

The effects of temperature changes on retinal ganglion cell responses to electrical stimulation

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Abstract — Little is known about how the retina's response to electrical stimulation is modified by temperatures. *In vitro* experiments are often used to inform *in vivo* studies, hence it is important to understand what changes occur at physiological temperature. To investigate this, we recorded from eight RGCs *in vitro* at three temperatures; room temperature (24°C), 30°C and 34°C. Results show that response latencies and thresholds are reduced, bursting spike rates in response to stimulation increases, and the spiking becomes more consistently locked to the stimulus at higher temperatures.

I. INTRODUCTION

Diseases such as retinitis pigmentosa and age-related macular degeneration result in blindness through loss of photoreceptor cells in the retina. Retinal ganglion cells (RGCs), the output neurons of the retina, have been shown to survive in large numbers in patients with retinitis pigmentosa [18]. Several research groups worldwide are investigating the feasibility of functional vision restoration through electrical stimulation that targets surviving RGCs via an implanted multi-electrode device. *In vitro* experiments that explore the effects of electrical stimulation on RGCs are instrumental to improving the effectiveness of these devices. However, to date, *in vitro* studies using explanted retina have been performed at a range of temperatures, leading to some difficulty in interpreting and comparing their results.

Studies on fish retinae [5], [13], [19], [20] have shown that spectral sensitivity is affected by changes in temperature. For example, Thorpe found that the response amplitude to green stimuli relative to red at 15°C is about half that at 25°C [19]. Ahlers and Ammermuller demonstrated that RGCs experience large changes in spike rate and response latency to flashes of light at temperatures ranging from 26° C to 36° C [1]. They found that the mean firing rate increased by a factor of ~2.8, and the response latency decreased by a factor of ~1.8 when the temperature was increased from 26°C to 36°C. Similarly, increasing the temperature from 25°C to 37°C lowers the contrast threshold for activating RGCs by a factor of ~2.5 [2]. There has been

little investigation of how changes in temperature affect the retina's ability to respond to electrical stimulation. *In vitro* experiments exploring electrical stimulation using explanted mammalian retina have been performed at room temperature [11], [14], [16], [21], at approximately physiological temperature [4], [9], [17], and at temperatures in between [7], [8], [10], [15]. Fohlmeister showed that temperature affects the intracellular spike width and the voltage dependence of channel gating between 23°C and 30°C in RGCs, demonstrating that firing properties of RGCs depend strongly on temperature [3]. It, therefore, seems likely that temperature may affect the ability of RGCs to respond to electrical stimulation.

In vitro experiments are often used to inform acute or chronic *in vivo* studies. Moreover, the charge injection capacities, size and features of electrode designs are also informed by *in vitro* studies. It is important to understand how the *in vitro* results will change once performed at physiological temperature, as this can bear large effects on neural response. Here we characterize the effect of temperature on the dynamics of RGC responses to electrical stimulation. We find that, at physiological temperatures, the sensitivity to electrical stimulation is increased, resulting in more stimulus-locked spikes for given amplitudes of biphasic pulse stimulation. This increase in sensitivity is accompanied by changes in the extracellular action potential shape, with spikes evoked at physiological temperature showing more rapid depolarization and shortened duration compared to spikes evoked at room temperature.

II. METHODS

A. Retinal whole mount preparation

Methods conformed to the policies of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of the University of Melbourne (Approval Number 1112084). Data were collected from the retinae of Long-Evans rats ranging from 1 to 6 months of age. The animals were initially anesthetized with a mixture of ketamine and xylazine prior to enucleation. After enucleation, the rats were euthanized with an overdose of pentobarbital sodium (350 mg intracardiac). Dissections were carried out in dim light conditions to avoid bleaching the photoreceptors. After hemisecting the eyes behind the ora serrata, the vitreous body was removed and each retina was cut into two pieces. The retinae were left in a perfusion dish with carbogenated Ames medium (Sigma) at room temperature until used. Pieces of retina were mounted on a micro-electrode array

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with ganglion cell layer up and were held in place with a perfusion chamber and stainless steel harp fitted with Lycra threads (Warner Instruments).

Once mounted in the chamber, the retina was perfused (4-6 mL/min) with carbogenated Ames medium (Sigma-Aldrich, St. Louis, MO). The chamber was mounted on the stage of an upright microscope (Olympus Fluoview FV1200) equipped with a x40 water immersion lens and visualized with infrared optics on a monitor with x4 additional magnification. The bath temperature was controlled using an inline heater (SDR scientific TC344B).

Extracellular recordings of cells were obtained using fine tipped carbon fiber electrodes (Carbostar, Kation Scientific). Recordings were obtained by placing the recording electrode in contact with the retina on the epiretinal side. Signals were digitized at 50 kHz (Tucker-Davis systems, PZ2) and stored for offline analysis.

B. Temperature and stimulation

For each experiment, the bath temperature was increased from room temperature (mean 23.9°C, std. dev. 1.1°C, range 22.2-25.1°C) to an intermediate temperature close to 30°C, then up to 34°C (the maximum achievable by the inline heater) and, finally, back down in the same steps. The bath temperature was measured at the inlet location, and experiments at each temperature began once the bath temperature had settled (10-15 min). At each temperature, light stimulation was applied to the cell, followed by electrical stimulation.

Light stimulation was achieved by focusing the light from a 600x800 monochrome OLED display (eMagin OLED-XL) at the level of the photoreceptors under the recorded cell. Stimulation consisted of 10 sec periods of light (OLED at full brightness) or darkness (OLED off), with the stimulus starting in darkness. The stimulus polarity was switched over every 50 sec and repeated 5 times. From these results, we could determine if the cell was an ON or OFF type cell.

Following light stimulation at each temperature, the effect of electrical stimulation was investigated. Electrical stimulation was applied via a 400 µm diameter electrode situated on the photoreceptor side of the tissue. The stimulus consisted of a train of biphasic pulses ranging in amplitude from 20-280 µA in 20 µA steps, with phase duration of 500 µs and no interphase gap. Stimulation was achieved using a multichannel stimulator with a 50 kHz resolution (Tucker Davis IZ2). The pulses were applied at 5 Hz for 30 seconds.

C. Data analysis

Stimulation artefacts were removed by first averaging the stimulus artefact in a train of pulses, then subtracting the averaged artefact from each stimulus. Recordings revealed extracellular spikes, from which a cluster analysis could be performed to extract the spiking information from individual cells. Spikes were detected and clustered using `wave_clus` [12]. If more than one cell was detected in the recording, only the cell with the highest spike amplitude was analyzed. Five variables were examined. From the spike times, changes in activation thresholds ($v1$), spike latencies ($v2$) and burst spiking ($v3$) were analyzed for each temperature.

Additionally, the change in spike width ($v4$) and amplitude ($v5$) were examined.

Thresholds were obtained by first calculating a spontaneous spike rate from a two second recording prior to stimulation. Next, the number of spikes produced between stimulus pulses was measured and the spontaneous rate was subtracted. From this a spike probability was calculated for each train of pulses. A sigmoid curve (Equation 1) was fitted to the data of response probability ($P(X)$) versus biphasic pulse amplitude (X), and the activation threshold (b) was defined as the amplitude resulting in a 50% response rate. To estimate threshold, we performed a constrained non-linear least squares fit of Equation 1 to the response probability data at each temperature. Only fits with a fit coefficient of variation (r^2) greater than 0.7 were used.

$$P(X) = \frac{1}{1 + e^{-a(X-b)}} \quad (1)$$

The change in spike latency was calculated by averaging the delay in the first spike time from stimulus onset for each stimulus pulse. The change in burst spike rate was analyzed by averaging the number of spikes produced in response to each stimulus pulse. $v2$ and $v3$ were only analyzed in response to pulse trains of 280 µA.

The spike width was measured by taking the width of the spike at half of the amplitude of the spike. The spike amplitude was calculated as the amplitude from the most negative point to the most positive point of the spike.

Statistical significance was tested by performing a correlated samples t-test using temperature as the independent variable and each of $v1$ - $v5$ as dependant variables. To visualize the changes produced by temperature, we took the ratio of the response at the higher temperature (T_h) divided by the response at the lower temperature (T_l), and plotted the log of the result. All variables were tested for equal variance and normality of distribution prior to running the t-test, and were only used if the test returned positive under 5% significance level. The test for normality used was MATLAB's chi-square goodness-of-fit test.

III. RESULTS

A. Cell identification and artefact removal

Cells were identified by their response to light stimulation. Cells that responded with an increase in spike rate at light onset were classified as ON cells, and cells with an increased spike rate at light offset were classified as OFF cells (Figure 1). No ON-OFF cells were encountered in these experiments. A total of six OFF and two ON cells were recorded.

Artefact removal revealed spikes that were clearly detectable using cluster analysis. Figure 2 depicts an example of the recorded response before and after artefact removal.

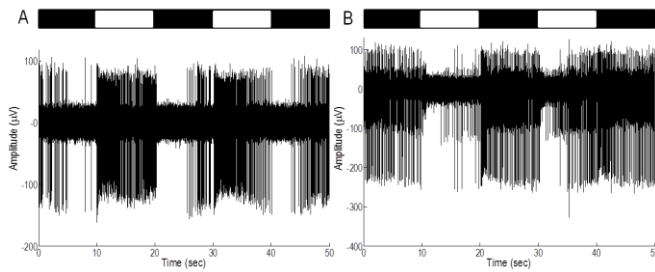


Figure 1: Cells were identified as ON or OFF by having an increased spike rate in response to light onset or offset, respectively. A) ON cell response. B) OFF cell response. Shown above each plot is the timing of the light stimulus (white represents light ON). Note that spikes from two cells were detected in (B), but only the cell with larger spikes was analyzed.

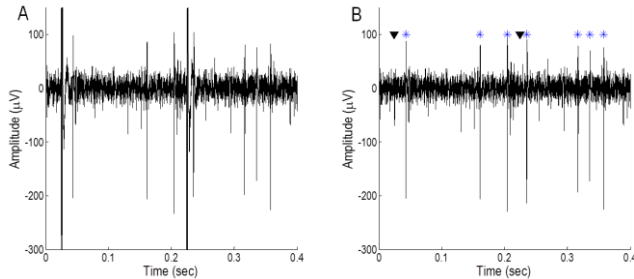


Figure 2: An example of artefact removal. A) Two large artefacts are visible and partially occlude spikes close to the artefact. B) Once the artefacts are removed (triangles), large and small spikes are clearly detectable. Large spikes are marked with stars.

B. Response to electrical stimulation

Increasing bath temperature resulted in enhanced RGC sensitivity to electrical stimulation. Cell response thresholds were reduced and cells responded with a higher probability for a given stimulus amplitude. Spikes occurred more consistently locked to the stimulus time and with a greater burst of spikes for each pulse. Additionally, the spike amplitude was increased and the spike width decreased at higher temperatures. Similar results to the room and intermediate temperature results were recovered when decreasing the temperature from 34°C back down to room temperature.

TABLE I: Correlated samples t-test (degrees of freedom (df), t-value)

Variable	$T_l=24, T_h=30$	$T_l=30, T_h=34$	$T_l=24, T_h=34$
threshold ($v1$)	(5, 5.90)	(5, 1.83)	(5, 6.85)
spike latency ($v2$)	(5, 2.58)	(5, 2.69)	(5, 3.11)
burst spiking ($v3$)	(5, 2.32)	(5, 2.79)	(5, 3.03)
spike width ($v4$)	(7, 7.90)	(7, 5.86)	(7, 8.84)
spike amplitude ($v5$)	(7, 2.90)	(7, 0.08)	(7, 1.37)

t-value > 2.571 achieves a statistical significance level < .05 for df=5
t-value > 2.365 achieves a statistical significance level < .05 for df=7

Figure 3A-C illustrates an example of the increased responsiveness of a RGC to electrical stimulation. This cell responded with an average of 0.98 spikes at 24.4°C and an average of 2.64 spikes at 33.8°C. Figure 3D shows an example of spike activation curves for the same cell at three different temperatures.

All variables produced significant changes in response due to temperature as shown in Table I. This suggests that temperature has a large effect on the response dynamics of RGCs to electrical stimulation. The mean changes produced

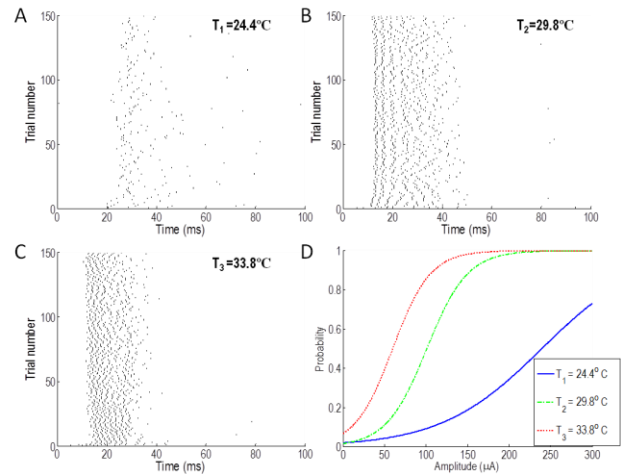


Figure 3: Cell latencies and spike thresholds. (A-C) Spike raster plots showing the spike latency from stimulus onset for three different temperatures. The stimulus amplitude for all plots is 280 μ A and the stimulus occurs at time zero. For higher temperatures, the spike latency is decreased and spiking becomes more regular. This cell responded with an average of 0.98, 1.57, and 2.64 spikes at temperatures $T_1=24.4^\circ\text{C}$, $T_2=29.8^\circ\text{C}$, and $T_3=33.8^\circ\text{C}$ respectively. (D) The sigmoidal fit to the spiking data from the same cell in A-C. At higher temperatures, the threshold for activation is reduced. The thresholds are 239, 101, and 60 μ A for temperatures T_1 , T_2 , and T_3 , respectively.

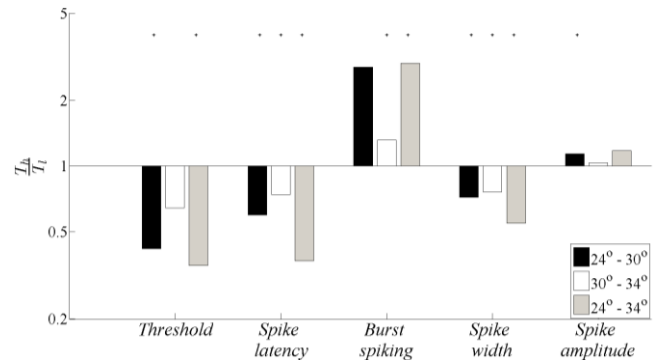


Figure 4: Changes in RGC responses due to temperature. Values shown in logarithmic scale for clarity. Black solid bars represent the change in responsiveness due to a change in temperature from $T_l=24^\circ\text{C}$ to $T_h=30^\circ\text{C}$. Clear bars represent changes in responsiveness due to a change in temperature from $T_l=30^\circ\text{C}$ to $T_h=34^\circ\text{C}$. Gray bars represent changes in responsiveness due to a change in temperature from $T_l=24^\circ\text{C}$ to $T_h=34^\circ\text{C}$. Stars above bars represent variables that significantly changed according to the t-test.

by each temperature for all 8 cells are shown in Figure 4. This gives an indication of how much each variable changed relative to the lower temperature response. At 34°C (T_h) compared to 24°C (T_l), $v1$ was reduced by a factor of 0.35 (standard deviation (SD) 0.11), while the spike latency ($v2$) was reduced by a factor of 0.37 (SD 0.19). Furthermore, a larger number of spikes were produced for a given stimulus amplitude. At the highest amplitude tested (280 μ A), the number of burst spikes ($v3$) increased on average by a factor of 3 (SD 1.7).

The spike waveform changed at higher temperatures: all cells had a narrower spike width ($v4$) and five cells produced spikes with higher amplitude ($v5$). Three OFF cells produced smaller spikes at higher temperatures. On average, the spike width decreased by a factor of 0.56 (SD 0.09, $n=8$), and the

spike amplitude increased in size by a factor of 1.21 (SD 0.28, n=8) at 34°C compared to 24°C.

IV. DISCUSSION

In vitro experiments exploring electrical stimulation of explanted retinal tissue are often performed at non-physiological temperatures. Many of these studies have investigated RGC thresholds for activation, burst spiking, and response probabilities. The study presented here demonstrates the dependence of RGC response properties on temperature during subretinal electrical stimulation. Our work shows that response thresholds, burst firing, and latencies are significantly changed at physiological temperature compared to room temperature. At higher temperatures, RGCs have a lower response threshold, fire with a higher number of spikes and have reduced response latencies in response to biphasic pulse stimulation.

Response thresholds were reduced by a factor of 0.35, spike latencies were reduced by a factor of 0.37, and burst response when stimulated with 280 μ A pulses increased by a factor of 3 at 34°C compared to room temperature. Similar findings have been discovered when investigating the effect of temperature on the light response of RGCs. Dhingra found that contrast response thresholds reduced by a factor of 2.5 from 25°C to 37°C [2]. Ahlers and Ammermuller found that response latencies reduced by approximately a factor of 1.8, and the average spike rate increased by more than a factor of 2.5 when the temperature was increased from 26°C to 36°C [1].

The extracellular spike waveform was found to change in response to temperature. The spike width was reduced by a factor of 0.56, and some cells were found to have a smaller spike at 34°C compared with room temperature. This has a large effect on the ability to record extracellular potentials at different temperatures.

Activation of neural tissue requires an understanding of the required charge densities needed for activation at physiological temperatures. This is used to inform electrode design, *in vivo* models and patient tests. It is important to understand how the changes in temperature will affect response thresholds, firing patterns, latencies, and the ability to record extracellular potentials. Therefore, the changes in response characteristics with temperature noted here should be taken into account when planning *in vivo* experiments.

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