSM-PC software. The brightness and contrast of the images were adjusted with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA). For presentation, all Photoshop manipulations (brightness and contrast only) were carried out equally across sections.
Data Analysis

Electrophysiology recording. Data were analyzed off-line with SciWorks and Matlab. Means and SE values were calculated in Microsoft Excel. Error bars shown in this study indicate SE. Statistical comparisons were done by performing ANOVA multiple comparison (Tukey test) among control and degenerate groups in SAS 9.2. Chronaxies were calculated by fitting exponential function \( y = H11002 b/(1 - e^{-x/a}) \) to the strength duration data (Lapicque 1907; Ranck 1975), where \( x \) represents the pulse duration and \( y \) represents the predicted threshold values of that pulse duration based on the function. The asymptote was defined as the rheobase (coefficient \( b \)); chronaxie was calculated as \( a/\ln 2 \). The condition of fit was evaluated by calculating the \( r^2 \) value.

Quantification of RGC density. We counted SMI-32-immunoreactive ganglion cells and cells counterstained with TOPRO-3 in whole-mount retinas. Cells were counted along both the nasal-to-temporal and dorsal-to-ventral retinal meridians in fields of 200 \( \mu \)m \( \times \) 200 \( \mu \)m per retina sampled in 1-mm steps. Densities were averaged in two opposite fields on each meridian per retina. The total number of SMI-32-immunoreactive ganglion cells per retina was also counted. Statistical comparisons were done by performing ANOVA multiple comparison (Tukey test) among control and degenerate groups in SAS 9.2.

RESULTS

Characteristics of Superior Colliculus Response to Light and Electrical Stimulation of the Retina

We first characterized the light-evoked response and the electrically elicited response (EER). Light-evoked responses were observed in all ages (P90–P700) of normal animals. Latency was typically \( >50 \) ms, and responses extending past 200 ms occurred infrequently (roughly 30%). Light-evoked responses were only observed in P84–P185 in the RD animals, with the exception of one observation in a single P648 RD animal. Both short (<5 ms)- and long (>10 ms)-latency EERs were observed, but short-latency responses were used for threshold. Long-latency responses were observed <50% of the time and at higher stimulus levels, while short-latency responses always appeared at lower stimulus levels than long-latency response. Others have reported inconsistent late response components to the EER (Chen et al. 2006). The threshold currents across all pulse durations were compared between the juxtazonal potentials and spikes. We found no consistent trend that would suggest one type of response is more sensitive, and hence juxtazonal potentials and spikes were treated as equivalent measures of retinal sensitivity to electrical stimulation. One outlier was observed in the group RD P700, and data from this experiment were not included in the electrophysiological or morphometric analysis.

Threshold Positively Correlates with Age in RD but Not in Normal

Strength-duration curves of the control and RD animals are shown in Fig. 3 with seven pulse durations varied from 100 \( \mu \)s to 2 ms. We tested whether the activation threshold correlated with age in the control groups but found no correlation (\( r = 0.14 - 0.25, P = 0.46 - 0.86 \)) across all pulse...
durations, suggesting that age does not contribute to any changes in threshold in the control group. However, we found a positive correlation of the activation threshold and age in the degenerate group for all pulse widths ($r = 0.72–0.84$, $P < 0.0001$). Figure 4 shows the progression of threshold increase in the RD group compared with the control group. Average threshold in normal and RD for each pulse duration is plotted as an $x,y$ pair (normal, RD). The identity diagonal has a slope of 1; data on the line indicate equal thresholds, data below the line indicate higher thresholds in normal, and data above the line indicate higher thresholds in RD.

Fig. 3. A and B: strength-duration (ms) curves obtained for RD (A) and normal (N, B) rats. C: charge density-duration graph shows that RD rats older than P500 exceed the electrochemical safety limit for platinum (0.35 mC/cm$^2$; shown as a horizontal line) for some pulse widths. D: individual data points at RD P600 and RD P700 only for the pulse durations when the average value was within the electrochemical safety limit.

Fig. 4. Averaged threshold for each pulse duration of degenerate vs. control at 4 age groups. The diagonal identity line indicates the same threshold values of degenerate and control (slope = 1). Red, blue, green, and orange denote P100, P300, P500, and P700, respectively.
RGC, retinal ganglion cell; N, normal retina; RD, S334ter line 3 retina; P, postnatal day. Overall ANOVA P value = 1.95e-07; significant values are in italics.

SMI-32-Labeled RGC Density in Ventral Temporal Retina Negatively Correlates with Age in RD but Not in Normal

The stimulating electrode was always placed in the ventral-temporal (VT) quadrant. Thus our detailed analysis only considers density measures taken from the ventral and temporal axes. When analyzing VT SMI-32-labeled RGC density, we found a negative correlation between age and density across P100, P300, P500, and P700 ($r = -0.74273$, $P = 0.0036$). Since it has been shown that RGC density does not change with age in rat (Harman et al. 2000), density measurements were only made in normal P500 and P700. SMI-32 is a relatively sparse label, so we used a second label (TOPRO-3) in a subset of animals to label all cells in the ganglion cell layer, which includes some amacrine cells. TOPRO-3 staining ($n = 2$ from both normal P700 and RD P700 groups) showed that control retinas had $5,975 \pm 975$ cells/mm$^2$ and RD retinas had 386 cells/mm$^2$. Thus the SMI-32 and TOPRO-3 labeling results are consistent. Similar TOPRO-3 results were reported by others (Jakobs et al. 2005).

We examined more closely the reduction of SMI-32-labeled ganglion cells in RD P500 and P700 by counting the total number of SMI-32-labeled ganglion cells in the whole-mount retina. The total number of cells was $5,580 \pm 1,105$ cells ($n = 2$) in P500 normal retina and $4,446 \pm 639$ cells ($n = 2$) in P700 normal retina. In contrast, we observed $3,381 \pm 510$ cells in RD P500 ($n = 2$) and $1,994 \pm 238$ cells in RD P700 ($n = 2$). The small sample sizes do not allow statistical comparison, but these data support the finding of a reduced number of ganglion cells with increasing age in RD.

Threshold and SMI-32-Labeled RGC Density Are Correlated

Correlation analysis of threshold and SMI-32-labeled VT RGC density showed statistically significant correlations for all pulse widths except 0.2 ms ($r = -0.58927$ to $-0.59375$, $P < 0.05$; for 0.2 ms, $r = -0.47792$, $P = 0.0716$). To examine this correlation in depth, we used ANOVA to compare the differences among groups in density and threshold. In general, P500 is the age at which RD animals began to show statistically significant differences versus both normal animals and younger RD animals. Density was only collected in RD P100, P300, P500, and P700, so threshold data from matching ages were used in the analysis. Density data were not collected in normal P100 and P300, as discussed above. The ANOVA $P$ values are shown in Tables 1 (density) and 2 (threshold). Density and threshold are generally in correspondence in terms of which age groups show statistically significant differences. Comparisons to normal P100 and normal P300 are excluded from the threshold table for brevity. These groups were the same as normal P500 and normal P700. RD P100 and RD P300 are different from RD P500 and RD P700 (with 1 exception as noted below). None of the normal groups are different from one another. RD P100 and RD P300 are not different from normal in either threshold or density. RD P500 is different from all normal groups for both threshold and density [with the exception of threshold normal P100 ($P = 0.0625$), data are not shown in Table 2]. RD P500 is different from RD P100 for both threshold and density. RD P700 threshold and density are different from all other groups. In contrast to the overall trend, RD P300 and RD P500 thresholds were not significantly different, whereas density was significantly different for these two groups.

Short Pulse Width Is Required to Maintain Safe Stimulation Limits in RD P600 and RD P700

We calculated the threshold charge density for normal and RD retinas for comparison with the electrochemical safety limit of platinum (0.35 mC/cm$^2$) (Rose 1990). In RD P600, average threshold at pulse durations of 1.5 ms and 2 ms exceeded the limit, and in RD P700 average threshold at pulse durations of 0.5 ms and above exceeded the limit. In P600, high average charge densities were due to one of the four experiments in this group, where charge

### Table 1. ANOVA $P$ values for SMI-32-labeled RGC density in ventral-temporal quadrant

<table>
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<tr>
<th></th>
<th>N P500</th>
<th>N P700</th>
<th>RD P100</th>
<th>RD P300</th>
<th>RD P500</th>
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<tr>
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<td></td>
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<td>0.554</td>
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<td>0.9257</td>
<td>0.9991</td>
<td>0.0099</td>
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<tr>
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<td>—</td>
<td></td>
<td>0.9798</td>
<td>0.0159</td>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
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Overall ANOVA $P$ value = 6.01912E-05; significant values are in italics.

### Table 2. ANOVA $P$ values for electrical stimulation thresholds

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<th>N P700</th>
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<td>&lt;0.0001</td>
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<td>0.1820</td>
<td>0.0015</td>
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<td>0.6261</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RD P500</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RD P700</td>
<td>—</td>
<td>—</td>
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Overall ANOVA $P$ value = 6.01912E-05; significant values are in italics.
densities at all pulse durations were higher than the platinum threshold. The remaining three experiments had threshold below the charge density limit at all pulse durations (Fig. 3D). In RD P700, all four retinas had thresholds below the safety limit, but only at the two shortest pulse durations (Fig. 3D).

The charge densities across all pulse durations for control groups N P100, P200, P500, and P700 stayed within the electrochemical limit of platinum (data not shown).

Rheobase Increases with Age but Chronaxie Does Not in RD Retina

We estimated the chronaxie and rheobase values by fitting the data in Fig. 3 to the model equation (see MATERIALS AND METHODS). The $R^2$ values ranged from 0.981 to 0.997, suggesting an excellent fit between the model and the data. The rheobases of the degenerate group increased monotonically. The rheobases of RD P100, P200, P300, P500, P600, and P700 were 4.2 ± 0.6 $\mu$A, 5 $\mu$A, 6.6 ± 1.4 $\mu$A, 7.5 ± 1.1 $\mu$A, 5.4 $\mu$A, and 21.25 ± 3.4 $\mu$A, respectively. The rheobases of the four control groups had no apparent trend (N P100, 5.3 ± 0.9 $\mu$A; N P200, 5.2 ± 0.2 $\mu$A; N P500, 4.7 ± 0.3 $\mu$A; N P700, 6.3 ± 1.3 $\mu$A). Although rheobase in RD increases monotonically with age, only at P700 is the difference statistically significant (ANOVA, overall $P$ value < 0.0001, P700 different from all other RD groups). The chronaxie of the degenerate group ranged from 0.28 to 0.41 ms, while that of the control group ranged from 0.32 to 0.36 ms. Chronaxies for all RD groups were statistically similar to their corresponding control groups (ANOVA $P = 0.247$).

SMI-32-Labeled RGC Density in Whole-Mount Retina

On the basis of ANOVA analysis, we combined some experimental groups for further analysis of SMI-32-labeled RGC density. Normal P500 and P700 were grouped as normal and RD P100 to P300 as young RD (from ANOVA, RD P100 and RD P300 were statistically the same as normal and the same as each other). Density was measured along the nasaltoperiodal and ventral-to-dorsal meridians in whole-mount retinas. Density (cells/mm²) from the pooled groups as well as RD P500 and RD P700 are shown in Fig. 5 as a function of distance from the optic disk and in Table 3 summed for each quadrant. As noted above, ventral and temporal quadrants show progressive decline in RD P500 and RD P700. Interestingly, no statistical difference was noted in RGC density along the nasal axis, indicating some heterogeneity of degeneration.

DISCUSSION

Our experiments demonstrate three major findings. First, there is an increase of stimulus threshold in the degenerate groups from RD P100 to RD P700. In the same retinas used for electrophysiology, a reduction in SMI-32-immunoreactive RGC density was observed from RD P100 to RD P700. Thus a correlation between degeneration stage and stimulus threshold has been shown. Second, the observation of similar chronaxie values among all groups suggests that the same class of cells is responding to stimulus regardless of the phase of degeneration. Third, the charge density required to elicit a response in severely degenerate retina (RD P700 group) with a small electrode (75 $\mu$m) stays within the traditional electrochemical safety limit of platinum electrodes, but only when short pulse duration (<0.5 ms) is used. These findings represent the first detailed measurements of both electrophysiological and morphometric properties across an extensive age range of degenerated retina.

Electrophysiological Measurement of Stimulus Threshold

Electrical threshold has been studied extensively in healthy retina. With small electrodes (between 10 and 125 $\mu$m), low...
charge densities were reported in several in vitro studies of healthy retinas (Ahuja et al. 2008; Jensen and Rizzo 2006; O’Hearn et al. 2006; Sekirnjak et al. 2006). The healthy state of the retina and the close contact between the microelectrode array and the retina in the in vitro setup may facilitate low electrical threshold. The charge densities ranged from 0.06 to 0.31 mC/cm² (Jensen et al. 2003; O’Hearn et al. 2006; Sekirnjak et al. 2006). This is in agreement with the low charge densities we reported in this study in the control retina group, which ranged from 0.06 to 0.3 mC/cm², suggesting that the impedance sensing method does position the stimulating electrode close to the retina.

Animal in vitro studies generally show higher threshold values in degenerate retinas. It was found that the threshold charge densities in 8- to 16-wk-old rd1 mouse were substantially higher than that in normal mouse (Chen et al. 2006; O’Hearn et al. 2006; Suzuki et al. 2004) with an array of platinum electrodes of 125-μm diameter. However, these studies only investigated the threshold charge densities at one age of degeneration. In a recent in vitro study, Jensen and Rizzo (2008) reported that the thresholds in rd1 mouse were consistently higher than in wild-type mouse, but that the threshold values were stable across the test age group, P25–P186, which, at first glance, conflicts with our present findings. However, Jensen’s study used subretinal stimulation, and the authors hypothesize that the immediate loss of photoreceptors in the rd1 mouse led to increased stimulus threshold. In contrast to most other studies, a recent study by Sekirnjak et al. (2009) found no difference in threshold for normal versus P23H rats even in very old rats (P700). No cell counting was done in this study, so the condition of these retinas is unknown. However, Kolomiets et al. (2010) have shown a decline in RGC density in P23H rats. In our study, a statistically significant increase in thresholds was only noted after a decrease in ganglion cells (at P500). In vitro studies allow precise positioning of the stimulating electrode to within several micrometers of RGCs, which is one explanation for the finding of no threshold difference in P23H rats. Achieving this position consistently in a clinical device will require significant advances in surgical technique as well as new materials for highly flexible electrode arrays and bio-glues to hold the tissue next to the array. If such advances can be made, then artificial vision may begin to approach natural vision, since it may be possible to selectively stimulate individual ganglion cells and even recreate temporal firing patterns that mimic natural patterns.

Threshold studies have also been carried out by measuring responses in higher visual centers elicited by retinal stimulation. These thresholds are generally greater than those measured by in vitro studies. Cortical response thresholds ranged from tens to hundreds of microamperes (Agnew et al. 1986; Beebe and Rose 1988; Weiland et al. 2002), well above the typical in vitro values (typically <10 μA). Nadig (1999) reported cortical response thresholds ranging from 50 to 600 μA with 0.2-ms pulse duration and a 250-μm epiretinal electrode, while Nakachi et al. (2005) reported thresholds ranging from 10 to 1000 μA with 0.5-ms pulse duration with a 2 × 4-mm electrode in a 3 × 5-mm sclera pocket. Relatively few studies have investigated stimulation of degenerate retina by recording from the central visual system (Sagdullaev et al. 2003). Similar to our studies, Kanda et al. (2004) recorded SC response at 20 μA with 0.5 ms, using a ball electrode placed on the sclera in a Royal College of Surgeons (RCS) model, which is consistent with our results ranging from 16 to 50 μA with 0.5 ms in the old RD group. Our threshold charge densities in degenerate retina are close to those studied in human perceptive thresholds. de Balthasar et al. (2008) reported that threshold charge density in human RP ranged from 0.1 to 0.64 mC/cm² with 1-ms pulse duration, whereas threshold charge density in our RD rodent ranged from 0.15 to 0.55 mC/cm² with 1-ms pulse duration in degenerate group P100–P700.

Chronaxie, a well-studied electrophysiological measure for tissue excitability, is the stimulus pulse duration for twice the rheobase current (rheobase is the theoretical current for an infinitely long stimulus pulse) (Geddes 2004; Lapicque 1907). Chronaxie relates to the time constant of the cell membrane; thus different cells will have different chronaxie dependent on membrane properties (Ranck 1975). Chronaxie estimates of ganglion cells in healthy retina performed by Sekirnjak et al. (2006) found a range of 0.2–0.4 ms, while our range of chronaxie in degenerate retina was 0.28–0.41 ms. This suggests that we were activating RGCs, regardless of retinal condition.

Reduction of Retinal Ganglion Cells in the Degenerated Retina

Morphometric analyses in postmortem human eyes showed cell reductions in the outer nuclear layer and ganglion cell layer in retinas with RP, but the cell count in the inner nuclear layer is similar to that in the control group (Santos et al. 1997; Stone et al. 1992). In RP retina, ganglion cells were reduced by 30% and 60% in the macular region and extramacular region, respectively. Animal model studies of ganglion cell loss in inherited retinal dystrophy started in the early 1970s. The first study demonstrated that in mutant mice lacking photoreceptors, the RGCs are decreased in size and number (Farber et al. 1994). Three recent studies show the decline in RGC counts in animal models of RP (Kolomiets et al. 2010; Marc and Jones 2003; Milam et al. 1998). Our data in ganglion cell reduction are consistent with these findings. Other studies showed that, aside from the changes in cell number and size, the morphology of the inner retinal layers, including the ganglion cell layer, is essentially normal on an electron microscope level (Caley et al. 1972; Karli 1952). One recent study confirmed this early finding that RGCs maintain dendritic morphology in the rd10 mouse model of photoreceptor degeneration (Mazzoni et al. 2008). However, this study demonstrated a stable population of RGCs in rd10 mouse up to P270, which is in conflict with the human studies, early animal studies about the decline in RGC count in late RD retinas, and our present study.

The inconsistency in the survival of RGC counts in animal models highlights the importance of choosing the age of the RD retina and the animal model with pathology resembling the human late-RP condition. Our study examined an extensive age range. In a separate study by our group using the same degeneration model, reorganization of the retina began as early as P90, including retraction of dendrites of bipolar cells after loss of photoreceptor cells and reduction of bipolar cell density (Ray et al. 2010). Our present study demonstrated that there was no statistically significant change in threshold up to P300 and threshold increased when ganglion cell density decreased. Since the same transgenic model was used, it is safe to
assume a similar pattern of bipolar cell loss before P300; thus these early changes in the retina did not affect threshold. It is important to note that the stimulating electrode was placed in the temporal-ventral quadrant, where a marked reduction in ganglion cell density was noted. An interesting future experiment would be to measure threshold by using alternative placements of the stimulating electrode in areas with better preservation of ganglion cell density (the nasal retina from our measurements).

Implications for Retinal Prosthesis

This study has major implications on the future development of a high-resolution retinal prosthesis.

de Balthasar et al. (2008) analyzed threshold data from humans with chronic retinal prosthetic implants and concluded that electrodes ~200 μm in diameter would be within the safe limit of the platinum electrode. However, in order to provide high-acuity vision (i.e., adequate for face and object recognition), it is estimated that 600–1,000 electrodes are required, necessitating electrodes with diameters on the order of 75 μm. In our present study, we measured the required current for activating degenerate retina with a small electrode. The results indicate that platinum electrodes as small as 75 μm in diameter are acceptable, but in a limited pulse range (<0.5 ms). This represents a challenge to maintain a safe stimulation level when stimulating with small electrodes, highlighting the importance of utilizing an electrode material with high charge delivery capacity, such as iridium oxide (Agnew et al. 1986; Beebe and Rose 1988) or titanium nitride (Weiland et al. 2002).

Our data have demonstrated a correlation between stimulus thresholds and ganglion cell density, suggesting that preservation of ganglion cells may reduce the stimulus output requirement and improve efficiency. However, the loss of retinal ganglion cells alone may not fully explain our results. The retinal remodeling scenario includes thickening of the glial membrane on the surface of the retina. While we did not observe an extensive epiretinal membrane in older RD eyes, we did not examine this membrane in a quantitative sense. It is possible that even a slight thickening of the membrane may increase the electrical resistance of the retina, resulting in a higher percentage of current being shunted through the more conductive vitreous. In this scenario, more current would need to be supplied through the electrode to maintain threshold current in the retina. Neuronal remodeling is common in the central nervous system (CNS) and is well documented in the fields of epilepsy and learning and memory (Sutula 2002; Van Reempts et al. 1992). Remodeling of the SC may contribute to the increased activation threshold. One study observed degenerative changes in mice, such as a reduction in neuronal density in the lateral geniculate nucleus and SC, secondary to the complete loss of photoreceptors and retinal remodeling (Ward 1982). The CNS plasticity in response to disease is unavoidable. It is not yet known whether electrical stimulation can halt or influence CNS remodeling.

We conclude that in a rat model of retinal degeneration electrical stimulation threshold increases with age and RGC density decreases with age. The changes in threshold and density are correlated, but a conclusive cause-and-effect relationship was not established. The linkage of structure and function is critical to understanding the mechanism behind increased perceptual thresholds in blind humans with implanted retinal prostheses. The animal model used in this study closely resembles the end stage of autosomal dominant RP in humans, particularly in the findings of loss of RGCs and the threshold charge density reported in retinal prosthesis clinical trials. In this animal model, electrodes with 75-μm diameter could evoke a response within the safe limit in a limited pulse range, supporting the continued development of a high-resolution retinal prosthesis for the blind.

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DISCLOSURES

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

REFERENCES


