Full-field Chromatic Pupillometry for the Assessment of the Postillumination Pupil Response Driven by Melanopsin-Containing Retinal Ganglion Cells

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Purpose. The postillumination pupil response (PIPR) is produced by intrinsically photosensitive retinal ganglion cells (ipRGCs). We aimed to refine the testing conditions for PIPR by investigating whether a greater PIPR can be induced using full-field light stimuli of shorter duration and lower intensity than that produced by existing protocols that use central-field stimuli.

Methods. Pupil response was recorded with an eye tracker in 10 visually-normal subjects. Red and blue light stimuli were presented using a Ganzfeld system. In Experiment 1 (intensity trials), PIPR was induced using 1-second full-field stimuli of increasing intensities from 0.1 to 400 cd/m^2 (11 steps). For comparison, PIPR also was induced using a 60° × 90° central-field blue stimulus of 400 cd/m^2. In Experiment 2 (duration trials), PIPR was induced using 100 and 400 cd/m^2 full-field stimulus of increasing duration from 4 to 1000 ms (10 steps).

Results. Results indicated that PIPR increased monotonically with increasing stimulus intensity. Full-field stimulation using blue light at 400 cd/m^2 intensity induced significantly more sustained PIPR than central-field stimulation ($P = 0.001$). In addition, PIPR increased as the stimulus duration increased from 4 to 200 ms; however, no further increase in PIPR was observed when the duration increased from 400 to 1000 ms.

Conclusions. Compared to existing central-field protocols, larger PIPR can be induced with a full-field stimulus with lower intensity and shorter duration, indicating that PIPR is a function of stimulus intensity, stimulus duration, and retinal area stimulated. The testing protocol can be refined with this new knowledge to target particular clinical populations.

Keywords: pupillometry, melanopsin-containing retinal ganglion cells, intrinsically photosensitive retinal ganglion cells, chromatic pupillometry

The melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) are a recently described specialized subset of photoreceptor cells in the retina.[1] Because melanopsin can absorb light energy directly and initiate the process of phototransduction, the ipRGCs are capable of generating and discharging an action potential in response to light exposure with or without synaptic input from the other two well-described photoreceptors, the rods and cones.[2–5] Activity of ipRGC provides the primary afferent signal for nonimage-forming light functions, including the pupillary light reflex and circadian rhythm regulation.[3,6–8] There also is growing evidence that ipRGCs provide the afferent signal for photophobia[9] and contribute to the image forming visual pathway,[10–12] but their exact roles in the image-forming visual pathway remain to be elucidated.

The postillumination pupil response (PIPR) is a sustained pupil constriction after the offset of a bright light stimulus. The PIPR has been long observed, but was poorly understood until the discovery of the ipRGCs and their characteristic pattern of activity upon light stimulation.[1,13] Electrophysiology studies have shown that melanopsin-driven intrinsic photo response of the ipRGC is characterized by steady-state activity that is sustained well beyond the cessation of the light stimulus.[1,11] The PIPR function remains largely unchanged after pharmacologic blockage of rod and cone functions in nonhuman primates,[14] indicating that PIPR is driven by the intrinsic melanopsin-driven activities of ipRGCs.

Several approaches have been used to induce and quantify PIPR. Kankipati et al.[15] showed that PIPR can be induced in vivo in visually-normal participants using a chromatic pupillometry technique. They also showed that PIPR was reduced in patients with advanced glaucoma compared to age-matched visually normal participants.[16,17] Kardon et al.[18,19] provided evidence that a clinically relevant chromatic pupillometry protocol could assess differentially the contributions of the rod, cone, and ipRGC pathways to the pupillary light response. Park et al.[20] further refined the protocol and provided evidence in favor of using dim blue stimuli to induce the rod-driven pupil response, bright red stimuli to induce the cone-driven pupil response, and bright blue stimuli of long duration to induce a sustained postillumination pupil constriction as an index of the intrinsic activity of the ipRGCs.

While using chromatic pupillometry to measure PIPR holds promise as a new diagnostic and therapeutic outcome

measurement tool to assess inner retinal function independent of visual photoreceptor input, testing conditions for PIPR have not been optimized for clinical use. All existing studies to our knowledge[14–16,18,19,21–23] have used central-field blue-light stimulation of high intensity and long duration to induce PIPR. The visual angle of the central-field stimuli ranged from 7.5° to 60° × 90°, which is not ideal because it does not stimulate all ipRGCs that are distributed across the entire retina.[3] Given the photon-counting properties of ipRGCs, it has been suggested that they collectively represent a system that measures the total retinal irradiance.[11] Inadequate stimulation of the peripheral retina may be a reason why all existing studies using central-field stimuli have required long duration stimulation (10–30 seconds) to induce a measurable PIPR. Although one protocol[20] reduced the duration of stimulation to 1 second, in our experience, most participants still found it challenging to tolerate the stimuli, especially at higher intensities, and they had difficulty keeping their eyes open. In the same study,[20] pupil size was measured at a single time point at 6 seconds after stimulus offset, making it more prone to artifact and noise, while other studies measured PIPR over a prolonged interval up to 50 seconds following the stimulation.[15–17,22]

In an effort to refine the existing PIPR testing methodologies, we sought to determine the optimal full-field stimulation duration and intensity to induce significant PIPR in visually-normal observers. We conducted two experiments to test the hypothesis that full-field stimulation induces a larger PIPR response, allowing testing to be performed at a lower stimulus intensity and shorter duration than central-field stimulation. We also compared the single time point postillumination pupil measurement technique[20] with prolonged interval measurement as an index of PIPR.

Methods

Participants

We tested 10 visually-normal participants for each experiment (not all subjects participated in both experiments). The experiments were conducted monocularly, with the right eye being stimulated and recorded. The left eye was patched. The study was approved by the Research Ethics Board at The Hospital for Sick Children. All the procedures adhered to the guidelines of the Declaration of Helsinki. Informed consent was obtained from each participant.

Apparatus

The chromatic pupillometry system consisted of two components, a Ganzfeld screen and an infrared video-based spectacle frame-mounted eye tracker. The Ganzfeld screen (Espion V5 system with the ColorDome LED full-field stimulator; Diagnosys LLC, Lowell, MA, USA) that was designed originally for full-field ERG testing was used to present full-field light stimulation. This system can generate a wide range of flash intensities from 0.0001 to 400 cd/m^2 (–4 to 2.6 log) for blue (467 ± 17 nm) light and 0.0001 to >400 cd/m^2 (–4 to >2.6 log) for red (640 ± 10 nm) light. The binocular eye-tracking camera system used near-infrared (940 nm) illuminating diodes (Arrington Research, Scottsdale, AZ, USA) to record the changes in pupil diameter at a sampling rate of 60 Hz. The chromatic pupillometry setup was identical to that described by Kardon et al.[18,19] and Park et al.[20] except for some modifications to our eye tracker. The scene camera was removed to allow the participants to place their forehead against the edge of the Ganzfeld screen for full-field stimulation. To increase the quality of the eye tracking, an extra miniature infrared illumination diode was added in front of each camera (peak wavelength, 940 nm; radiant intensity, 40 mw; 18° angle of emitted light; Fairchild Semiconductor, San Jose, CA, USA) to provide additional infrared illumination to each eye.

Experimental Conditions and Procedure

Overview. This study consisted of two experiments. Experiment 1 (intensity trials) compared the PIPR in response to full-field stimulation of increasing intensities from 0.1 to 400 cd/m^2 at a fixed 1-second duration. For comparison, PIPR also was induced using a 60° × 90° central-field blue stimulus of 400 cd/m^2 as used by Park et al.[20] Experiment 2 (duration trials) was conducted on two separate days testing two specific flash intensities (100 cd/m^2 red and blue stimuli on one day, and 400 cd/m^2 red and blue stimuli on the second day) with 10 different durations from 4 to 1000 ms. The PIPR at various durations was compared for the two specific intensities. All experiments were conducted during the day between 8 AM and 2 PM.

Experiment 1: Intensity Trials. Ten visually-normal subjects participated (7 females; mean age, 31 years; range, 22–56 years). After 10 minutes of dark adaptation, alternating red and blue full-field stimuli of 1-second duration of increasing intensity from 0.1 (–1.0 log) to 400 (2.6 log) cd/m^2 (11 steps: 0.1, 0.32, 1, 3.16, 10, 31.6, 75, 100, 150, 200, and 400 cd/m^2) were presented in a darkened room. For each of the intensity steps, a red flash was presented first followed by a blue flash 45 seconds after the offset of the red flash. Participants were provided with a short break at 45 seconds after the offset of the blue flash to allow the pupil size to return to baseline and to prevent fatigue before the presentation of the next intensity level. The duration of the short break varied (20–90 seconds) depending on the time it took for the pupil diameter to return to baseline. During the break, the lights were kept extinguished and participants were allowed to move their chin off the chin-rest. To compare with previously published protocols, 400 cd/m^2 red and blue central-field flashes subtending a visual angle of 60° × 90° then were presented for a duration of 1 second with the participants' eyes positioned 75 mm away from the opening of the Ganzfeld screen as described by Kardon et al.[18,19] and Park et al.[20]

Experiment 2: Duration Trials. Ten visually-normal subjects participated (6 females; mean age, 32 years; range, 22–56 years). After 10 minutes of dark adaptation, alternating red and blue full-field stimuli of constant intensity and increasing duration from 4 to 1000 ms (10 steps: 4, 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ms) were presented. Similar to Experiment 1, the red flash was presented first and then the blue flash was presented 45 seconds after the offset of red flash. At 45 seconds after the blue flash, recording was paused to allow the pupil diameter to return to baseline and to provide a short break for the participant.

Data Analysis

Data from the eye tracker were analyzed offline using a custom-written script (MatLab; MathWorks, Inc., Natick, MA, USA). A median (window length of 0.5 second) and low-pass (fourth-order, zero-phase Butterworth) filter with a cut-off frequency of 5 Hz were applied to remove eye blink artifacts. The filtered data were inspected visually in a graphical user interface (GUI) to ensure data quality and detect artifacts. The filtered data then were normalized to the baseline pupil size calculated from the mean pupil size during a 1-second period

before the onset of each stimulus (i.e., normalized pupil size = ratio of absolute pupil size to baseline pupil size). Two parameters were measured: $PIPR_{(10-30seconds)}$, mean of the normalized pupil size over a 20-second period from 10 to 30 seconds after the offset of light stimuli (i.e., prolonged interval measurement) and $PIPR_{(6seconds)}$, normalized pupil size at 6 seconds after the offset of light stimuli (i.e., single time point measurement). For both parameters, a smaller value represents greater pupil constriction. The intersubject coefficient of variation (CV) for $PIPR_{(10-30seconds)}$ and $PIPR_{(6seconds)}$ for blue stimuli were calculated to quantify the relation between signal and noise in responses. The CV is defined as the ratio of the SD to the mean changes of pupil diameter: CV = SD/(1 − mean PIPR).

Statistical analyses were performed on the normalized pupil size data using SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). Differences in PIPR between different testing conditions were compared by 1-way ANOVA. Post hoc analysis was corrected for pairwise multiple comparisons using the Tukey–Kramer method. A value of $P < 0.05$ was considered statistically significant.

Results

Experiment 1: Intensity Trials

Figure 1 shows the mean ($n = 10$) waveforms for pupil responses to 1-second full-field stimulation using red and blue flashes for intensities from 0.1 to 400 cd/m^2 (Figs. 1a–k), and to 1-second central-field (60° × 90°) stimulation using red and blue flashes of 400 cd/m^2 intensity (Fig. 1l). The pupil response is shown from 1 second before stimulus onset to 45 seconds after the stimulus offset. In general, the red stimulus induced a rapid constriction of the pupil, which quickly redilated to reach baseline approximately 10 seconds after stimulus offset. In contrast, the blue stimulus of photopically-matched luminance induced a higher peak pupil constriction response and a much more sustained PIPR, especially when the intensity was 3.16 cd/m^2 and above. When central-field stimulation was compared to full-field stimulation, the PIPR induced by the 400 cd/m^2 blue central-field stimuli (Fig. 1l) was less sustained than the responses induced by 200 and 400 cd/m^2 blue full-field stimuli (Figs. 1j, 1k). The response induced by the 400 cd/m^2 blue central-field stimulation was comparable to those induced by the 100 and 150 cd/m^2 blue full-field stimuli (Fig. 2).

The mean $PIPR_{(10-30seconds)}$ measurements were significantly different across intensity steps ($F_{[11,99]} = 62.20$, $P < 0.0001$). The $PIPR_{(10-30seconds)}$ for the blue full-field stimulation increased monotonically with increasing stimulation intensity. The highest $PIPR_{(10-30seconds)}$ was induced by 400 cd/m^2 blue full-field stimuli (mean ± SD, 0.54 ± 0.06 normalized pupil diameter), which was significantly greater than the mean $PIPR_{(10-30seconds)}$ induced by the 400 cd/m^2 blue central-field stimulus (0.64 ± 0.08, $P = 0.0239$) as shown in Figure 3. However, $PIPR_{(10-30seconds)}$ for 200 cd/m^2 (0.59 ± 0.07), 150 cd/m^2 (0.62 ± 0.10), 100 cd/m^2 (0.66 ± 0.08), and 75 cd/m^2 (0.68 ± 0.13) full-field blue stimulations did not differ significantly from that of the 400 cd/m^2 blue central-field stimuli (0.64 ± 0.08, $P > 0.1000$). In contrast, following red stimulation (full and central-field), the induced $PIPR_{(10-30seconds)}$ was small and did not differ significantly across all the stimulus intensities ($F_{[11,99]} = 1.71$, $P = 0.0825$).

The $PIPR_{(6seconds)}$ (single time-point analysis) showed that $PIPR_{(6seconds)}$ to blue full-field stimulation increased steeply from 1 to 31.6 cd/m^2 (Fig. 3), appearing to saturate by 31.6 cd/m^2. Post hoc analysis showed that $PIPR_{(6seconds)}$ to 31.6 cd/m^2 was significantly greater than $PIPR_{(6seconds)}$ for 1 to 10 cd/m^2 ($P < 0.001$). From 31.6 to 400 cd/m^2, $PIPR_{(6seconds)}$ did not differ significantly in any pairwise comparisons. The PIPR induced by the central 400 cd/m^2 blue stimulus was not significantly different from those induced by 31.6 to 400 cd/m^2 full-field blue stimuli at 6 seconds ($P > 0.9$). The $PIPR_{(6seconds)}$ for the red stimuli showed a gradual and significant increase with increasing intensities ($F_{[11,99]} = 2.58$, $P = 0.0064$).

The intersubject CV of $PIPR_{(10-30seconds)}$ and $PIPR_{(6seconds)}$ for blue stimuli in Experiment 1 was plotted in Figure 4. The CV of $PIPR_{(6seconds)}$ is lower than that of $PIPR_{(10-30seconds)}$ at all intensity steps. The CV of $PIPR_{(10-30seconds)}$ to 200 cd/m^2 (CV = 0.17) and 400 cd/m^2 (CV = 0.13) blue full-field stimuli have lower CV than that of $PIPR_{(10-30seconds)}$ to 400 cd/m^2 blue central-field stimulus (CV = 0.21).

Since the largest PIPR was induced with 400 cd/m^2 blue full-field stimulation, we evaluated this intensity further in Experiment 2, in which we varied the duration of stimulation. The 100 cd/m^2 stimulus also was tested in Experiment 2 for comparison purposes.

Experiment 2: Duration Trials

Mean waveforms of pupil light responses to 100 and 400 cd/m^2, red and blue full-field flashes of increasing duration from 4 ms to 1000 ms are shown in Figure 5. For the 400 cd/m^2 blue stimuli, pupil constriction was increasingly sustained when duration increased from 4 to 400 ms; however, further increases in duration (greater than 400 ms) did not result in a more sustained pupil response, as shown in Figure 6. This is verified quantitatively by the mean $PIPR_{(10-30seconds)}$, which increased gradually until the 200 ms duration step, with the last five duration steps (200, 400, 600, 800, and 1000 ms) not differing significantly among one another (see Table). When 400 cd/m^2, 1000 ms central-field data from experiment 1 were compared to the data from experiment 2, the $PIPR_{(10-30seconds)}$ induced by the last four duration steps (400, 600 , 800, and 1000 ms) of 400 cd/m^2 full-field blue stimuli were significantly greater than the $PIPR_{(10-30seconds)}$ induced by 1000 ms central-field stimuli of the same wavelength and intensity (0.54 ± 0.09, 0.53 ± 0.08, 0.52 ± 0.07, 0.52 ± 0.07 vs. 0.64 ± 0.08, $P < 0.007$). For the 100 cd/m^2 blue stimulation, on the other hand, $PIPR_{(10-30seconds)}$ increased monotonically with increased duration, indicating that 100 cd/m^2 blue stimuli were not strong enough to induce saturated PIPR for durations between 4 and 1000 ms. The 100 and 400 cd/m^2 red stimuli induced no or very little $PIPR_{(10-30seconds)}$ that did not change significantly with increased duration (Fig. 7A).

The $PIPR_{(6seconds)}$ to blue stimuli increased rapidly with increasing duration, reaching its maximum at 200 ms for the 400 cd/m^2 blue stimulus and at 400 ms for the 100 cd/m^2 stimulus, with no significant increases in $PIPR_{(6seconds)}$ with further increases in duration. The $PIPR_{(6seconds)}$ to red stimuli increased slowly, but monotonically, reaching a maximum at 1000 ms for 100 and 400 cd/m^2 stimuli ($F_{[9,81]} = 7.75$, $P < 0.0001$ for 100 cd/m^2; $F_{[9,81]} = 4.29$, $P = 0.0001$ for 400 cd/m^2; Fig. 7B).

Intersubject CV of $PIPR_{(10-30seconds)}$ and $PIPR_{(6seconds)}$ to blue stimuli in Experiment 1 is plotted in Figure 8. Similar to the results of Experiment 1, CV of $PIPR_{(6seconds)}$ generally is lower than that of $PIPR_{(10-30seconds)}$. Compared to central-field stimulation, full-field stimulation has a lower CV of $PIPR_{(10-30seconds)}$, yet higher CV of $PIPR_{(6seconds)}$. The CV of $PIPR_{(10-30seconds)}$ to the last 5 duration steps of full-field blue stimuli and that of 400 cd/m^2, 400 ms central-field blue stimulus were 0.20, 0,19, 0.16, 0.15, 0.15, and 0.21, respectively.

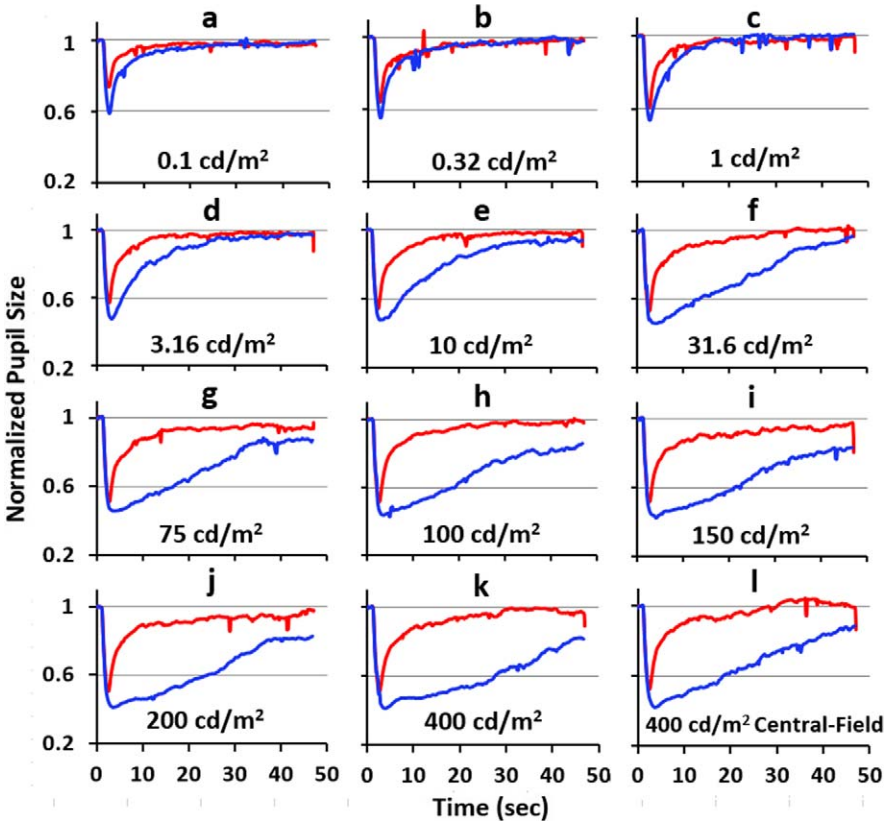

Figure 1. Mean normalized PIPR tracings in response to 1-second stimulation of varying intensity from 10 visually-normal participants. (**a**–**k**) Show PIPR to full-field red and blue stimuli at 11 intensity levels from 0.1 to 400 cd/m^2. (**l**) Shows PIPR generated by a 400 cd/m^2 central-field stimulus.

Discussion

One of the major findings of this study is that a large PIPR can be induced using full-field stimulation of lower intensity and shorter duration than existing central-field protocols.[20] The results from Experiment 1 showed that compared to central-field stimulation at 400 cd/m^2 intensity (1-second duration), full-field stimulation induced significantly greater PIPR with a stimulus of the same intensity and duration. In addition, full-field stimulation induced an equally large PIPR with stimulus intensities as low as 100 to 150 cd/m^2 (compared to central-field stimulation at 400 cd/m^2 intensity), indicating that full-field stimulation is more effective in inducing PIPR. We used a slightly modified eye tracker configuration to allow participants to be positioned optimally for full-field stimulation.

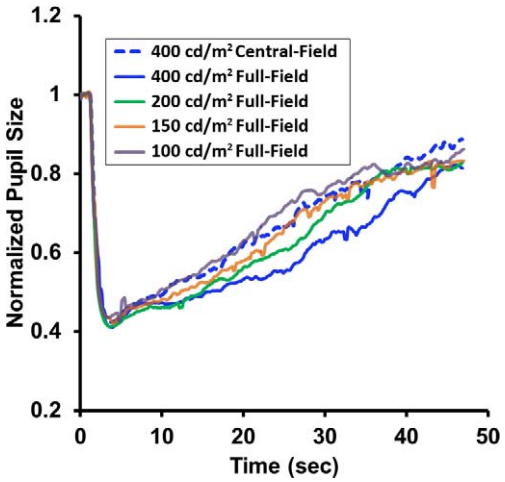

Figure 2. Comparison of PIPR induced using 400 cd/m^2 central-field stimuli (*dashed line*) versus 100 to 400 cd/m^2 full-field stimuli (*solid lines*). The response induced by the 400 cd/m^2 blue central-field stimulation (*dashed line*) was comparable to those induced by the 100 and 150 cd/m^2 blue full-field stimulus.

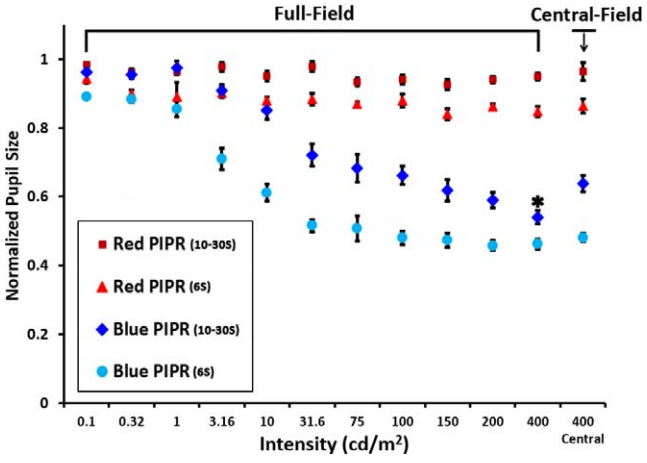

Figure 3. Mean normalized pupil size from 10 to 30 seconds after stimulation offset ($PIPR_{[10-30seconds]}$) and pupil size at 6 seconds after stimulation offset ($PIPR_{[6seconds]}$). The highest $PIPR_{(10-30seconds)}$ was induced by 1-second, 400 cd/m^2 blue full-field stimuli, which was significantly greater than the mean $PIPR_{(10-30seconds)}$ induced by the 1-second, 400 cd/m^2 blue central-field stimulus (*far right column* indicated by the *arrow*). *Error bars* represent ±SE.

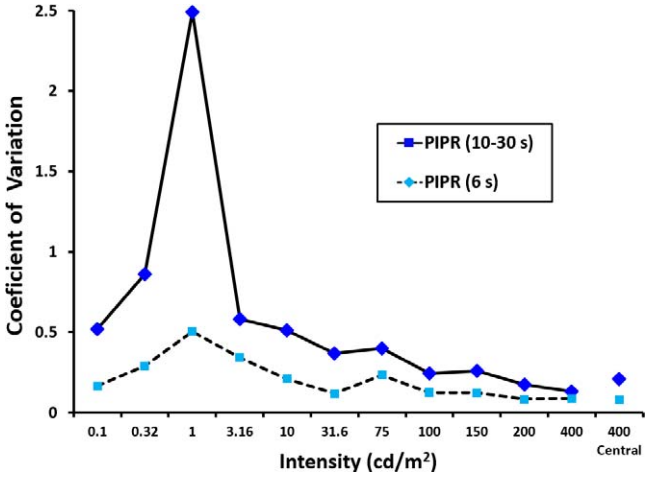

FIGURE 4. Coefficient of variation (CV = SD/1 − mean PIPR) to $PIPR_{(10-30seconds)}$ and $PIPR_{(6seconds)}$ to blue light stimuli in Experiment 1. For $PIPR_{(10-30seconds)}$ and $PIPR_{(6seconds)}$, CV decreases with increasing intensity of blue light stimulation, indicating that higher intensity induces more greater PIPR (higher amplitude, lower between subject variability). There is no substantial difference in CV between corresponding full-field and central-field stimulation (400 cd/m^2 full-field versus 400 cd/m^2 central-field).

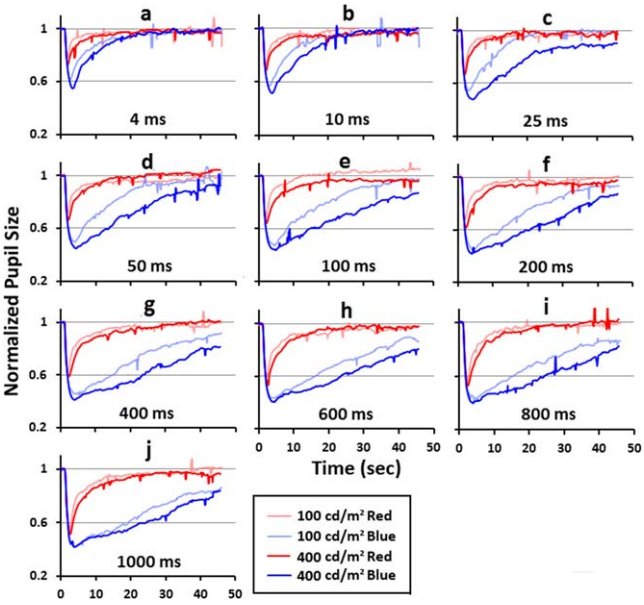

FIGURE 5. (a–j) Mean PIPR to 100 and 400 cd/m^2 full-field stimulation of varying duration from 10 visually-normal observers.

Although subjects were closer to the ColorDome screen when the eyes were stimulated with full-field versus central-field stimuli, the difference in viewing distance between these conditions was only 75 mm. The physical attenuation of light intensity over this distance is negligible; thus, the stimulus light projected onto the central part of the retina can be considered equally intense in both conditions. The difference in PIPR induced by full-field versus central-field conditions most likely is attributable to additional retinal recruitment.

Since the largest PIPR was induced with 400 cd/m^2 full-field stimulation, this intensity level was chosen for Experiment 2, in which we compared the effects of varying stimulus duration. Previous studies in a rat model[1,13] have shown that the intrinsic light response of ipRGCs has low light sensitivity. In addition, the response is slow to start spiking (typically from several hundred milliseconds to several seconds depending on stimulus intensity)[1] and slow to reach peak firing (3–20 seconds).[11] Because of these findings, most previous pupillometry studies in humans[14–16,18,19,21–23] have used long duration stimuli (10–20 seconds). Although Park et al.[20] titrated the duration down to 1 second based on single time point measurement, $PIPR_{(6seconds)}$ is not an ideal index of ipRGC activity (see further discussion below). Our study is the first to our knowledge to demonstrate that a large PIPR recordable 10 to 30 seconds after the offset of the stimulus can be induced in vivo with a strong blue flash of only a few hundred milliseconds. Specifically, we found that PIPR from 10 to 30 seconds after cessation of the stimulus became saturated at approximately 200 to 400 ms with 400 cd/m^2 blue full-field stimuli. Because 400 cd/m^2 is the maximum intensity our apparatus can generate, it remains unknown whether similarly strong PIPR can be induced with even shorter exposure if higher light intensities are used, although higher light intensity will be more uncomfortable for the subjects, particularly for photophobic patients.

In our experiment, detectable $PIPR_{(10-30seconds)}$ emerged at approximately 3.16 cd/m^2 (approximately equivalent to 12 log quanta/cm^2/seconds,[20,24] given a mean resting pupil diameter of 6.4 mm in our subjects), and increased steadily with increasing stimulus intensity up to 400 cd/m^2 (14.3 log quanta/cm^2/s). The action spectrum of these responses corresponds well with the observed dynamic range of melanopsin for 470 nm light in vitro,[1,11] and matches the response range of PIPR induced with 10 seconds blue light stimulation in human subjects (approximately 12–15 log quanta/cm^2/s).[14] In addition, the PIPR recorded in this study is selectively sensitive to short wavelength light. Similar to pupillometry study on human using 10-second long exposure of bright blue light to induce PIPR,[14] we also measured pupil constriction to 0.35 to 0.4 of normalized pupil size at 30 seconds after 400 cd/m^2 full-field blue light stimulation for 200 to 1000 ms. Therefore, the chromatic sensitivity and kinetics are consistent with the known features of the melanopsin-driven photoresponse in vitro.[1,11] These findings suggested that the PIPR induced with our short duration testing conditions most likely is mediated by the melanopsin-driven ipRGC activity.

Previous studies[15,16,18,19,22] using central-field bright blue light stimuli of 10 to 20 seconds in duration induced a response profile characterized by a rapid pupil constriction upon stimulus onset, followed by a sustained component of pupil constriction under constant illumination, then a rapid dilation at stimulus offset, followed by a sustained postillumination pupil constriction of lesser magnitude. It should be noted that the characteristics of the pupillary responses to a short bright blue flash used in the current study are fundamentally different—the response is characterized by a rapid constriction that is sustained well after the offset, and lacks a rapid dilation upon stimulus offset. The rapid dilation upon stimulus offset during central-field stimulation of long duration is likely due to the photoreceptors' (rods and cones) OFF effect being superimposed on the intrinsic melanopsin-driven activity. This is supported by the study of Gamlin et al.[14] where the OFF effect was recorded by pupillometry and intracellular recording from an ipRGC. When the synaptic input from the image-forming photoreceptors was blocked pharmacologically, the OFF effect was absent.[1,11,14] We hypothesized that under our testing conditions, the spikes of intrinsic melanopsin-driven ipRGC firing occur after the offset of the blue flash, so that the intrinsic firing can carry on without being affected by the OFF effect. If this hypothesis is true, PIPR induced by a short flash of blue light would represent a "pure" intrinsic ipRGC activity

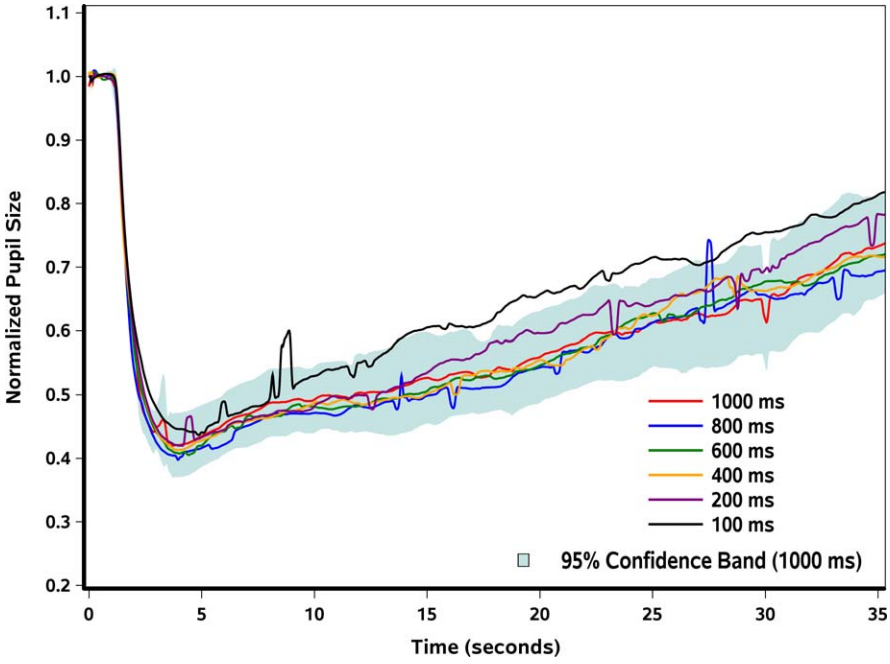

FIGURE 6. Comparison of PIPR to blue stimuli of 400 cd/m^2 intensity of the six longest duration steps (100, 200, 400, 600, 800, and 1000 ms). The PIPR time courses for 400 cd/m^2 blue stimuli of the four longest durations are essentially overlapping.

that is not being "contaminated" by the image-forming photoreceptor OFF effect.

The findings in our experiment also supported the idea that PIPR is a function of stimulus intensity, stimulus duration, and retinal area stimulated—higher stimulus intensity, longer stimulus duration, and larger stimulated retinal area all contribute to a larger and more sustained PIPR. This phenomenon is attributable to the precise photon-counting ability of ipRGCs that was first described by Dacey et al.[11] using intracellular recording, which demonstrated a highly linear relation between the total number of cell firing spikes and light intensity. It has been suggested that long, sparsely branching dendrites of ipRGCs form a large overlapping network that cover the entire retina as an irradiance detector.[11] The relations between PIPR and stimulus intensity, stimulus duration, and retinal area stimulated found in our study supported the idea that the ipRGC network codes intensity-dependent spatial and temporal summation of retinal irradiance.

The ability of our protocol to induce significant sustained PIPR with full-field stimulation of 200 to 400 ms is of considerable clinical significance. First, a short flash greatly decreases participant discomfort compared to long duration bright light stimulation in darkness. Second, a short flash enhances the consistency of the amount of light exposure, since it is practically impossible for participants to keep their eyes open throughout a 10- to 20-second duration of bright light exposure. Eye blinking and squinting cause inconsistent light exposure, and both can be avoided largely with our protocol using a 200 to 400 ms short flash. The pupil constriction during light stimulation also is minimized with a short flash, so the retinal irradiance during exposure is more consistent. In addition, the shorter the exposure, the less likely it is that the pupillary response will be affected by other factors, such as attention, accommodation, and fatigue. Third, compared to central-field stimulation, full-field stimulation provides stimulation to the entire retina, and may be useful in assessing ipRGC activity as an input signal to other biological functions, such as circadian rhythm regulation, or as an index of remaining inner retinal function in end-stage diseases. With this new understanding of the relation between PIPR and stimulus intensity, stimulus duration, and retinal area stimulated, investigators may now tailor their PIPR testing paradigm to target a particular clinical or research question.

In this study, two different measurement intervals were evaluated as indicators of the postillumination pupil response: $PIPR_{(6seconds)}$ and $PIPR_{(10–30seconds)}$. The results showed that in both experiments, $PIPR_{(6seconds)}$ responses to blue stimuli of varying intensity and duration are less linear and saturated much earlier than those of $PIPR_{(10–30seconds)}$ (Figs. 3, 7B). In contrast, red light stimulation, which primarily induces a cone response, generated a slow increase in $PIPR_{(6seconds)}$ with increasing intensity and duration (Figs. 3, 7B). We observed that the pupil response to red light stimulation subsided to a plateau near baseline levels 10 seconds after illumination. In addition, it has been well established that the response latency of the melanopsin-driven ipRGC activity typically is several hundred milliseconds to several seconds[1,11,25] (negatively correlated with stimulus intensity), so at 6 seconds after the offset of light, the melanopsin may not be fully activated yet, especially when the stimulus intensity is low. These results suggested that at 6 seconds after illumination, there still is a significant proportion of cone-driven responses contributing to the pupillary light response. In contrast, the $PIPR_{(10–30seconds)}$ to red stimulation was minimal at all stimulation levels. Furthermore, as discussed earlier, the $PIPR_{(10–30seconds)}$ induced in our experiment closely matches the action range of melanopsin. Therefore, we concluded that $PIPR_{(10–30seconds)}$ is the more appropriate index of the ipRGC-driven postillumination pupil responses tested. In Experiments 1 and 2, the coefficient of variation of $PIPR_{(10–30seconds)}$ to full-field stimulation is lower than that of central-field stimulation (Figs. 4, 8), suggesting that full-field $PIPR_{(10–30seconds)}$ is less variable between subjects. The intrasubject test–retest variability of the current full-field, short duration protocol remains to be investigated.

Table. Summary of *P* Values for Paired Comparisons (Post Hoc Analysis Corrected for Multiple Comparisons With Tukey-Kramer Method) of $PIPR_{(10-30seconds)}$ to 400 cd/m² Full-Field Blue Stimuli

	P Values, Post Hoc Tukey-Kramer Test							
100 ms (0.63 ± 0.09)								
200 ms (0.57 ± 0.09)	0.4597							
400 ms (0.54 ± 0.09)	0.0269*	0.9755						
600 ms (0.53 ± 0.08)	0.0098*	0.8914	1.0000					
800 ms (0.52 ± 0.07)	0.0016*	0.5815	0.9987					
1000 ms (0.52 ± 0.07)	0.0026*	0.6787	0.9997	1.0000				
Duration steps (mean ± SD)	100 ms (0.63 ± 0.09)	200 ms (0.57 ± 0.09)	400 ms (0.54 ± 0.09)	600 ms (0.53 ± 0.08)	800 ms (0.52 ± 0.07)	1000 ms (0.52 ± 0.07)		

The values in parentheses represent mean ± SD.

* $P < 0.05$.

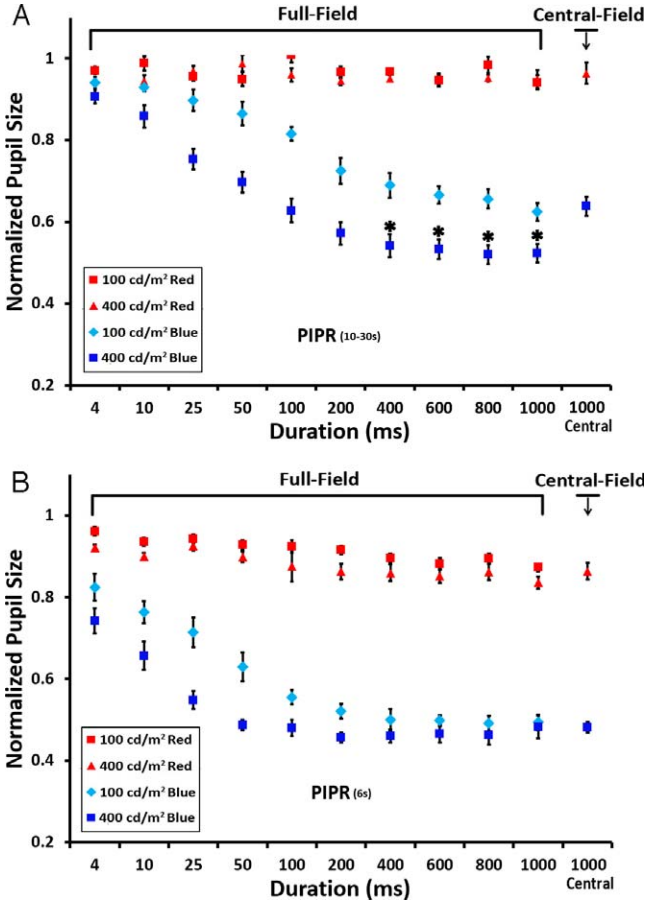

Figure 7. The $PIPR_{(10-30seconds)}$ **(A)** and $PIPR_{(6seconds)}$ **(B)** as a function of stimulus duration. *Error bars* represent ±1 SE. **(A)** Full-field blue stimulation at 400 cd/m² for 400 to 1000 ms induced significantly greater $PIPR_{(10-30seconds)}$ than the central-field 400 cd/m² stimulation for 1000 ms. **(B)** Postillumination pupil $response_{(6seconds)}$ to 1000 ms full-field blue stimulation at 400 cd/m² does not differ from $PIPR_{(6seconds)}$ to central-field blue stimulation of the same intensity and duration.

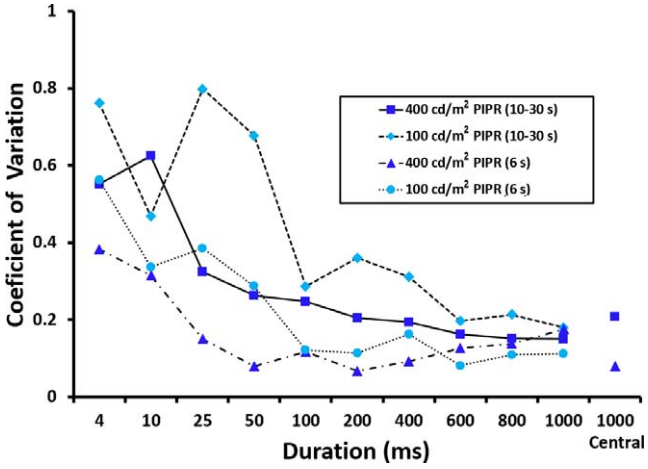

Figure 8. Coefficient of variation (CV = SD/1 − mean PIPR) to $PIPR_{(10-30seconds)}$ and $PIPR_{(6seconds)}$ to blue light stimuli in Experiment 2. The CV decreases with increasing duration of blue light stimulation. Compared to central-field stimulation, corresponding full-field stimulation (1000 ms full-field versus 1000 ms central-field) yields lower CV for $PIPR_{(10-30seconds)}$, yet higher CV for $PIPR_{(6seconds)}$.

In summary, compared to the existing protocols that used central-field stimulation, full-field stimulation induces a larger PIPR with lower stimulus intensities and dramatically shorter durations, indicating that PIPR represents an intensity-dependent spatial and temporal summation of retinal irradiance coded by melanopsin-driven ipRGC activity. This new understanding of the relation between PIPR and stimulus intensity, stimulus duration, and retinal area stimulated will allow investigators to tailor their PIPR testing paradigm to target a specific research question, and facilitate the development of a reliable, convenient, and comfortable technique to assess ipRGC function for emerging clinical use.

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