SUSTAINED PUPIL CONSTRICTION FOLLOWING BRIEF LIGHT EXPOSURE: 
RELATION TO RETINAL HEALTH

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RELATIONSHIP TO RETINAL HEALTH

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VISION SCIENCE

ABSTRACT

The pupillary light reflex (PLR) is an indispensable clinical measure of visual, neurological and autonomic function which, until recently, was thought to be driven by only rods and cones. In 2000, a novel subset of retinal ganglion cells (RGCs) was discovered that express melanopsin and are intrinsically photosensitive (ipRGC). These ipRGCs contribute to the PLR and are responsible for the sustained pupilloconstriction observed following light offset (the post-illumination pupil response (PIPR)). The primary goal of this project was to examine the PIPR in a broad sample of the general human population. Using a newly-developed, wide-field optical system, we demonstrate that all normal subjects display a post-illumination pupil response in response to a 10-second, 470nm light stimulus. We demonstrated that this PIPR was not correlated with subject characteristics such as age, race and gender, and that the only factor affecting the magnitude of the PIPR was the baseline pupil diameter. In most normal individuals, the PIPR was substantial (mean = 1.4 mm), and this test therefore has the potential to be utilized as a tool in evaluating subjects with either retinal or melanopsin-related disorders.
Glaucoma is a group of diseases of the optic nerve that causes optic neuropathy (GON) associated with visual field loss. The second goal of the project was to test the PIPR in a group of patients with GON and compare the results with normal subjects and visual fields. Our results indicate that there was a significant difference between the GON patients and age-matched controls (p<0.05). We also demonstrated that the loss of PIPR correlated with the severity of visual field loss (as evidenced by the mean deviation (MD) loss).

In conclusion, using a newly-developed, wide-field optical system, we have demonstrated that all normal subjects display a post-illumination pupil response which is reduced in patients with GON. Thus, testing for PIPR has the potential to be utilized as a clinical tool in evaluating and following patients with GON or melanopsin-related disorders.

Key words: ipRGC, glaucoma, optic neuropathy, pupillary light reflex
For my wife, Priya
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<td>Eye Foundation Hospital</td>
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<td>EW</td>
<td>Edinger-Westphal nucleus</td>
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<td>GHT</td>
<td>glaucoma hemifield test</td>
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<td>GON</td>
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<td>IpRGCs</td>
<td>intrinsically-photosensitive retinal ganglion cells</td>
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<td>LabView</td>
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<td>PSD</td>
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<td>RGC</td>
<td>retinal ganglion cell</td>
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<tr>
<td>SAS</td>
<td>statistical analysis software</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<td>SITA</td>
<td>Swedish Interactive Threshold Algorithm</td>
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PIPR (mm) = [Baseline pupil diameter (mm) – sustained pupil diameter (mm)]

PIPR change (%) = [(PIPR*100)/Baseline pupil diameter]

Net PIPR (mm) = [Blue PIPR – Red PIPR]

Net PIPR change (%) = [Blue PIPR change (%) – Red PIPR change (%)]

PIPR area (mm$^2$) = [Baseline pupil area (mm$^2$) – sustained pupil area (mm$^2$)]

PIPR area change (%) = [(PIPR area*100)/Baseline pupil area]

Net PIPR area (mm$^2$) = [Blue PIPR area (mm$^2$) – Red PIPR area (mm$^2$)]

Net PIPR area change (%) = [Blue PIPR area change (%) – Red PIPR area change(%)]

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The pupillary light response (PLR) is an indispensable clinical measure of visual, neurological and autonomic function. Until recently, it was thought that the PLR was driven by rods and cones (Alpern and Campbell 1962; Loewenfeld and Lowenstein 1993). However, recent findings now suggest that the PLR is substantially driven by a novel class of retinal ganglion cells – the melanopsin-containing, intrinsically photosensitive retinal ganglion cells (ipRGCs) (Gamlin et al., 2007; Lucas et al., 2001; Gooley et al., 2003).

Pupillary light reflex

The PLR is a reflexive constriction of the pupil in response to an increase in ocular illumination. Lesion or disease in any of the areas traversed by the reflex produces specific defects in the PLR (Kardon 1998). The retinal ganglion cells (RGCs) conveying light information from the eyes to the brain via the optic nerve form the afferent limb of the PLR (Oyster 1999). A small subset of the RGCs project to the pretectal nucleus of the midbrain which in turn project bilaterally to the Edinger-Westphal nuclei (EW), also found in midbrain. This midbrain
segment of the reflex allows the PLR to be utilized in clinical assessment of midbrain function. Pupil diameter at any given time is determined by the relative activation of the iris sphincter and dilator muscles, which are driven respectively by the parasympathetic and sympathetic branches of the autonomic nervous system (Oyster 1999).

Intrinsically photosensitive retinal ganglion cells (ipRGCs) and their role in pupillary light response

Until recently, the PLR was assumed to be driven by RGCs that received light signals exclusively from rod and cone photoreceptors, and the response characteristics of the PLR could be directly attributable to the photic responses of these photoreceptors (Alpern and Campbell 1962; Loewenfeld and Lowenstein 1993). But it now appears that a previously undiscovered photoreceptive mechanism also influences the PLR. Recently, a novel photopigment melanopsin was discovered in the inner retina of both primates and rodents (Provencio et al., 2000). It was later determined that a unique class of RGCs expresses melanopsin (Gooley et al., 2003), and that in addition to receiving classical photoreceptor input, they are intrinsically photosensitive (Hattar et al., 2002).

Intracellular recording from the ipRGCs in both rodents and macaques have provided details of the light signals conveyed to the pretectum and
suprachiasmatic nucleus (SCN) (Kawasaki 2007; Hattar et al., 2002; Dacey et al., 2005). Although ipRGCs receive traditional rod and cone inputs, their unique intrinsic photosensitivity allows them to transmit light signals unlike that of any other retinal ganglion cell type directly to the afore-mentioned brain centers via the optic nerve. When a pulse of light is presented to these cells, they show a characteristic transient burst of firing at stimulus onset that decays to a plateau of sustained firing that continues well past the stimulus offset (Berson et al., 2002; Hattar et al., 2002; Dacey et al., 2005).

By distinguishing the three photoreceptive inputs to this cell type, rod, cone and intrinsic, Dacey et al., (2005) determined the relative contribution of these inputs to the firing pattern of the ipRGCs. The melanopsin-driven responses of ipRGCs are present at higher irradiances (>11 log quanta/cm²/s) during the light stimulus and these cells continue to show a prolonged increase in firing rate even after the light offset. When the spectral sensitivity of this isolated intrinsic response is measured, it closely matches a single pigment action spectrum with peak at approximately 480nm (Dacey et al., 2005; Qiu et al., 2005).

It is widely known that when a short wavelength light is presented the pupil showed a sustained pupilloconstriction even after light offset. This paradoxical pupilloconstriction was thought to be an interplay of varying rod and cone inputs
(Newsome 1971). It was found in people with no rods or cones that the ipRGCs may contribute not only to circadian physiology but also rudimentary visual awareness thus challenging the assumption that rod and cone based photoreception mediates all visual responses to light (Zaidi et al., 2007). Preliminary data in humans suggest that under photopic conditions, cones primarily drive the transient phase of the pupil light reflex, whereas intrinsic activation of the ipRGCs contributes heavily to sustained pupilloconstriction (Kawasaki and Kardon 2007; McDougal 2008). The paradoxical pupillary constriction is thus ipRGC-induced and not the result of rod and cone interactions, henceforth this constriction will be termed the post-illumination pupil response (PIPR).

In behaving macaques, following pharmacological blockade of the “classical” photoreceptors, pupillary responses to equal irradiance light stimuli at different wavelengths was tested and significant pupillary responses were demonstrated to persist during continuous (10sec) light and following light offset (Gamlin et al., 2007). This observation was extended to a limited sample of human subjects indicating that the ipRGCs play an important role in the pupillary light reflex and are responsible for the PIPR in both humans and non-human primates (Gamlin et al., 2007).
In the present study we extended this observation by examining the magnitude of PIPR for a given retinal irradiance in a large sample of the general human population. The use of light stimuli to elicit such sustained pupil responses may be useful as a clinical test that would allow differentiation between disorders affecting photoreceptors and those affecting retinal ganglion cells (Kawasaki and Kardon 2007).

Glaucoma: Potential clinical significance of ipRGCs

“Glaucoma is a group of diseases that have in common a characteristic optic neuropathy with associated visual field loss for which elevated intraocular pressure (IOP) is one of the primary risk factors.” (American Academy of Ophthalmology). It is one of the leading causes of blindness, worldwide. Untreated glaucoma damages the optic nerve and resultant visual field loss can progress to irreversible blindness. This irreversible loss of visual field often occurs gradually over a long time and may only be symptomatic when it is already quite advanced. Although raised intraocular pressure (IOP) is a significant risk factor for developing glaucoma, there is no set threshold for IOP that causes glaucoma and IOP measurement alone is not an adequate screening test for glaucoma. One person may develop nerve damage at relatively low IOP, while another with high IOP may never develop the same (Shields 1998).
Ocular hypertension is the largest risk factor in most glaucomas. Though, in some populations only 50% of patients with primary open angle glaucoma have elevated ocular pressure (Sommer et al., 1991). Screening for glaucoma is usually performed as part of a standard eye examination performed by ophthalmologists and optometrists. Evidence suggests that visual dysfunction in eyes with early glaucoma varies significantly between individuals and no single technique is superior to the others in all patients (Sakata et al., 2007).

A multimodal functional assessment may be more effective in detecting/quantifying visual impairment associated with early glaucoma. Testing for glaucoma usually includes measurement of the intraocular pressure, detailed eye examination for changes in size or shape of the eye, anterior chamber angle examination, examination of the optic nerve to look for any visible damage, changes in the cup-to-disc ratio, and also rim appearance and vascular changes. A formal visual field test should also be performed. The retinal nerve fiber layer can also be assessed qualitatively and with quantitative imaging techniques such as optical coherence tomography, scanning laser polarimetry, and/or scanning laser ophthalmoscopy or Heidelberg Retina Tomography (Thomas et al., 2006).

The management of the disease involves limiting optic nerve damage, preserving visual field and improving the overall quality of life for patients with minimal side effects (Noecker 2006; Parikh et al., 2008). This requires
appropriate diagnostic techniques and follow-up examinations and judicious selection of treatments for the individual patient. Standard visual field testing in United States is performed most commonly using a Humphrey field analyzer (Humphrey model 750 Carl Zeiss Meditec, Dublin, CA, USA) with SITA (Swedish Interactive Threshold Algorithm) standard 24-2 program. A visual field is considered reliable when the fixation losses are less than 20% and false negatives and false positive rates are less than 25% (Ojima et al., 2007). Normal visual field is defined as a mean deviation (MD) and pattern standard deviation (PSD) within 95% confidence limits, with fewer than three non edge contiguous points identified as significant (P < 0.05) on the same side of the horizontal meridian in the pattern deviation plot, and within the normal limits of the glaucoma hemi-field test.

Reduction in the peripheral visual field is the usual initial functional finding in glaucomatous optic neuropathy (GON) followed by progressive loss of the field, which is correlated to the retinal ganglion cell (RGC) loss (Harwerth et al., 2004 and 2006). There is debate about the type of RGC that are damaged primarily by glaucoma. For example, it has been suggested that RGC with larger soma are preferentially affected in GON especially in rodents (Quigley et al., 1988 and 1999; Glovinsky et al., 1991 and 1993). In rodents, it has been suggested that the ipRGCs (large RGC) are preferentially spared (Li et al., 2006). However recent studies in rodents suggest that there is no preference in RGC soma size but a topological influence of RGC cell death in glaucoma (Jakobs et
al., 2005; Wang et al., 2008). Studies in macaques and humans have demonstrated that the ipRGCs possess large cell bodies with extensive dendritic arbors (Dacey et al., 2005). To investigate whether ipRGCs are affected by GON, we tested the PIPR (a measure of ipRGC activity) in patients with advanced GON and compared the PIPR with their visual field loss as well as to a normal population.

The research project presented in this document is a result of the renewed interest in the scientific field about the role of ipRGCs in the PLR and the PIPR. The major goals of this project were: 1) to investigate the PIPR in a broad sample of the general population. This would allow us to document that this is a robust phenomenon across the population and also develop this as a potential tool for identifying subjects with OPN4 (melanopsin gene) defects, which would be expected to affect circadian rhythms and sleep-wake patterns; and 2) to investigate the potential reduction of the PIPR in patients with GON and compare it to normal subjects. In addition we investigated whether PIPR was correlated with overall visual field loss as a psychophysical measure of the reticulo-geniculate RGC population.

The products of this research are presented in the following chapters, the layout of which is as follows. Chapter 2 is composed of the study on normal subjects that will be submitted for publication to the journal *Investigative*
*Ophthalmology and Visual Science.* This demonstrated the presence of PIPR among a sample of the general population and investigated the subject characteristics (age, race, gender, and baseline pupil diameter) that affected the magnitude of the response.

Chapter 3 consists of the final phase of the current research project, which will be submitted for publication to the journal *Investigative Ophthalmology and Visual Science,* and is presented in the same format as would be submitted to the journal. The research presented in this chapter demonstrates the loss of PIPR in a sample of patients with GON compared to normal subjects. It also demonstrates that loss of PIPR in the patients is well correlated with visual field loss (Mean Deviation loss in decibels) in this group. These findings provide valuable insights into the progression of GON, especially the RGC loss due to the disease progression.

Chapter 4 provides a summary of the findings of the research project as a whole. In this chapter I address how these findings have contributed to the advancement of the understanding of the role of ipRGC in pupillary response to light in normal subjects and in patients with GON. This section also contains a discussion of future research projects that could be further investigated.
POST-ILLUMINATION PUPIL RESPONSE: NORMAL SUBJECTS

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ABSTRACT

PURPOSE:

The intrinsic photoresponse of melanopsin-containing, intrinsically-photosensitive retinal ganglion cells (ipRGCs) underlies the sustained pupilloconstriction observed following light offset (the post-illumination pupil response (PIPR)). However, to date, this effect has only been reported in two subjects, so the present study examined the PIPR in a much broader sample of the general population.

METHODS

Subjects (N=37; 19 female and 18 male, mean age 48.6 years) were tested by presenting a 60°, 10-second light stimulus (13 log quanta/cm²/s retinal irradiance), and recording pupillary responses for 50 seconds after light cessation. The light stimuli were presented in an optical system to one eye dilated with 2.5% Phenylephrine/1% Tropicamide, while the consensual pupil response of the fellow, undilated eye was recorded by an infrared camera and computer. The pupillary responses to 470nm (optimal wavelength for melanopsin-driven responses) and 623nm (control for non-specific pupillary responses) light were compared.
RESULTS

A positive PIPR was seen in all subjects tested. Overall, the population average of the PIPR for 470nm light was 1.5 mm (Standard error of mean(SEM) 0.10, p<0.05) and the net PIPR (blue PIPR - red PIPR) was 1.4 mm (SEM 0.09, p<0.0001). The net PIPR area was 8.9 mm² (SEM 0.7, p<0.05). Net PIPR and net PIPR area were positively correlated (p<0.05) with baseline pupil diameter, but were not significantly correlated (p>0.05) with age, race or gender for the test population.

CONCLUSIONS

Using a newly-developed, wide-field optical system, we have demonstrated that all normal subjects display a post-illumination pupil response for a 10-second, 470nm light stimulus. In most normal individuals, this response is substantial, and this test therefore has the potential to be utilized as a tool in evaluating subjects with either inner retinal or melanopsin-related disorders.
INTRODUCTION

The pupillary light reflex (PLR), a reflexive constriction of the pupil in response to an increase in ocular illumination, is an indispensable clinical measure of visual, neurological and autonomic function. Until recently, it was thought that the PLR was driven by rods and cones. In 2000, a novel photopigment, melanopsin, was discovered in the inner retina of both primates and rodents that was later determined to be expressed by a unique class of RGCs. These cells in addition to receiving classical photoreceptor input, are intrinsically photosensitive and henceforth named melanopsin-containing, intrinsically photosensitive retinal ganglion cells (ipRGCs).

Intracellular recording from the ipRGCs in both rodents and macaques have shown that ipRGCs exhibit a characteristic transient burst of firing at stimulus onset that decays to a plateau of sustained firing that continues well past the stimulus offset. Dacey et al., distinguished the three photoreceptive inputs to these cells (rod, cone and intrinsic) and determined the relative contribution of these inputs to the firing pattern of the ipRGCs. The melanopsin driven responses of ipRGCs are present at higher irradiances (＞11
log quanta/cm$^2$/s) during the light stimulus and these cells continue to show prolonged firing even after the light offset. When the spectral sensitivity of this isolated intrinsic response is measured, it closely matches a single pigment action spectrum with peak at 480nm. It was found in people, with no rods or cones, that these ipRGCs may contribute not only to circadian physiology but also rudimentary visual awareness thus challenging the assumption that rod and cone based photoreception mediates all visual responses to light. Preliminary data in humans suggests that under photopic conditions, cones primarily drive the transient phase of the PLR, whereas intrinsic activation of the ipRGCs contributes heavily to sustained pupilloconstriction. In behaving macaques, following pharmacological blockade of the “classical” photoreceptors, pupillary responses to equal irradiance light stimuli at different wavelengths were demonstrated to persist during continuous (10sec) light and following light offset (henceforth termed Post-Illumination Pupil Response – PIPR). This observation was extended to two human subjects indicating that the ipRGCs play an important role in the pupillary light reflex in higher primates.

The present study was conducted to determine the characteristics of the ipRGC mediated PIPR in a broad sample of normal individuals. The use of visible light stimuli to elicit transient and sustained pupil light reflexes may be used as a clinical test that would allow differentiation between disorders affecting photoreceptors and those affecting retinal ganglion cells.
MATERIALS AND METHODS

Subject selection

Normal subjects for the study were recruited as part of ongoing studies within the Glaucoma Service of the Eye Foundation Hospital (EFH) at University of Alabama at Birmingham, Birmingham, Alabama. The study adhered to the declaration of Helsinki and approved by the Institutional Review Board, informed consent was obtained from all the subjects. Normal subjects between the ages of 19 to 80 years were enrolled with the understanding and written consent of each subject. All individuals had a best-corrected visual acuity 20/30 or better in each eye and a normal slit lamp examination, applanation tonometry, gonioscopy and dilated fundus examination. Standard visual field testing was performed using a Humphrey field analyzer (Humphrey model 750 Carl Zeiss Meditec, Dublin, CA, USA) with SITA (Swedish Interactive Threshold Algorithm) standard 24-2 program. A visual field was considered reliable when the fixation losses are less than 20% and false negatives and false positive rates are less than 25%. Normal visual field was defined as a mean deviation (MD) and pattern standard deviation (PSD) within 95% confidence limits, with fewer than three non edge contiguous points identified as significant (P < 0.05) on the same side of the horizontal meridian in the pattern deviation plot, and within the normal limits of
the glaucoma hemifield test (Carl Zeiss). Individuals with repeatable and reliable visual fields were included in the study. Fundus photographs were obtained and reviewed in a masked fashion to verify normality. 11 subjects used in the study were volunteers recruited from the clinic and laboratory who had a normal eye examination within the past year.

Testing apparatus

The optical system used for the study was an extended Maxwellian-view system consisting of LED light sources imaged in the pupil plane of the eye via two Fresnel lenses (Edmund Optics) each of a diameter 10cm, focal length 7cm, separated by 14cm. The two lenses were mounted within an enclosure; at one end of the enclosure the blue (470nm) and red (623nm) LEDs (25nm, full width at half maximum, Optek Technologies Inc.) were positioned at the focal point of the first Fresnel lens using a beam splitter (50x50x1 mm, Edmund Optics) (Fig. 1). At the other end of the enclosure, a 5° light shaping diffuser (Physical Optics Corporation) was placed in front of the second Fresnel lens. The effective field of view of this optical system was ±30°. One eye of the subject was dilated with 1% Tropicamide (Mydral, Bausch & Lomb) and 2.5% phenylephrine (Neofrin, Bausch & Lomb), and the light stimulus was presented to this eye while the consensual pupil response in the undilated eye was recorded via infrared camera (Digivue EC-PC-Cam Elyssa Corporation) and computer.
Stimulus presentation

The experiment was conducted under LabView software (National Instruments) control. Light stimuli were adjusted to present a retinal irradiance of 13 log quanta/cm^2/s assuming normal prereceptoral filtering. Each test was run as 2 epochs (either blue or red stimulus), each of 80 seconds duration and separated by up to five minutes. During each epoch, after a 20 second fixation period, the stimuli were presented to the dilated eye for a period of 10 seconds. The red stimulus primarily served as a control for non-specific influences such as fatigue on the post-illumination pupil response. A total of 3 or 4 tests were conducted, and the duration of the entire session was approximately 45 minutes.

Statistical analysis

Data from all tests were stored and analyzed off-line. Traces showing pupillary diameter were displayed, regions of the data were selected for further analysis by Microsoft EXCEL, SAS and MATLAB software. Data plots were generated using SigmaPlot or Creative DOCS.NET.

Data analyses

For the data analyses, we defined the following measures. Baseline pupil diameter was the average pupil diameter, 7 seconds before light stimulus. Sustained pupil diameter was the average pupil diameter for a period of 30
seconds, starting 10 seconds after the light offset. The variables used for subsequent analyses were:

- \( \text{PIPR (mm)} = [\text{Baseline pupil diameter (mm)} - \text{sustained pupil diameter (mm)}] \);

- \( \text{PIPR change (\%)} = \frac{(\text{PIPR} \times 100)}{\text{Baseline pupil diameter}} \);

- \( \text{Net PIPR (mm)} = [\text{Blue PIPR} - \text{Red PIPR}] \);

- \( \text{Net PIPR change (\%)} = [\text{Blue PIPR change (\%)} - \text{Red PIPR change (\%)}] \);

- \( \text{PIPR area (mm}^2) = [\text{Baseline pupil area (mm}^2) - \text{sustained pupil area (mm}^2)] \);

- \( \text{PIPR area change (\%)} = \frac{(\text{PIPR area} \times 100)}{\text{Baseline pupil area}} \);

- \( \text{Net PIPR area (mm}^2) = [\text{Blue PIPR area (mm}^2) - \text{Red PIPR area (mm}^2)] \);

- \( \text{Net PIPR area change (\%)} = [\text{Blue PIPR area change (\%)} - \text{Red PIPR area change (\%)}] \).
RESULTS

A total of 45 subjects with normal corrected vision were tested for the study. 8 interviewed subjects were excluded from the analysis due to detected ocular abnormality (5) or unusable data (3). Hence a total of 37 subjects were used for the analysis (9 males of European ancestry, 8 males of African ancestry, 9 females of European ancestry and 11 females of African ancestry). The subjects’ average age was 48.6 years ranging from 26-80 years.

Average pupil diameters of the subjects were plotted against time for both control and test stimuli (623 and 470 nm) respectively (Fig. 2A, and 2B). As expected, given the spectral sensitivity of melanopsin (Fig. 2C), the 470nm stimulus evoked a substantial post-illumination pupil response following light offset. However, despite producing substantial light-evoked pupillary responses at both 13 and 13.5 log quanta/cm²/s (Fig. 2A, and 2B), the 623nm stimulus produced no significant post-illumination pupil response. The PIPR values for both the blue and red stimuli were plotted against their respective baseline pupil diameters (Fig. 2D) (blue light $R^2 =0.354$ and red light $R^2 =0.157$ respectively). The slope of PIPR for blue light was significantly different from zero ($p<0.05$).
The linear and area values for the baseline pupil diameter, sustained pupil diameter and PIPR measures for red (control) and blue (test) lights respectively are shown in Table 1. The mean response for the blue stimulus was 1.5 mm (SEM 0.10, p<0.05), with a net PIPR of 1.4 mm (SEM 0.09, p<0.001). The net PIPR area was 8.9 mm$^2$ (SEM 0.70, p<0.05).

To investigate further the influence of baseline pupil diameter on the PIPR values, we grouped the study population into tertile groups (3.3-4.3 mm (group 1), 4.4-5.4 mm (group 2) and 5.5-6.5 mm (group 3)), and plotted them against PIPR as box plots with error bars (Fig. 3). An analysis of Covariance - autoregressive model (ANCOVA) showed that PIPR was significantly different between the test and control for all the groups (p<0.05), though the power of the test substantially increased (90%, p<0.001) when the baseline pupil diameters were greater than 4.4 mm (groups 2 and 3).

The proportional changes in pupil diameter for the red and blue PIPR are shown in Table 2. The net PIPR change showed a reduction of 27% (SEM1.44, p<0.001) and the net PIPR area change was 44.4% (SEM 2.04, p<0.001).
We calculated the difference between the test (blue) and control (red) measures (net PIPR) and used this for further analysis. Figure 4 demonstrates the net PIPR (Fig. 4A, $R^2 = 0.345, p<0.05$), net PIPR change (Fig. 4B, $R^2 = 0.066, p>0.05$), net PIPR area (Fig. 4C, $R^2 = 0.557, p<0.05$) and net PIPR area change (Fig. 4D, $R^2 = 0.044, p>0.05$) measures as a function of baseline pupil diameter. The offset values for all the above measures were significantly different from zero ($p<0.05$).

We further examined subject demographics to determine which factors affected the baseline pupil diameter and PIPR measures. To examine the effect of age, we plotted baseline pupil diameter, net PIPR values and net PIPR area against age in years (Fig. 5). The baseline pupil diameters decreased with increasing age ($R^2 = 0.166, p<0.05$), (Fig. 5A) but there was no correlation of the net PIPR ($R^2 = 0.04, p>0.05$) (Fig. 5B) or net PIPR area ($R^2 = 0.069, p>0.05$) with age.

We then examined the potential influence of gender and race on the net PIPR measures (Fig. 6). The slopes of males and individuals with European ancestry were significantly different from zero ($P>0.05$). There was no statistically significant difference between males or females ($p>0.05$) (Fig. 6A) or individuals of European or African ancestry (Fig. 6B) ($p>0.05$) in the net PIPR values in the sample population.
In five subjects, to examine the repeatability of the test, we repeated the entire experiment. We found a very high correlation ($R^2 = 0.98$) for both the baseline pupil diameters and the PIPR values measured on different days.
DISCUSSION

The goal of this study was to investigate the post-illumination pupil response in a broad sample of the general population. Previously, Gamlin and colleagues had reported a melanopsin-driven PIPR in a small sample of macaques and humans. Our results demonstrate a positive melanopsin-driven PIPR in all 37 subjects tested. Overall, we found the average PIPR for a 470nm stimulus to be 1.5mm (SEM = 0.10 mm), which is consistent with the results of the original study. More specifically, the stimulus used in the present study was 13 log quanta/cm²/sec and had an area 0.44 log units greater than that used in the original study. Assuming linear summation, this corresponds to a retinal irradiance of ~13.5 log quanta/cm²/sec in the original study; this retinal irradiance resulted in an average PIPR for a 470nm stimulus of 1.7 mm in humans and 1.5mm in monkeys.

We found that the magnitude of the net PIPR varied from 0.5mm to 2.3mm in our sample population. The small PIPR values seen in some subjects were not the result of unresponsive pupils, since these subjects showed substantial light-evoked pupilloconstriction. However, our initial analyses did show that
people with smaller baseline pupils tended to display smaller PIPR values. To remove this interaction from our subsequent analyses, we found that the data could be normalized by plotting percent PIPR change (either linear or area) as a function of the baseline pupil measure. Even after this correction, we observed a significant range in the values of percent PIPR change. In particular, a few subjects displayed minimal PIPR values. It is possible that these subjects represent a small percentage of the general population that possesses a reduced PIPR. Such reduced responses could be seen in individuals with defects in the OPN4 gene that codes for melanopsin. They could also be seen in subjects in whom the intrinsic photoresponse was reduced in magnitude due to a reduced expression of melanopsin. In either of these cases, given such reduced melanopsin-driven pupillary responses, it is likely that these subjects could also display circadian or sleep/wake abnormalities.

It is well known that baseline pupil diameters tend to get smaller with age, and this age-related miosis is seen over a wide range of illuminance levels. Furthermore, it has been suggested that the magnitude of light-evoked pupillary responses are reduced with age. Our results indicate that, after controlling for baseline pupil diameter, age per se is not a significant contributing factor to the magnitude of the PIPR. However, a recent study of light-induced melatonin suppression showed an age-related loss in sensitivity to short wavelength light. It is thought that such light-induced melatonin suppression is driven by the ipRGCs, and this result therefore suggests that there is an age-
related reduction in ipRGC signaling. Interestingly, such a decrease in ipRGC signaling might be expected to result in age-related mydriasis rather than the miosis that is actually seen. It is clear that more extensive study is needed to investigate the effect of age on pupillary responses. In addition, there have been some previous reports of racial differences, particularly as they relate to iris color, in pupillary response magnitude.\textsuperscript{24,25} However, we found no such effect of race or gender on the net PIPR.

In conclusion, using a newly-developed, wide-field optical system, we have demonstrated that all normal subjects display a post-illumination pupil response. This response is substantial in most normal individuals and this system therefore has the potential to be utilized as a clinical tool in evaluating patients either with inner retinal or melanopsin-related disorders.

ACKNOWLEDGEMENTS

The authors would like to thank all the subjects for participating; Bobbie Hill (Glaucoma Service, EFH) for invaluable help in recruitment; Jerry Millican, David Mc Dougal, Abidin Yildrim and Mark Bolding for their help in the development of the test apparatus.
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Table 1. Pupil measurements in test subjects (N=37). Baseline measure is average pupil diameter (mm), 7 seconds prior to light onset. Sustained measure is average pupil diameter (mm) for a period of 30 seconds, starting 10 seconds after light offset. The difference between the two measures gave the PIPR value. Absolute measures shown on the left (mm) and pupil area values on the right side (mm$^2$). * indicates statistical significance (p<0.05). ** indicates p<0.001. Values rounded to 1 decimal point.

Table 2. Proportional change in the pupil diameters for the red and blue PIPR. ** indicates statistical significance (p<0.001). Values rounded to 1 decimal point.
Figure 1. Schematic diagram showing the essential components of the optical system used in the study.

Figure 2. Time trace plots of the pupillary response to the control (red) and test (blue) LEDs (N=37). Bar indicates light stimulus duration. (A) Average pupil diameter (mm) plotted against time (seconds) for all subjects. The red and blue traces depict pupil diameter for red and blue lights respectively. (B) Maximum response for red light generated by increasing the retinal irradiance by half a log unit. Average pupil diameter (mm) plotted against time for 5 subjects. The red and blue traces depict pupil diameter for red and blue lights respectively. (C) Spectral sensitivity nomogram for Melanopsin showing the expected relative PIPR sensitivity for the 470 nm (blue trace) and 623 nm (red trace) stimuli reprinted with permission from Gamlin et al. 4 (D) Linear regression plots for the control (red, ●, R² = 0.157) and test (blue, ●, R² = 0.354) with 95% confidence intervals (dashed lines). Baseline pupil diameters (mm) are plotted against PIPR values (mm) for the subjects (N=37).

Figure 3. Box plot of tertile groups with error bars. The baseline pupil diameters for red light grouped into tertiles namely 3.3 – 4.3mm, 4.4-5.4mm and 5.5-6.5mm
and plotted against PIPR (mm). Control responses are shown in red and test values in blue. Boxes indicate mean and 1 standard error. Whiskers indicate 2 standard error. O indicate outliers and * indicate extreme values.

**Figure 4.** Net PIPR plotted against baseline pupil diameters, with linear regression lines (solid line) and 95% confidence intervals (dashed lines). (A) net PIPR values (mm) ($R^2 = 0.345$, $p<0.05$). (B) net PIPR change (%) ($R^2 = 0.066$, $p>0.05$). (C) net PIPR area (mm$^2$) ($R^2 = 0.557$, $p<0.05$). (D) Net PIPR area change (%) ($R^2 = 0.044$, $p>0.05$). The offset for all the parameters was significantly different from zero ($p<0.05$).

**Figure 5.** Influence of age on baseline pupil and PIPR. (A) Average baseline pupil diameter (mm) plotted against age in years ($R^2 = 0.166$, $p<0.05$). (B) Net PIPR (mm) plotted as a function of age (years) ($R^2 = 0.04$, $p>0.05$). (C) Net PIPR area (mm$^2$) plotted against age (years) ($R^2 = 0.069$, $p>0.05$). p values are for the slopes of the regression lines.

**Figure 6.** Influence of gender and race on PIPR. (A) Net PIPR (mm) plotted against baseline pupil diameters based on gender distributions. Males are indicated by open triangles ($R^2 = 0.65$, $p<0.05$) and females by filled inverted triangles ($R^2 = 0.11$, $p>0.05$). There was no statistically significant difference
between the two means (p>0.05). (B) Net PIPR measures (mm) plotted against
their respective baseline pupil diameters (mm) based on racial distributions.
Individuals of African ancestry are indicated by filled diamonds (R^2 = 0.14,
p>0.05) and individuals of European ancestry indicated by open diamonds (R^2 =
0.633, p<0.05). There was no statistically significant difference between the two
means (p>0.05). The offsets for all the regression lines were not significantly
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Table 1.

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Figure 1.
Figure 2.

(C) Adapted from “Human and macaque pupil responses driven by melanopsin-containing retinal ganglion cells” by Gamlin PD, McDougal DH, et al., 2007, Vis Res, 47, p.952. Copyright 2007 by Elsevier Ltd. Adapted with permission.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
POST-ILLUMINATION PUPIL RESPONSE IS REDUCED IN GLAUCOMA PATIENTS

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ABSTRACT

PURPOSE

The intrinsic photoresponse of melanopsin-containing, intrinsically-photosensitive retinal ganglion cells (ipRGCs) underlies the sustained pupilloconstriction observed following light offset (the post-illumination pupil response (PIPR)). We have shown that there is a significant PIPR in the normal population. Retinal ganglion cells, including ipRGCs, are affected in glaucoma. Therefore, in a group of glaucomatous patients, the present study examined whether the PIPR is affected by ipRGC loss.

METHODS

The test was conducted by presenting a 60°, 10-second light stimulus (13 log quanta/cm²/s retinal irradiance), and recording the pupillary response for 50 seconds after light offset. The light stimuli were presented in an optical system to one eye that was dilated with 2.5% Phenylephrine/1% Tropicamide, while the consensual pupil response of the fellow, undilated eye was recorded by an infrared camera and computer. The pupillary responses to 470nm (optimal
wavelength for melanopsin-driven responses) and 623nm (control for non-specific pupillary responses) light were compared.

RESULTS

Among the glaucoma patients (N=16; 12 female and 4 male, mean age 63.7 years) the PIPR value was 0.7 mm (13% change, p<0.05) in response to 470nm light. All measures of the PIPR were significantly less in the patient population than the equivalent PIPR values for age-matched control subjects. In addition, the decrease in the post-illumination pupil response correlated with the measured Mean Deviation (in decibels (dB)) visual field loss amongst the test population.

CONCLUSIONS

Using a newly-developed, wide-field optical system, we have demonstrated that there is a significant decrease in the PIPR in glaucomatous patients. As the severity of GON increases, there is a correlated decrease in the PIPR. The differences in the PIPR between the normal subjects and the glaucomatous subjects are substantial. Therefore, this test has the potential to be utilized as a clinical tool in evaluating patients either with inner retinal or melanopsin-related disorders.
INTRODUCTION

The pupillary light reflex, until recently, was thought to be primarily driven by rods and cones. In 2000, a novel class of retinal ganglion cells – the melanopsin containing, intrinsically photosensitive retinal ganglion cells (ipRGCs) were discovered that in addition to receiving classical photoreceptor input, are intrinsically photosensitive. The ipRGCs are large bodied cells and have shown also to be responsible for circadian rhythm. Studies in macaques and a limited sample of humans showed that the ipRGCs are responsible for the sustained pupilloconstriction following light offset, termed the post-illumination pupil response. Using a newly-developed, wide-field optical system, we had previously demonstrated that all normal subjects display a post-illumination pupil response following a 10-second, 470nm light stimulus.

Glaucoma is a group of diseases that have in common a characteristic optic neuropathy associated with visual field loss. There are multiple factors contributing to Glaucoma, increased intraocular pressure (IOP) being the most significant risk factor. Serial visual field measurements is one of the standard method used to follow progression of the disease in patients in the United States.
Reduction in the peripheral visual field is the usual initial functional finding in glaucomatous optic neuropathy (GON) followed by progressive loss of the field, which is correlated to the retinal ganglion cell (RGC) loss. There is debate about the type of RGC that are damaged primarily by glaucoma. For example, it has been suggested that RGC with larger soma are preferentially affected in GON. Studies in macaques and humans have demonstrated that the ipRGCs possess large cell bodies with extensive dendritic arbors. In rodents, Li et al., (2006) showed that ipRGCs are preferentially spared in glaucoma. However, recent studies in rodents showed that there is no preference in RGC soma size but a topological influence of RGC cell death in glaucoma. To investigate whether ipRGCs are affected in GON, we tested the PIPR (a measure of ipRGC activity) in patients with advanced GON and compared it with their visual field loss as well as with normal population.
MATERIALS AND METHODS

Subject selection

Patients with glaucoma for the study were recruited as part of ongoing studies within the Glaucoma Service of the Eye Foundation Hospital (EFH) at University of Alabama at Birmingham, Birmingham, Alabama, in accordance with the declaration of Helsinki and approved by the Institutional Review Board, informed consent was obtained from all the subjects. Patients were recognized for this study by comparing their visual field measurements (Humphrey field analyzer, 750 Carl Zeiss Meditec, Dublin, CA, USA) with SITA ((Swedish Interactive Threshold Algorithm) standard 24-2 program on previous visits. A visual field was considered reliable when the fixation losses are less than 20% and false negatives and false positive rates are less than 25%. Normal visual field was defined as a mean deviation (MD) and pattern standard deviation (PSD) within 95% confidence limits, with fewer than three non edge contiguous points identified as significant (P < 0.05) on the same side of the horizontal meridian in the pattern deviation plot, and within the normal limits of the glaucoma hemi field test (Carl Zeiss). Individuals with repeatable and reliable visual fields were included in the study. Fundus photographs were obtained and reviewed in a masked fashion to verify severity of glaucoma. Only those subjects were
included in the study whose visual field tests showed a difference of MD of at least 4dB between the two eyes. During their visit to the clinic, along with general examination their IOP was also measured.

Testing apparatus

A novel optical system that has previously been described was utilized for this study. The viewing eye of the subject was dilated with 1% Tropicamide (Mydral, Bausch & Lomb) and 2.5% phenylephrine (Neofrin, Bausch & Lomb), and the light stimulus was presented to this eye while the consensual pupil response in the undilated eye was recorded via infrared camera (Digivue EC-PC-Cam Elyssa Corporation) and computer.

Stimulus presentation

The experiment was conducted under LabView software (National Instruments) control. Light stimuli were adjusted to present a retinal irradiance of 13 log quanta/cm²/s assuming normal prereceptoral filtering. Each test was run as 2 epochs (either blue or red stimulus), each of 80 seconds duration and separated by up to five minutes. During each epoch, after a 20 second fixation period, the stimuli were presented to the dilated eye for a period of 10 seconds. The red stimulus primarily served as a control for non-specific influences such as fatigue
on the post-illumination pupil response. A total of 3 or 4 tests were conducted, and the duration of the entire session was approximately 45 minutes.

Statistical analysis

Data from all tests were stored and analyzed off-line. Traces showing pupillary diameter were displayed, regions of the data selected for further analysis by Microsoft EXCEL, SAS and MATLAB software. Data plots were generated using SigmaPlot or Creative DOCS.NET.

Data analyses

For the data analyses, we defined the following measures. Baseline pupil diameter was the average pupil diameter, 7 seconds before light stimulus. Sustained pupil diameter was the average pupil diameter for a period of 30 seconds, starting 10 seconds after the light offset. The variables used for subsequent analyses were:

PIPR (mm) = \[\text{Baseline pupil diameter (mm)} - \text{sustained pupil diameter (mm)}\];

PIPR change (%) = \[\text{(PIPR*100)} / \text{Baseline pupil diameter}\];

Net PIPR (mm) = \[\text{Blue PIPR} - \text{Red PIPR}\];

Net PIPR change (%) = \[\text{Blue PIPR change (%)} - \text{Red PIPR change (%)}\];
PIPR area (mm$^2$) = [Baseline pupil area (mm$^2$) – sustained pupil area (mm$^2$)];

PIPR area change (%) = [(PIPR area*100)/Baseline pupil area];

Net PIPR area (mm$^2$) = [Blue PIPR area (mm$^2$) – Red PIPR area (mm$^2$)];

Net PIPR area change (%) = [Blue PIPR area change (%) – Red PIPR area change (%)].
RESULTS

A total of 16 people with advanced glaucoma were recruited for the study (10 females of European ancestry, 2 females of African ancestry, 1 male of European ancestry and 3 males of African ancestry). The subjects' average age was 63.7 years ranging from 42-88 years.

Average pupil diameters of the patients were plotted against time for both control and test stimuli (623 and 470 nm) respectively (Fig. 1A). For comparison, pupil traces for the same stimuli in normal subjects are presented in Fig. 1B. The patients had smaller baseline pupils compared to normal subjects. They also showed a smaller sustained pupil constriction when compared to the normal subjects. To examine the influence of baseline pupil diameter on the response magnitude, PIPR values for the patient group were plotted as a function of baseline pupil diameter (Fig. 1C). The slope of PIPR for blue light was significantly different from zero (R^2 = 0.437, p<0.05) compared to the red light (R^2 = 0.034).
The linear and area values for the baseline pupil diameter, sustained pupil diameter and PIPR measures for red (control) and blue (test) lights respectively are shown in Table 1. The mean response for the blue stimulus was 0.7 mm (standard error of mean (SEM) 0.14, p<0.05)) with a net PIPR of 0.6 mm (SEM 0.16, p<0.05). The net PIPR area was 4.2 mm² (SEM 0.95, p<0.05).

For subsequent analyses, we used net PIPR values. Figure 2 shows the relationship between the net PIPR (Fig. 2A, $R^2 = 0.433$, p<0.05), net PIPR change (Fig. 2B, $R^2 = 0.239$, p=0.055), net PIPR area (Fig. 2C, $R^2 = 0.553$, P<0.05) and net PIPR area change (Fig. 2D, $R^2 = 0.236$, p=0.057) and the baseline pupil diameter. Linear regression lines generated from our previously collected normal data¹ are overlaid on the respective plots, for comparison. The offset values for all the above measures were not significantly different from zero (p>0.05). The proportional PIPR measures were not significantly correlated with the baseline pupil diameter.

The proportional change in pupil diameter and pupil area for the red and blue PIPR are shown in Table 2. The net PIPR change showed a reduction of 13% (SEM 2.0., p<0.05), while the net PIPR area change was 23% (SEM 3.25, p<0.05).
We then examined the effect of visual field loss, as measured by the MD (in dB), on linear PIPR measurements (Fig. 3). We noted that as the visual field loss became worse, baseline pupil diameter became smaller (Fig. 3A, $R^2 = 0.379$, $p<0.05$), as did net PIPR and net PIPR area measures. This can be seen in the plot of net PIPR (Fig. 3B, $R^2 = 0.395$, $p<0.05$) and net PIPR area (Fig. 3C, $R^2 = 0.338$, $p<0.05$) as a function of MD.

To control for the influence of the baseline pupil diameter on PIPR measures, we examined the relationship between MD and proportional PIPR measures (Fig. 4). As before, we found that as visual field loss increased in severity there was a reduction in the net PIPR change (Fig. 4A, $R^2 = 0.386$, $p<0.05$) and also the net PIPR area change (Fig. 4B, $R^2 = 0.375$, $p<0.05$).

We compared the results from our glaucomatous patients with age-matched controls ($N=19$, mean age 59 years) extracted from data collected in our previous study done in our laboratory. We found that there was a significant difference between the patient and normal groups for all PIPR measures (Table 3). Specifically, although the baseline pupil diameter was similar in both populations, the PIPR measures were significantly lower in the patient population ($p<0.001$).
DISCUSSION

The main goal of the present study was to investigate the PIPR in a sample of patients with GON. Prior studies have shown that ipRGCs are responsible for the PIPR in normal subjects. \cite{15} Our results demonstrate small, but significant PIPR in most patients (net PIPR 0.6mm, SEM 0.125, p<0.05). We compared these results with age-matched controls, and found that there was a significant difference between the patient and normal groups for all PIPR measures (p<0.05).

When compared to the normal subjects in previous study, \cite{1} our sample had smaller baseline pupil diameters. This smaller pupil may be reflective of the older age group of the patients (mean age 63.7 years) when compared to the normal group (mean age 48.6 years) as it is well known that pupil size decreases as age progresses. \cite{27,29,32}

The loss of PIPR in these patients was well correlated with their MD values as determined by visual field measurements. As the MD values decreased we noticed a decline in PIPR. This correlation was present for both
the net linear and area PIPR values and also the normalized percent area measures (slope for all regression lines p<0.05) thus demonstrating that MD was a determinant of PIPR independent of baseline pupil diameters.

Among the patient data we noted one patient who had a PIPR value of 2.1 mm which is comparable to normal subjects. We noted that this outlier subject had a MD loss of 4.5 dB. The robust PIPR that this patient displayed may therefore be a result of the relative sparing of ipRGCs in the central field where they are more abundant. Alternatively, this individual could have a relative sparing of ipRGCs compared to other RGCs. 21-25

The present study demonstrates clearly a loss of the PIPR (ipRGC loss) in GON patients. It is not clear from this study if the ipRGCs are lost due to GON at the same rate as the rest of the RGCs or are preferentially spared. To determine a stronger correlation between disease progression and loss of PIPR, other parameters that are used to follow glaucoma patients namely serial fundus photos, retinal nerve fiber layer thickness measurements, and ocular coherence tomography, need to be studied along with this test. For this, it would require a larger multi-control study. Intra-subject and technician variability also needs to be done. This test does have a potential value in following patients with glaucoma along with other traditional measures.
In conclusion, our study demonstrated a loss of PIPR in patients with GON that is correlated with the visual field loss. Thus, our novel, wide-field optical system has the potential to be used as a clinical screening tool in following patients with glaucoma, along with other clinical parameters.

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Table 1. Pupil measurements in glaucoma patients (N=16). Baseline measure is average pupil diameter (mm), 7 seconds prior to light onset. Sustained measure is average pupil diameter (mm) for a period of 30 seconds, starting 10 seconds after light offset. The difference between the two measures gave the PIPR value. Absolute measures shown on the left (mm) and pupil area values on the right (mm$^2$). Values rounded to 1 decimal point. * indicates values significantly different from zero (p<0.05).

Table 2. Proportional change in the pupil diameters for the red and blue PIPR. Values rounded to 1 decimal point. * indicates values significantly different from zero (p<0.05).

Table 3. Comparison of PIPR values between glaucomatous subjects and age-matched controls. The baseline pupil diameter was similar in both populations, but all PIPR measures are significantly lower in the patient population. Values rounded to 1 decimal point.
**Figure 1.** Time trace plots of the pupillary response to the control (red) and test (blue) LEDs. Bar indicates light stimulus duration. (A) Average pupil diameter (mm) plotted against time for all patients (N=16). The red and blue traces depict pupil diameter for red and blue lights respectively. (B) pupil time course for normal subjects as a comparison (N=37), (reprinted from Kankipati et al., with permission). (C) PIPR values (mm) plotted against baseline measurements (mm) for the patient population (N=16) with linear regression lines (solid line) and 95% confidence intervals (dashed lines) for both red (♦ $R^2 = 0.034$) and blue (♦ $R^2 = 0.437$) light stimuli.

**Figure 2.** Net PIPR plotted against baseline pupil diameters, with linear regression lines (solid line) and 95% confidence intervals (dashed lines). (A) Baseline diameter plotted against absolute net PIPR values ($R^2 = 0.433$, p<0.05). (B) net PIPR change (%) plotted against baseline diameter ($R^2 = 0.239$, p=0.055). (C) Baseline diameter plotted against net PIPR area (mm$^2$) ($R^2 = 0.553$, p<0.05). (D) Net PIPR area change (%) plotted against baseline diameter ($R^2 = 0.2358$, p=0.057). The offset for all the parameters was not significantly different from zero (p>0.05). The regression lines for normal subjects' data overlaid on the respective plots (green lines).
**Figure 3.** Linear regression plots of Mean deviation (MD) values in decibels (dB), as determined by visual field charts, plotted against baseline (A), net PIPR (B) and net PIPR area (C) values for the patient group (N=16). As the MD reduced (dB loss increased) so did the baseline ($R^2 = 0.379$, $p<0.05$), net PIPR ($R^2 = 0.395$, $p<0.05$) and PIPR area (mm$^2$) ($R^2 = 0.338$) ($p<0.05$). The offsets for all the plots were significantly different than zero ($p<0.05$).

**Figure 4.** Linear regression analysis plots of percentage changes in net PIPR values as a function of MD (dB). (A) Percent change in the net PIPR value plotted against MD showed a positive correlation between the two variables ($R^2 = 0.386$, $p<0.05$). (B) Percentage change in PIPR area plotted against MD values also showed a positive correlation between the two ($R^2 = 0.375$, $p<0.05$). The offsets for both plots were significantly different than zero ($p<0.05$).
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<td>PIPR</td>
<td>0.1</td>
<td>0.7</td>
<td>0.6*</td>
<td>0.7</td>
<td>4.9*</td>
<td>4.2*</td>
</tr>
<tr>
<td>SEM</td>
<td>0.05</td>
<td>0.14</td>
<td>0.12</td>
<td>0.39</td>
<td>1.11</td>
<td>0.95</td>
</tr>
</tbody>
</table>
Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Red (R) (%)</th>
<th>Blue (B) (%)</th>
<th>Net (B-R) (%) (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPR change</td>
<td>2.1</td>
<td>14.9</td>
<td>13.0* (2.0)</td>
</tr>
<tr>
<td>PIPR area change</td>
<td>4.0</td>
<td>26.9</td>
<td>23.0* (3.25)</td>
</tr>
</tbody>
</table>
Table 3.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Age-matched</th>
<th>Difference</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline pupil (mm)</td>
<td>4.5</td>
<td>4.54</td>
<td>0.04</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Net PIPR (mm)</td>
<td>0.6</td>
<td>1.3</td>
<td>0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Net PIPR change (%)</td>
<td>13.0</td>
<td>27.3</td>
<td>14.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Net PIPR area (mm²)</td>
<td>4.2</td>
<td>8.4</td>
<td>4.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Net PIPR area change (%)</td>
<td>23.0</td>
<td>44.6</td>
<td>21.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4.
SUMMARY

At the commencement of this research project, our laboratory had previously shown that the ipRGCs were responsible for PIPR in two human subjects (Gamlin et al., 2007). But it was yet to be shown that this response was present in the general population. Also, yet to be determined, was the clinical significance of the PIPR in diseases affecting the retina such as glaucomatous optic neuropathy. The overall goal of this project was to extend the observation of the PIPR to a sample of general population. In addition, we sought to test the PIPR in patients with GON as a potential screening tool for following patients with glaucoma. At the conclusion of the project, we can state that our goals have been met.

Our initial experiments included continuation of the previous work done in our laboratory, that showed that ipRGCs are primarily responsible for the post illumination pupil response in non-human primates and 2 normal human subjects (Gamlin et al., 2007). It was shown that the ipRGCs show a maximum response to a short wavelength stimulus. In this project, we extended this observation to a broad sample of the general population by presenting a short wavelength (test - 470nm) and a long wavelength (control – 623nm) light stimuli for a short duration (10 seconds) at equal retinal irradiance (13 log quanta/cm²/sec) in a
representative sample of the population (N=37). We demonstrated that the PIPR is a demonstrable reaction for the short wavelength stimulus, in our test population. We then tested for parameters that may have affected the observed response.

Our initial analyses did show that people with smaller baseline pupils tended to display smaller PIPR values. To remove this interaction from our subsequent analyses, we found that the data could be normalized by plotting percent PIPR change (either linear or area) as a function of the baseline pupil measure. Even though it was noted that few of the subjects had smaller pupils and a smaller response, it was not the result of unresponsive pupils, since these subjects showed substantial light-evoked pupilloconstriction. We also noted that age is a determinant of baseline pupil diameters but not a determinant of PIPR (after controlling for baseline pupil diameters). Furthermore we observed that other subject characteristics like race and gender did not affect the PIPR in our test population. Nevertheless, we suspect that these characteristics need to be addressed in a larger population based study. Our demonstration should lead to a better understanding of the physical function of the ipRGCs and therefore PIPR. Testing for PIPR, in a clinical setting, using this newly-developed, wide-field optical system, has the potential for evaluating patients either with retinal or melanopsin-related disorders such as circadian rhythm disorders, jet lag, glaucoma and age-related macular degeneration. This test could be done in a short duration, with minimal subject fatigue compared to the more traditional time-taking tests like visual field measurements. Also this test is an objective
measurement of pupil thus reducing false positive and false negative results that are observed with subjective tests like visual field tests. Hence, this test system has the potential of both being a screening tool in the diagnosis of these conditions and also as a tool in serial follow-up management in clinical settings.

The next part of the project was to look at PIPR in a group of patients with GON. There is debate about the type of retinal ganglion cells that are damaged by the advancing optic neuropathy in glaucoma. Quigley et al., (1988 and 1999) reported that large soma retinal ganglion cells were preferentially affected in glaucoma compared to the other RGCs. These studies were supported by Glivensky et al., (1991, 1993). In rodents, Li et al., (2006) demonstrated that ipRGCs are primarily spared. But Jakobs et al., (2005) and Wang et al., (2008) demonstrated that retinal cell death is not size dependant but depends upon the location of the cells on the retina, in rodents. They also suggested that RGCs in periphery are preferentially effected compared to those in central retinal areas. Studies in macaques and humans have demonstrated that the ipRGCs possess large cell bodies with extensive dendritic arbors (Dacey et al., 2005). We tested 16 glaucoma patients with moderate to advanced stage of disease based on visual fields, fundus photos and clinical examination, to investigate if PIPR (measure of ipRGC function) is affected in GON.

Our results indicated that there was a loss of PIPR among the patient population compared to the normal subjects. We also demonstrated that the loss of PIPR correlated well with the severity of visual field loss (as evidenced by the mean deviation loss). This suggested that there was a loss of ipRGCs consistent
with the severity of optic neuropathy, and that these cells are not preferentially spared in GON. We believe these findings give a better insight into disease progression of glaucoma. Following patients with glaucoma with this test, along with traditional tests, could give a valuable insight into the inner retinal health. Also this test could be beneficial in designing other projects that look into the disorders of melanopsin function like circadian rhythm disorders, jetlag and seasonal affective disorders.
REFERENCES


Form 4: IRB Approval Form  
Identification and Certification of Research  
Projects Involving Human Subjects  

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on January 23, 2012. The Assurance number is FWA0005960.

Principal Investigator: GIRKIN, CHRISTOPHER ANTHONY  
Co-Investigator(s): GAMLIN, PAUL D  
KANKIPATI, LAXMIKANTH  
Protocol Number: X070222014  
Protocol Title: Subcortical Luminance Pathways in Optic Neuropathy

The IRB reviewed and approved the above named project on 3/6/09. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.  
IRB Approval Date: 3-6-09  
Date IRB Approval Issued: 3-6-09  

Marilyn Doss, M.A.  
Vice Chair of the Institutional Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.
Consent Form

Consent to Participate in a Research Study
at The University of Alabama at Birmingham
Department of Ophthalmology

Title of Project: Subcortical Luminance Pathways in Optic Neuropathy
IRB Protocol Number: X070222014
Sponsor: UAB Department of Ophthalmology
Investigators: Christopher A. Girkin, MD
Laxmikanth Kankipati, M.B.B.S, M.S.
Paul Gamlin, PhD

Introduction:
Optic nerve disorders are a major cause of blindness. Usually, the disease is advanced before damage is noticed in clinical tests. Measuring the reaction of the pupil to light may be a better method to detect early damage when other tests fail to do so.

Drs. Girkin and Gamlin and Laxmikanth Kankipati at UAB, are conducting a research study designed to evaluate how impairment in visual function and the optic nerve from optic nerve disorders affect pupil responses. The purpose of this study is to determine pupil responses to luminous visual targets varying in color, intensity and location in healthy eyes and in eyes with optic neuropathy.

Explanation of Procedures:
You have been asked to take part in this study because you have an optic neuropathy (for example, glaucoma) or because your eyes are healthy.

If you agree to be in this study, the following will happen to you:
1. Drops will be placed in one of your eyes to dilate your pupil.

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03/02/09
UAB – IRB
Consent Form Approval 2-02-07
Expiration Date 3-02-10
Participant’s Initials
2. You will be asked to look at a target inside the can-shape device held in front of your eye that has the dilated pupil. This device will present a series of images varying in color, intensity, and location. A video camera will be held in front of your other eye to record pupil responses to each of the light stimuli. Neither the device nor the video camera will touch your eyes.

The time involved for all testing is one visit of approximate 30 minutes. In some cases, it may be necessary to return for another 30-minutes visit if the test needs to be repeated for valid results.

Testing results and demographic data will be saved on a password-secured database. Your coded study ID and date of birth will be provided for this database.

**Risks and Discomforts:**
Participation in this study does not involve any serious risks, but may involve some discomforts, which are similar to those encountered in any complete eye examination. Dilation of your eye may result in blurred near vision and sensitivity to bright light. You will be provided with sunglasses, if needed. If you are unusually sensitive to bright light, you may wish to arrange for alternative transportation from the clinic. These drops may cause some irritation to the eye. Fatigue or anxiety from testing would be minimal, since testing will take about 30 minutes to complete. You may ask for a break at any time or reschedule the testing if needed.

**Benefits:**
Benefits to you from these study procedures will be the assessment of your visual function and your optic nerve, which may or may not allow a better understanding of the condition of your eye. Your participation in this research may also help the investigators learn more about optic nerve
disorders and to develop better methods for diagnosis and follow-up care of these disorders.

**Alternative Treatments:**
This protocol does not provide treatment. The alternative is to choose not to participate in this protocol.

**Confidentiality:**
Information obtained about you for this study will be kept private to the extent allowed by law. However, research information that identifies you may be shared with the UAB Institutional Review Board (IRB) and others who are responsible for ensuring compliance with laws and regulations related to research, including people on behalf of the Office for Human Research Protections (OHRP). The results of the study may be published for scientific purposes. However, your identity will not be given out.

**Refusal or Withdrawal without Penalty:**
Your taking part in this study is your choice. There will be no penalty if you decide not to be in the study. If you decide not to be in the study, you will not lose any benefits you are otherwise owed. You are free to withdraw from this research study at any time. Your choice to leave the study will not affect your relationship with this institution.

**New Findings:**
Any significant new findings that develop during the course of the research study, which may affect your willingness to continue in the research, will be provided to you by Dr. Girkin or his staff.

**Cost Of Participation In Research:**
There will be no cost to you for participation in the research study.
Payment for Participation in Research:
We will pay you $10.00 for one study visit. However, if you are required to return for repeat testing, we will pay you an additional $10.00.

Payment for Research – Related Injuries:
UAB has not provided for any payment if you are harmed as a result of taking part in this study. If such harm occurs, treatment will be provided. However, this treatment will not be provided free of charge.

Questions:
If you have any questions, concerns, or complaints about the research or a research-related injury including available treatments, please contact Dr. Christopher Girkin. He will be glad to answer any of your questions. Dr. Girkin’s number is 205-325-8660.

If you have questions about your rights as a research participant, or concerns or complaints about the research, you may contact Ms. Sheila Moore. Ms. Moore is the Director of the Office of the Institutional Review Board for Human Use (OIRB). Ms. Moore may be reached at (205) 934-3789 or 1-800-822-8816. If calling the toll-free number, press the option for “all other calls” or for an operator/attendant and ask for extension 4-3789. Regular hours for the Office of the IRB are 8:00 a.m. to 5:00 p.m. CT, Monday through Friday. You may also call this number in the event the research staff cannot be reached or you wish to talk to someone else.

Legal Rights:
You are not waiving any of your legal rights by signing this consent form.
**Please initial as appropriate**

I agree that my data from this study may be used in other studies on optic neuropathy.

I do not agree that my data from this study may be used in other studies on optic neuropathy

**Signatures:**
Your signature below indicates that you agree to participate in this study. You will receive a copy of this signed consent document.

Signature of Participant: ______________________________ Date: __________

Signature of Investigator: __________________________ Date: __________

Signature of Witness: ______________________________ Date: __________

Signature of Person Obtaining Consent (if other than the investigator): ______________________________ Date: __________
University of Alabama at Birmingham

AUTHORIZATION FOR USE/DISCLOSURE OF HEALTH INFORMATION FOR RESEARCH

What is the purpose of this form? You are being asked to sign this form so that UAB may use and release your health information for research. Participation in research is voluntary. If you choose to participate in the research, you must sign this form so that your health information may be used for the research.

Participant name: ____________________________

UAB IRB Protocol Number: X070222014

Research Protocol: Subcortical Luminance Pathways in Optic Neuropathy

Sponsor: UAB Department of Ophthalmology

Principal Investigator: Christopher Girkin, M.D., Paul Gamlin, PhD, Laxmikanth Kankipati, M.B.B.S., M.S.

What health information do the researchers want to use? All medical information and personal identifiers including past, present, and future history, examinations, laboratory results, imaging studies and reports and treatments of whatever kind related to or collected for use in the research protocol.

Why do the researchers want my health information? The researchers want to use your health information as part of the research protocol listed above and described to you in the Informed Consent document.

Who will disclose, use and/or receive my health information? The physicians, nurses and staff working on the research protocol (whether at UAB or elsewhere); other operating units of UAB, HSF, The Children’s Hospital of Alabama, Callahan Eye Foundation Hospital and the Jefferson County Department of Public Health, as necessary for their operations; the IRB and its staff; the sponsor of the research.
and its employees; and outside regulatory agencies, such as the Food and Drug Administration.

**How will my health information be protected once it is given to others?** Your health information that is given to the study sponsor will remain private to the extent possible, even though the study sponsor is not required to follow the federal privacy laws. However, once your information is given to other organizations that are not required to follow federal privacy laws, we cannot assure that the information will remain protected.

**How long will this Authorization last?** Your authorization for the uses and disclosures described in this Authorization does not have an expiration date.

**Can I cancel the Authorization?** You may cancel this Authorization at any time by notifying the Director of the IRB, in writing, referencing the Research Protocol and IRB Protocol Number. If you cancel this Authorization, the study doctor and staff will not use any new health information for research. However, researchers may continue to use the health information that was provided before you cancelled your authorization.

**Can I see my health information?** You have a right to request to see your health information. However, to ensure the scientific integrity of the research, you will not be able to review the research information until after the research protocol has been completed.

Signature of participant: __________________________________________

Date: ______

or participants’ legally authorized representative:______________

Date: ______

Printed Name of participant’s representative: __________________________

Relationship to the participant: ____________________________________