Effects of *Test Flash Duration on the Photopic Negative Response (PhNR) of the Flash Electroretinogram*

By

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Thesis

In partial satisfaction of the requirements for the degree of

Master of Science in Vision Science

State University of New York

College of Optometry

May, 2023

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ABSTRACT

Purpose: The Photopic Negative Response (PhNR) of the cone mediated electroretinogram (ERG) is a slow potential with negative polarity that appears after the b-wave. The PhNR originates from the electrical activity of retinal ganglion cells (RGCS) and has clinical utility. The PhNR is typically recorded to a brief (<4ms) test flash, we explored the effect of increasing the stimulus duration on the PhNR amplitude of normal subjects in an ongoing attempt to optimize the stimulus conditions for its clinical use.

Methods: ERGs were recorded with DTL electrodes from normal subjects (N=10) in the age range 23-53 years using the ColorBurst handheld ganzfeld stimulator and hardware from Diagnosys (Lowell, MA). The stimuli consisted of red test flashes on constant blue background (8 phot cd.s/m.sq). The test flashes were either brief stimuli (<4 ms duration) in the range of 0.00625 - 6.4 phot cd.s/m.sq or longer duration (20-80 ms) in the range of 0.125-1500 cd/m.sq. A new algorithm in the Espion software with objective sweep selection based on various noise and artifact identification criteria was used to average repeated responses at each test flash intensity. The PhNR amplitude of the averaged waveform was plotted as a function of test flash intensity and fitted with the standard Naka-Rushton equation. The saturated amplitude (Vmax), slope (n) and semisaturation constant (K) derived from the fits were analyzed. The student t-test was performed to compare the fit parameters across different test flash durations with correction for multiple comparisons using the Holm's method.

Results: Vmax for the brief stimulus was 20 ± 7 microvolts and increased to 23 ± 2 microvolts for 20 ms duration stimuli. With further increase in stimulus duration the

PhNR Vmax was 34±8 μv, 42±10 μv and 37±10 μv for 40 ms, 50 ms and 60ms duration stimuli and thereafter reduced to 29±7 μv for an 80 ms stimulus duration. The Vmax amplitude differences between the brief and longer duration stimuli were statistically significant only for the 40 ms ($p=0.04$), 50 ms ($p=0.001$) and 60 ms ($p=0.01$) after correcting for multiple comparisons. Responses to stimulus durations up to 60 ms demonstrated a single PhNR trough and for longer duration stimuli two PhNR troughs were observed one following light onset after the b-wave and another following light offset.

Conclusions: The saturated PhNR amplitude is larger for longer duration stimuli and is maximal in the range of 40-80 ms duration. The larger PhNR amplitude at the intermediate test flashes likely reflect the summation of the PhNR to stimulus onset and offset and could potentially have more value in assessing retinal ganglion cell function in patients with disease affecting the optic nerve and/or inner-retinal neurons.

INTRODUCTION

German physiologist Hermann von Helmholtz first observed in 1873 the electrical potential changes in the retina of animals in response to light stimuli. In 1907, the Czech physiologist František Wenckebach used the term "electroretinogram" (ERG) to describe the electrical activity of the retina recorded from the surface of the eye in response to light stimulation (Wenckebach, 1907). Since then, the technique has been refined and developed for use in both clinical and research settings.

The cellular origins of the different components of the scotopic and photopic ERG recorded to a full-field flash stimulus have been identified and cell characterized over the years. Figure 1 illustrates a typical photopic flash ERG waveform. The a-wave of the photopic flash ERG, an initial negative deflection whose amplitude is measured at its trough from baseline is believed to be generated by the activity of cone photoreceptors and off-cone bipolar cells. The b-wave, the positive deflection measured at its peak from the preceding trough is from on- and off- cone bipolar cells. (for review, Frishman et al, 2018). The focus of my thesis work relates to the Photopic Negative Response (PhNR), a negative-going waveform that occurs after the b-wave and whose amplitude is measured at its trough from baseline, that reflects the activity of retinal ganglion cells (RGCs) (Viswanathan et al, 1999).

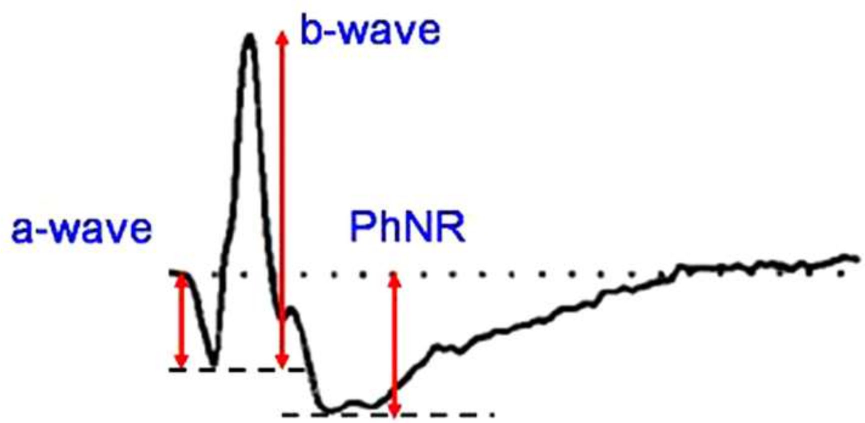

Figure 1: Photopic flash ERG waveforms illustrating the a-wave, b-wave and the Photopic Negative Response (PhNR) and how their amplitudes are determined.

In the clinic, ERGs are a non-invasive electrophysiological technique used to assess the integrity and function of the retina and are widely used to diagnose and monitor various retinal disorders. The International Society for Clinical Electrophysiology of Vision provides standard clinical protocols for the use of electroretinography in clinical settings with the goal of a set of universal protocols for measuring various retinal and disease conditions. In patients with glaucoma, for example, the PhNR amplitude has been found to be reduced, suggesting that it may serve as a biomarker for RGC dysfunction and disease progression (Viswanathan et al, 2001, Prencipe et al, 2020). The current ISCEV standard for recording PhNR involves a brief (<4ms duration) test flash (Frishman et al, 2018). Despite the growing field of clinical electrophysiology in vision health care, the stimulus parameters for recording the PhNR for routine clinical testing has not been fully optimized.

In my thesis I examine the effect of test stimulus duration on the PhNR amplitude to increasing stimulus strengths in normal healthy subjects.

METHODS

Full field ERGs were recorded from a total of 7 males and 3 females ranging in age from 22 to 53 years. Inclusion criteria stipulated that all subjects have healthy eyes, with no known retinal pathology with the spherical equivalent of the refractive error of less than 5 diopters.

ERGs were recorded monocularly with one eye chosen at random. The pupil of the tested eye was maximally dilated with 1% tropicamide. Both eyes were anesthetized with 0.5% proparacaine hydrochloride before placing corneal DTL electrodes. The non-tested eye was covered with a light-proof patch and its electrode served as the reference. The ground electrode was clipped to the earlobe ipsilateral to the tested eye.

The visual stimuli were delivered using a ColorBurst™ Handheld stimulator (Diagnosys LLC) and consisted of red (640 nm) test stimuli delivered on a constant rod saturating blue (440 nm) background following 15 minutes of light adaptation to the background illumination (Figure 2). The strength of the background stimuli was 8 phot cd/m^2 for all experiments. The red test stimuli were brief flashes <4ms in duration increasing in intensity from 0.00625 to 6.4 phot cd.s/m^2, or longer duration test flashes (10, 20, 40, 50, 60, 80 and 125 ms) of stimulus strengths in the range of 0.25 to 1000 phot.cd/m^2 for each stimulus duration.

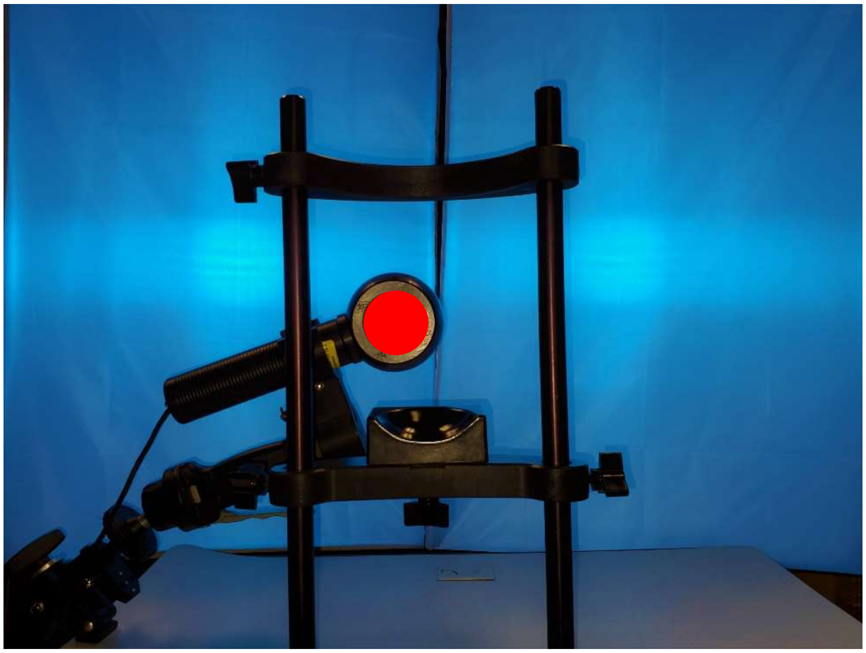

Figure 2: Experimental setup illustrating a red test stimulus on a constant blue background (underlying the red stimulus) and a blue surround behind the stimulator created with a photographer's photoshoot lighting.

ERG signals were sampled at 1 KHz, amplified, digitized and bandpass filtered in the range of 0.3 to 300 Hz. Proprietary software from the manufacturer applied an algorithm for objective sweep selection based on various noise and artifact identification criteria prior to averaging 25 responses at each test flash intensity. A 3-point smoothing function was applied to reduce noise and extract the PhNR amplitude. The a-wave and b-wave implicit times and amplitudes were derived without the application of the smoothing function.

The raw and smoothed waveforms at test flash strength was imported into Sigmaplot software (Systat Software, San Jose, CA, USA) for analysis. The PhNR, amplitude of the averaged waveform was plotted as a function of test flash strength and fitted with a general form of the Naka-Rushton equation of the form,

$$V(I) = (V_{max} * I^n) / (I^n + K^n)$$

Where I is the test flash strength, V(I) the amplitude at that given flash strength, Vmax the saturated amplitude, n the slope of the linear portion of the function and K the semisaturation constant or the test flash strength where the response amplitude is one half of the saturated amplitude. The student t-test was performed to compare the fit parameters of Vmax, n and K between the current standard, namely the brief (<4ms) test flash condition and the longer test stimulus durations. A correction factor was applied for multiple comparisons using the Holm's criteria.

RESULTS

Figure 3 illustrates the average ERG response traces to increasing flash strengths for the standard brief (<4ms) test stimuli from one representative subject. The black traces represent the actual signal before the 3-point smoothing used to derive the a- and b-wave implicit times and amplitudes and the red traces represents the smoothed waveforms used to derive the PhNR amplitude. The a-wave, b-wave and the PhNR amplitudes increased as the luminance of the test flashes was increased and the b-wave and the PhNR appear to saturate at higher test flash strengths. This figure illustrates how the a- and b-waves can be clearly identified in the unsmoothed traces whereas the smoothing allows easier identification of the PhNR trough that is quite broad and noisy in the unsmoothed waveform. At lower test flash strengths when the a-wave, b-wave or the PhNR were not prominent, their amplitudes were derived at the timing in the successive bright flashes where the waveforms can be clearly identified.

Figure 4 illustrates the stimulus strength response function of the PhNR fit with the Naka-Rushton equation for the same subject as in Figure 3 for PhNR amplitudes derived for the stimulus strengths from the unsmoothed (black) and smoothed (red) waveforms. The values of the Naka-Rushton fit parameters are quite close with both techniques. Figure 5 illustrates the comparison of the Naka-Rushton fit parameters of Vmax, n and K (Figures 5A, 5B and 5C) for the PhNR from all 10 subjects in the study for the unsmoothed (black) and smoothed waveforms (red). While the mean values of the fit parameters of n and K were not statistically different between the unsmoothed and smoothed data there was a statistically significant difference between Vmax for the two sets of data ($p=0.004$). However, while the Vmax difference was statistically significant, the difference in the mean values is not clinically significant. The unsmoothed data can be expected to have higher amplitudes as the trough tends to be noisy with slight excursions of the waveform contributing to slightly larger amplitudes when the most negative point is chosen.

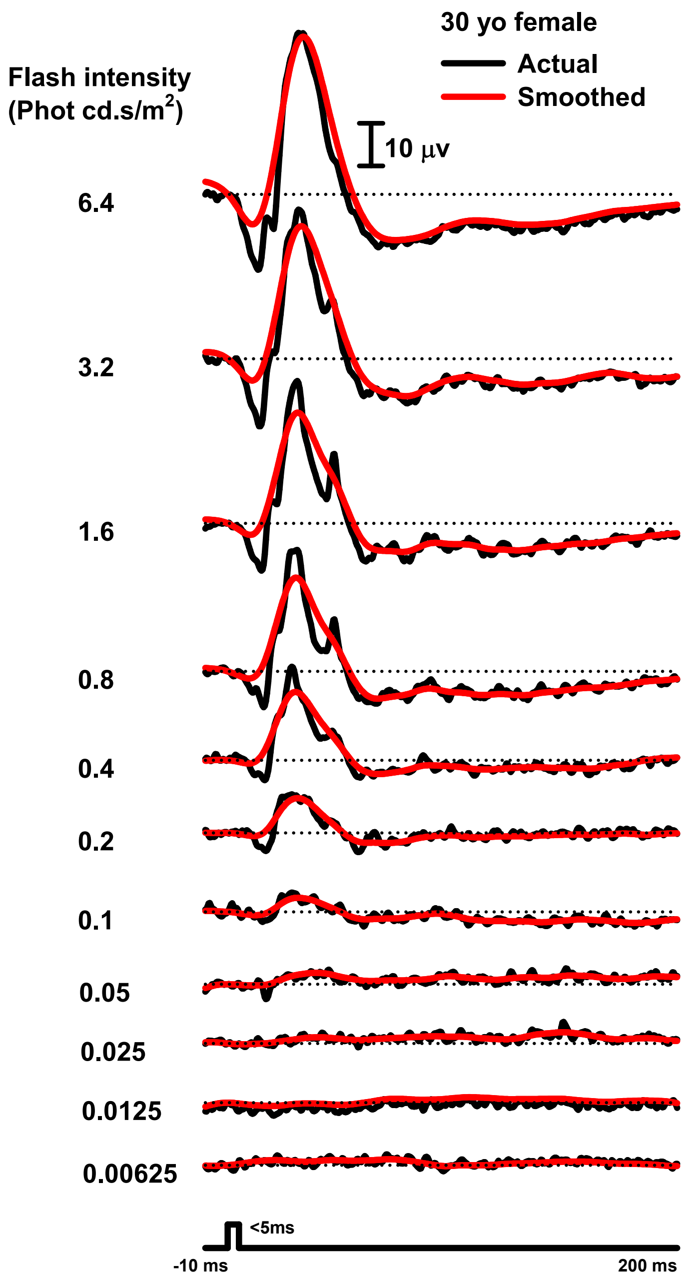

Figure 3: Electroretinogram intensity series to a brief flash for one subject. Black traces represent the raw ERG signal, whereas the red traces represent traces after 3-point smoothing.

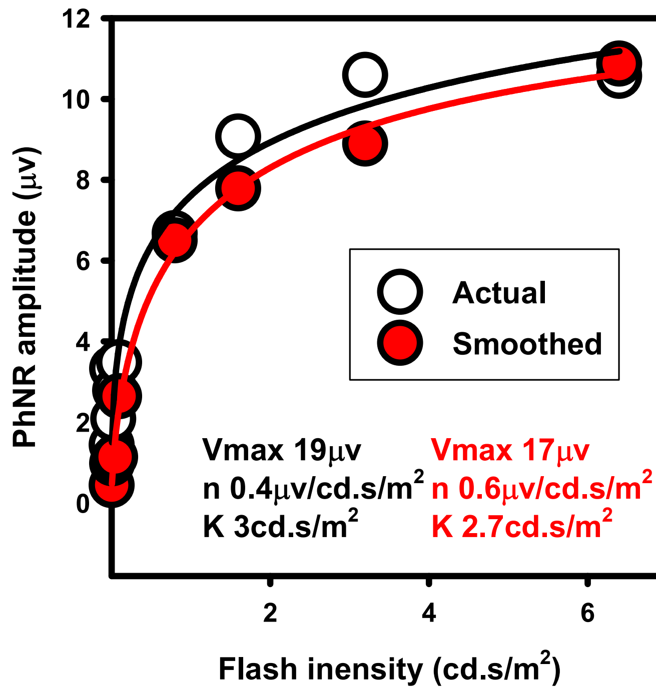

Figure 4: Intensity response function fit with the naka-Rushton equation for the same subject as in Figure 3.

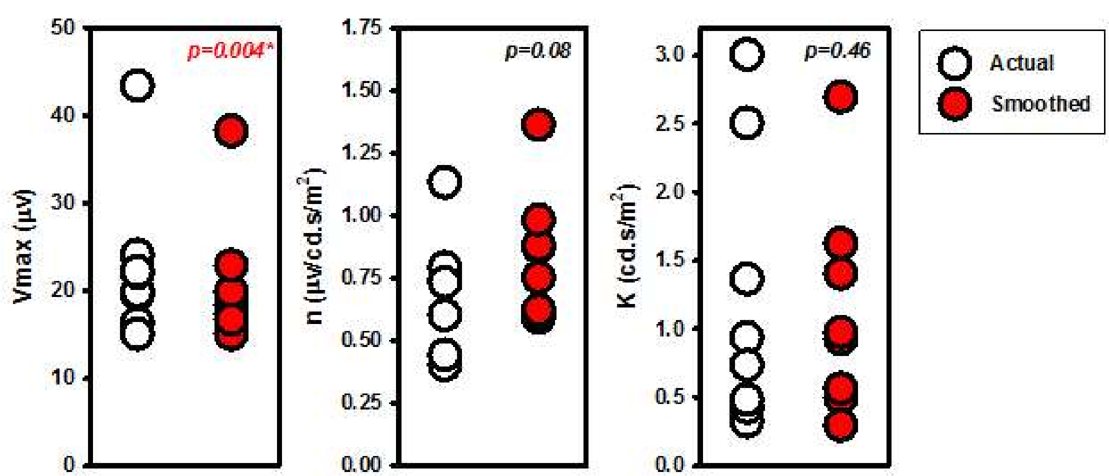

Figure 5: PhNR Naka-Rushton fit parameters from all study subjects for data extracted from unsmoothed (black) and smoothed (red) waveforms.

Figure 6 illustrates the intensity response functions for one subject for test flash duration ranging from 10 to 125 ms. Expectedly the amplitudes of all ERG components increased with stimulus strength for all test flash durations. Across test flash durations, the PhNR amplitude tended to increase from 10ms to durations in the range of 40 to 60ms. However, at longer test flash durations two troughs became apparent, on the $PHNR_{on}$ following the b-wave after stimulus onset and another, the $PhNR_{off}$ following stimulus offset (green arrows in Figure 6) and the PhNR tended to have smaller amplitude compared to the amplitude of the single trough seen at the intermediate test flash durations.

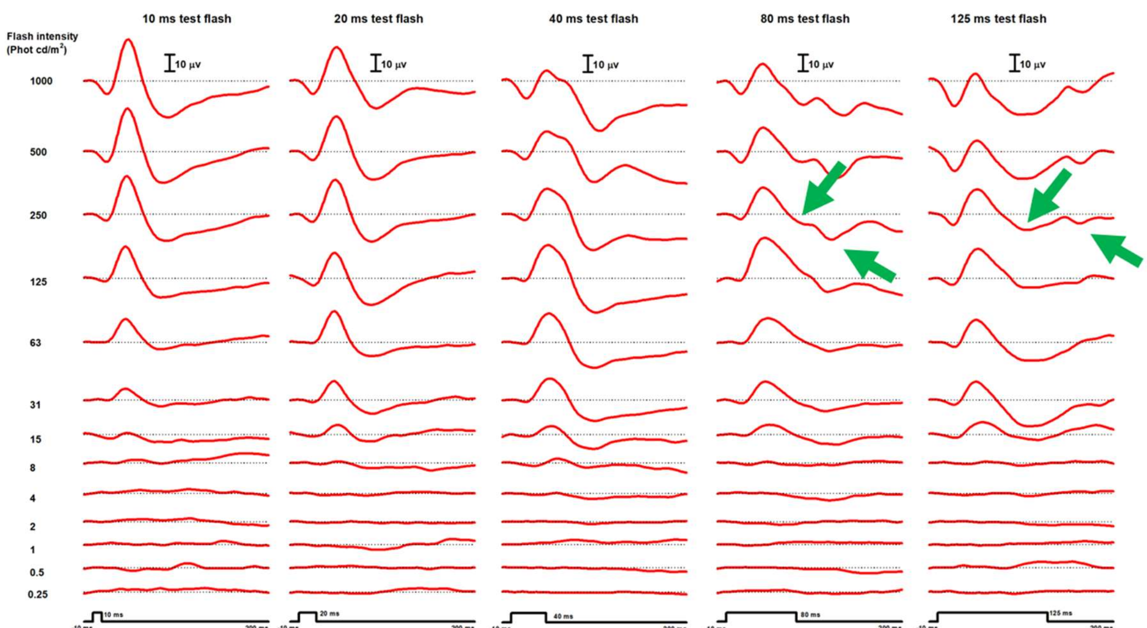

Figure 6: Photopic flash ERG responses to increasing stimulus strengths for test flash durations increasing from 10 ms to 125 ms. The green arrows indicate the separation of the $PHNR_{on}$ and $PhNR_{off}$ at longer stimulus durations.

Figure 7 illustrates the mean ($\pm$ standard deviation) amplitude of the PhNR Vmax for all study subjects plotted as a function of test flash duration. For test flash durations where the separation of the $PHNR_{on}$ and $PhNR_{off}$ was visible only the $PHNR_{on}$ values were used in this plot. The mean value of the PhNR Vmax was 20 $\pm$7 μv for the brief (<4ms) stimulus condition. For test flash durations of 10 ms, 20 ms, 40 ms, 50 ms, 60 ms, 80 ms and 125 ms the mean values of the PhNR Vmax were 23$\pm$5 μv, 23$\pm$2 μv, 34$\pm$8 μv, 42$\pm$10 μv, 37$\pm$10 μv, 29$\pm$7 μv and 28$\pm$12 μv respectively. While there was a trend for average Vmax of longer duration stimuli to be greater than the mean value obtained for the brief stimuli, the differences were statistically significant only for the 40 ms ($p=0.04$), 50 ms ($p=0.001$) and 60 ms ($p=0.01$) after correcting for multiple comparisons. As illustrated in Figure 8, the PhNR amplitudes measured at tow fixed test flash strengths for the different flash durations showed a similar trend with maximum amplitude for a 40 ms duration stimulus and amplitudes reducing for shorter and longer stimulus durations.

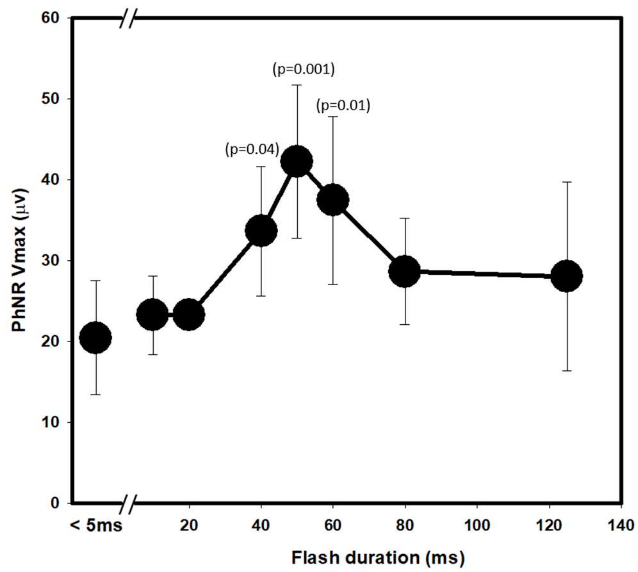

Figure 7: Mean $\pm$ standard deviation of PhNR saturated amplitude (Vmax) as a function of test flash duration.

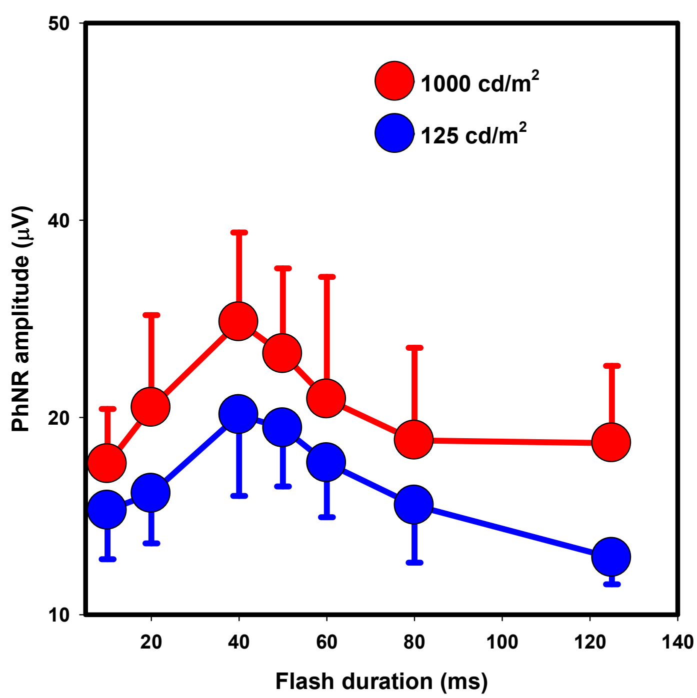

Figure 8: Mean $\pm$ standard deviation of PhNR amplitude as a function of test flash duration for an intermediate (blue 125 phot cd/m^2) and high (red 1000 phot cd/m^2) test lash strength.

DISCUSSION

In our investigation, we found the PhNR amplitude is significantly larger for stimulus durations in the range of 40-60 ms as reflected by an increase in the Vmax. We also found that there is a single PhNR trough in the ERG responses at these test flash durations and that two separate PhNRs can be observed at longer test flash durations, a $PhNR_{on}$ following the b-wave after stimulus onset and the second, the $PhNR_{off}$ following stimulus offset it is very likely that the larger PhNR amplitude observed at intermediate test flash durations is due to the overlap of the $PhNR_{on}$ and $PhNR_{off}$.

The amplitude of the $PhNR_{off}$ tends to be smaller than that of the $PhNR_{on}$ and consequently it may be argued that the $PhNR_{off}$ adapts to the test flash for longer duration stimuli. However, studies that have used ramped stimuli to record PhNR responses found that the $PhNR_{off}$ response elicited to ramped on/rapid off stimuli is smaller in amplitude compared to the $PhNR_{on}$ response elicited to rapid on/ramped off stimuli (Pangeni et al., 2012). Since the rapid on and rapid off stimuli are considered to minimize the effects of neuronal light adaptation it is very unlikely that the amplitudes differences we find between $PhNR_{on}$ and $PhNR_{off}$ are due to adaptation of the light offset response. It may also be argued that the increase in PhNR amplitude with test flash duration may be the result of some form of temporal integration that augments the neuronal responses. While we cannot completely rule out the possibility that there might have been some degree of temporal integration, that alone cannot explain our findings as the PhNR amplitude reduces with increase in stimulus durations greater than 60 ms. For all the reasons highlighted above, summation of PhNR responses generated to stimulus onset and offset is likely the major factor contributing to the enhancement of the amplitudes in the intermediate stimulus durations. Furthermore, our findings with healthy human subjects are consistent with previous demonstrations in non-human primate studies using focal and full filed flash ERGs (Kondo et al.,2008), Kinoshita et al., 2016).

The PhNR Vmax for the 50 ms duration stimulus was on average twice as large as the Vmax obtained with the brief (<4ms) test flash that is recommended for clinical use by the International Society for Electrophysiology of Vision (Frishman et al., 2018) for clinical use. Now it remains to be seen whether intermediate test flash durations may be more sensitive than the than the brief stimuli for early detection or monitoring treatment effects and/or disease progression in patients with retinal ganglion cell pathology.

CONCLUSION

The saturated PhNR amplitude is larger for longer duration stimuli and is maximal in the range of 40-80 ms duration. The larger PhNR amplitude at the intermediate test flashes likely reflect the summation of the PhNR to stimulus onset and offset and could potentially have more value in assessing retinal ganglion cell function in patients with disease affecting the optic nerve and/or inner-retinal neurons.

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