Effect of Pupil Size on Flicker ERGs Recorded With RET*eval* System: New Mydriasis-Free Full-Field ERG System

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Submitted: December 28, 2014
Accepted: April 28, 2015

Citation: Kato K, Kondo M, Sugimoto M, Ikesugi K, Matsubara H. Effect of pupil size on flicker ERGs recorded with RET*eval* system: new mydriasis-free full-field ERG system. *Invest Ophthalmol Vis Sci*. 2015;56:3684–3690. DOI:10.1167/iovs.14-16349

Purpose. We studied whether pupil size affects the flicker electroretinograms (ERGs) recorded by RET*eval*, a new mydriasis-free full-field flicker ERG system.

Methods. We studied 10 healthy subjects. The RET*eval* manufacturer claims that the system delivers a constant flash retinal illuminance by adjusting the flash luminance to compensate for changes in the pupil size. Two experiments were performed. First, the flicker ERG was recorded every 3 minutes after the instillation of mydriatics. Second, the flicker ERG was recorded while the subjects wore soft contact lenses with two different artificial pupil sizes.

Results. The first experiment showed that as pupil size increased, the amplitudes of the fundamental component of the flicker ERG did not change significantly, but the implicit times of the fundamental component were significantly prolonged for larger pupil sizes. There was a significant positive correlation between the pupillary area and implicit time of the fundamental component ($r = 0.93$, $P < 0.001$). The second experiment showed that the implicit times of the fundamental component in the flicker ERG were significantly longer with larger artificial pupil.

Conclusions. The results suggest that the effective retinal illuminance of the stimulus delivered by the RET*eval* system decreases for large pupil sizes. However, in most clinical testing situations, patients' undilated pupils will likely be sufficiently small to fall within the range for which the system delivers a stimulus of constant retinal illuminance.

Keywords: electroretinogram (ERG), flicker ERG, pupil, implicit time, mydriasis, RET*eval*, fundamental component

Electroretinograms (ERGs) elicited by fast flicker rates, usually approximately 30 Hz, are used widely to assess the function of the retinal cone pathway in clinical and experimental situations.[1] The fast flicker rates are used because the rod-driven responses cannot follow frequencies above approximately 15 Hz.[2] The flicker ERGs are valuable not only for monitoring residual cone function in degenerative retinal diseases, such as retinitis pigmentosa or cone dystrophy,[3–7] but also for evaluating the retinal ischemic status in diabetic retinopathy,[8,9] central retinal vein occlusion,[10–13] and other retinal vascular disorders.[14]

The retinal origin of the fast flicker ERGs has not been determined definitively, but studies using glutamate analogs in macaque monkeys have shown that the fast flicker ERGs were dominated by activity originating from the postphotoreceptoral ON- and OFF-pathway neurons regardless of whether the flicker stimulus was a sine-wave, square-wave, or brief pulses.[15–17]

The guideline for eliciting and recording full-field ERG by the International Society of Clinical Electrophysiology of Vision (ISCEV) Standards[1] recommends that the pupils be fully dilated before the ERG recording to eliminate any effect due to changes in pupil size as well as to maximize the area of retina stimulated. However, mydriatics cannot be used on subjects with allergic reactions to mydriatics. In addition, it would be more practical and convenient for the clinician and/or the patient/subject if pupil dilation was not necessary.

Recently, a full-field flicker ERG recording system called the RET*eval* system (LKC Technologies, Inc., Gaithersburg, MD, USA) was developed. In this system, a small, hand-held Ganzfeld dome and a special skin electrode array are used. The manufacturer claims that flicker ERGs can be recorded without mydriasis because the device delivers a stimulus with constant retinal illuminance (photopic Td-s) by adjusting the luminance (photopic cd-s/m^2) to compensate for changes in the pupillary area (mm^2). However, the range of pupil sizes for which the retinal illuminance actually remains constant has not been specified. This range can be defined by determining the pupil sizes for which the flicker ERG remains unchanged.

The purpose of this study was to determine the range of pupil sizes over which the RET*eval* system presents a stimulus with constant retinal illuminance by evaluating the effects of increasing pupil size on the amplitude and implicit time of the flicker ERG.

Subjects and Methods

Subjects

We studied 10 healthy volunteers (median age, 33 years; range, 25–46 years). All subjects had neither known ocular and systemic diseases nor myopia of –6.0 diopters or more.

The research was conducted in accordance with the Institutional Guidelines of Mie University Hospital and was approved by the Institutional Ethical Review Board (number 2595). The procedures conformed to the tenets of the World Medical Association's Declaration of Helsinki, and a written

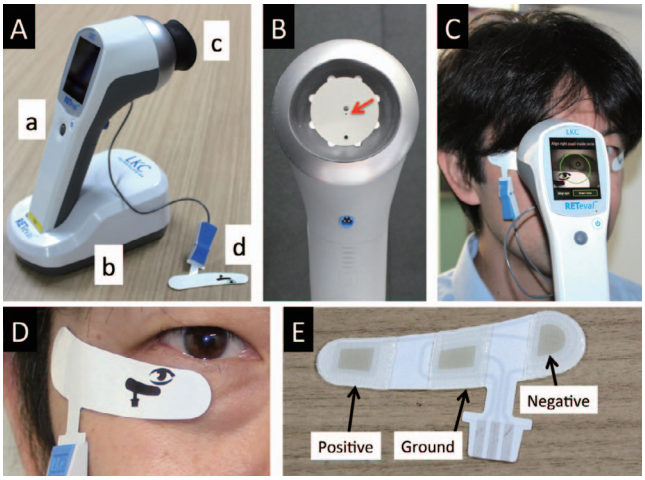

FIGURE 1. New hand-held flicker ERG recording system (RETeval) used in this study. (**A**) This system consists of a hand-held stimulator, recording, and analysis apparatus (**a**), a docking station for charging and downloading results to a computer (**b**), a soft eye cup that contacts the bony regions around the eye (**c**), and disposable skin electrode array (Sensor Strip) to record electrical response (**d**). (**B**) Small Ganzfeld dome for full-field stimuli. A small red fixation spot is provided at the center of dome (*red arrow*). (**C**) Recording of full-field flicker ERG using the RETeval system. During the recording, the subject's pupillary area is automatically measured by a built-in automated pupillometer, and a constant flash retinal illuminance (Td-s) is delivered. (**D**) Special skin adhesive tape containing the electrodes (Sensor Strip). The tape was placed on the orbital rim 2 mm from the margin of the lower eyelid. (**E**) Back surface of the electrode array which contains three electrodes; the active (positive), reference (negative), and ground electrodes in a single adhesive tape.

informed consent was obtained from all subjects after they were provided with sufficient information on the procedures to be used.

Pupil Measurement

Two pupil measurement techniques were used. During flicker stimulation, the pupil size (mm^2) was automatically measured in real time by the RETeval system using a built-in infrared pupillometer. In the recent version of RETeval, the raw data of the stimulus intensity (cd-s/m^2) at each time point were obtained using a software provided by LKC Technologies, Inc. (available in the public domain at http://www.lkc.com/products/RETeval/index.html). We estimated the pupillary areas at each time point during ERG recording using the equation: photopic flash retinal illuminance (Td-s) = photopic flash luminance (cd-s/m^2) × pupillary area (mm^2). Using this software and equation, we calculated the average pupillary area during flicker ERG recordings.

The pupillary area (mm^2) also was measured by another automated pupil size measuring device (OPD Scan III; Nidek Co. Ltd., Hiroishi, Japan) under room light (850 lux) immediately before each ERG recording.

To compare the values of pupillary areas obtained by the RETeval system and OPD Scan III system, the pupillary area was measured by these two systems after mydriatic instillation in one subject.

Flicker ERG Recordings by RETeval System

The RETeval system consists of a hand-held stimulator, recording device, and analyzing device (7 × 10 × 23 cm; weight, 232 g; Fig. 1A-a). There also is a docking station for charging and downloading the recorded results to a computer (Fig. 1A-b), a soft eye cup that contacts the bony regions around the eye (Fig. 1A-c), and disposable skin electrode arrays to pick up the electrical signals (Fig. 1A-d).

Full-field stimuli are presented by a small (60-mm) diameter dome (Fig. 1B). Visible white stimuli (CIE 1931 chromaticity, $x = 0.33$, $y = 0.33$) are created by a combination of three colored light emitting diodes (LEDs; red, 622 nm; green, 530 nm; blue, 470 nm; CLV6A-FKB; Cree, Inc., Durham, NC, USA). The frequency of the flicker stimulus is 28.306 Hz, and the pulse duration is less than 1 msec (confirmed by recording the LED responses with a photodiode). A small red fixation spot is present at the center of the Ganzfeld dome (Fig. 1B, red arrow), and subjects are instructed to fixate this spot during the ERG recordings.

During flicker stimulation, the pupil size (mm^2) is automatically measured in real time (Fig. 1C), and according to the manufacturer, the stimulus flash luminance (cd-s/m^2) is adjusted continuously to keep a constant flash retinal illuminance (Td-s) according to the following equation: Photopic flash retinal illuminance (Td-s) = photopic flash luminance (cd-s/m^2) × pupillary area (mm^2).

The RETeval picks up ERGs using a special skin electrode array (Sensor Strip; LKC Technologies, Inc.) placed on the orbital rim 2 mm from the margin of the lower eyelid (Figs. 1A-d, 1D). This electrode array contains three electrodes, an active (positive), reference (negative), and ground in a single adhesive tape (Fig. 1E). The electrical potentials are DC-amplified and digitized at a 2 kHz sampling rate. The data resolution is 24 bits for ±0.6 V, which is equal to approximately 0.07 μV.

The RETeval was used according to the instructions provided with the instrument. We chose a fixed stimulus flash retinal illuminance of 8 photopic Td-s, which is the recommended default stimulus setting for flicker ERGs for nondilated eyes in the RETeval system. This stimulus setting also is used for multicenter studies of diabetic retinopathy screening with RETeval (e.g., Ref. 18). This stimulus intensity, 8 Td-s, is much lower than the ISCEV standard for flicker stimulation, which is approximately 150 Td-s. No background illumination was used.

The flicker stimulus has a period of 35.328 ms (28.306 Hz), and the duration of each "sweep" is 1 period long. The flicker ERG recording time ranged from 5 to 15 seconds depending on the reliability of the results, assessed by estimating the standard error of the mean estimate of the implicit time from all the sweeps. Thus, the ERGs elicited by 141 to 425 flashes were analyzed for each recording. If the subject blinked, as determined by the infrared camera, the data were removed from the analysis by the RETeval system.

The amplitudes or magnitudes and implicit times of the fundamental component were automatically measured and displayed by the RETeval system using a special algorithm using discrete Fourier transformation (DFT) and cross-correlation analysis.[18] Because the response to a periodic stimulus is composed of sinusoidal components that are multiples of the stimulus frequency, it is possible to reconstruct a less noisy version of the raw flicker ERG waveform by determining the amplitude and phase of each of the harmonics and summing them.[19] In this system, two flicker ERG waveforms, the fundamental component and the reconstructed flicker ERG waveform using the first eight harmonics, are presented.

The values of the raw luminances and ERG data are retained in the RETeval system as a rff file, and this file can be analyzed off line using software provided by LKC Technologies, Inc. (available in the public domain at http://www.lkc.com/products/RETeval/index.html).

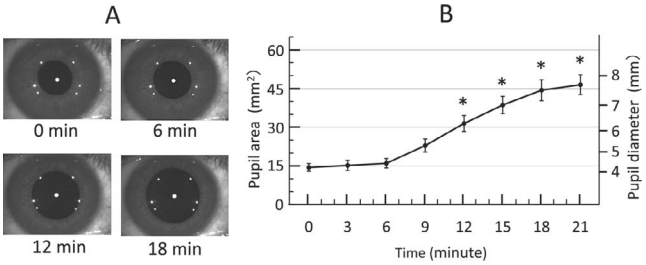

FIGURE 2. Change in pupil size after the instillation of mydriatics. The pupillary areas were measured by OPD Scan III immediately before RET*eval* flicker ERG recordings. (**A**) Infrared images of the pupils obtained from a representative subject. The photographs were taken at 0, 6, 12, and 18 minutes after mydriatic instillation. (**B**) Graph showing the changes in mean pupillary area (mm²) as a function of time after mydriatic instillation. *Significant change from baseline ($P < 0.05$, $F = 42.4$, $df = 7$). The pupillary area increases slowly after the instillation of mydriatics. *Error bars*: SEM for 10 subjects.

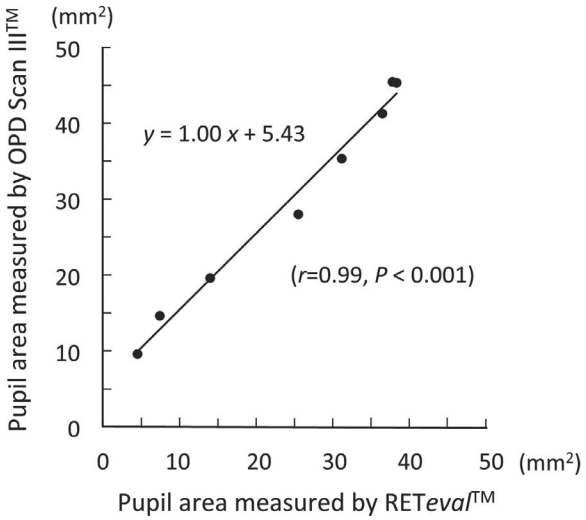

FIGURE 3. Comparison of pupillary area measured by OPD Scan under room light (850 lux) and by RET*eval* during flicker ERG recording following mydriatic instillation in one subject. A regression line is fit to the data ($r = 0.99$).

Changes in RET*eval* Flicker ERGs After Installation of Mydriatics

After 10 minutes of light-adaptation under room lights (850 lux), mydriatic drops were instilled (0.5% tropicamide and 0.5% phenylephrine, Mydrin P; Santen Co. Ltd., Osaka, Japan) and flicker ERG was recorded from the right eye every 3 minutes over a period of 21 minutes.

RET*eval* Flicker ERGs With Fixed Artificial Pupils

We also recorded the flicker ERG while the subjects wore soft contact lens (SCL) with artificial irides of two different pupil sizes. These SCLs were especially made for this experiment. The artificial irides were colored by metallic oxide and phthalocyanine compounds, and the average luminous transmittance of the artificial iris was 1.2% as measured by UV-VIS Spectrophotometer (UV-2550; Shimadzu Co. Ltd., Tokyo, Japan). The pupil diameters of the two SCLs were measured with a profile projector (V12A; Nikon Co. Ltd., Tokyo, Japan) and were 1.6 mm (small) and 5.8 mm (large).

The subjects' pupils were dilated, and after 10 minutes of light-adaptation under the room light (850 lux), we confirmed that pupil diameter was greater than 5.8 mm (the size of the larger artificial pupil). One of the artificial iris SCLs was inserted and the flicker ERG was recorded. Five subjects used the small pupil SCL first and then the large pupil SCL, and the other five subjects wore the large pupil SCL first and then the small pupil SCL. Before this experiment, we confirmed that the real-time pupillometer which was built into the RET*eval* detected the pupil sizes of these two types of SCLs correctly.

Statistical Analyses

Repeated measured 1-way layout ANOVA was performed to examine if there was a significant change in the pupillary areas, and in amplitudes and implicit times of the fundamental component of flicker ERG with increasing time after dilation. Then, the Bonferroni multiple comparison tests were used to identify specifically which time points were significantly different from the baseline value. Pearson product-moment correlation coefficient was used to examine if there was a significant correlation between the pupillary area and the implicit times of the fundamental component. Differences in the amplitudes or implicit times of the fundamental component between the eyes while wearing small or large artificial pupils were determined by paired *t*-tests, and statistical significances also were confirmed by nonparametric Wilcoxon signed-rank test. Results were considered statistically significant when $P < 0.05$.

RESULTS

Pupil Size

The changes in the size of the pupil with time after instilling the mydriatics, which were measured by OPD Scan III immediately before the flicker ERG recordings, are shown in Figure 2A. The mean pupillary area gradually increased, and at 21 minutes it was approximately 3.2 times larger than that at the baseline (Fig. 2B). The mean pupil area at 12 through 21 minutes was significantly larger than at the baseline ($P < 0.05$).

The pupillary area measurements obtained during RET*eval* ERG recording were significantly correlated with the OPD Scan measurements ($r = 0.99$, $P < 0.001$), but they were approximately 5.4 mm² smaller, presumably due to the adaptational effect of the flickering stimulating light. Figure 3 compares the data for the two pupil measurement methods.

RET*eval* Flicker ERGs During Pupillary Dilation

Changes in the flicker ERG after the instillation of the mydriatics are shown in Figure 4. Representative flicker ERGs recorded from a subject at 0, 12, and 21 minutes are shown in Figure 4A. The fundamental component (colored dotted line) is superimposed on the reconstructed flicker ERG (solid black line) using the first eight harmonics. A magnified view of the fundamental component shown in Figure 4A is shown in Figure 4B.

The means ± SEMs of the amplitudes (Fig. 4C) and implicit times (Fig. 4D) of the fundamental component are plotted as a function of the time after applying the mydriatics. The mean amplitude of the fundamental component tended to decrease slightly with time, but the change was not statistically significant ($P > 0.1$, Fig. 4C). In contrast, mean implicit time of the fundamental component increased gradually with time and was significantly different from that at baseline at 15 through 21 minutes ($P < 0.05$, Fig. 4D).

The mean implicit time of the fundamental component for the 10 subjects is plotted against the mean pupil area at the

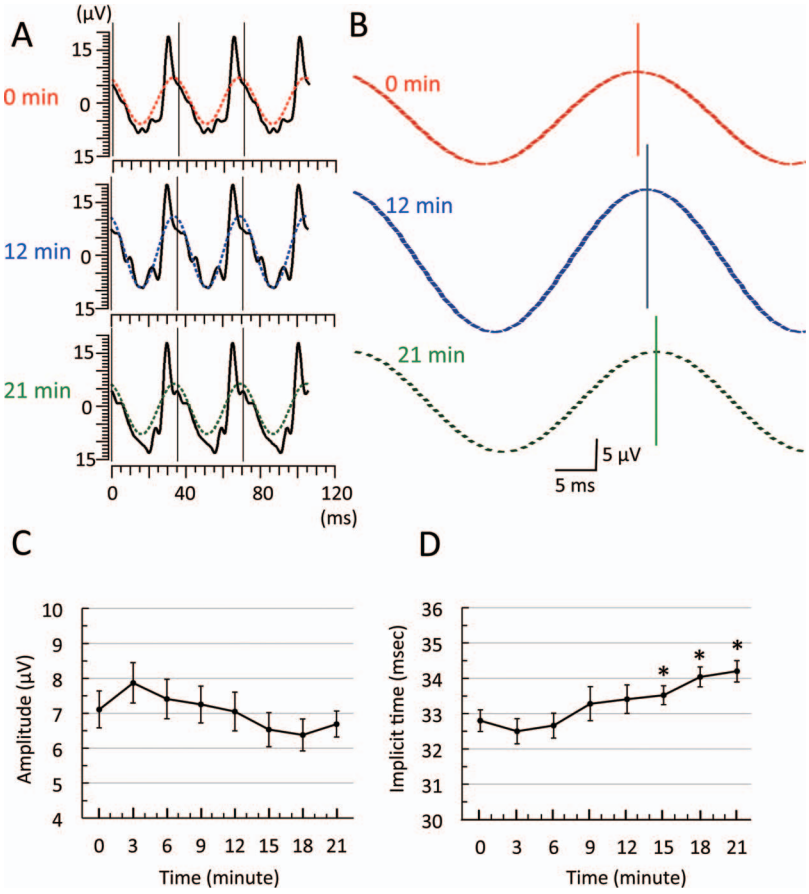

Figure 4. Changes in the flicker ERGs recorded with the RET*eval* system after the instillation of the mydriatics. (**A**) Representative flicker ERGs recorded from a subject at 0, 12, and 21 minutes after the instillation of the mydriatics. The fundamental component (*dotted colored line*) is superimposed on the reconstructed flicker ERG waveform using the first eight harmonics (*solid black line*). The *vertical lines* indicate the center of the light flashes. (**B**) Magnified image of fundamental component of (**A**). Note that the implicit time of fundamental components increases with time. (**C**) Mean amplitude of the fundamental component as a function of time after mydriatic instillation. There was no significant change in mean amplitude with time ($P > 0.1$, $F = 4.3$, $df = 7$). *Error bars*: SEMs for 10 subjects. (**D**) Mean implicit time of the fundamental component as a function of time after mydriatic instillation. The increase in mean implicit time at 15 through 21 minutes was statistically significant, as indicated by the asterisks ($P < 0.05$, $F = 17.6$, $df = 7$). *Error bars*: SEMs for the 10 subjects.

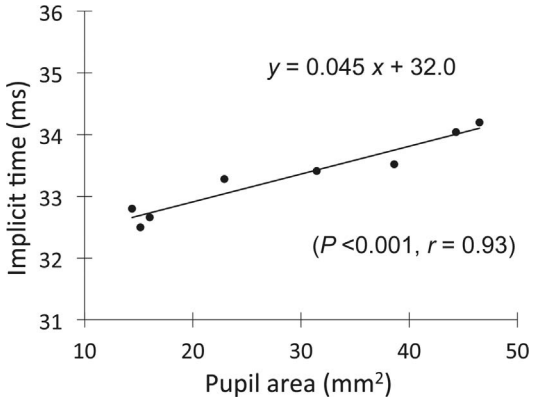

Figure 5. Implicit time of the fundamental component as a function of pupillary area. The mean implicit time of the fundamental component for the 10 subjects is plotted against the mean pupillary area at the eight different measuring points. There was a significant correlation between the pupillary area and implicit time of the fundamental component ($r = 0.93$, $P < 0.001$). The best fit linear regression line also is drawn on this Figure.

eight different measuring points in Figure 5. There was a significant positive correlation between the pupillary area and implicit time of the fundamental component ($r = 0.93$, $P < 0.001$). A linear regression fit to the data indicates that a 1 mm^2 increase was accompanied by an approximately 0.045 msec delay in the implicit time of the fundamental component of the flicker ERG.

RET*eval* Flicker ERGs While Wearing Artificial Pupils

The changes in the amplitude and implicit time of the fundamental component of flicker ERG while wearing a SCL with a small (1.6 mm) or a large (5.8 mm) artificial pupil are shown in Figure 6. Representative flicker ERGs recorded from a subject while wearing small and large artificial pupils are shown in Figures 6A and 6B.

In Figures 6C and 6D, the means ± SDs of the amplitude and implicit time of the fundamental component for the 10 subjects are plotted. The mean amplitude of the fundamental component was slightly smaller for the larger artificial pupil, but the difference was not statistically significant ($P = 0.31$, Fig. 6C). In contrast, the mean implicit time of the fundamental component for large artificial pupil was significantly longer than that for small artificial pupil ($P < 0.01$, Fig. 6D).

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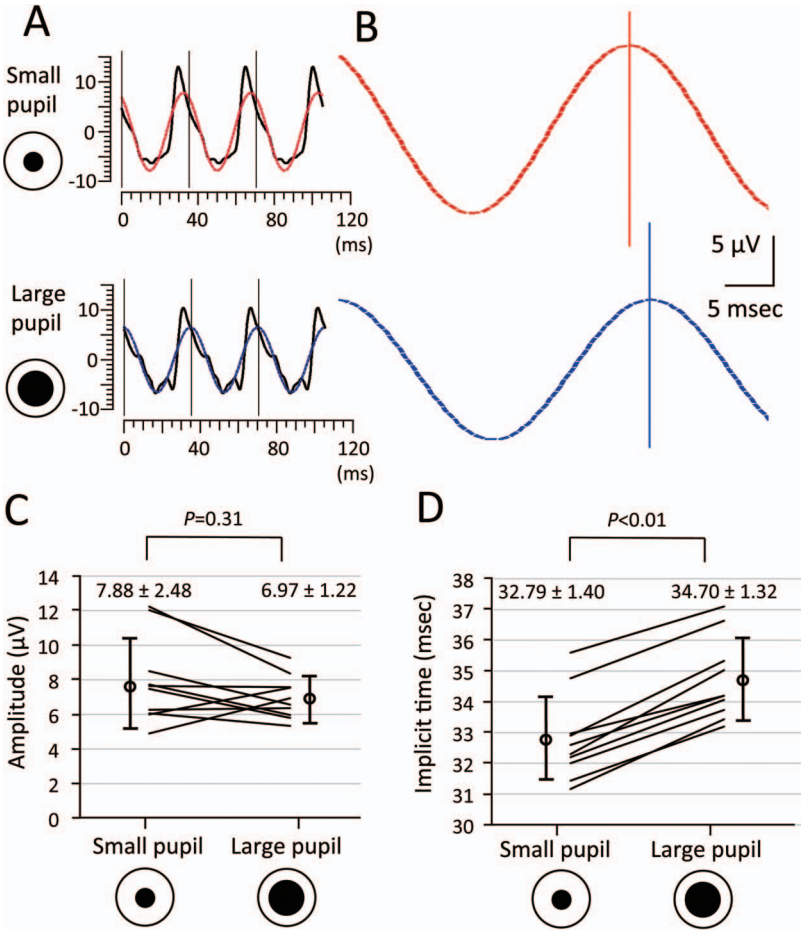

Figure 6. Comparison of flicker ERGs recorded with small (1.6 mm) versus large (5.8 mm) artificial pupils. (**A**) Representative flicker ERGs recorded from a subject while wearing soft contact lenses with small or large artificial pupils. The fundamental components (*colored dotted lines*) are superimposed on the reconstructed flicker ERG waveforms using the first eight harmonics (*black solid line*). (**B**) Magnified image of fundamental components of Figure 6A. Note that the implicit time of the fundamental component while wearing large artificial pupil is longer than that while wearing small artificial pupil. (**C**) Comparison of the amplitude of fundamental component between small and large artificial pupils. Means (± SDs) and individual values from each of the 10 subjects are plotted. The mean amplitude was not significantly different for small versus large artificial pupils ($P = 0.31$). (**D**) Comparison of the implicit time of fundamental component between small and large artificial pupils. Means (± SDs) and individual values from each of the 10 subjects are plotted. The mean implicit time was significantly longer for the large artificial pupil than for the small artificial pupil ($P < 0.01$).

Discussion

We found that the implicit time, but not amplitude, of the fundamental component of the RETeval flicker ERG increased significantly for large pupil sizes. We also found that implicit time was significantly longer with a large artificial pupil than with a small artificial pupil. These results indicated that despite the adjustment of stimulus luminance to compensate for changes in pupillary area in the RETeval system, a constant retinal stimulus is not maintained across all pupil sizes.

Why are the implicit times of the fundamental component in the RETeval system longer for larger pupil sizes even after adjustments were made to deliver the same flash retinal illuminance? We do not have a simple explanation for this question, but we suggest that at least two factors may be involved. The first factor is the Stiles-Crawford effect of the cone system. It is known that the response of cone photoreceptors is different when the angle of light stimulating the cones is changed.[20,21] Because of this Stiles-Crawford effect, the effective retinal illuminance to the cone system is not simply the product of luminance × pupil area for large pupil areas. Paupoo et al.[22] reported that when the pupil is fully dilated, the effective retinal illuminance of the cone system may be calculated by taking the pupil area to be approximately 20 mm^2. McCulloch and Hamilton[23] also showed that the rays of light entering 2.5 mm away from the pupil center have approximately one-half the effectiveness of those entering the pupil center (see their fig. 4). Therefore, in photopic electrophysiological testing with dilated pupils, the Troland values overestimate the effective retinal illumination. Thus, for large pupil size, the effective retinal illuminance would be dimmer than the specified Troland value, that is, the same total amount of retinal illuminance is less effective if it passes through a larger pupil. Therefore, since the RETeval uses the formula photopic flash retinal illuminance (Td-s) = photopic flash luminance (cd-s/m^2) × pupillary area (mm^2) in its determination of stimulus strength, it apparently does not take the Stiles-Crawford effect into account, and the lower effective retinal illuminance for large pupils could explain the significant increase in the implicit times when the pupil is large (Figs. 4–6).

What is the range of pupil sizes over which the RETeval delivers constant illuminance based on the data of ERG and pupil size? The ERG results indicated that amplitude and

implicit time remained constant until 15 minutes after instillation of mydriatic drops (Fig. 4), and the OPD Scan pupil data indicated that at 15 minutes after drop instillation, the mean pupillary area was 38.6 mm^2 (pupil diameter = 7.0 mm, Fig. 2B). Based on the graph plotting OPD Scan versus RET*eval* measurements (Fig. 3), the corresponding RET*eval* pupil area would be approximately 33.2 mm^2 (diameter = 6.4 mm). The corresponding diameter is approximately 6.5 mm. Based on these data, it can be concluded that for pupil diameter less than approximately 6.5 mm, the RET*eval* system delivers a stimulus with constant retinal illuminance; for larger pupil diameters, it is necessary to compensate for the Stiles-Crawford effect. Because the majority of patients are likely to have an undilated pupil diameter less than 6.5 mm, the manufacturer's claim that the RET*eval* system delivers constant retinal illuminance usually will be valid when testing is performed without mydriasis.

Another factor that may have contributed to the variation in implicit time is that even if the amount of light that enters the eye is kept constant for the different pupil sizes, the distribution of light across the retina may not remain constant, especially when pupil size is small.[24] Such an uneven distribution of stimulus intensities on different retinal areas may produce complex flicker ERG waveforms, which can be different from those from the eyes with large pupils. In addition, the different local distributions of the light may affect the ON and OFF system of the cone-pathway differently causing shifts in the phase of the flicker ERG.

A limitation of the study is that only the fundamental component of the flicker ERG, which is displayed automatically by the RET*eval* system, was studied quantitatively. It would be interesting also to analyze the other harmonic components and the reconstructed waveforms. For example, in Figure 4A, there seem to be only slight differences in the implicit times for the reconstructed waveform, in contrast with the obvious implicit time differences in the fundamental component. Similar findings have been reported in patients with the complete-type congenital stationary night blindness (CSNB).[25] If this observation is true for all subjects, it may suggest that the changes in ERG recording conditions affect the ERG harmonics differently, as reported previously.[26,27]

In conclusion, we found that the implicit time of the fundamental component of the RET*eval* flicker ERG is significantly affected by pupil size. We suggested that this is due to reduced effective retinal illuminance resulting from the Stiles-Crawford effect when the pupil is large (approximately >6.5 mm) and possibly to unequal distribution of light across the retina especially when the pupil is small. We believe that the RET*eval* system is a promising device for screening for retinal disease, but we caution that pupil size should be carefully monitored and stimulus intensity adjusted when necessary.

Acknowledgments

We thank John G. Robson of University of Houston College of Optometry (Houston, TX, USA); Eberhart Zrenner of Tubingen University (Tubingen, Germany) for valuable discussions; and Hidetaka Kudo, Daigo Yoshikawa, and Masao Yoshikawa of Mayo Company (Inazawa, Japan) and Quentin Davis of LKC Technologies for technical help. We also thank Duco I. Hamasaki of the University of Miami (Miami, FL, USA) and anonymous reviewers for critical discussion and final manuscript revisions.

Supported by Grant-in-Aid for Scientific Research C (#20592603) from Ministry of Education, Culture, Sports, Science and Technology of Japan (available in the public domain at http://www.jsps.go.jp/). The authors alone are responsible for the content and writing of the paper.

Disclosure: **K. Kato**, None; **M. Kondo**, None; **M. Sugimoto**, None; **K. Ikesugi**, None; **H. Matsubara**, None

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