Article

Factors Affecting Photopic Negative Response Recorded with RET*eval* System: Study of Young Healthy Subjects

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Purpose: To determine whether there is a significant correlation between the amplitude of the photopic negative response (PhNR) and the peripapillary retinal nerve fiber layer thickness (pRNFLT) in eyes of young, healthy subjects.

Methods: We analyzed 136 eyes of 136 young, healthy subjects (89 males and 47 females; age, 20–29 years). The PhNRs were recorded with the RET*eval* system without mydriasis using red flashes on a blue background. PhNR amplitude was measured at two points: at 72 ms (P_{72}) and at the negative trough following the b-wave (P_{min}). Univariate and multivariable linear regression analyses were performed to identify the independent variables that were significantly correlated with P_{72} and P_{min}. The variables included age, sex, axial length, pRNFLT, intraocular pressure (IOP), a-wave amplitude, b-wave amplitude, and pupillary area during the electroretinogram recordings.

Results: The amplitudes of P_{72} and P_{min} were significantly larger in female subjects ($P = 0.021$ and $P = 0.001$, respectively). Univariate analyses showed that PhNR amplitudes were significantly correlated with pRNFLT (P_{72}: $r = 0.246$, $P = 0.004$; P_{min}: $r = 0.219$, $P = 0.011$). Female sex was significantly and negatively correlated with P_{72} ($r = -0.206$; $P = 0.016$) and P_{min} ($r = -0.271$; $P = 0.001$). Multivariable regression analyses showed that greater pRNFLT was an independent factor significantly associated with a larger P_{72} ($r = 0.283$; $P = 0.004$) and P_{min} ($r = 0.299$; $P = 0.002$). Female sex was an independent factor that was significantly associated with a larger P_{min} ($r = -0.208$; $P = 0.022$).

Conclusions: These findings indicate that PhNR amplitude is significantly associated with pRNFLT and female sex in young, healthy subjects.

Translational Relevance: The amplitude of the PhNR recorded with RET*eval* is smaller in subjects with thinner pRNFLT not only in glaucoma patients but also in young healthy subjects.

Introduction

The photopic negative response (PhNR) of the full-field electroretinogram (ERG) is a negative wave following the b-wave that originates from the spiking activity of the retinal ganglion cells and amacrine cells with some possible contribution from the retinal glial cells.[1] In a clinical study, the PhNR amplitude was found to be reduced in eyes with glaucoma, and the decrease was significantly correlated with the degree of visual field loss and a reduction in the peripapillary retinal nerve fiber layer thickness (pRNFLT).[2]

There are several electrophysiological examinations, such as pattern ERGs and PhNR, that can detect the presence of glaucoma prior to visual field losses.[3] However, the use of conventional ERG recordings as a screening tool for glaucoma is time consuming and somewhat invasive for patients. A full-field ERG recording system called the RET*eval* system (LKC Technologies, Gaithersburg, MD) was recently introduced. This system is comprised of a hand-held Ganzfeld dome and a special single-use skin electrode array referred to as the Sensor Strip. This system can record International Society for Clinical Electrophysiology of Vision (ISCEV)-compliant five- and six-step

protocols based on the adaptational state of the eye and the stimulus luminance. The device can also record the PhNR and on/off responses of the ERGs. The device delivers stimulus flashes with constant retinal illuminance (Td-s) by adjusting the luminance (cd·s/m^2) to compensate for changes in the pupillary area (mm^2) in real time, and the ERGs can be recorded without mydriasis.[4]

Several clinical studies have used the RETeval system as an ERG recording device, and the results showed that the PhNRs recorded by RETeval are reliable and can be used for clinical and research purposes.[5–7] Although there have been studies showing a significant correlation between PhNR and pRNFLT in patients with glaucoma and optic nerve disorders, there has not been a study to determine whether the PhNR is significantly correlated with pRNFLT in young, healthy individuals. Thus, the purpose of this study was to determine whether there is a significant correlation between PhNR amplitude and pRNFLT. In addition, we determined the relationship between PhNR amplitude and the age, axial length, pupillary diameter, sex, and a-wave and b-wave amplitudes of the photopic ERGs recorded with the RETeval system from young, healthy Japanese subjects.

Methods

Study Design

This was a prospective, single-center study conducted at the Mie University Hospital between March 2017 and February 2019. The Medical Ethics Committee of Mie University Hospital approved the procedures used, and the procedures conformed to the tenets of the Declaration of Helsinki. All participants signed a written informed consent form after they were provided with information on the procedures to be used.

Subjects

One hundred and fifty-six individuals whose age ranged from 20 to 29 years were recruited from the medical students of Mie University. The students who had any ocular or systemic diseases were excluded.

Protocols of Ocular Examinations

The examinations were performed on the left eyes and consisted of measurements of the best-corrected visual acuity (BCVA), fundus examination by indirect ophthalmoscopy, and nonmydriatic color fundus photography (AFC-330; Nidek, Gamagori, Japan). The axial length was measured by partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Jena, Germany), and pRNFLT was measured by optical coherence tomography (OCT; RS-3000, Nidek). The pRNFLT measurements were performed using the Disk Map protocol (6 × 6 mm) without mydriasis. Only reliable results with a signal strength index ≥ 7/10 with no artifacts were used in the statistical analyses.

PhNR Recordings by RETeval

Full-field PhNRs were recorded with the RETeval system. The device uses a built-in infrared camera system that can measure the pupil size in real-time and adjusts the flash luminance (cd·s/m^2) continuously to deliver constant retinal illuminance stimuli throughout the measurement period. The stimulus intensity was set at 38 Td-s red flash on a 380-Td blue background. The stimuli were presented at a rate of 3.4 Hz, and 100 sweeps were averaged for each recording. A small red fixation spot was present at the center of the dome. During the stimulation, the pupil size (mm^2) was automatically measured in real time, and the stimulus flash luminance (cd·s/m^2) was continuously adjusted to maintain a constant flash retinal illuminance (Td-s) using the following equation:

$$\text{Photopic flash retinal illuminance (Td} - \text{s)}$$
$$= \text{Photopic flash luminance (cd} \times \text{s/m}^2)$$
$$\times \text{pupillary area (mm}^2)$$

The averaged pupil diameters during the Electroretinogram results recorded by RETeval system. The ERGs were obtained using LKC Sensor Strips, special skin electrode arrays placed 2 mm from the margin of the lower eyelid. Before application of the skin electrode array, the skin of the lower lid was cleaned with alcohol-soaked cotton. This electrode array contained an active, a reference, and a ground electrode in a single adhesive tape. The elicited electrical potentials were direct current (DC) amplified and digitized with a sampling rate of 2 kHz. The data resolution was 24 bits for ±0.6 V, which is equal to approximately 0.07 μV.

To record the PhNRs, ISCEV standard processing steps were used, except that the 0-phase high-pass filter (HPF) had a corner frequency of 1 Hz rather than 0.3 Hz and the 0-phase low-pass filter (LPF) had a corner frequency of 100 Hz rather than 300 Hz. The change in the HPF made the baseline more stable, which is important because the PhNR occurs

about 70 ms after the stimulus onset. The change in the LPF reduced the noise, which was better for the relatively broad PhNR trough. An extended pre-stimulus baseline was used so that the baseline stability could be better assessed. To prevent the common-mode signal from being recorded by the device, the RET*eval* used an active ground (right leg drive) and a shielded electrode cable. To prevent common-mode signals from turning into difference-mode signals measurable by the data acquisition system, all of the electrodes in the Sensor Strips had the same area to match their impedances, and the data acquisition system had high-impedance analog input at the power line frequencies.

Signal Analysis

Individual traces were detrended with a third-order polynomial fitted to the entire response to provide optimal balance in reducing the baseline drift.[8] The PhNR time series dataset consisting of 430 data points (time interval from −100 to 120 ms) was extracted with RET*eval* rff browser software. An approximate curve was generated from this 430-point time series dataset by selecting the third-order polynomial approximation with the approximate curve format setting for the Excel 2019 graph tool (Microsoft, Inc., Redmond, WA). The detrending was performed by subtracting this approximate curve from the original PhNR. We excluded the ERGs of subjects when we could not reduce the baseline drift in the third-order polynomial fitted ERGs.

The methods used to measure each ERG component are shown in Figure 1. The a-wave amplitude was measured from the pre-stimulus baseline to the first negative trough. The b-wave amplitude was measured from pre-stimulus baseline to the positive peak. The PhNR amplitude was measured at 72 ms from the pre-stimulus baseline (P_{72}), and P_{min} was measured from the pre-stimulus baseline to the negative trough immediately after the b-wave. The ratio of PhNR voltage at 72 ms to b-wave peak was designated as the P ratio (P_{72} voltage/b-wave voltage).[3] The ratio of the b-wave peak voltage to the PhNR trough voltage to b-wave amplitude was designated as the W ratio (b-wave voltage − P_{min} voltage)/(b-wave voltage − a-wave voltage).[9]

Statistical Analyses

Univariate and multivariable linear regression analyses were used to determine the factors that were significantly correlated with the PhNR amplitude recorded by the RET*eval* system. The amplitudes of P_{72} and P_{min} and the P ratio and W ratio that represent
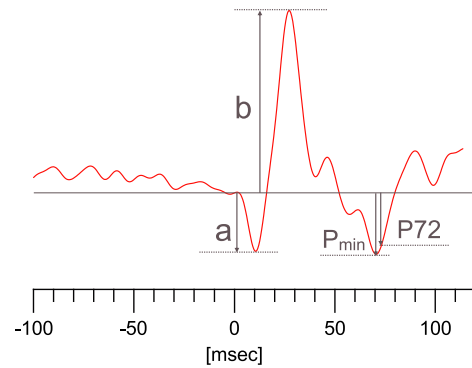
P ratio = -P72/b
W ratio = (b-P_{min})/(b-a)

a : a-wave peak voltage
b : b-wave peak voltage
P72 : The negative voltage at 72msec
P_{min} : The negative trough voltage following the b-wave

Figure 1. Illustration of the methods to measure various components of the ERG waveform. Arrows represent the voltage of each parameter. The a-wave voltage was measured from the pre-stimulus baseline to the first negative trough. The b-wave voltage was measured from the pre-stimulus baseline to the positive peak. The P_{72} voltage was measured at 72 ms after the stimulus onset, and the voltage of P_{min} was measured at the negative trough voltage following the b-wave.

the PhNRs were used as dependent variables, and the independent variables were the age, sex, axial length, pRNFLT, a-wave and b-wave amplitudes of the ERGs, intraocular pressure (IOP), and pupillary area during the ERG recordings. These variables were selected based on past ERG studies,[4,9–11] the ISCEV extended protocol,[12] and medical points of view. The coefficients of correlation (r) and P values were calculated for the univariate linear regression analyses, and the standardized partial regression coefficients (β) and P values were calculated for the multivariable linear regression analyses for the eight independent variables. After confirming that the data were approximately normally distributed by the Shapiro–Wilk test, the differences between the male and female subjects were compared with unpaired t-tests. The results were considered statistically significant when $P < 0.05$.

Results

Clinical Characteristics of Subjects

We initially examined 156 subjects, but 16 subjects were excluded because of baseline drift of the PhNRs, presumably due to many blinks or narrow palpable fissures. We also excluded two subjects because the

Table 1. Demographics of Subjects

Variables	Mean ± SD			P
	Overall ($N = 136$)	Males ($n = 89$)	Females ($n = 47$)	
Age (y)	23.54 ± 1.62	23.55 ± 1.68	23.53 ± 1.52	0.919
Axial length (mm)	25.40 ± 1.25	25.63 ± 1.26	24.97 ± 1.14	0.004[*]
Pupil diameter (mm)	2.27 ± 0.25	2.30 ± 0.26	2.21 ± 0.22	0.041[*]
pRNFLT (μm)	99.28 ± 9.99	98.54 ± 8.95	100.68 ± 11.69	0.392
P_{72} (μV)	3.69 ± 2.19	3.37 ± 1.94	4.31 ± 2.50	0.021[*]
P_{min} (μV)	5.19 ± 2.31	4.74 ± 1.91	6.05 ± 2.73	0.001[*]
P ratio	0.31 ± 0.19	0.29 ± 0.17	0.33 ± 0.21	0.406
W ratio	1.00 ± 0.15	0.99 ± 0.13	1.04 ± 0.18	0.110
a-Wave amplitude (μV)	5.19 ± 1.43	4.98 ± 1.36	5.58 ± 1.47	0.016[*]
b-Wave amplitude (μV)	17.56 ± 4.66	16.60 ± 4.02	19.22 ± 5.70	0.009[*]
IOP (mm Hg)	13.8 ± 2.9	13.3 ± 3.0	14.8 ± 2.4	0.003[*]

[*]$P < 0.05$ was considered significant (difference in means between males and females student t-test).
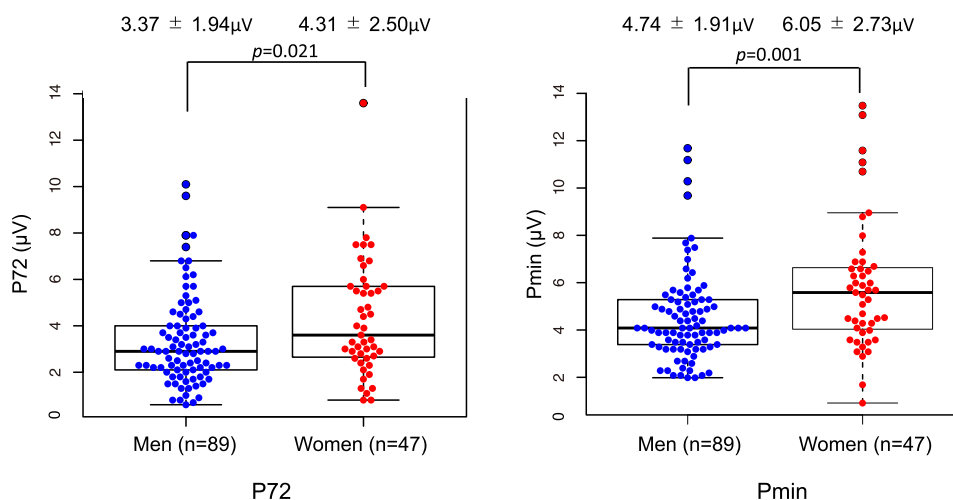

Figure 2. A beeswarm plot of the PhNR measured at 72 ms and the negative trough following the b-wave in 89 male subjects (*blue dots*) and 47 female subjects (*red dots*). Bars are mean ± SD. The difference in PhNR amplitude between the male and female subjects was significant ($P = 0.021$ and $P = 0.001$, respectively; unpaired t-tests).

signal index of their OCT images was low and two other subjects because their IOPs were not recorded. In the end, 136 eyes were used for the final statistical analyses. The demographic information of the 136 eyes of 136 young healthy subjects are shown in Table 1. The mean age of the subjects was 23.5 ± 1.6 years (range, 20–29 years). The number of male subjects was 89, and the number of female subjects was 47; the predominance of male subjects reflected the population of young medical students. The axial length was significantly longer in the male subjects ($P = 0.004$), and the pupillary diameter during the ERG recordings was significantly larger in the male subjects ($P = 0.041$). The a- and b-wave amplitudes were significantly larger in the female subjects ($P = 0.016$

and $P = 0.009$, respectively). We also found that the amplitudes of P_{72} and P_{min} were significantly larger in the female subjects ($P = 0.021$ and $P = 0.001$, respectively) (Fig. 2). The IOPs were slightly, but significantly, higher in the female subjects ($P = 0.003$).

Univariate and Multivariable Analysis of PhNR Amplitudes and Independent Variables

Univariate and multivariable linear regression analyses were used to determine the factors that were significantly correlated with the P_{72}, P_{min}, P ratio, and W ratio (Tables 2, 3). Univariate analyses showed that P_{72}, P_{min}, P ratio, and W ratio were significantly associated with pRNFLT (Table 2). The pRNFLTs

Table 2. Univariate Correlations of PhNR and Other Variables

Variable	P_{72}		P_{min}		P Ratio		W Ratio	
	r	P	r	P	R	P	r	P
Age	−0.006	0.940	0.031	0.721	0.009	0.921	0.035	0.687
Sex	−0.206	0.016*	−0.271	0.001*	−0.092	0.289	−0.134	0.119
Axial length	−0.055	0.524	0.057	0.509	−0.080	0.353	−0.020	0.817
pRNFLT	0.246	0.004*	0.219	0.011*	0.221	0.010*	0.201	0.019*
Pupil diameter	−0.158	0.066	−0.232	0.007*	−0.146	0.090	−0.201	0.012*
a-Wave amplitude	0.219	0.010*	0.197	0.022*	−0.077	0.375	−0.398	<0.001*
b-Wave amplitude	0.242	0.005*	0.261	0.002*	−0.195	0.023*	−0.247	0.004*
IOP	0.162	0.059	0.094	0.278	0.186	0.030*	0.166	0.054

Multiple regression analysis was performed by replacing the sex with a number; women were replaced with 1 and men with 2.
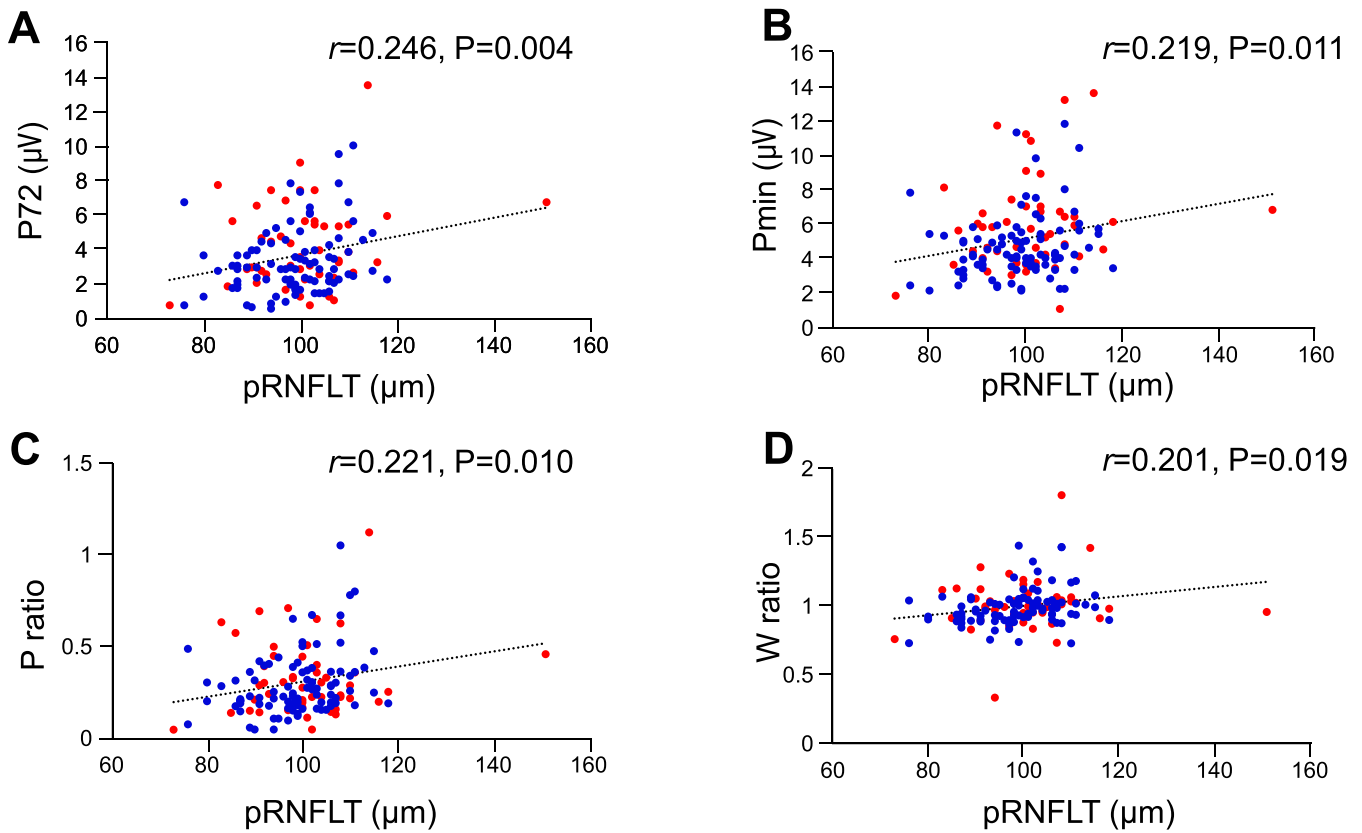
*$P < 0.05$ was considered significant.

Figure 3. Graph showing the relationship between PhNR and pRNFLT in 89 male subjects (*blue dots*) and 47 female subjects (*red dots*). Scatterplots show (**A**) PhNR at 72 ms (P_{72}), (**B**) PhNR at the negative trough after the b-wave (P_{min}), (**C**) the ratio of PhNR voltage at 72 ms to the b-wave peak (P ratio), (**D**) the ratio of b-wave peak to PhNR trough voltage to b-wave amplitude (W ratio) versus pRNFLT.

are plotted against PhNRs in Figure 3. The PhNRs recorded from two representative men are shown in Figure 4. The amplitude of the PhNR was larger in eyes with the thicker pRNFLT (Fig. 4, upper waveform).

There was a weak but significant correlation between sex and P_{72} ($r = -0.206$; $P = 0.016$) and between sex and P_{min} ($r = -0.271$; $P = 0.001$) (Table 2). The amplitude of the PhNRs was significantly larger in the female subjects. There was a moderate and

Table 3. Multivariable Analysis of the Associations Among PhNR and Other Variables

Criterion Variable	Variables	Partial Regression Coefficient	β	P	Variance Inflation Factor	R
P_{72}	Age	−0.015	−0.011	0.892	1.034	0.430
	Sex	−0.369	−0.081	0.431	1.317	
	Axial length	0.185	0.106	0.277	1.475	
	pRNFLT	0.062	0.283	0.004*	1.430	
	Pupil diameter	−0.893	−0.101	0.239	1.128	
	a-Wave amplitude	0.187	0.122	0.353	2.682	
	b-Wave amplitude	0.058	0.123	0.359	2.774	
	IOP	0.144	0.194	0.024*	1.124	
P_{min}	Age	0.013	0.009	0.908	1.034	0.483
	Sex	−1.003	−0.208	0.022*	1.317	
	Axial length	0.507	0.275	0.004*	1.475	
	pRNFLT	0.069	0.299	0.002*	1.430	
	Pupil diameter	−1.572	−0.168	0.044*	1.128	
	a-Wave amplitude	−0.007	−0.004	0.923	2.682	
	b-Wave amplitude	0.094	0.191	0.143	2.774	
	IOP	0.069	0.088	0.289	1.124	
P ratio	Age	−0.002	−0.016	0.848	1.034	0.413
	Sex	−0.033	−0.084	0.368	1.317	
	Axial length	0.015	0.100	0.309	1.475	
	pRNFLT	0.005	0.280	0.004*	1.430	
	Pupil diameter	−0.065	−0.086	0.320	1.128	
	a-Wave amplitude	0.033	0.251	0.060	2.682	
	b-Wave amplitude	−0.017	−0.417	0.002*	2.774	
	IOP	0.012	0.186	0.031*	1.124	
W ratio	Age	−0.004	−0.042	0.578	1.034	0.547
	Sex	−0.060	−0.189	0.029*	1.317	
	Axial length	0.026	0.214	0.019*	1.475	
	pRNFLT	0.004	0.253	0.005*	1.430	
	Pupil diameter	−0.091	−0.148	0.064	1.128	
	a-Wave amplitude	−0.055	−0.517	<0.001*	2.682	
	b-Wave amplitude	0.003	0.098	0.432	2.774	
	IOP	0.005	0.083	0.293	1.124	

β, standardized partial regression coefficients. Multiple regression analysis was performed by replacing the sex with a number; women were replaced with 1 and men with 2.

*$P < 0.05$ was considered significant.

significant correlation between a-wave amplitude and P_{72} ($r = 0.219$; $P = 0.010$), P_{min} ($r = 0.197$; $P = 0.022$), and the W ratio ($r = -0.398$, $P < 0.001$) (Table 2). There was a weak but significant correlation between pupillary diameter and P_{min} ($r = -0.232$; $P = 0.007$) and the W ratio ($r = -0.201$; $P = 0.012$) (Table 2). There was also a weak but significant correlation between the IOP and the P ratio ($r = 0.186$; $P = 0.030$).

Multivariable linear regression analyses showed that the factors that were significantly correlated with PhNRs varied depending on which criterion variables were used. pRNFLT was the only variable that was significantly correlated with all of the PhNR measures: P_{72} ($r = 0.283$; $P = 0.004$), P_{min} ($r = 0.299$; $P = 0.002$); P ratio ($r = 0.280$; $P = 0.004$), and W ratio ($r = 0.253$; $P = 0.005$) (Table 3). P_{min} and the
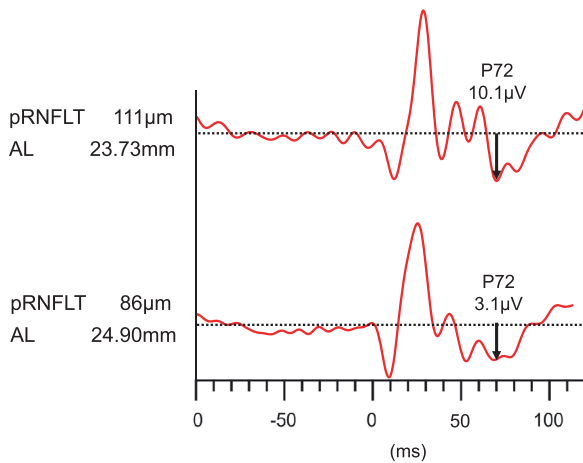
Figure 4. Representative PhNR data recorded from two males who had pRNFLT values of 111 μm and 86 μm. PhNR amplitude was measured from the baseline (*dotted black line*) at 72 ms. PhNR amplitude is larger in eyes with a thicker pRNFLT.

W ratio were significantly correlated with axial length ($r = 0.275$, $P = 0.004$ and $r = 0.214$, $P = 0.019$, respectively) (Table 3), and female sex ($r = –0.208$, $P = 0.022$ and $r = –0.189$, $P = 0.029$, respectively) (Table 3). We also found that P_{72} and the P ratio were weakly but significantly correlated with the IOP ($r = 0.194$, $P = 0.024$ and $r = 0.186$, $P = 0.031$, respectively) (Table 3). The b-wave amplitude was significantly correlated with the P ratio ($r = –0.417$; $P = 0.002$), and the a-wave amplitude was significantly correlated with the W ratio ($r = –0.517$, $P < 0.001$) (Table 3). However, we believe that the correlations between the PhNR and a-wave or b-wave were not significant because the b-wave amplitude and a-wave amplitude were used for calculation of the P ratio and W ratio (Fig. 1), and it was expected there would be a significant correlation between them.

Discussion

In this study, PhNR amplitude was significantly correlated with pRNFLT in young, healthy subjects. In addition, PhNR amplitude was significantly larger in female subjects. As best we know, this is the first study to detect a significant sex difference in PhNR amplitude. Several studies have examined PhNR amplitudes in patients with glaucoma or optic nerve atrophy induced by trauma,[13] compression,[14] inflammation,[15] or ischemia.[16] These studies showed that PhNR amplitude was selectively or predominantly reduced by these disorders. In glaucoma patients, significant correlations have been found between the amplitude of the

full-field PhNR and the visual field sensitivities determined by static automated perimetry, the morphological indicators of RNFLT surrounding the optic disc, the optic disc rim area, and cup/disc area ratio.[2,17] Our results indicate a significant correlation between PhNR amplitudes recorded by the RETeval system and pRNFLT in subjects without any eye disease.

The retinal ganglion cells are selectively damaged by diseases of the optic nerve and inner retina. Several studies have analyzed the correlation between PhNR amplitude and pRNFLT. In healthy subjects, our results indicate a weak but significant correlation between PhNR amplitude and pRNFLT (Tables 2, 3). However, the results of an earlier study on glaucoma patients and healthy subjects showed that the coefficient of correlation was stronger ($r = 0.53$, $P < 0.001$).[13] The results of another study that examined the relationship between PhNR amplitude and pRNFLT in eyes with optic nerve atrophy and in the unaffected contralateral eyes reported that the coefficient of correlation was strong ($r = 0.88$, $P < 0.001$).[14]

The question then arises: Why was the correlation weaker in our study? We suggest two possible reasons. First, the range of pRNFLT values was small because we studied only young, healthy subjects with a limited age range. In two earlier studies, the mean $\pm$ SD values for pRNFLT were 81.2 ± 36.8 μm[13] and 81.92 ± 8.8 μm,[14] whereas it was 99.28 ± 9.9 μm in our study. Second, the signal-to-noise ratio was probably lower in our study because we used skin electrodes. According to a past study, the correlations between the PhNR and structural parameters such as pRNFLT was weak at the early stage of glaucoma and stronger in advanced glucoma,[17] which perhaps could be the reason for the weak correlation between the PhNR and pRNFLT in our cohort.

We found that the difference in PhNR amplitude between male and female subjects was significant (P_{72}, $P = 0.021$; P_{min}, $P = 0.001$) (Table 1, Fig. 2). Thus, the mean PhNR amplitude in female subjects was 28% larger than that of male subjects. Univariate correlation analysis showed that the sex of the subject was significantly correlated with the amplitude of P_{72} ($r = –0.206$; $P = 0.016$) and P_{min} ($r = –0.271$; $P = 0.001$) (Table 2). However, these sex differences were not significant when the ratio of the PhNRs for a-waves and/or b-waves was used (P ratio, $P = 0.406$; W ratio, $P = 0.110$) (Table 1). These results indicate that the larger PhNR amplitudes in the female subjects may be because the amplitudes of the ERGs are generally larger in females than in males. Actually, the a-wave and b-wave amplitudes were larger in females than in males (Table 1).

A difference in the amplitudes of the ERGs between males and females has been reported in earlier studies. For example, Birch and Anderson[18] reported that the amplitudes of the rod and cone b-waves were slightly larger in the female subjects than in age-matched male subjects. In addition, it has been reported that the amplitudes of scotopic b-waves were significantly larger in female subjects,[10,19] and the amplitudes of the multifocal ERGs were also larger in female subjects.[20] In animal experiments, the amplitudes of the scotopic and photopic ERGs were larger in female rats,[21] and the amplitudes of the multifocal ERGs were larger with shorter implicit times in female monkeys.[22]

Several reports have discussed differences in ERG amplitudes between males and females, and the authors have hypothesized that these sex-related differences were due to differences in axial length,[18] ratio of L cones,[20] and estrogen levels.[19,21] Recently, Chaychi et al.[21] reported that ERGs were larger in premenopausal than menopausal female rats and were also larger than those for age-matched male rats. They suggested that these sex differences might be due to the effects of the estrogen hormone on retinal physiology. It is known that estrogen receptors are expressed on retinal ganglion cells and retinal pigment epithelial cells, and they play essential roles in normal retinal physiology.[23] If this is the case, the fact that we studied only young subjects who have higher sex hormone levels might be a reason why our analysis identified male/female differences as being a significant factor in PhNR amplitude. It is well known that there are male and female differences in hemoglobin levels in venous blood. Our findings indicate that separate normal ERG values for males and females may be necessary when we interpret the ERG results quantitatively. If we analyzed data with consideration of sex differences, we would be able to obtain more accurate results.

We also found that there was a weak but significant correlation between axial length and P_{min} ($r = 0.275$; $P = 0.004$) and the W ratio ($r = 0.214$; $P = 0.019$) by multivariable linear regression analysis (Table 3). However, these correlations were not significant by univariate analysis (P_{min}, $P = 0.524$; W ratio, $P = 0.817$) (Table 2), which indicated that there was not a significant linear correlation between the PhNR and axial length, but there was a significant nonlinear correlation between them. Our cohort consisted of subjects with relatively longer axial length, and we believe that additional studies are necessary to determine if there is a significant correlation between the PhNR and axial length.

We found that there was a weak but significant correlation between the P ratio and the IOP ($r = $ 0.186; $P = 0.030$) (Table 2) by univariate analysis. In addition, multivariable linear regression analysis showed that the IOP was an independent factor that was significantly correlated with the amplitude of P_{72} ($r = 0.194$; $P = 0.024$) and the P ratio ($r = 0.186$; $P = 0.031$). These results indicate that PhNR amplitude at 72 ms can be smaller in healthy subjects with lower IOPs. Considering a past report that lowering IOP made the PhNR larger in glaucoma patients who had poor IOP control[24] and the common idea among ophthalmologists that lower IOP is better for maintaining optic nerve health, our results appear to contradict such observations. We offer a hypothesis on the positive correlation between IOP and PhNR amplitude. In eyes with normal tension glaucoma (NTG), which is more common in Japan, it is known that reduced ocular blood flow can be one of the factors that accelerate glaucomatous optic neuropathy.[25] If the reduced ocular blood flow causes lower IOP in NTG, then lower IOP could be one of the risk factors for increased degeneration of the optic nerve. This would then result in a thinning of the pRNFLT and a reduction of PhNR amplitude in healthy subjects. Clearly, further study is necessary to determine whether there is a significant relationship between IOP and PhNR amplitude in healthy subjects and patients with NTG.

Finally, we should mention the weak but significant correlation between pupil diameter during ERG recordings and P_{min} ($r = -0.232$; $P = 0.007$) and the W ratio ($r = -0.201$; $P = 0.012$). Multivariable linear regression analysis showed that pupil diameter was an independent factor that was significantly correlated with P_{min} ($r = -0.168$; $P = 0.044$) (Table 3). This indicates that PhNR amplitude at the negative trough is smaller in eyes with larger pupillary diameter. This phenomenon could be explained by the Stiles–Crawford effect of the cone system.[4,26] However, considering the fact that the coefficient correlation between the PhNR and pupil diameter was very weak by multivariable analysis, pupil diameter during the ERG recording may not affect PhNR amplitude much. However, if we remove the effect of pupil diameter on the amplitudes of the ERGs, we need to consider recording the PhNR under mydriasis.

There are four limitations in this study. The first limitation is that we did not examine the static visual fields, so we cannot eliminate the possibility that early-stage glaucoma was present in some of our cohort. However, we did measure IOP, and none had elevated an IOP that would suggest early glaucoma. In addition, a glaucoma specialist examined the fundus photographs and confirmed that none of the subjects had optic disc signs of early-stage glaucoma.

The second limitation is that the test–retest variability of PhNR amplitudes is not as reliable as the a- and b-wave amplitudes of the photopic ERGs.[27] To reduce this limitation, we averaged the PhNRs from a larger number of stimuli (up to 100 responses) for each measurement. Also, the examination of a large number of subjects mitigated this limitation. In addition, we used P_{min} and the P ratio and W ratio as criterion variables, which had better repeatability than P_{72} when we performed statistical analyses.[9,27,28] The analyses using the P ratio and W ratio showed almost the same results as the analysis using P_{72} and P_{min} (Tables 2, 3).

The third limitation was that the RETeval system uses a LPF to reduce noise caused by standard settings, but the LPF may change the characteristics of the a-waves and reduce the a-wave amplitudes slightly. However, these changes would have occurred in all subjects and would not affect the results of the comparative analyses. The fourth limitation was the higher proportion of male to female subjects. The high proportion of male subjects was because we recruited the subjects from among the medical students of our university.

In conclusion, our results suggest that a thicker RNFL is independently correlated with larger amplitude PhNR recorded with the RETeval system. In addition, larger PhNR amplitudes were recorded for the female sex with the RETeval. These findings indicate that PhNR amplitude is associated with structural characteristics of the RNFL in healthy subjects. Because a decrease in PhNR amplitude has been reported to precede pRNFLT thinning in glaucoma patients,[13] an undiagnosed patient with reduced PhNR should be examined carefully for retinal disorders likely to affect the retinal ganglion cells. Furthermore, we should consider sex differences when we interpret the ERG results.

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