Published in final edited form as:

Exp Eye Res. 2018 October ; 175: 207–211. doi:10.1016/j.exer.2018.08.007.

Retinal Tissue Oxygen Tension and Consumption during Light Flicker Stimulation in Rat

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Abstract

Light flicker stimulation has been shown to increase inner retinal oxygen metabolism and supply. The purpose of the study was to test the hypothesis that sustained light flicker stimulation of various durations alters the depth profile metrics of oxygen partial pressure in the retinal tissue (tPO₂) but not the outer retinal oxygen consumption rate (QO₂). In 17 rats, tPO₂ depth profiles were derived by phosphorescence lifetime imaging after intravitreal injection of an oxyphor. tPO₂ profile metrics, including mean inner retinal tPO₂, maximum outer retinal tPO₂ and minimum outer retinal tPO₂ were determined. QO₂ was calculated using a one-dimensional oxygen diffusion model. Data were acquired at baseline (constant light illumination) and during light flicker stimulation at 10 Hz under the same mean illumination levels, and differences between values obtained during flicker and baseline were calculated. None of the tPO₂ profile metric or QO₂ differences depended on the duration of light flicker stimulation ($R^2 = 0.03$). No significant change in any of the tPO₂ profile metrics was detected with light flicker compared with constant light ($P = 0.078$). Light flicker decreased QO₂ from 0.53 ± 0.29 to 0.38 ± 0.30 mL O₂/(min*100 gm), a reduction of 28% ($P = 0.022$). The retinal compensatory responses to the physiologic challenge of light flicker stimulation were effective in maintaining the levels of oxygen at or near baseline in the inner retina. Oxygen availability to the inner retina during light flicker may also have been enhanced by the decrease in QO₂.

Keywords

Light flicker stimulation; Retinal oxygen tension; Retinal oxygen consumption; Oxygen

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Declarations of interest: none

1. INTRODUCTION:

The processes conducted by the retina to enable vision require energy, and this energy requirement can vary depending on the conditions of retinal illumination.(Bill and Sperber, 1990; Birol et al., 2007; Lau and Linsenmeier, 2012; Linsenmeier, 1986; Palkovits et al., 2015; Teng et al., 2014; Wang and Bill, 1997; Wang et al., 2010) For example, diffuse light flicker stimulates inner retina neural activity, (Lee et al., 1989; Riva, 1998) and, accordingly, inner retinal oxygen metabolism (MO_2) increases in rats(Teng et al., 2014) and humans. (Palkovits et al., 2015) Although increased MO_2 is expected to reduce the intraretinal tissue oxygen tension (tPO_2), there are, in fact, two compensatory responses that counteract this reduction of tPO_2 . First, neurovascular coupling, which is active in both the inner retina and optic nerve,(Kornfield and Newman, 2014; Kur et al., 2012; Newman, 2013; Riva et al., 2005; Trick and Berkowitz, 2005) leads to vasodilation(Formaz et al., 1997; Kotliar et al., 2004; Mishra et al., 2011; Noonan et al., 2013; Palkovits et al., 2014; Polak et al., 2002) and increased blood velocity,(Michelson et al., 2002; Mishra et al., 2011; Scheiner et al., 1994) both of which augment retinal blood flow,(Garhofer et al., 2004; Kiryu et al., 1995; Shih et al., 2013) and inner retinal oxygen delivery (i.e., the product of retinal blood flow and arterial oxygen concentration). Second, the inner retina can change the rate at which oxygen is removed from the blood, thereby altering the arteriovenous oxygen concentration difference,(Hammer et al., 2011; Shakoor et al., 2006) and the oxygen extraction fraction (OEF).(Felder et al., 2015; Teng et al., 2014) While several groups have measured tPO_2 , (Braun et al., 1995; Felder et al., 2017b; Felder et al., 2018; Linsenmeier, 1986; Pournaras, 1995; Shahidi et al., 2010; Wanek et al., 2012; Yu and Cringle, 2005) to the best of our knowledge, the only information available on how effective these compensatory mechanisms are in terms of stabilizing the retinal tPO_2 during light flicker stimulation is from Lau and Linsenmeier(Lau and Linsenmeier, 2012) in rats. In their study, oxygen sensitive microelectrodes that had been positioned within the inner retina (9 - 18% of the retinal depth from the internal limiting membrane) were used to measure tPO_2 during light flicker stimulation. They found that light flicker lowered tPO_2 by an average of 1.33 mm Hg. This is consistent with the observation that the tissue PO_2 in the optic nerve head decreases during light flicker.(Ahmed et al., 1994; Buerk and Riva, 2002)

The energy requirements of the outer retina also can vary depending on the conditions of retinal illumination. Dark adaptation is known to increase oxygen consumption in the outer retina (QO_2).(Braun et al., 1995; Lau and Linsenmeier, 2012; Linsenmeier, 1986; Linsenmeier and Braun, 1992) The outer retina is primarily supplied by the choroidal circulation, but, unlike the retinal circulation, substantial changes in choroidal blood flow were not found during light flicker.(Garhofer et al., 2002; Shih et al., 2011; Shih et al., 2013) This implies either that QO_2 did not change, thereby requiring no vascular compensation, or that QO_2 changed, but choroidal blood flow compensation was not provoked.

Thus far, the knowledge regarding the effect of light flicker stimulation on retinal oxygenation and oxygen consumption is incomplete. First, although Lau and Linsenmeier(Lau and Linsenmeier, 2012) measured inner retinal tPO_2 at a single retinal depth location, there is no information on how light flicker affects tPO_2 at multiple retinal depths or on the effect of flicker duration on these alterations. Second, the effect of sustained

light flicker stimulation on QO_2 has not been investigated. Accordingly, we tested the following hypotheses: 1) sustained light flicker stimulation of various durations alters the tPO_2 depth profile metrics, and 2) sustained light flicker stimulation does not affect QO_2 .

2. METHODS:

2.1 Animals:

Seventeen male Long-Evans pigmented rats (450–650 g) were studied. The animals were treated in compliance with the National Institutes of Health guide for the care and use of Laboratory animals. The rats were anesthetized using ketamine (85 mg/kg IP) and xylazine (3.5 mg/kg IP). An oxygen-sensitive phosphor (Oxyphor R2; Oxygen Enterprises, Ltd., Philadelphia, PA) was dissolved in saline and 3 μL (0.5 mM) were injected intravitreally. The phosphor diffused throughout the vitreous and into the retina over 24 hours. Then the animals were re-anesthetized and an imaging procedure was undertaken to measure the lifetime of phosphorescence originating within the retinal tissue. To ensure normal systemic blood gas levels, rats were mechanically ventilated with room air (or room air and supplemental oxygen) using a small-animal ventilator (Harvard Apparatus, Inc., South Natick, MA) connected to an endotracheal tube. Normal body temperature was maintained with an animal holder connected to a closed loop water heater. Before imaging, the pupils were dilated with 2.5% phenylephrine and 1% tropicamide, 1% hydroxypropylmethylcellulose was applied to the cornea, and a glass coverslip was placed on the cornea to eliminate its refractive power and to prevent corneal dehydration.

2.2 Image Acquisition:

The instrument and methods for retinal tPO_2 imaging have been described. (Felder et al., 2017b; Felder et al., 2018; Shahidi et al., 2010; Wanek et al., 2012) Briefly, the illumination light of a slit lamp biomicroscope was fitted with a 570 nm filter to illuminate a 30 degree retinal field of view, centered on the optic nerve head. Prior to image acquisition, the rats were under constant illumination for approximately 10 minutes during image alignment. Imaging was performed under the same light illumination level before and during flicker. An excitation laser beam at 532 nm was projected into the eye to form a slit 20 μm in width and 1 mm in height on the retina, and the phosphorescence emission from the retinal tissue was imaged. Because of the angle between the incident laser beam and the imaging path, the phosphorescence signals throughout the retinal depth were displaced laterally on the image. Phosphorescence was selectively imaged by placing a high-pass (>650 nm) emission filter in the imaging path and captured using an intensified charge coupled device camera. Phosphorescence images were acquired at retinal locations within approximately three disc diameters nasal or temporal to the optic nerve head. The laser light and the sensitivity of the camera were modulated independently to acquire multiple phase-delayed images. The phosphorescence lifetime was measured from the phase-delayed images with a frequency-domain approach. Three consecutive images were acquired in 30 seconds both at baseline (i.e., constant illumination before light flicker stimulation) and during light flicker at 10 Hz (50% duty cycle). By doubling the light power during the “on” phase of flicker, the time-averaged mean light illumination (25 μW) was the same at baseline and during flicker stimulation. Thus, rat eyes were in a state of constant light adaptation throughout data

acquisition at baseline and during light flicker. In each rat, data were collected at one duration of light flicker stimulation ranging from 31 to 180 seconds. This interval was thought to be reasonable on the basis of previous experiments on related physiologic parameters, such as PO₂ in the optic nerve and the oxygen extraction fraction with flicker. (Buerk and Riva, 2002; Felder et al., 2017a)

2.3 Intraretinal tPO₂ Profile Metrics:

Phase-delayed phosphorescence images were registered to eliminate eye motion artifacts during acquisition, flattened to correct for the curvature of the globe, and smoothed using a 6×4 pixel averaging filter. Phosphorescence lifetime was calculated for every pixel from the set of phase-delayed images and converted to PO₂ values using the Stern-Volmer expression, (Shahidi et al., 2006) thereby generating a map of tPO₂ through the retinal depth. Each retinal tPO₂ map displayed tPO₂ values across the retinal depth along a vertical length of approximately 1.5 mm. Three repeated tPO₂ maps were averaged together to generate one map for each light flicker condition in each rat. From the mean tPO₂ maps, tPO₂ depth profiles were derived by vertically averaging pixel values within a central region of interest extending approximately 500 μm vertically. The profile depth location corresponding to the maximum value of tPO₂ was designated the chorioretinal interface, and 200 μm (total retinal thickness) anterior to this position was assigned as the vitreoretinal interface. (Lozano and Twa, 2012; Ruggeri et al., 2007; Ryals et al., 2017) The following three tPO₂ profile metrics were calculated: mean inner retinal tPO₂ (calculated from the inner 50% of the retinal depth), maximum outer retinal tPO₂ (calculated from the outer 50% of the retinal depth), and minimum outer retinal tPO₂ (Figure 1).

2.4 Outer Retinal Oxygen Consumption Rate:

QO₂ was calculated from tPO₂ depth profiles as described previously. (Wanek et al., 2012) Briefly, tPO₂ depth profiles were fit to a three-layer, one-dimensional steady state model of oxygen diffusion and consumption in the outer retina according to Linsenmeier and coworkers. (Braun et al., 1995; Linsenmeier and Braun, 1992) In this model, oxygen diffuses in one dimension from the choroidal circulation across the retinal depth (some oxygen may diffuse from the inner retina to the outer retina (Lau and Linsenmeier, 2012)). Anatomically, the three outer retinal layers represent the photoreceptor outer segments (layer 1), the photoreceptor inner segments (layer 2), and the rest of the outer half of the retina (layer 3). Layers 1, 2 and 3 extended from 100% to 85% (0 – 30 μm), 85% to 75% (30 – 50 μm), and 75% to 50% (50 – 100 μm) of the 200 μm total retinal thickness, respectively. QO₂ has been shown to be negligible except in layer 2, which has a high concentration of mitochondria. (Braun et al., 1995; Linsenmeier and Braun, 1992) This results in a quadratic dependence of tPO₂ on retinal depth in layer 2, while tPO₂ varies linearly with depth in layers 1 and 3. The oxygen diffusion model was fit to the outer retina portion of the tPO₂ profile using a non-linear least squares algorithm. The location of the center of layer 2 was identified as the depth position of the tPO₂ profile with the maximum curvature, which was found by fitting quadratic functions to segments of the profile as previously described. (Wanek et al., 2012) and this location was assigned to 80% of the total retinal depth. Oxygen consumption in layer 2 was calculated from the quadratic term of the oxygen diffusion model and values of the diffusion coefficient (1.97×10^{-5} cm²/sec) and oxygen solubility (2.4 ml O₂/(ml retina

mmHg)).(Linsenmeier and Braun, 1992) Last, QO_2 was determined by weighting the consumption value in layer 2 according to the thickness of layer 2 relative to the thickness of the outer retina, taken to be 100 μm .

2.5 Statistical Analysis:

Since measurements were obtained at a single duration of light flicker stimulation in each rat, we controlled for inter-animal variability by calculating differences in both tPO_2 profile metrics and QO_2 . These differences were obtained by subtracting the baseline value from the value obtained during light flicker. The relationships between tPO_2 metric or QO_2 differences and durations of light flicker were determined by linear regression. Values obtained during light flicker stimulation and those obtained at baseline were compared by the paired Student's t-test. All statistical analyses were performed with IBM SSPS Statistics, Version 24 (IBM Armonk, New York). Statistical significance was accepted at $P = 0.05$.

3. RESULTS:

3.1 Relation of Intraretinal tPO_2 Profile Metric and QO_2 Differences to Light Flicker Duration:

The linear relationships of the profile metric and QO_2 differences on duration of light flicker are displayed in Table 1. The slopes, R^2 and P values indicate that there was no significant dependence of any of the metric differences on duration of light flicker.

3.2 Effect of Light Flicker on Intraretinal tPO_2 Profile Metrics and QO_2 :

The means and SD of the three tPO_2 profile metrics and QO_2 in all rats at baseline, during flicker (all durations) and their differences are presented in Table 2. Light flicker had no statistically significant effect on any of the tPO_2 profile metrics ($P = 0.078$). In contrast to the tPO_2 profile metrics, the effect of light flicker stimulation on QO_2 was statistically significant, and reduced QO_2 from 0.53 ± 0.29 to 0.38 ± 0.30 mL O_2 /(min*100 gm), a decrease of 28% ($P = 0.022$).

4. DISCUSSION:

This study tested two hypotheses regarding the effects of light flicker stimulation on the physiology of oxygen in the retinal tissue. First, we were not able to confirm the hypothesis that sustained light flicker stimulation from 31 to 180 seconds alters the tPO_2 depth profile metrics in rats. Second, we rejected the hypothesis that light flicker stimulation had no effect on outer retinal oxygen consumption, but rather led to a decrease in it, which did not depend on the duration of flicker.

We sought to determine the time courses of changes in the tPO_2 profile metrics and QO_2 based on the assumption that light flicker would induce a varying, transient state that would lead to a new steady state. In future experiments, we would prefer to make measurements after the steady state had been established and the values had become independent of time. In the case of QO_2 , the new steady state had been approximated prior to 31 seconds after the onset of light flicker stimulation. This may provide guidance for planning investigations of

photoreceptor function using light flicker. On the other hand, with respect to the tPO_2 profile metrics, instead of identifying when a new steady state had been established, we were unable to identify any change from the baseline steady state because there was no discernible effect of light flicker on them. Retinal vessel diameter, the oxygen saturation of hemoglobin (SO_2) and OEF are related to tPO_2 . Felder and coworkers recently described the temporal dynamics of these factors upon stimulation with light flicker in humans.(Felder et al., 2017a) They found the time constants (i.e., the time required for metrics to reach 63% of their maximum change as a result of light flicker) for retinal arterial diameter, venous diameter, venous SO_2 and OEF to be 14, 15, 39 and 34 seconds, respectively. However, the study did not provide information on the temporal dynamics of tPO_2 .

No measurements of MO_2 are available in rats during flicker to date. However, Teng and coworkers estimated that MO_2 would be expected to increase by about 48% based on measurements of a flicker-induced increase in OEF(Teng et al., 2014) and a previously published flicker-induced increase in retinal blood flow.(Shih et al., 2013) In humans, Palkovits and coworkers found an increase in oxygen extraction (similar to MO_2) of 34.6% during light flicker stimulation.(Palkovits et al., 2015) This suggests that there would have been a marked increase in oxygen flux from the blood in the retinal vessels to the inner retinal cells, and without compensatory responses this would have resulted in a precipitous reduction in tPO_2 . Nonetheless, we did not find a statistically significant difference between the mean inner retinal tPO_2 values at baseline and during light flicker. On the other hand, the possibility of a Type 2 statistical error prevents us from declaring that no change occurred. The difference in mean inner retinal tPO_2 (flicker–baseline) was -1.6 ± 7.1 mm Hg (mean $\pm$ SD). To the best of our knowledge, the only other determination of retinal tPO_2 during light flicker is from Lau and Linsenmeier.(Lau and Linsenmeier, 2012) With the use of intraretinal microelectrodes in rats, they found a reduction in tPO_2 of 1.33 mm Hg, which was statistically significant. We also note that tPO_2 in the optic nerve, in which neurovascular coupling behaves in a way similar to that in the retina,(Riva et al., 2005) was shown to decrease by 1-7 mm Hg during light flicker.(Ahmed et al., 1994; Buerk and Riva, 2002) Together, these observations indicate a robust capacity of the retina to compensate for the increase of oxygen demand and maintain or nearly maintain tPO_2 . This compensation is attributed to efficient neurovascular coupling by which vessel diameter and blood flow increase(Formaz et al., 1997; Garhofer et al., 2004; Kiryu et al., 1995; Kornfield and Newman, 2014; Kotliar et al., 2004; Kur et al., 2012; Michelson et al., 2002; Mishra et al., 2011; Newman, 2013; Noonan et al., 2013; Palkovits et al., 2014; Polak et al., 2002; Riva, 1998; Riva et al., 2005; Scheiner et al., 1994; Shih et al., 2013; Trick and Berkowitz, 2005) and alterations in the arteriovenous oxygen concentration difference and OEF occur.(Felder et al., 2015; Hammer et al., 2011; Shakoor et al., 2006; Teng et al., 2014) Recently, we described a method in which dual phosphors with different spectral characteristics were used to measure tPO_2 and intravascular PO_2 simultaneously.(Felder et al., 2017b) Further modifications of this method to also measure retinal blood flow and MO_2 is expected to contribute to elucidating the relationships among these factors and their time courses in response to light flicker stimulation.

During light flicker stimulation, QO_2 became reduced. Investigators have focused more on the influence of light flicker stimulation in the inner retina than in the outer retina, and we

have found no previous measurements of QO_2 during light flicker in vivo. On the other hand, QO_2 has been shown to decrease when the illumination changes from darkness to constant light.(Braun et al., 1995; Lau and Linsenmeier, 2012; Linsenmeier, 1986; Linsenmeier and Braun, 1992) This could explain our finding if the time-averaged illumination were higher during light flicker stimulation. However, our data were collected with the same time-averaged illumination at baseline and during flicker. One potential effect of a flicker-induced reduction of QO_2 would be that more oxygen might be made available to the inner retina from the choroid. The outer retina has a high QO_2 , and to supply it some oxygen may be derived from the inner retina during constant light adaptation in rats.(Lau and Linsenmeier, 2012) However, during light flicker QO_2 is reduced so that less oxygen needs to be supplied to the outer retina from inner retina, and some oxygen derived from the choroid might become available to the inner retina where MO_2 is increased. Thus, reduced QO_2 could represent another flicker-induced compensatory mechanism to meet the simultaneous increased metabolic demand in the inner retina, possibly mediated by signaling between the inner and outer retina. Of note, anatomical and geometric differences in the retinal tissue among species can alter intraretinal PO_2 profiles.(Linsenmeier and Zhang, 2017) For example, compared to the cat, the rat has thinner photoreceptor inner segments and higher minimum PO_2 under dark-adapted conditions(Lau and Linsenmeier, 2012) and the macaque showed a smaller reduction in QO_2 with light.(Birol et al., 2007)

There were several limitations of the current study. First, as with all animal experiments, the results may have been influenced by species differences and use of anesthesia. Second, the systemic blood pressure and gases were not measured. However, perturbations in these systemic physiological parameters most likely would have led to reduced tPO_2 , which was not observed, since the tPO_2 measurements obtained in the current study were consistent with those from previous studies. Third, intraretinal phosphorescence scattering may reduce depth resolution and alter the tPO_2 depth profiles, though the general trend of tPO_2 retinal depth profiles was similar to that obtained by previous techniques. Fourth, the calculation of tPO_2 from phosphorescence lifetime used oxyphor constants that were derived from *ex vivo* experiments, which may be different from those under in vivo conditions. This may affect absolute tPO_2 values, the possibility of which cannot be excluded in this study. Fifth, calculation of QO_2 required the use of oxygen diffusion and solubility constants obtained from the literature. While this may have impacted the absolute values, the relative change induced by light flicker would not have been altered. Sixth, retinal blood flow was not measured in these experiments. Future studies are planned to comprehensively evaluate flicker-induced hemodynamic and metabolic responses and assess their individual effects on tPO_2 . Seventh, there is the possibility of light injury to the retina during imaging after intravitreal injection of the phosphor. However, this appears to be unlikely based on our previous study in which no progressive effects were seen with repeated measurements, and no abnormalities in retinal structure and function were observed by optical coherence tomography and electroretinography, respectively.(Shahidi et al., 2010) Finally, light flicker resulted in vasodilation, which in turn could have resulted in higher phosphorescence scatter within the tissue, smoother tPO_2 profiles and lower QO_2 .

5. CONCLUSIONS:

Overall, the retinal compensatory responses to the physiologic challenge of light flicker stimulation were effective in maintaining the levels of oxygen at or near baseline in the inner retina. Access of oxygen to the inner retina during light flicker may also have been enhanced by the decrease in outer retinal QO_2 .

ACKNOWLEDGEMENT:

The authors would like to acknowledge the contributions of Mrs. Tara Nguyen for animal care and preparation.

FUNDING:

This work was supported by NIH grants EY017918, and EY001792, Senior Scientific Investigator award (MS) and an unrestricted departmental grant from Research to Prevent Blindness.

ABBREVIATIONS:

MO_2	Inner retinal rate of oxygen metabolism (nL O_2 /min)
tPO_2	Intraretinal tissue oxygen tension (mm Hg)
OEF	Oxygen extraction fraction
QO_2	Outer retinal rate of oxygen consumption (mL O_2 /(min*100 gm))
SO_2	Oxygen saturation of hemoglobin (%)

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Highlights of Retinal Tissue Oxygen during Light Flicker in Rat

- Retinal tissue PO₂ was studied at baseline and during light flicker in rats.
- No changes in the three tissue PO₂ metrics studied were found with flicker.
- Light flicker reduced outer retinal O₂ consumption.
- Flicker duration (31-180 s) did not impact PO₂ metrics or outer O₂ consumption.

Tissue oxygen tension (tPO_2) profiles generated by plotting tPO_2 as a function of retinal depth in a rat under baseline conditions (blue line) and during light flicker stimulation (red line). The vitreoretinal interface is at the left (0% depth) and the chorioretinal interface is at the right (100% depth). The arrows to the right indicate the locations of the maximum outer retinal tPO_2 , and the arrows toward the center display the locations (in this case at 50% depth) of the minimum outer retinal tPO_2 . The black, horizontal line indicates the region (the inner 50% of the retinal depth) in which tPO_2 values were used to obtain the mean inner retinal tPO_2 .

Table 1

Effect of Duration of Light Flicker Stimulation on Intraretinal Tissue Oxygen Tension (tPO₂) Profile Metric and Outer Retinal Consumption Rate (QO₂) Differences in Rats (N=17)

	Slope	R ²	P
Mean Inner Retinal tPO ₂	-1.08 mm Hg *min ⁻¹	0.03	0.50
Maximum Outer Retinal tPO ₂	0.48 mm Hg *min ⁻¹	0.005	0.80
Minimum Outer Retinal tPO ₂	0.54 mm Hg *min ⁻¹	0.01	0.69
QO ₂	0.06 (mL O ₂ /(min*100 gm))*min ⁻¹	0.03	0.50

Table 2

The Effect of Light Flicker Stimulation on Intraretinal Tissue Oxygen Tension (tPO₂) Profile Metrics and Outer Retinal Oxygen Consumption (QO₂) in Rats (N=17)

	Baseline		During Flicker		Difference		P
	Mean	SD	Mean	SD	Mean	SD	
Mean Inner Retinal tPO ₂ (mm Hg)	22.2	12.8	20.6	11.4	-1.6	7.1	0.35
Maximum Outer Retinal tPO ₂ (mm Hg)	39.5	20.9	35.6	19.2	-3.9	8.5	0.078
Minimum Outer Retinal tPO ₂ (mm Hg)	22.6	13.9	21.3	14.4	-1.3	5.4	0.35
QO ₂ (mL O ₂ /(min*100 gm))	0.53	0.29	0.38	0.30	-0.15	0.25	0.022