Effect of inner retinal dysfunction on slow double-stimulation multifocal electroretinogram

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ABSTRACT

Purpose

This study investigated the retinal adaptive mechanism in inner retinal dysfunction using the slow double-stimulation multifocal electroretinogram (mfERG) paradigm.

Methods

Slow double-stimulation mfERG responses were recorded from 15 eyes of 15 4-month-old Mongolian gerbils in control conditions and after suppression of inner retinal responses with injections of tetrodotoxin (TTX) and N-methyl-D-aspartic acid (NMDA). The stimulation consisted of five video frames: the two initial frames with multifocal flashes were triggered by two independent m-sequences, followed by three dark video frames. The results were compared with findings in humans: 7 subjects with glaucoma and 31 age-matched normal subjects were measured using the same mfERG protocol.

Results

The stimulation generates two responses (M1 and M2) from the two independent multifocal frames. The M1:M2 ratio showed a significant reduction after administration of TTX+NMDA in the animal study. This matched with the human glaucoma findings. Glaucoma subjects generally have a reduced M1:M2 ratio; this ratio showed a sensitivity of 86%, with a specificity of 84% for differentiating normal

eyes from glaucomatous eyes.

Conclusion

This stimulation paradigm provides a method of measuring temporal visual characteristics. The M1:M2 ratio acts as an indirect functional indicator of retinal adaptation, which may be abnormal in the diseased retina. Further development of this method may help to describe the functional variation in the diseased retina and to predict the occurrence of a range of retinopathies.

INTRODUCTION

Glaucoma is a major health concern throughout the world because it is the second leading cause of blindness globally,[1] causing around 12% of all cases of total blindness.[2] Glaucoma refers to a group of eye diseases with a characteristic pattern of optic neuropathy involving the loss of retinal ganglion cells,[3] which results in irreversible visual field constriction and ultimately in the loss of central vision. Temporal visual properties are affected in the early stages of glaucoma.[4] These losses are believed to be related to damage of the magnocellular system in glaucomatous eyes.[4] However, the pathophysiology of this complex disease is still not well understood.

Electrophysiological tests are objective tools for measuring the functional responses

of the visual system. A number of studies have shown that electrophysiological tools can detect early functional changes in glaucoma and that it is possible to detect early functional changes before significant loss of nerve fibres.[5-13] The multifocal electroretinogram (mfERG) is a well-developed technology used to localise, study and diagnose diseases of the human retina.[6,7] Its fast-flickering stimulation (75 Hz) allows examination of retinal temporal properties. The mfERG response is contributed to by different retinal cells, which respond to the rapid stimulation sequence, and any impairment of retinal temporal mechanisms will result in impaired successive responses. The adaptive retinal mechanism generates non-linear responses, which can be obtained as 'higher order kernel' responses.[8] The higher order kernel response is influenced by adaptation to successive flashes, but these responses have relatively low signal to noise ratios that are difficult to measure. The global flash mfERG stimulation paradigm can be used to elicit a large non-linear mfERG component,[9] which also represents adaptive changes in the response. This nonlinear component is believed to be generated predominantly from the inner retina.[10] It may be used as an indicator of inner retinal dysfunction due to glaucoma,[11,12] and its compromise shows that the fast-adaptive mechanisms from the retina are affected in inner retinal disease.[13]

Rodents, such as rats, have been used as animal models for inner retinal study for

years.[14] However, the rod-dominated retina of the rat has limited its usefulness for evaluating the retinal photopic adaptive function. The gerbil is a small diurnal mammal reported to have better photopic vision than other rodents as it has a well-developed cone system: about 13% of its photoreceptors are cones[15] and its acuity is about 2 cycles/degree.[16] The gerbil retina consists of the same layers as the human retina.[17] It also has a cone-dominated avascular macula-like region called visual streak. There is enhanced thickness of the inner retinal layer within the streak region compared with the periphery, which is thought to indicate greater synaptic interaction in this area.[18]

In this study, we explored a new paradigm, slow double-stimulation mfERG, to investigate the retinal adaptive mechanism in inner retinal dysfunction. We also explored the possibility of using the gerbil as a new animal model for eye research; it is possible to pharmacologically suppress the inner retinal response of the gerbil using tetrodotoxin (TTX) and N-methyl-D-aspartic acid (NMDA). Having done this, we applied the new mfERG paradigm to the model and to human glaucoma subjects and controls, to determine whether the animal model adequately represents the electrophysiological response of human glaucoma subjects.

METHODS

Animals

The mfERG recordings were obtained from 15 eyes of 15 normal, 4-month-old Mongolian gerbils (Meriones unguiculatus; Laboratory Animal Unit, The University of Hong Kong, Hong Kong SAR, China). The gerbils were reared in a temperature-controlled room on a 12-hour light/12-hour dark cycle. All the animals were anaesthetised by inhalation of isofluane (2%) with 100% oxygen supply during the experiment, and the right eye was used for testing. The mfERG was measured before and after the intravitreal injections. All experimental and animal care procedures adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Subcommittee of The Hong Kong Polytechnic University.

Human subjects

Seven Chinese primary open angle glaucoma patients (mean age 49.14±11.5 years) without any other ocular and systemic diseases were recruited. All had diagnosed glaucoma of more than 2 years' duration. They were being treated bilaterally with either latanoprost (Pfizer, New York, USA) or timolol maleate (Alcon, Fort Worth, Texas, USA), which was prescribed by their own ophthalmologists. One eye of each

patient with greater glaucomatous visual field loss as measured by the central 30-2 threshold test (Humphrey; Carl Zeiss Meditec, Dublin, California, USA) was selected for testing (mean defect = -10.44±4.97 dB). These patients were compared with 31 age-matched Chinese normal subjects (mean age 50.87±6.17 years) with no systemic or ocular disease. One eye of each control subject was randomly selected for testing. The range of refractive errors for all subjects was between +1.0 and -4.0 D sphere and ≤ -1.0 D cylinder. All research procedures adhered to the tenets of the Declaration of Helsinki and were approved by the ethics committee of The Hong Kong Polytechnic University. All subjects were fully informed of the possible risks and gave written, voluntary consent.

Stimulation conditions

The stimulus pattern was presented on a 22-inch liquid crystal display monitor (model: vx2260wm; ViewSonic, Walnut, California, USA), and the mfERG stimulation was driven by VERIS (V.5.01) from Electro-Diagnostic-Imaging (San Mateo, California, USA). The mfERG stimulation was followed by a slow double-stimulation paradigm (sequence of video frames: M1 M2 OOO), as shown in figure 1. In this paradigm, the stimulus contained five video frames (each frame lasts 13.3 ms with a frame rate of 75 Hz). During stimulation of the first two video frames with multifocal flashes (M1 and

M2), each hexagon was either flashed (200 cd/m^2) or dark (3 cd/m^2) according to two independent selected pseudo-random binary m-sequences. In addition to the two multifocal flashes frames, the slow double-stimulation mfERG paradigm also contained three more dark frames (OOO) (3 cd/m^2) before the next cycle of stimulation. The average luminance of the multifocal flash frames was about 100 cd/m^2 and the background was set to this value.

In the animal study, the working distance from the liquid crystal display monitor to the tested eye was 20 cm, so the stimulus pattern subtended the visual angle close to 608. A 37 non-scaled hexagons pattern with 211e1 pseudo-random binary m-sequence was used for measurement (figure 1A). The recording time was approximately 2.3 min.

In the human study, the working distance from the monitor to the tested eye was 30 cm, so the stimulus pattern subtended the visual angle close to 45°. A 103 scaled hexagons pattern (scale factor, 10.46) with 2^{12}-1 pseudo-random binary m-sequence was used (figure 1B). Recordings were divided into 16 slightly overlapping recording segments approximately 17 s in length. The recording time for the measurement was about 4.5 min.

Recording conditions

Before testing, pupils were fully dilated with 1% tropicamide, and the ocular surface was anaesthetised with 0.4% benoxinate HCl. The refractive error of the tested eye was fully corrected for the viewing distance.

In the animal study, the eyelids of the tested eye were held by an eye speculum. A monopolar contact lens electrode (Mayo, Inazawa, Japan) was used as an active electrode and Grass subdermal F-E7 electrodes (Astro-Med, West Warwick, Rhode Island, USA) were applied subcutaneously at the pelvic region and the temporal canthus of the tested eye as ground and reference electrodes, respectively. Before each recording, in order to maintain the same alignment between the eye and the stimulator, a short conventional mfERG recording was performed and the three-dimensional topography was used to locate and align the positions of the visual streak.

In the human study, a Dawson-Trick-Litzkow electrode was used as the active electrode and gold-cup surface electrodes were used at the temporal canthus of the test eye and the forehead as the reference and the ground electrodes, respectively.

The mfERG signals were amplified by a Grass amplifier (model CP122 bench-top style amplifier; Grass Instruments, Quincy, Massachusetts, USA) with band pass 1-300 Hz and gain x20 000 and band pass 10-300 Hz and gain x100 000 for both animal and human studies, respectively. The recording was monitored using the

real-time signals shown by the VERIS mfERG program. Any recording segments contaminated with blinks or small eye movements were rejected and immediately re-recorded.

Intravitreal injections for animals

In the animal study, intravitreal injections (2 ml) were made 1 mm posterior to the superior limbus with a sterile 30-gauge needle attached to a 25 ml Hamilton microsyringe (Hamilton Company, Reno, Nevada, USA) inserted through the sclera and at an angle of 458 to avoid contact with the crystalline lens. Assuming that the vitreal volume is 40 ml, the intravitreal concentrations of the pharmacologic agents (Sigma-Aldrich, St Louis, Missouri, USA) used were: TTX 5 mM and NMDA 4 mM. These concentrations are sufficient to have the desired effects on the flash ERG or mfERG in primates,[19] pigs[10] and rats.[20] The two injections were given separately 1 h apart. All the animals were anaesthetised during each intravitreal injection and were allowed to recover after that in order to minimise the effect of prolonged anaesthesia. Recordings were made at least 1 h after the last drug administration for the stabilisation of the effect.

Data analysis

The responses were processed and analysed separately at two ‘time slices’ for the m-sequence frames using the VERIS 5.01 mfERG program. The peak-to-peak response amplitudes (P1) of the two m-sequence frames (M1 and M2) were measured (figure 2B, D). The ratios of the response amplitude (M1:M2) from the two independent multifocal frames were also calculated for comparison. The data for implicit times were not shown in this paper due to the insignificant difference between the conditions either in the animal or in the human study. In the animal study, the individual mfERG responses from the eye of each gerbil with amplitudes within the top 25 percentile were grouped to represent the visual streak (figure 2B).10 The effects of drug administration on the mfERG responses at the visual streak region were compared using a paired t test. In the human study, the individual mfERG responses were grouped into three concentric ring responses, which covered 10°, 10 - 27° and 27 - 45° fields of view, respectively (figure 2D). The ring responses between control and glaucoma subjects were compared using ANOVA with Bonferroni post-hoc correction. The level of significance was set at 0.05.

RESULTS

The slow double-stimulation mfERG generates two responses from the two independent multifocal frames. The typical waveforms triggered by the first (M1) and

second (M2) m-sequence stimuli from the gerbil eye's visual streak area were shown in figure 2B. As with the conventional mfERG response, the M1 waveform contains a trough at around 35 ms and followed by a major positive component (P1) at around 60 ms, whereas the M2 waveform shows a similar feature but the amplitude was significantly reduced ($p<0.0001$). After injection of TTX together with NMDA, there was no remarkable change in the waveform; however, the amplitude showed a significant reduction in both M1 ($p<0.0001$) and M2 ($p<0.001$) responses (figure 3A). The M1 :M2 ratio also showed a significant reduction ($p<0.5$) after administration of TTX+NMDA (figure 3B).

Typical response waveforms from the control and glaucoma subjects are shown in figure 2D and the individual clinical characteristics are shown in table 1. Generally, the human mfERG waveform is similar to that of the gerbil, but with a faster implicit time, where the human M1 waveform contains a trough at around 25 ms and a peak (P1) at around 40 ms. In the control group, the amplitudes of the M1 responses were significantly larger than those of the M2 responses for ring 1 and ring 2 ($p<0.001$). In the glaucoma group, the M1 amplitude was larger than the M2 amplitude for ring 1 only ($p<0.001$). In the sequence of M1 , there was no significant difference in amplitude between the glaucoma and control subjects in any rings; however, the M2 amplitude for ring 1 of the glaucoma group was significant larger than that of the

control group ($p<0.01$) (figure 4A,B). The M1 :M2 ratios for ring 2 showed smaller values than those of the control group ($p<0.05$) (figure 4C).

The M1:M2 ratio for ring 2 showed good differentiation between the two groups. Figure 4D shows the receiver operating characteristic (ROC) curve based on different cut-off values of the M1:M2 ratio. The ROC curve illustrates the balance between sensitivity and specificity for the discrimination of subjects with glaucoma from normal subjects. The area under the ROC curve provides an index for quantifying the accuracy of the test (where 1.0 is a perfect result). The area under this ROC curve is 0.864 and the sensitivity would be 86% with a specificity of 84% using the best cut-off M1:M2 ratio of 1.4 based on this ROC curve.

DISCUSSION

Our results illustrated that the slow double-stimulation mfERG paradigm can detect the retinal damage in glaucoma patients and the pharmacological suppression of inner retinal activity in the animal model. This paradigm provides a platform for measurement of temporal visual characteristics. The two multifocal flashes in this new paradigm refer to the concept of double-flash stimulation in the conventional full field flash ERG.[21] However, because two independent m-sequences are used, the chance that a hexagon will contain a double flash stimulation is only 0.25, so the

temporal adaptive effect on the M2 response was reduced by half (figure 5). The interleaving of three dark frames (approximately 53 ms) between the successive double-stimulation allows recovery from the prior stimulations, as periods of three dark frames between the multifocal flash presentations have been reported to eliminate higher order adaptive effects superimposed on the response.[22]

Retinal signal processing involves multiple levels of adaptation. The retinal adaptive mechanisms begin at the photoreceptors, which are followed by postreceptoral feedback[23] and lateral interaction mechanisms. The slow double-stimulation mfERG paradigm adopted in this study emphasises the recovery of retinal sensitivity. Our recent study has shown an enlarged M1:M2 ratio in diabetic subjects, indicating a more severe reduction in the M2 response. This suggests that the recovery of photoreceptor function after the desensitisation may be altered in patients with diabetes.[24] The advantage of this paradigm is its ability to assess the integrity of the adaptive mechanism at the photoreceptor level, and to access the fast adaptive mechanism, which is believed to be generated from the inner retina.[12]

This study has demonstrated an effect on the slow double-stimulation of the gerbil mfERG after using an established pharmacological suppression method to inhibit the inner retinal contributions to the mfERG. TTX inhibits the voltage-gated sodium channel in the ganglion cells and some amacrine cells and is powerful in removing the

large inner retinal contribution of the mfERG[10,19]; NMDA (an ionotropic glutamate agonist) removes the remaining inner retinal activity that is not suppressed by TTX. After the inner retinal influences have been essentially removed, there was a general reduction of both M1 and M2 responses. However, the reduction in the M1:M2 ratio suggested lesser reduction in the M2 response, which may be caused by an altered fast retinal adaptive mechanism. The hypothesis here is that if the slow double-stimulation does not cause any fast adaptive effect (the suppression of the M2 response due to the prior flash stimulation), the retina would show an equal reduction in the M1 and M2 responses after suppression of the activity of the inner retina.

The human glaucoma results in our study were also comparable with those of the animal experiment. The general reduction of the M1:M2 ratio was found in the glaucomatous retina where the inner retinal function is believed to be compromised. The reduction of the M1:M2 ratio in glaucoma subjects also suggested an alteration of the fast retinal adaptive mechanism. The significant reduction of the M1:M2 ratio in the ring 2 region means that the altered fast adaptive effect may be compromised more in the mid-periphery (where glaucomatous visual field defects usually occur) than in the macular region. The ROC curve of the M1:M2 ratio showed that this method provides good sensitivity and specificity in differentiating normal subjects from those with glaucoma. Although the sensitivity to inner retinal dysfunction is

slightly better than other electrophysiological techniques such as photopic-negative response or pattern electroretinogram,[25] the glaucoma subjects with marked visual field defect in this study may definitely provide better differentiation and this may also be biased by the small number of glaucoma subjects used. A further large-scale study investigating preperimetric glaucoma is necessary for assessing the sensitivity of this new technique in glaucoma diagnosis.

In conclusion, the M1:M2 ratio acts as an indirect functional indicator of retinal adaptation, which is believed to be abnormal in the diseased retina. The significant difference in the M1:M2 ratios between normal and diseased retinal conditions suggests that this modified mfERG paradigm can detect pathological changes; higher M1:M2 ratios may suggest altered recovery of photoreceptor function24 and lower M1:M2 ratios may suggest altered inner retinal function. However, inner retinal function can only be assessed using this paradigm if the recovery of photoreceptor function is robust. Further advancement of this method may help to monitor the functional variation in diseased retinas and predict the occurrence of different retinopathies.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by the Ethics Committee of The Hong Kong Polytechnic University.

Contributors All authors have contributed to the design and interpretation of data, drafting of the article and final approval of the version to be published.

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Subject	Eye	Mean deviation	p-value	Pattern SD	p-value	Visual acuity
1	RE	-9.07	<0.5%	12.73	<0.5%	6/7.5+
2	LE	-10.81	<0.5%	11.55	<0.5%	6/6
3	RE	-3.53	<1%	4.6	<0.5%	6/6
4	RE	-12.51	<0.5%	16.31	<0.5%	6/6
5	LE	-17.84	<0.5%	13.32	<0.5%	6/6-
6	LE	-14.05	<0.5%	15.83	<0.5%	6/7.5+
7	RE	-5.3	<1%	4.23	<0.5%	6/6-

Table 1 The individual clinical visual field characteristics of the seven glaucoma subjects (LE – left eye; RE – right eye)

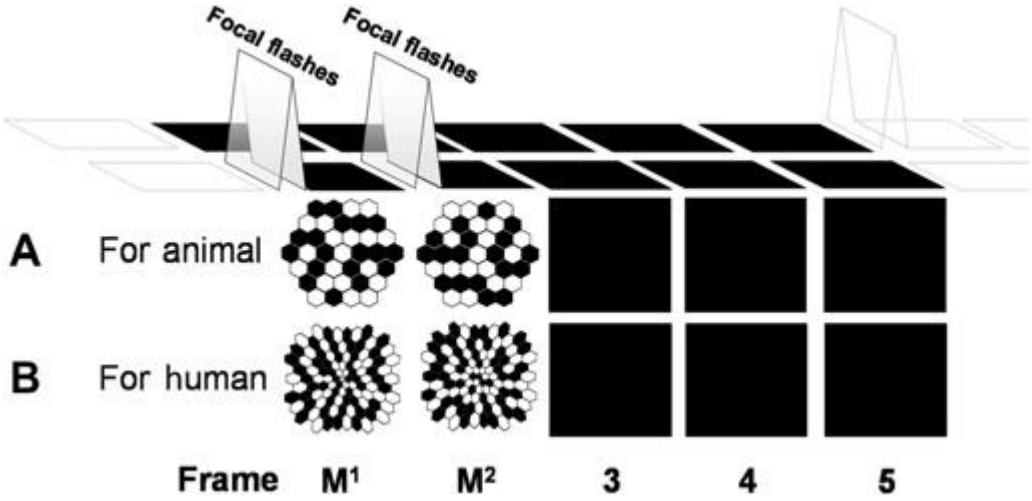

Figure 1 The slow double-stimulation multifocal electroretinogram (mfERG) stimulus sequence contains five frames. The two initial frames (multifocal flash) alternated between bright and dark according to two independent pseudo-random binary m-sequences. The mfERG was measured using (A) a 37 non-scaled hexagons pattern for the animal study and (B) a 103 scaled hexagons pattern for the human study. After the M1 and M2 frames, three dark frames were presented.

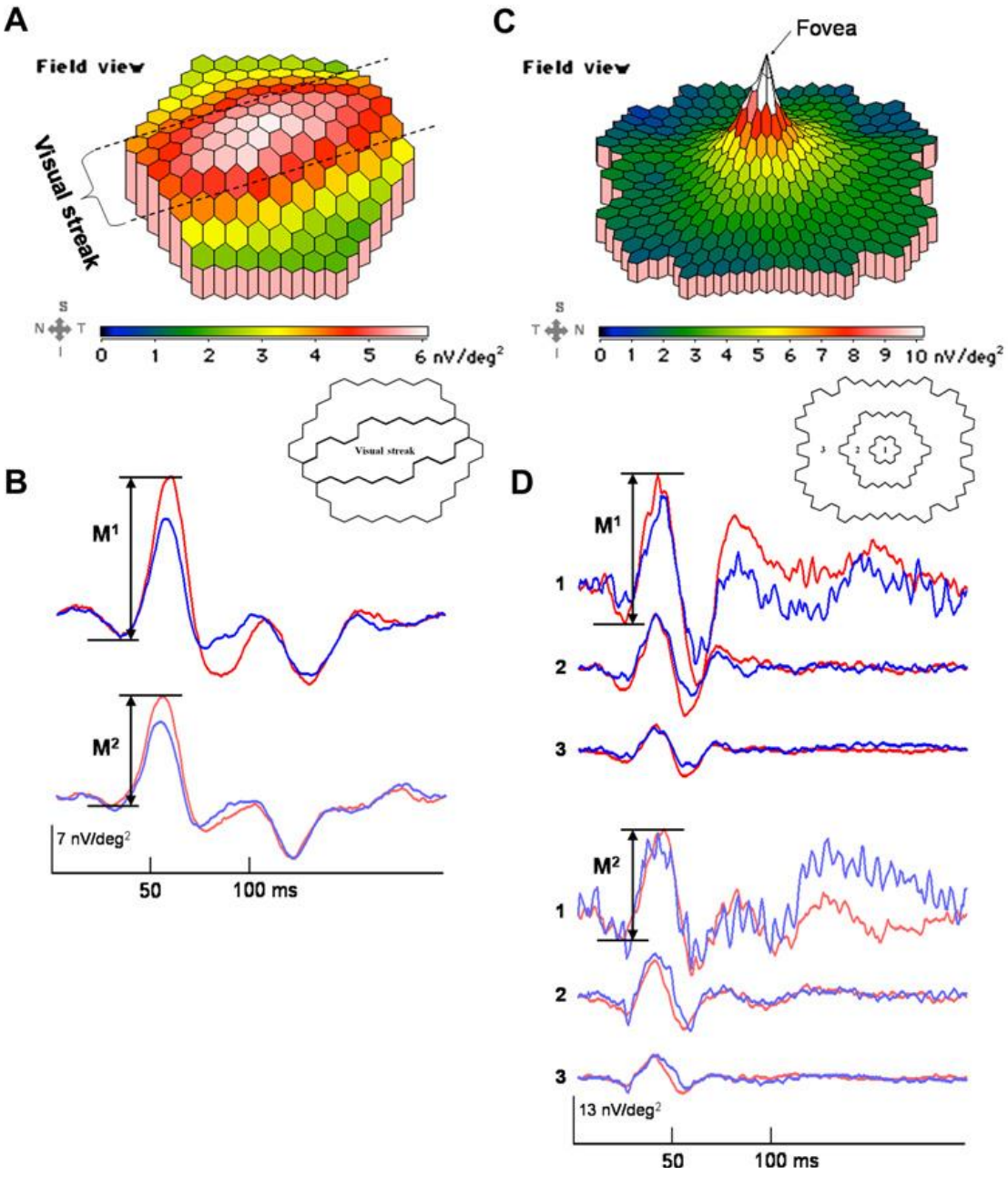

Figure 2 The typical slow double-stimulation multifocal electroretinogram (mfERG) result from the gerbil and human eye. (A) Three-dimensional field-view topography of M1 response across the retina from the right eye of a gerbil in the control group, where the visual streak is shown. (B) Superimposed comparisons of the typical averaged responses from the visual streak of a gerbil's eye for the first (upper panel) and second (lower panel) m-sequences. Red traces show the responses obtained under the control condition while blue traces show responses under the influence of

tetrodotoxin + N-methyl-D-aspartic acid. (C) Three-dimensional field-view topography of M1 response across the retina from the left eye of a human in the control group, where the fovea is shown. (D) Superimposed comparisons of the typical averaged concentric ring responses of the first (upper panel) and second (lower panel) m-sequences. Red traces show the responses obtained from a control subject while blue traces show responses from a glaucoma subject; M1 and M2 show the measurement of peak-to-peak amplitude.

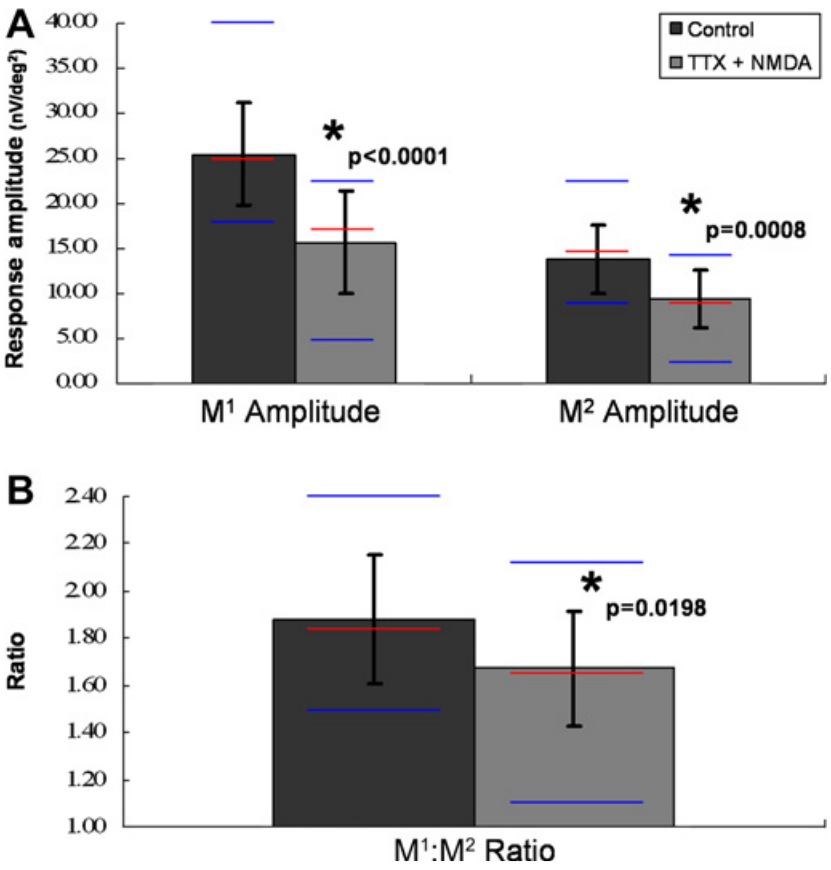

Figure 3 Histograms showing the results of the visual streak response of the gerbil eye. (A) Averaged peak-to-peak amplitudes of the first and second m-sequence stimulation before and after the administration of tetrodotoxin (TTX)+N-methyl-D-aspartic acid (NMDA). (B) Comparison of the averaged M1:M2 amplitude ratios between the control and under the influence of TTX+NMDA. Error bars are ±SD; red lines are medians and blue lines are maximum and minimum of the data.

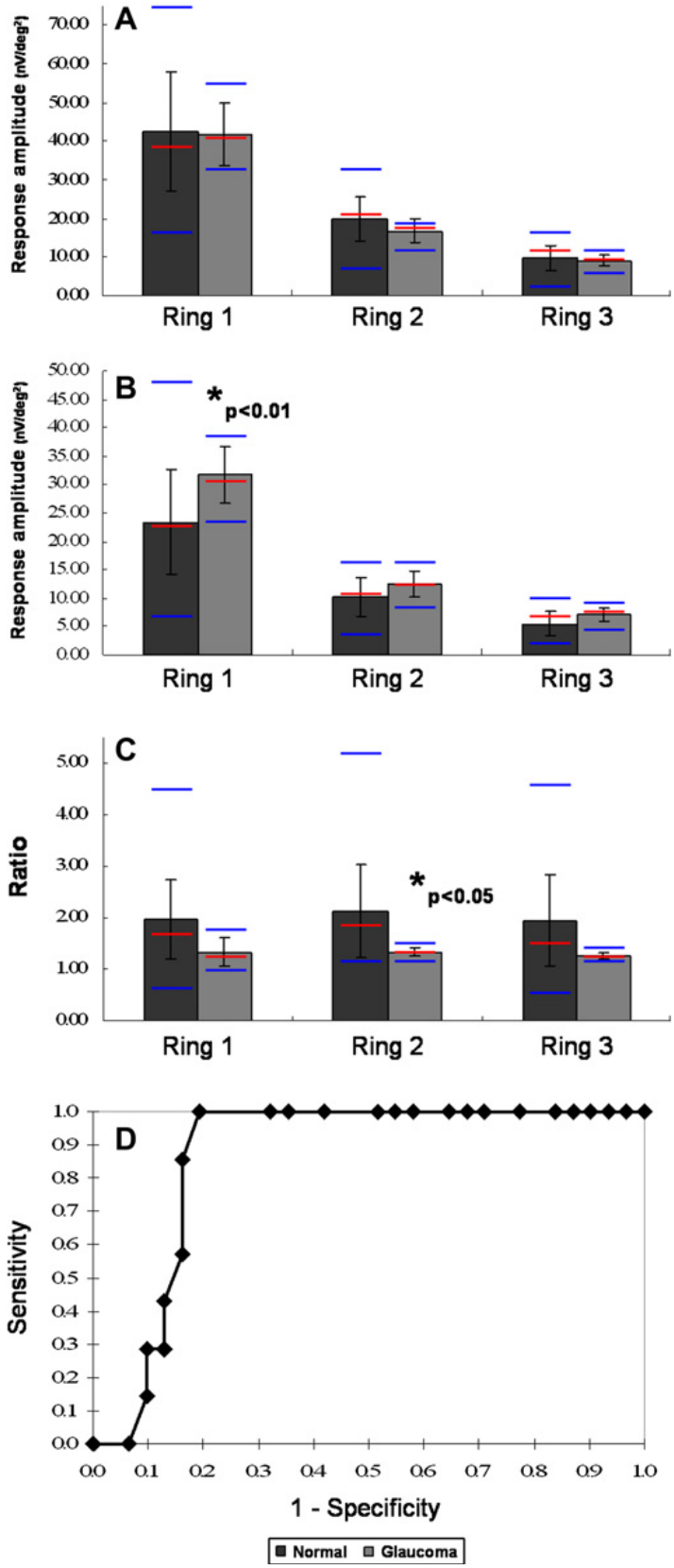

Figure 4 Results from three concentric ring responses for the human study. (A) The averaged M1 amplitude of the first m-sequence stimulation for the control and

glaucoma subjects. (B) The averaged M2 amplitude of the second m-sequence stimulation for the control and glaucoma subjects. (C) Averaged M1:M2 amplitude ratios. (D) The receiver operating characteristic plot derived from different cut-off values of the M1:M2 ratio at ring 2. Error bars are ±SD; red lines are medians and blue lines are maximum and minimum of the data.

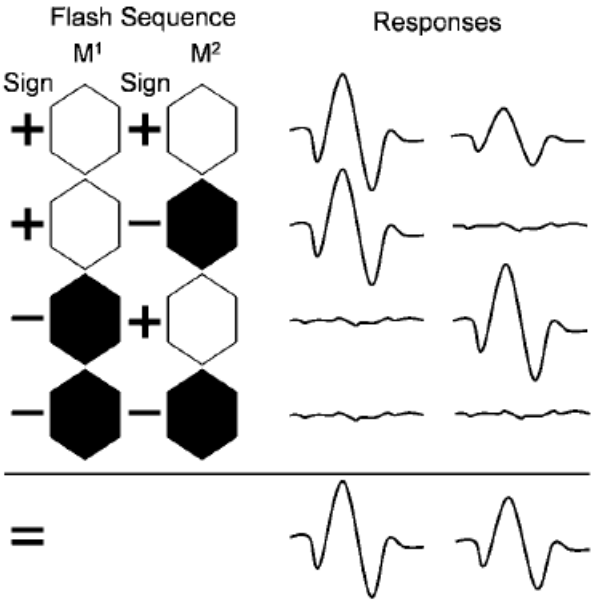

Figure 5 Schematic diagram illustrating the signal derivation of the first order kernels of the slow double-stimulation multifocal electroretinogram. The probability that a hexagon will contain a double flash stimulation in one cycle of m-sequence is only 0.25, so the temporal adaptive effect on the M2 response is reduced by half.