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The decline of the photopic negative response (PhNR) in the rat after optic nerve transection

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Abstract

Purpose: To investigate the contribution to the photopic negative response (PhNR) of the electroretinogram (ERG) by retinal ganglion cells (RGCs). The PhNR was assessed longitudinally following optic nerve transection (ONTx). *Methods*: Photopic ERGs were recorded from each eye of an anesthetized (ketamine/xylazine, 60 mg/kg and 5 mg/kg) Brown Norway rat using custom made electrodes (PT-IR Tef., A-M System Inc). ERGs were elicited using green Ganzfeld flashes (11.38 scd/m^2, 22.76 cds/m^2) and a rod suppressing green-background (40 cd/m^2). PhNRs were compared before and after optic nerves were transected. Cresyl violet stained retinal flatmounts were used to estimate cell loss in the ganglion cell layer 3 and 15 weeks after optic nerve transection. The pharmacological effect of 1.3 μM intravitreal TTX on the PhNR was also evaluated. *Results*: There was a significant loss ($p < 0.05$) in the PhNR of 20, 36, 34, 35, 48, 48 and 56% for ONTx eye versus the contralateral eye, at post ONTx times of 24 h, 1, 2, 3, 4, 8 and 15 weeks. B-wave amplitudes of ONTx eyes were not significantly different from the control eyes. In ONTx eyes, mean cell loss in the retinal ganglion cell layer was 27 and 55% at the 3 week and 15 week time periods. In the eyes with ONTx, the decline of PhNR amplitudes was correlated positively with RGC loss ($r = 0.98$; $p < 0.01$). Thirty minutes after intravitreal TTX injection, the PhNR was significantly reduced (57%, $p < 0.01$). *Conclusions*: There was a time-dependent decline in the PhNR after ONTx, as exemplified by a 35% reduction from 1–3 weeks, a 48% decline for 4–8 weeks and a 56% decline after 15 weeks. The correlation between the decline in the PhNR and retinal ganglion cell loss suggests that the PhNR depends on inner retina integrity and the PhNR may be important biological signal or detecting glaucomatous damage and the monitoring of RGC function changes in early glaucoma.

Abbreviations: PhNR – Photopic Negative Response; ONTx – Optic Nerve Transection.

Introduction

Glaucoma is a progressive optic neuropathy characterized by a specific pattern of optic nerve head and visual field damage due to the death of retinal ganglion cells. It may often go undiagnosed early in the disease process due to the slow decline in vision. It has been estimated that up to 50% of the nerve fibers may be lost before obvious clinical symptoms [1]. However, because the damage is irreversible, diagnosis and treatment early in the course of the disease are crucial.

Studies of the retinal function can be evaluated objectively by electroretinography (ERG). Although well established as a measure of photoreceptor and bipolar cell activity, the full-field flash ERG was thought to contain little, if any contribution from ganglion cells, as these tests are sensitive to widespread retinal damage only. However, a response has been newly identified that originates from the third-order neurons that receive signals from cones. This response was named that photopic negative response (PhNR) [2] by Frishman and colleagues who have

demonstrated that the PhNR is dependent on ganglion cell activity and is reduced in experimental and human glaucoma. Tetrodotoxin (TTX) [2, 3], is a drug known to suppress sodium dependent action potentials of retinal ganglion cells and amacrine cells. Since TTX selectively blocks retinal activity it has been used to evaluate the cellular contributions to PhNR responses that relates to electrical activity in the proximal layers of the retinal. Published findings with TTX support the hypothesis that the PhNR of the primate cone ERG originates from the inner retina, directly from retinal ganglion cells, or perhaps, through mediation by glia [2]. This raises the possibility that this component, similar to the pattern electroretinogram (PERG)[4–9] could be useful to evaluate inner retinal function in human glaucoma and thus serve as a diagnostic functional endpoint measure of glaucomatous neuropathy.

In the present study, the PhNR of the cone-mediated ERG was evaluated in normal rats, as well as rats treated with TTX and in rats whose its optic nerve transected. The purpose of our study was to determine the magnitude of the PhNR signal in the rat and to determine whether it was altered by damage to retinal ganglion cells (i.e. RGCs) and finally, whether there is a correlation between the magnitude of the PhNR and RGC counts determined by a standard flatmount method. The results showed that PhNR, unlike other ERG components, was significantly altered in eyes in which there was RGC damage and that loss of the PhNR signal was correlated with the decrease in RGC number in rats that had undergone optic nerve transection.

Methods

Thirty-nine adult male Brown Norway rats (Charles River Labs, Raleigh, NC) weighting 300–350 g were used in the present study. The treatment of all animals was in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. They were anesthetized with an intraperitoneal injection of a mixture of Ketamine (60 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine (5 mg/kg; Vetus, Phoenix Scientific, Inc., St. Joseph, MO). The depth of anesthesia was sufficient to prevent the animals from blinking or moving. Pupils were fully dilated to approximately 3.8 mm in diameter with two drops of 1% Atropine (Alcon) and 2.5% Mydfrin (Alcon) were applied at an interval of 10 min, followed if necessary by further drops after intervals of about other 10 min. No differences in pupil size were observed between eyes of control rats and eyes of rats undergoing optic nerve transection. Body temperature was maintained between 36 and 38 °C with a heating pad.

Surgical Procedures

Optic nerve transection (i.e. ONTx) was conducted on one eye, and the contralateral eye served as an untreated control. With the use of a surgical microscope, a 6 mm lateral incision was made along the superior conjunctiva about 3 mm posterior to the limbus. The eye was rotated downward using forceps to grip the anterior portion of the conjunctiva. The bulbar fascia was separated by blunt dissection, using the superotemporal and superonal vortex veins as guides, until the optic nerve sheath was accessed at the posterior pole. Once the optic nerve was clearly visualized, the dura sheath of the nerve was cut open longitudinally by means of a 15° cataract blade (Alcon), taking care to avoid injury to the ophthalmic artery. The ON was then gently separated from the sheath and completely transected approximately 2 mm posterior to the eyeball with a microsissor (Katena products, Inc., Denville, NJ07834, U.S.A.). Completeness of ONTx was confirmed by visualization of the posterior end of the nerve stump in cross-section during surgery. Normal retinal vascular perfusion was confirmed with direct ophthalmoscopy immediately following the operation and at each ERG measured over time.

Intravitreal injection

TTX (Sigma Chemical Co., St Louis, MO, USA) and vehicle were delivered in a 3 µl (1.3 µM) aliquot into the vitreal chamber of the rat's eye using a 30 gauge needle attached to a 10 µl Hamilton micro-syringe (Hamilton Company, Reno, NV, USA). The needle was inserted through the sclera superiorly, 1 mm behind the limbus, at an angle of 45° to avoid contact with the lens. The TTX was diluted in a balanced salt solution (BSS, Alcon). The concentrations given

represented the estimated final vitreous concentrations calculated by assuming a vitreous chamber volume of 40 μl for the rat[10, 11].

Counting of cell in RGC layer

Briefly, the retina was removed immediately after the rats were euthanized with CO_2 and retinal flatmounts were prepared. The eyecup was dissected in 0.01 M phosphate buffer, postfixed in 4% paraformaldehyde for 60 min, rinsed three times in 0.01 M phosphate buffer, cut into four quadrants, flatmounted on a glass slide. To evaluate RGC profiles after complete transection of the ON, microphotoes of retinal flat mount were examined. The focus was in the retinal RGC layer, eight areas from one eye were obtained (40×) along the midlines of the four quadrants starting from the optic disc to the peripheral border of the retina with a field of 340×270 μm^2. They were counted in a double-blind manner. The density of labeled RGCs in the whole retina was calculated by averaging the number of all sampled fields in each retina. Data are expressed as the number of cells per square millimeter at each time point, and results are expressed as mean ± SEM.

ERG recording

Ganzfeld electroretinograms in response to 50 trial green ($\lambda_{max} = 514$ nm) flashes of a Xenon discharge tube were recorded using the Espion Diagnosys system (Diagnosys LLC, Littleton, MA 0146–0670, USA). A 15 cm in diameter Ganzfeld stimulus was positioned very close to one eye. Two stimulus conditions were tested: 11.38 and 22.76 cds/m^2 were presented on a 40 cd/m^2 green background. The intensity values given above were those found to provide optimal conditions evoking a large negative response following the positive B-wave. To suppress the electrical activity of the rods, a continuous green background of 40 cd/m^2 was maintained uninterrupted throughout the lengthy recording sessions.

After topical corneal anesthesia with Alcaine (Alcon), the photopic ERG was recorded with the Espion system with thread electrodes (PT-IR Tef., A = M system, Inc., Everett, WA) placed in contact with the corneal surface of the eye. The electrode wires (The test eye = positive electrode; other eye = reference electrode) were shaped into a circle (approximately 3 mm in diameter) and embedded in putty molded specially for each eye, thus enabling recording of responses from the same contact points on the cornea throughout an experiment. The cornea was intermittently irrigated with balanced saline solution (BSS; Alcon) to maintain adequate electrical contact and to prevent exposure keratopathy. A grounding electrode was placed subcutaneously on the animal. The sample frequency was 1 kHz, and 50 responses were averaged, an interval in between two flashes was 1000–3000 ms. Recording sessions lasted approximated 1 h (15 min adaptation time each eye and 15 min collecting data each eye), after which the animals were allowed to recover.

Measurement of the response

For all rats, the PhNR and B-wave were measured semiautomatically on the screen by shifting cursors interactively along the traces. In this way the amplitude between the deepest negative point following the B-wave and the baseline was read out by the computer software. The peak latency of the negative response was defined as the time elapsing between the moment of the flash and the point of maximal depression following the B-waves. For each record, amplitude and time to peak of on components were measured. The following parameters were evaluated: B-wave amplitude, and time to peak, and PhNR amplitude and time to peak.

Statistical analysis

Results from normal Brown Norway rats were analyzed by one-way analysis of variance (ANOVA) with Dunnett's tests for multiple comparisons. Pearson's correlation and linear correlation analysis was used to correlate amplitudes of the various PhNR components with the corresponding RGC recorded in the individual rat with ON transected. In all the analyses, $p < 0.05$ was considered significant.

Result

PhNR waveform in normal eyes

Figure 1 shows a representative normal rat PhNR and photopic B-wave to stimulus flashes of increasing intensity correlation has been

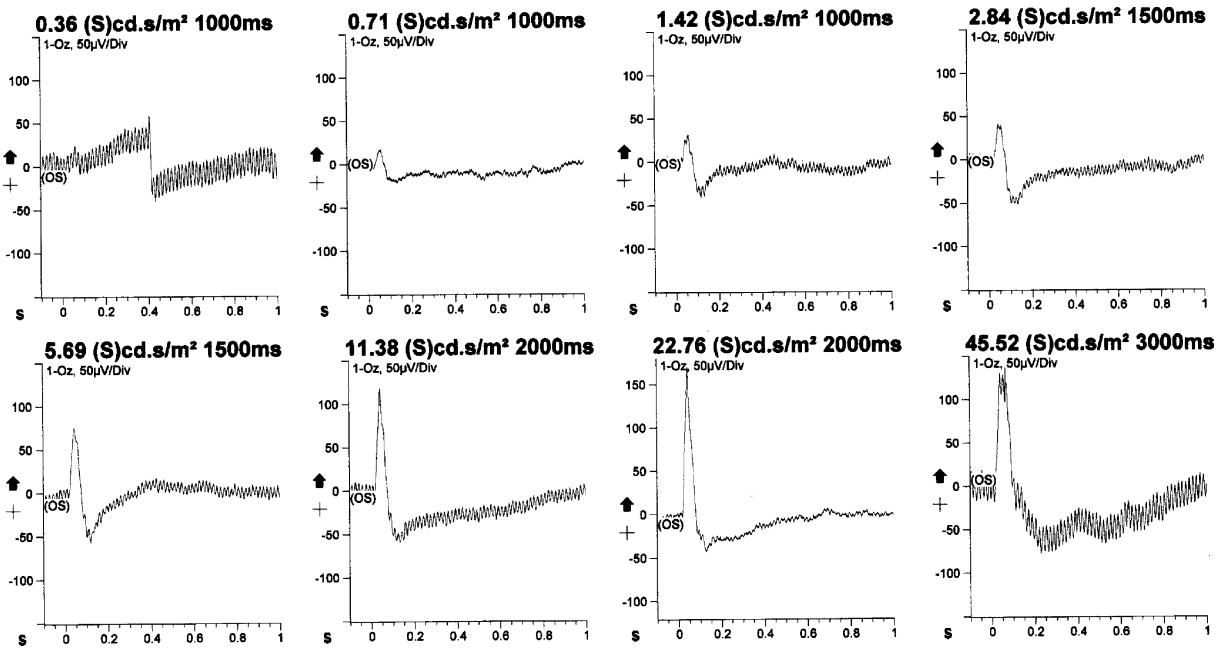

Figure 1. Photopic ERG changes induced by increasing intensity. The eight panels from the top left to the bottom right, the intensity was increase from 0.36 to 45.52 scd/m^2.

established. With increasing stimulus intensity, both the PhNR and B-wave grew in amplitude. The amplitude of the PhNR saturated at approximately 11.38 and 22.76 cds/m^2, whereas the B-wave continued to grow. Intensities of 11.38 and 22.76 cds/m^2 appeared optimal for the PhNR wave. Thus we choose 11.38 and 22.76 cds/m^2 for graphical analysis. As shown in Figure 1, the emergence of a shallow PhNR wave was consistently observed at about 0.71 cds/m^2. For these and higher intensities, the PhNR became more prominent. There were no differences between right and left eye group means during the baseline session. The implicit time of PhNR became longer with an increase in intensity, however, the B-wave' implicit time began to compress by flash intensities.

Time course of PhNR changes after optic nerve transection (i.e. ONTx)

In rodents, nearly all RGCs die and degenerate 1–4 weeks after ONTx[12–15] although the precise time course can very depending on the target projection, or cell type [16, 21], and the distance between the injury and the soma [17]. Therefore, longitudinal data on retinal function were collected at 24 h and 1, 2, 3, 4, 8, and 15 weeks after ONTx in the rats, as shown in Figure 2 and Table 1. Results for the PhNR were obtained at each time point. The group data confirm that ONTx did decrease the PhNR amplitude and the PhNR was significantly reduced at all time points ($p < 0.05$). Figure 3 shows a representative animal where the OD traces represent a reduction in the amplitude of the PhNR for all intensities

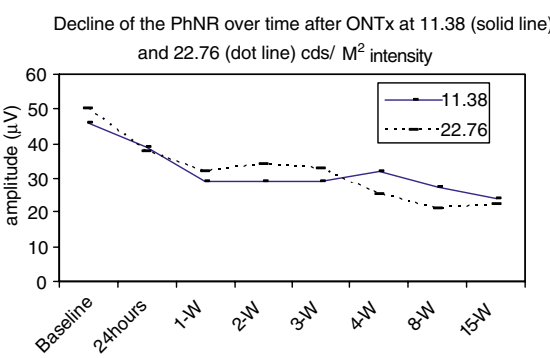

Figure 2. Time course of PhNR changes after ONTx. The PhNRs were obtained after ONTx 24 h, 1, 2, 3, 4, 8 and 15 weeks. The amplitudes of PhNR are shown as the value μV, but in fact, they were negative in value.

Table 1. PhNR-amplitude of normal rats compare with ON transected' rats

	11.38 cds/m^2	22.76 cds/m^2
Baseline	-46 ± 1.3 (μV) $N=87$	-50 ± 1.3 (μV) $N=87$
P-24 hours	-39 ± 6.5 (μV) $N=10$	-38 ± 4.6 (μV) $N=10$
P-week 1	-29 ± 1.3 (μV) $N=10$	-32 ± 4.6 (μV) $N=10$
P-week 2	-29 ± 3.6 (μV) $N=10$	-34 ± 3.4 (μV) $N=10$
P-week 3	-29 ± 4.9 (μV) $N=10$	-33 ± 5.8 (μV) $N=10$
Month-1	-32 ± 5.5 (μV) $N=4$	-25 ± 6.2 (μV) $N=4$
Month-2	-27 ± 3.0 (μV) $N=4$	-21 ± 8.7 (μV) $N=4$
Month-3$^+$	-24 ± 7.6 (μV) $N=3$	-22 ± 1.0 (μV) $N=3$

$p < 0.05$ in comparing baseline with rest group.

measured at 3 week post-ONTx (OS traces show control responses from the fellow eye described above).

ONTx reduced the PhNR component over time, leaving only a small residual negative potential at week-15. PhNR declined by 33 and 55 % at week 3 and week 15, respectively, in the ONTx eye, but normal PhNR wave obtained in an unaffected eye. Both time points revealed that ONTx had no appreciable effect on Photopic B-waves over this intensity range.

The Tukey statistical test showed significant differences in PhNR mean amplitude between normal rat (or before cut ON) and rats with ON transected ($p < 0.05$). Mean amplitude changed significantly across whole follow up period ($p < 0.05$).

B-waveforms after ONTx

The B-wave profile following the optic nerve transection revealed that ONTx did not significantly affect photopic B-wave amplitude at any time point, but did produce a trend towards a decrease in amplitude without being statistically significant at post-24 h. The surgical trauma (e.g. edema), not ONTx per se, appeared to cause a mild reduction of photopic B-wave. Overall, however there were no significant differences between baseline and post –ONTx.

ERGs profile after TTX

The PhNR was obtained before and after selective pharmacological suppression of voltage-gated sodium currents (action potentials) using TTX. The effect of TTX on the PhNR is shown in Figure 4, which compares baseline to post-TTX waveforms for two intensities. The differences between these waveform pairs are plotted in Figure 4. After TTX injection, there was a dramatic reduction in the PhNR. TTX effects became evident at 15, 30 min and 1 h after injection and were maximal at the 30 min-time point. TTX did not completely abolish the PhNR in naïve rats, but it did alter the amplitude considerably. Our TTX data over time show that our results for TTX inhibition of retinal function in this study were similar to the

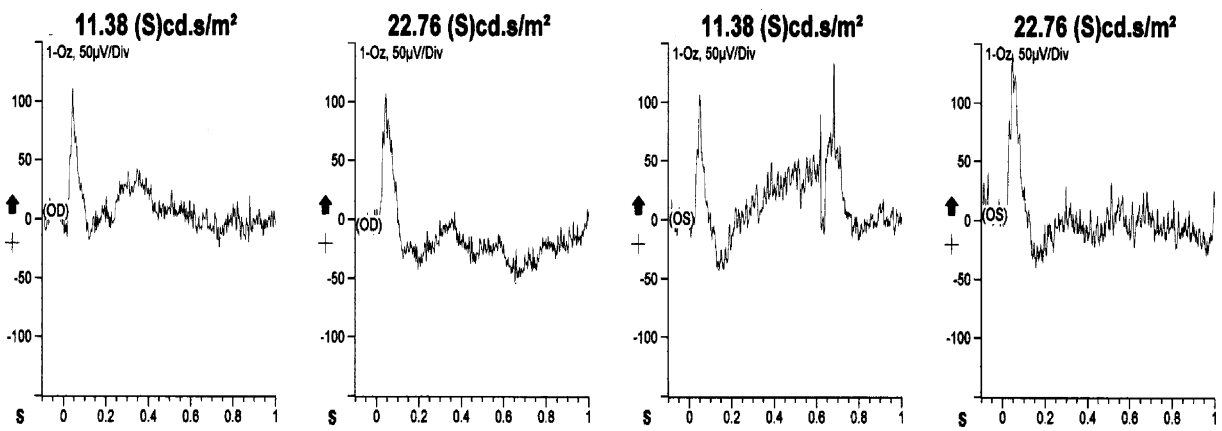

Figure 3. A rat PhNR recording at week-3 time point. First two panels were ONTx eyes at 11.38 and 22.76 scd/m^2, respectively. The right two panels were normal eye (OS) at as same intensity as other.

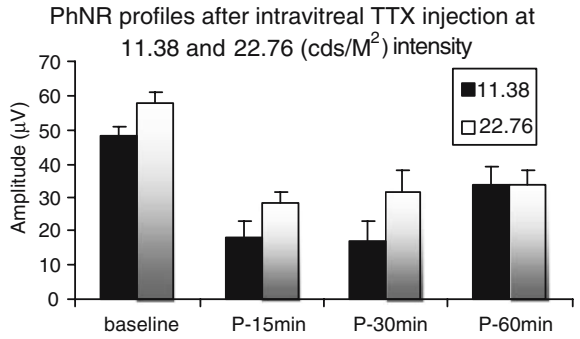

Figure 4. Effect of TTX intravitreal injection on the PhNR changes in the one-hour observation period. (Mean ± SE; $N = 11$; $p < 0.001$).

published results of other [18, 19]. After TTX application, inspection of the baseline waveforms revealed that the amplitude of the B waveforms appeared partially affected after TTX in Figure 5.

Control data: PhNR after vehicle injection

To ensure that the injection technique, volume or vehicle used did not contribute to the function changes described above, the PhNR was assessed following intravitreal injection of 3 μl vehicle ($N = 12$). In all rats there was no significant effect of the vehicle injection on the measured endpoints Figure 6.

Histological findings after ONTx

At the third week after ONTx, the density of RGCs was reduced to nearly 30% that observed in

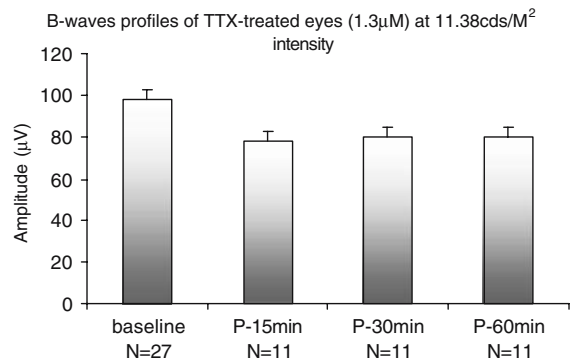

Figure 5. Effect of TTX intravitreal injection on the B-wave changes in the one-hour observation period. (Mean ± SE; $N = 11$) $p = 0.002$ (Baseline Vs. 15 min); $p = 0.009$ (Baseline Vs. 30 min); $p = 0.004$ (Baseline Vs. 60 min).

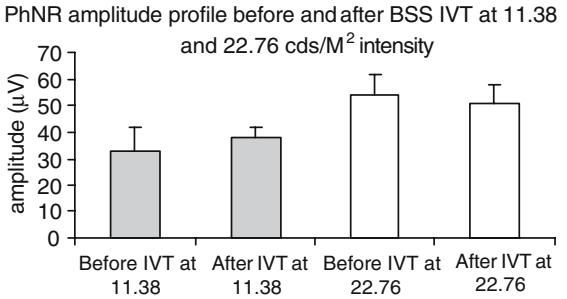

Figure 6. Effect of BSS intravitreal injection on the PhNR changes. (Mean ± SE; $N = 12$.)

fellow control eyes at equivalent eccentricities. Expressed as a percentage of the fellow eye, the total number of RGCs remaining in the RGC layer at week-3 after ONTx was 73% and at 15 weeks after ONTx was 45%, respectively.

In summary, there was a significant correlation between the decline in the PhNR amplitude at week-3 and -15 and the RGC number in retinal flatmounts at week-3 and -15 (r values and corresponding slopes of linear regressions, fitted to the data points, are reported in the Figure 7). PhNR amplitudes were positively correlated with central RGC loss ($p < 0.01$). In contrast to the flash ERG component and the PERG, neither the A-or B-wave amplitude was correlated with central RGC loss induced by ONTx.

Discussion

The results of this study show that the PhNR component of the green cone photopic ERG was, on average, significantly reduced in amplitude in eyes undergoing ONTx in which RGC count was reduced, compared with the responses obtained from either normal control or control eyes of rat. This reduction appeared to be specific for PhNR, because the mean peak amplitudes of the B-wave did not differ at all between control eye and affected eyes. An average reduction of 55% in the PhNR amplitude was strongly correlated with a 55% loss in RGCs in ON transected eyes, indicating a predominant, or even selective, effect of optic nerve damage on the green PhNR component. The present findings are in agreement with those previously reported in monkeys [2] showing that PhNR was selectively reduced in

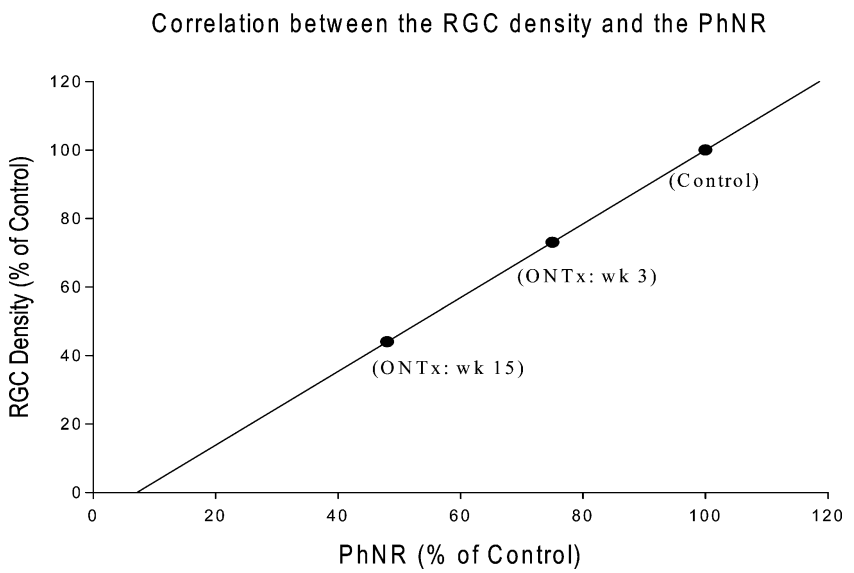

Figure 7. The correlation between decline in the PhNR and RGC loss ($Y = 1.08X - 7.7$; $r^2 = 0.999997$).

eyes with experimentally induced glaucoma, with respect to contralateral, untreated eyes.

The cellular origin of the PhNR component has not been clearly established. However, the available evidence in cats and primates suggests that this component originates from the inner retina. In the cat [20], intraretinal recordings showed that TTX-sensitive negative potentials, with a time course similar to the PhNR of the ERG, are present in the proximal retina of the area centralis, where ganglion cell density is high. In addition, these components are particularly pronounced at recording sites approaching the optic nerve head. In the monkey, intravitreally injected TTX [2] abolishes the PhNR selectively, leaving the other ERG components relatively unaltered. Because TTX is a voltage-gated Na^+ blocker, and the retinal ganglion cells, their axons, and the amacrine and interplexiform cells are the major retinal elements that possess voltage-gated Na^+ channels, these neurons would be, as suggested previously [2], those most likely responsible for PhNR generation. In human, PhNR amplitude showed a maximum response density in the foveal region, where ganglion cell density is highest, and its distribution differed from that of the B-wave, whose response density was much less pronounced in the same foveal area [17]. The results in humans, indicating that PhNR spatial distribution may follow that of retinal ganglion cells, are consistent with the experimental data in cats and lend further support to the hypothesis that the PhNR signal is generated from the inner retina.

In this study, we found that losses in PhNR amplitude observed in ON transection eyes were significantly correlated with central RGC loss assessment by the retinal flatmount technique of quantifying RGC counts. The present results in rats and the published monkey [2] data suggest that PhNR and RGC losses in glaucomatous neuropathy share, at least in part, a common mechanism, presumably related to retinal ganglion cell dysfunction.

In our rats with ON transection, a significant correlation was found between PhNR and RGC losses. This suggests that PhNR may be directly related to retinal ganglion cell activity, because the B-wave was normal, meanwhile others [2, 3] have reported that they thought the PhNR reflects specifically functional integrity of these neurons.

In the present study, the density of RGCs was found to be higher closer to the optic disc, consistent with previous reports [21–23]. After ON transection, the absolute number, as well as, the percentage of RGC loss was greater nearer to the optic disc. Apparently, this is because the site of transection of the ON was closer to the somata of RGCs near the optic disc, thus the RGCs were more seriously damaged [12].

The association found in this study between PhNR and RGC loss support the hypothesis that PhNR losses may represent a specific indicator of glaucomatous damage. Clearly, further cross-sectional and longitudinal studies, in glaucomatous animal models, are needed before the accuracy and predictive value of PhNR in RGC changes can be fully established.

Compared with PERG, and STR, the PhNR wave may possess some practical advantages. PhNR recording does not require full refractive correction for the test distance, which is otherwise critical for PERG measurement. Furthermore, the response loss should be specific for glaucomatous damage also in eyes with moderate optical media opacities, which precludes recording of the PERG. Indeed, PhNR response losses in glaucomatous monkey eyes did not show a dependence on either mean luminance or modulation depth [2]. Therefore, it is presumable that the PhNR, unlike the PERG, may not be significantly affected in nonglaucomatous eyes with early or moderate media opacities. These technical advantages, in addition to the presumed retinal origin, further point to a potential clinical use of the PhNR as a tool in clinical detection of glaucomatous damage. The PhNR may provide a better measure of abnormal retinal function in glaucoma and is easier and faster to acquire than STR.

Commercial relationship policy: N.

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