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Pupillary light responses in type 1 and type 2 diabetics with and without retinopathy

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ABSTRACT.

Objective: **We assessed the function of rod/cones and melanopsin in type 1 (T1DM) and type 2 diabetes mellitus (T2DM) with and without non-proliferative diabetic retinopathy (NPDR).**

Methods: **We performed pupillometry on 22 healthy controls and four diabetic groups: 12 T1DM patients without NPDR and 12 with moderate NPDR, and 16 T2DM patients without NPDR and 12 with moderate NPDR. Monocular stimulations of 20 seconds with red (λ = 633 nm) and blue light (λ = 463 nm) at ~15 log quanta/cm^2/second were performed. The primary outcome was the melanopsin-mediated late redilation phase of postillumination pupillary light response ($PIPR_{Late}$) to blue light. The secondary outcomes were the mixed rod/cone and melanopsin responses, that is maximal pupil constriction and the early redilation phase of PIPR ($PIPR_{Early}$).**

Results: **Late redilation phase of PIPR ($PIPR_{Late}$) to blue and red light stimuli was not significantly different between healthy control and the four diabetic groups (n.s.). The maximal pupil contractions to blue light stimulus were significantly reduced in T1DM patients as well as in T2DM patients with NPDR ($p \leq 0.02$), whereas for red light stimuli, the maximal pupil constriction was only reduced in T2DM with NPDR ($p < 0.01$). Early redilation phase of PIPR ($PIPR_{Early}$) to blue and red light stimuli was not significantly different between healthy controls and diabetic patients (n.s.).**

Conclusion: **Neither the $PIPR_{Early}$ nor the $PIPR_{Late}$ was significantly reduced in diabetics with or without NPDR compared to healthy controls. The reduced maximal pupil constrictions in diabetics with NPDR indicate decreased mixed rod/cone and melanopsin responses.**

Key words: cone – diabetic retinopathy – ipRGCs – melanopsin – pupillary light reflex – pupillometry

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Introduction

Intrinsically photosensitive retinal ganglion cells (ipRGCs) contain the photopigment melanopsin, which is sensitive to bright blue light (Berson et al. 2002; Dacey et al. 2005). In addition, these specialized retinal ganglion cells receive signals from rods and cones (Guler et al. 2008). Both the intrinsic signals and the rod/cone signals are conveyed through the ipRGCs to the suprachiasmatic nucleus to entrain the inner biological clock to environmental light levels and to the olivary pretectal nucleus, which is the control centre for pupillary light response (PLR) (Hattar et al. 2002). Interestingly, recent research demonstrates that melanopsin also contribute to image-forming vision in humans (Zele et al. 2018).

Pupillometry can be used to preferentially stimulate rods, cones and ipRGCs, and the response of the different cell types contributing to the PLR can be quantified from the recorded PLR (Kardon et al. 2009; Herbst et al. 2011). The ipRGC-controlled PLR is characterized as slow pupillary redilation following stimulation with blue light. This slow redilation phase of the pupil after termination of blue light exposure is termed postillumination pupillary light response (PIPR) (Gamlin et al. 2007; Kardon et al. 2011; Adhikari et al. 2015). Recent studies have shown attenuated PIPR to blue light in different neuroretinal diseases including glaucoma (Feigl et al. 2011; Kankipati et al. 2011; Nissen et al. 2014), autosomal dominant retinitis pigmentosa (Kawasaki et al. 2012), non-arteritic anterior ischaemic optic neuropathy (Herbst et al. 2013; Tsika et al. 2015) and idiopathic intracranial hypertension with longer disease duration (Park et al. 2016; Ba-Ali & Lund-Andersen 2017).

Diabetic retinopathy (DR) is estimated to develop in 56% of patients

with type 1 diabetes mellitus (T1DM) and 30% of patients suffering from type 2 diabetes mellitus (T2DM) (Thomas et al. 2015). With an expected global increase in the prevalence of diabetes from 415 million in 2015 to 642 million by 2035, DR continues to be a major economic and healthcare challenge in the upcoming decades (International-Diabetes-Federation, 2015).

Diabetic retinopathy (DR) is characterized by microvascular as well as neuronal damage in the retina, and increasing evidence emerges that neuroretinal damage can occur prior to microvascular abnormality (Juen & Kieselbach 1990; Barber 2003; Klemp et al. 2005; Carpineto et al. 2016; Chen et al. 2016; El-Fayoumi et al. 2016; Gundogan et al. 2016).

Pupillometric studies on T2DM without DR have shown no significant reduction in the melanopsin-mediated PIPR (Feigl et al. 2012; Park et al. 2017, 2019; Dumpala et al. 2019). However, in patients with manifest non-proliferative DR (NPDR), a significantly reduced PIPR has been reported (Park et al. 2017, 2019). Meanwhile, to the best of our knowledge, the function of ipRGCs in patients with T1DM has not been investigated yet.

In the current study, we tested the functions of rod/cone and ipRGCs in patients with T1DM and T2DM grouped into with and without NPDR.

Methods

Design

We conducted a cross-sectional, comparative study between June 2016 and March 2017 at the Department of Ophthalmology, Rigshospitalet, Glostrup, Denmark. The study was approved by the Regional Committee on Health Research Ethics (H-15013160) and performed according to the Declaration of Helsinki. Prior to the study inclusion, the participants received detailed written and oral information about the study, and written informed consent was obtained subsequently.

Subjects

Diabetic patients were recruited from Steno Diabetes Center, Gentofte, which is a multidisciplinary outpatient diabetes clinic, offering care for around 5700 patients from the Capital Region of Denmark. The clinic has a unique diabetes-specific electronic medical record, Eye Care database (Hansen et al. 2005), which was used to recruit the patients. From a list of 1012 diabetic patients, visiting Steno Diabetes Center within the latest year, we screened 86 patients. Based on the following inclusion criteria and exclusion criteria, we excluded 32 patients. The inclusion criteria were as follows: patients suffering from either T1DM or T2DM, without or with moderate NPDR, diabetes duration ≥15 years and age between 40 and 80 years. The exclusion criteria were retinal diseases except DR, ocular or systemic disease affecting the PLR pathway, bilateral retinal laser photocoagulation, pregnancy and lack of cooperation during ophthalmological examination. Hence, patients without retinopathy or moderate NPDR were included. The healthy controls underwent the same selection criteria and were also recruited from the Capital Region of Denmark.

Clinical examination

Demographics including age, sex, body mass index (BMI), diabetes duration and tobacco use were provided by all participants through a questionnaire. We performed a detailed ophthalmological evaluation including slit-lamp examination, intraocular pressure (IOP) measurement and visual acuity determination using Early Treatment DR Study (ETDRS) chart. We measured blood pressure with an automatic monitor (Microlife AG, 9443, Widnau, Switzerland). Venous blood samples were collected to measure the glycated haemoglobin (HbA_{1c}). The patients were scanned with spectral domain optical coherence tomography (SD-OCT; Topcon Corporation, Tokyo, Japan) or Heidelberg SD-OCT (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany), and the healthy controls were scanned with Heidelberg SD-OCT. Mean retinal thickness was calculated using the standard OCT output, displaying the retinal thickness for each of the nine retinal topographic area according to ETDRS. A non-mydriatic colour fundus photography was used to capture five non-stereoscopic images of the retina (Topcon TRC-NW8 Non-Mydriatic Retinal Camera; Topcon Corporation). The retinopathy was graded by highly trained health personals applying a standard grading system used at the Steno Diabetes Center, Gentofte. The photographic protocol and grading system have previously been shown to be comparable to ETDRS seven-field stereoscopic imaging (Hansen et al. 2004). Specifically, patients without DR were characterized by no lesion in the retina, whereas those with moderate NPDR had microaneurysms, intraretinal haemorrhages or venous beading that do not reach the severity of the standard photographs 2A, 6A and 8A according to the ETDRS, and in addition, the patients with NPDR had soft exudates or hard exudates periphery to one disc diameter of the macula (Early Treatment Diabetic Retinopathy Study Research Group, 1991).

Chromatic pupillometry

Subjects were dark-adapted for 5 min followed by binocular pupillary measurement at *30 Hz* with a chromatic pupillometer (DP-2000 Human Laboratory Pupillometer; NeurOptics, Inc., Irvine, CA, USA). Pupil measurement was performed continuously for 90 seconds including 10 seconds prior to light stimulus, 20 seconds during stimulus and 60 seconds after light termination, Fig. 1. The light stimulus colour was red at 100 lux (300 CD/m^2 or 15.23 log quanta/cm^2/second) and blue at 100 lux (332 CD/m^2 or 15.27 log quanta/cm^2/second). The luminance was measured for each light colour with RP-655 spectrophotometer (Photo Research, Chatsworth, CA, USA). The peak wavelengths and bandwidths for red and blue light were λ_{max} = 623 nm (25 nm) and λ_{max} = 463 nm (17 nm), respectively. We presented the light monocularly to the study eye, and the testing was conducted through an undilated pupil. Red light was always presented prior to blue light, and the patients were always dark-adapted for 5 min prior to each light stimulus session. The test was performed between 12 and 8 PM to avoid the diurnal variation on the melanopsin-controlled PIPR (Zele et al. 2011). The light was presented through a screen at approximately 50°×35° of visual angle (van de Kraats & van Norren 2007). The patients were asked to look far inside the ocular to account for the effect of accommodation on the PLR. The

measured pupillary diameter was imported into R-statistical program, and a custom algorithm was used to remove blink artefacts. To avoid inter-individual variation in pupil size and age-related decrease in pupil size, the pupil diameter was normalized, that is pupil diameter was expressed as ratio relative to the baseline pupil diameter (BPD) during the prestimulus period (Muppidi et al. 2013).

The pupillometric metrics calculated in the current study were BPD (in mm), maximal pupillary constriction amplitude during light stimulus, and early and late redilation phases of PLR after light offset, termed $PIPR_{Early}$ and $PIPR_{Late}$, respectively, Fig. 1 (Ba-Ali et al. 2017a). The BPD was calculated as mean of pupil diameter before normalization from 0 to 10 seconds. The maximal pupil contraction was the mean difference between 1 and normalized pupil size from 3 to 6 seconds immediately following stimulus onset. The $PIPR_{Early}$ was the mean difference between 1 and pupil size from 0 to 10 seconds following light offset, and the $PIPR_{Late}$ was the mean difference between 1 and pupil size from 10 to 30 seconds after light offset. The rationale for the times used to define the $PIPR_{Early}$ and $PIPR_{Late}$ is that $PIPR_{Late}$ is solely controlled by melanopsin-expressing ipRGCs (Adhikari et al. 2015).

Study outcomes

The primary outcome was $PIPR_{Late}$ to bright blue light, which is driven by ipRGCs (Adhikari et al. 2016; Kostic et al. 2016; Yeh et al. 2017).

The secondary outcomes were $PIPR_{Early}$ and maximal pupillary constriction. Both $PIPR_{Early}$ and maximal pupillary constrictions are transient pupillary response, immediately after light ON and light OFF, respectively. Early redilation phase of PIPR ($PIPR_{Early}$) is dominated by rods and melanopsin (with minor cone contributions) (Adhikari et al. 2016; Kostic et al. 2016; Ba-Ali et al. 2017a; Yeh et al. 2017). All three photoreceptors contribute to maximal pupil constriction (Yeh et al. 2017).

Statistics

We conducted statistical analysis with R-statistical package version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). Data are reported for the study eye, which was the right eye, except when the right eye had a history of monocular laser treatment. For all outcomes, unless stated differently, mean ± SD are reported. A one-way analysis of variance (ANOVA) was used to compare the overall means of pupillometric outcomes between patients and healthy controls. If ANOVA test returned statistically significant results, post hoc analysis was performed using the Tukey's honestly significant difference to test differences between healthy controls and the patients stratified into four subgroups according to the diabetes type and retinopathy (T1DM without NPDR, T1DM with NPDR, T2DM without and T2DM with NPDR). Two outliers were excluded because they drove the statistical significance in almost all cases except on two parameters, and hence, the data will be reported with the outliers excluded. For demographic analysis, ANOVA was used for numerical dependent variables and chi-squared test was applied to categorical data. In the latter case, Fisher's exact test was used when sample sizes were small. For all tests, significance level was set to 0.05. p-values were adjusted to control for age, sex, diabetes duration and when relevant also BPD by including these parameters as covariate in the ANOVA model. The adjusted p-values, taking the multiple comparisons into account, are reported.

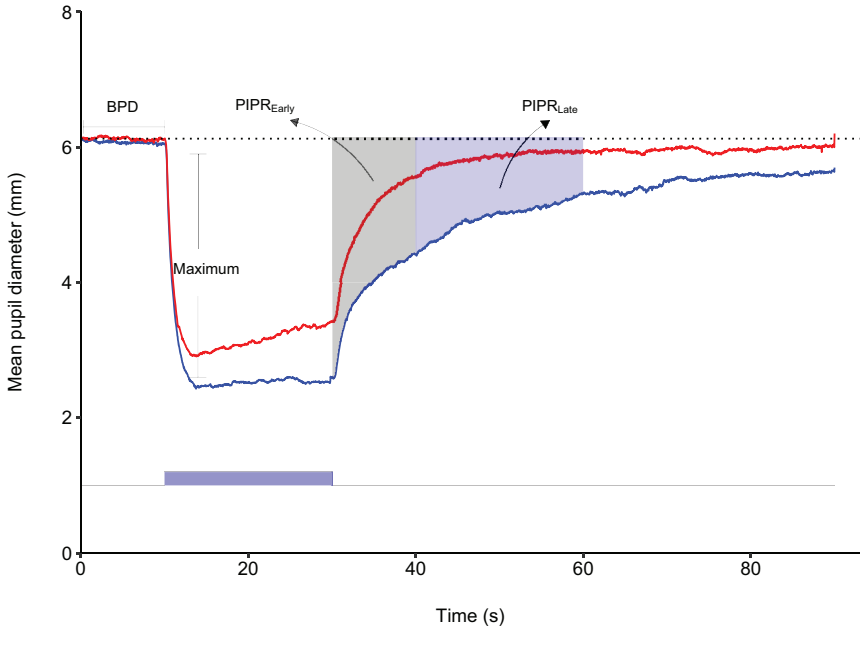

Fig. 1. An example of pupillary light reflex to blue (λ_{max} = 463 nm) and red light (λ_{max} = 623 nm) at 100 lux in a healthy subject. The pupillometric outcomes were baseline pupil diameter (BPD), maximal pupillary constriction (maximum), early redilation phase ($PIPR_{Early}$) and late redilation phase ($PIPR_{Late}$) of the postillumination pupillary response (PIPR). The blue bar under the curve indicates the light exposure. The pupil size is expressed as ratio of the actual pupil size to the BPD.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Demographics

A total of 74 participants, 52 with diabetes mellitus and 22 healthy controls, were included, Table 1. The patients were grouped according to diabetes type and whether they had moderate NPDR. Among patients with T1DM, 12 of them were without NPDR and 12 had moderate NPDR, whereas in T2DM diabetes, 16 was without NPDR and 12 had moderate NPDR. Two of T2DM patients with moderate NPDR had previous history of anti-vascular endothelial growth factor (VEGF) treatments, and one had previously received laser treatment in her fellow eye.

Diabetes duration was significantly longer in patients with T1DM regardless of the status of retinopathy compared to patients with T2DM ($p < 0.05$). However, there was no significant difference in the diabetes duration between T1DM patients with and without NPDR ($p > 0.05$). We found neither any significant difference in diabetes duration between T2DM with and without NPDR ($p > 0.05$).

Patients with T2DM without NPDR were significantly older than both healthy controls ($p = 0.02$) and patients with T1DM without NPDR ($p = 0.01$). Body mass index (BMI) was significantly higher in T2DM patients with NPDR compared to both healthy controls ($p < 0.001$) and T1DM patients with ($p = 0.002$) and without NPDR ($p = 0.006$). The HbA_{1c} (mmol/mol) was significantly higher in all four diabetic subgroups compared with healthy controls ($p < 0.00001$). And the HbA1c in T2DM patients with NPDR was significantly higher than both T1DM patients without NPDR ($p = 0.004$) and T2DM patients without NPDR ($p = 0.001$). The visual acuity was significantly lower in T2DM patients with NPDR compared to healthy controls ($p = 0.02$) as well as in T1DM patients without NPDR ($p = 0.01$). Two T1DM patients with NPDR, three T2DM patients without NPDR and three T2DM patients with NPDR had intraocular lenses (IOL) and thus a previous history of cataract surgery. None of the healthy controls or T1DM patients without NPDR had undergone cataract surgery. The proportions of men in T2DM patients without NPDR were significantly higher than in the healthy controls ($p = 0.02$). Blood pressure not significantly different between healthy controls and diabetic patients, regardless of diabetes type of retinopathy (for all comparisons; n.s.). Mean IOP was significantly higher in T2DM-patients with NPDR compared to both healthy controls ($p = 0.02$) and T1DM patients without NPDR ($p = 0.01$). Mean retinal thickness was significantly thinner both in T1DM ($p < 0.001$) and in T2DM with NPDR ($p = 0.01$), but not in T1DM and T2DM without NPDR (n.s.).

Pupillometric outcomes

The dark-adapted BPD before exposure to blue light was not significantly different between healthy controls and the diabetic groups (n.s.), Table 2. Baseline pupil diameter (BPD) decreased significantly with increasing age in all subjects including patients and healthy controls ($r = -0.58$, $p < 0.01$).

The maximal pupil constriction during blue light stimuli was significantly reduced both in T1DM with NPDR ($p = 0.02$) and T2DM with NPDR ($p < 0.01$). However, no significant difference was found between healthy control and the diabetics without NPDR (n.s.). Maximal pupil constriction was positively correlated with BPD in all subjects ($r = 0.41$, $p < 0.01$).

Early redilation phase of PIPR ($PIPR_{Early}$) after blue light exposure was not significantly different between diabetic groups and healthy controls (n.s.), Table 2. Early redilation phase of PIPR ($PIPR_{Early}$) was positively correlated with BPD in all subjects ($r = 0.28$, $p = 0.03$). Similarly, $PIPR_{Late}$ after blue light stimuli was not significantly different between healthy controls and diabetic patients (n.s.), Table 2.

There was no significant difference in BPD prior to red light between healthy controls and diabetic patients regardless of diabetes type and retinopathy ($p \geq 0.10$), Table 2. Like BPD prior to blue light stimulus, BPD before red light was also negatively correlated with age in all subjects ($r = -0.37$, $p < 0.01$). There was also a negative correlation between BPD prior to red light and diabetes duration in the diabetic group ($r = -0.29$, $p = 0.04$).

The maximal pupil constriction to red light stimuli was only reduced in T2DM patients with NPDR ($p < 0.01$), whereas no significant difference between healthy controls and the remaining diabetic groups was found (n.s.). Maximal pupil constriction was positively correlated to BPD in all subjects ($r = 0.35$, $p < 0.01$).

Neither the $PIPR_{Early}$ nor the $PIPR_{Late}$ to red light stimuli was significantly different between healthy and the diabetic groups (n.s.) There was a

Table 1. Demographics and clinical characteristics of healthy controls and patients with type 1 (T1DM) and type 2 diabetes mellitus (T2DM), with no diabetic retinopathy (DR) and moderate non-proliferative DR (NPDR).

		T1DM ($n = 25$)		T2DM ($n = 29$)	
	Controls ($n = 22$)	No DR ($n = 12$)	Moderate DR ($n = 12$)	No DR ($n = 16$)	Moderate DR ($n = 12$)
Sex (female:male)	15:7	7:5	6:6	2:14*	4:8
Age (years, mean ± SD)	59 ± 11	57 ± 7	59 ± 10	68 ± 5*	64 ± 8
BMI (kg/m^2, mean ± SD)	25 ± 4	26 ± 3	25 ± 4	28 ± 3	32 ± 7*
Blood pressure (mmHg)					
Systolic (mean ± SD)	131 ± 13	136 ± 8	137 ± 15	141 ± 17	138 ± 14
Diastolic (mean ± SD)	82 ± 7	76 ± 10	78 ± 12	78 ± 9	81 ± 3
Smoking (yes: no)	1:21	1:12	1:11	3:13	1:12
HbA_{1c} (mmol/mol)(mean ± SD)	35 ± 3	58 ± 8*	63 ± 11*	58 ± 14*	73 ± 11*
Diabetes duration (years, mean ± SD)	–	35 ± 9	33 ± 12	24 ± 6	25 ± 4
Visual acuity (ETDRS, mean ± SD)	87 ± 3	89 ± 4	83 ± 6	85 ± 4	81 ± 9*
IOP (mmHg, mean ± SD)	14 ± 3	15 ± 2	16 ± 2	13 ± 2	16 ± 3
Retinal thickness (μm)[†](mean ± SD)	314 ± 19	297 ± 18	280 ± 27*	291 ± 23	287 ± 28*

BMI = body mass index, ETDRS = Early Treatment Diabetes Retinopathy score, HbA1c = glycated haemoglobin, IOP = intraocular pressure.
* Indicates significant difference between healthy controls and the diabetic subgroup.
[†] Optical coherence tomography was performed in all healthy controls and T2DM with NPDR, but only in seven T1DM patients without NPDR, 11 T1DM patients with NPDR and 10 T2DM without NPDR.

Table 2. Comparisons between healthy controls and patients with type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), with and without diabetic retinopathy (DR).

		T1DM (n = 25)				T2DM (n = 29)			
	Controls (n = 22)	No DR (n = 12)	p	Moderate DR (n = 12)	p	No DR (n = 16)	p	Moderate DR (n = 16)	p
Blue light									
BPD (mm)	6.08 ± 0.86	5.57 ± 1.21	0.81	5.88 ± 0.85	0.68	5.44 ± 0.83	0.17	5.27 ± 1.08	0.08
Maximum	0.59 ± 0.04	0.58 ± 0.05	0.92	0.55 ± 0.04	0.02	0.57 ± 0.04	0.55	0.54 ± 0.05	0.004
$PIPR_{Early}$	0.36 ± 0.07	0.30 ± 0.06	0.11	0.31 ± 0.06	0.26	0.34 ± 0.08	0.79	0.32 ± 0.08	0.48
$PIPR_{Late}$	0.18 ± 0.09	0.11 ± 0.03	0.11	0.14 ± 0.07	0.63	0.16 ± 0.10	0.97	0.16 ± 0.09	0.96
Red light									
BPD (mm)	6.14 ± 0.89	5.61 ± 1.21	0.83	5.70 ± 0.63	0.31	5.39 ± 0.89	0.10	5.36 ± 1.02	0.11
Maximum	0.52 ± 0.04	0.51 ± 0.05	0.99	0.47 ± 0.05	0.07	0.51 ± 0.05	0.98	0.44 ± 0.082	<0.01
$PIPR_{Early}$	0.20 ± 0.05	0.18 ± 0.03	0.99	0.18 ± 0.05	0.87	0.18 ± 0.05	0.79	0.19 ± 0.06	0.68
$PIPR_{Late}$	0.05 ± 0.03	0.05 ± 0.02	0.87	0.04 ± 0.02	0.99	0.03 ± 0.04	0.45	0.08 ± 0.13	0.95

BPD = baseline pupil diameter (mm), maximum = maximal pupillary constriction, $PIPR_{Early}$ = early redilation phase of postillumination pupillary light response, early phase, $PIPR_{Late}$ = late redilation phase of postillumination pupillary light response.

positive correlation between $PIPR_{Late}$ and HbA1c (r = 0.37, p = 0.01).

Discussion

In the current study, we tested the function of rod, cone and ipRGCs by monochromatic pupillometry in 22 healthy controls and 52 diabetic patients consisting of four groups: 12 T1DM patients without NPDR and 12 with NPDR; 16 T2DM patients without NPDR and 12 with NPDR. Although the melanopsin-mediated $PIPR_{Late}$ was smaller in all four diabetic groups compared with healthy controls, it did not reach statistically significance. Nor the $PIPR_{Early}$ (rod and melanopsin) to blue and red light stimuli was significantly reduced in the diabetic groups. Maximal pupil contraction to blue light stimuli was reduced in T1DM patients as well as in T2DM patients with NPDR. To red light stimuli, we only found reduced maximal pupil contraction in T2DM patients with NPDR.

This is the first study, which investigated the function of ipRGC in patients with T1DM. In contrast, four previous pupillometric studies have investigated the functions of ipRGCs in patients with T2DM (Feigl et al. 2012; Park et al. 2017, 2019; Dumpala et al. 2019). In the following, we will discuss the results of these studies for each photoreceptor separately.

Melanopsin-mediated $PIPR_{Late}$

The slow kinetic of melanopsin-expressing ipRGCs becomes evident as the slow redilation phase of PLR following termination of bright blue light stimulus (Kostic et al. 2016; Ba-Ali et al. 2017a; Yeh et al. 2017). We have previously, by using blue and red light stimuli, shown that our pupillometry protocol can differentiate ipRGC- and rod/cone-mediated pupil responses in an x-linked retinal disease called choroideremia, which lead to degeneration of outer retina (rod and cones), while inner retinal structures including ipRGCs are preserved (Ba-Ali et al. 2017a). Additionally, the larger PIPR to blue light in comparison with PIPR by red light stimulus in the current study may owe to the melanopsin-expressing ipRGC activity (Fig. 1).

Although the mean melanopsin-mediated $PIPR_{Late}$ was smaller both in T1DM patients and in T2DM patients with or without NPDR, it did not reach statistical significance. The melanopsin-mediated PIPR in patients with T2DM has also been investigated in four previous studies (Feigl et al. 2012; Park et al. 2017, 2019; Dumpala et al. 2019). In a pupillometric case series involving seven T2DM patients without NPDR, Feigl et al. (2012) showed a trend of reduced melanopsin-mediated PIPR in T2DM patients without retinopathy. This case series was followed by a larger study by the same group (Dumpala et al. 2019) and, in agreement with our study, showed no significant reduction in the melanopsin-mediated $PIPR_{Late}$ in T2DM without DR.

Two other pupillometric studies have been conducted by Park et al. (2017, 2019), involving T2DM patients without NPDR (n = 15 and 17), mild NPDR (n = 15 and 16) and moderate–severe degree of NPDR (n = 15 and 17). Similar to our findings, Park et al. (2017) showed reduced, although non-significant, melanopsin-mediated PIPR in T2DM patients without DR compared to healthy controls. However, the reduced melanopsin-mediated PIPR in T2DM with mild and moderate/severe NPDR reached significance in Park's studies (Park et al. 2017, 2019), whereas in our study, it did not. The discrepancy between the two studies on melanopsin function in T2DM with mild and moderate/severe NPDR could be that Park et al. (2017, 2019) calculated melanopsin-mediated PIPR as the difference between normalized BPD and the median normalized PLR from 5 to 7 seconds following light offset. And even though we calculated the $PIPR_{Late}$ from 5 to 7 seconds after light offset, we did not find any statical difference between healthy subjects and T2DM patients with moderate NPDR (n.s.). Another important factor, which could affect the PIPR in Park's studies, is the effect of the treatments with anti-VEGF or focal laser. Even though they showed reduced melanopsin function after excluding patients with anti-VEGF in the 2017 study, the remaining patients in that particular group had a history of laser treatment, which is known to affect retinal photoreceptors (Ogden et al. 1976; Hoshino 1995; Kim et al. 2012; Messias et al. 2012; Najjar & Milea 2017). Moreover, three of the patients in the 2019 study had a diabetic macular oedema, which could also have impacted on their results (Park et al. 2019).

Many studies have shown the robustness of ipRGCs to anatomical changes until later stages of diabetes; however, this does not preclude changes in the physiological response of ipRGCs during early stages of disease, which can lead to measurable functional deficits before the detection of anatomical changes.

Maximal pupillary constriction and $PIPR_{Early}$

Peak pupil constriction (immediately following stimulus onset) to high intensity red and blue stimuli is mediated by rod/cone and melanopsin (Lucas et al. 2003; McDougal & Gamlin 2010; Gooley et al. 2012; Kostic et al. 2016; Ba-Ali et al. 2017a). The reduced pupillary constriction amplitude to blue and red light in diabetics with NPDR could be due to the reduced rod/cone and/or melanopsin contribution (Adhikari et al. 2016; Kostic et al. 2016; Yeh et al. 2017). Since bright red light mainly stimulates M and L cones, and the bright blue light stimulates melanopsin and S cones, the reduced maximal constriction in T1DM patients as well as in T2DM patients with NPDR may owe to S, M and L cones and/or melanopsin dysfunction. Moreover, recent studies indicate that rods make significant contributions to both the pupil constriction and redilation (even at bright light levels); thus, we cannot exclude rod dysfunction in diabetics with NPDR (Adhikari et al. 2016; Kostic et al. 2016).

The significant reduction in the maximum pupil constriction amplitude in response to a high blue stimulus in T2DM with NPDR and no effect in those without DR in the current study is in accordance with Dumpala et al.'s (2019) recent findings. However, the $PIPR_{Early}$ to blue light is not affected in the current study and thus contradicts the findings reported by Dumpala et al. (2019) with a cohort twice the size of this study. The latter could be due to different definitions of $PIPR_{Early}$ in the two studies: Dumpala et al. defines $PIPR_{Early}$ from 0 to 1.7 seconds based on spectral sensitivity measures by Adhikari et al. (2016), whereas we use a 10s cut-off, that is 0–10 seconds after light termination. The reason that we use the 10s cut-off is because the 0–1.7 seconds is narrow and thus susceptible to noise contamination. Furthermore, because $PIPR_{Early}$ could be affected by maximal pupil constriction during illumination, particularly in diabetics who could have response delays.

Park et al. (2017) also showed reduced cone-mediated PLR in patients with T2DM (moderate–severe DR), which was also in agreement with our study. However, in Park's study, no statistical difference was found between healthy controls and patients with moderate–severe NPDR after exclusion of patients with history of anti-VEGF treatment (71%) (Park et al. 2017). The latter could be due to the low statistical power caused by a small sample size (after exclusion of 2/3 of the patients). In our study, only two patients had previously received anti-VEGF treatments and our results remained unchanged after excluding these two patients.

We could not compare our results regarding T1DM patients with other pupillometric studies, because previous pupillometric studies have not investigated the function of rod/cone or melanopsin in T1DM. However, using OCT and electroretinography, other researchers have shown outer as well as inner retinal damage in T1DM (Juen & Kieselbach 1990; El-Fayoumi et al. 2016; Gundogan et al. 2016). In our study, we found reduced retinal thickness by means of OCT in T1DM patients with NPDR ($p < 0.01$), but not in T1DM patients without NPDR ($p = 0.36$). Likewise, the retinal thickness was also reduced in T2DM patients with NPDR ($p < 0.01$), but not in those without NPDR (n.s.).

The normal response of maximal pupil constriction both in T1DM and in T2DM patients without NPDR in the current study contrasts with some previous electrophysiological findings but confirms the results of other studies (Phipps et al. 2004; Luu et al. 2010; Jansson et al. 2015). It is worth mentioning that the neuroretinal damage in diabetics without retinopathy usually refers to the degeneration of the amacrines, and not to the rod and cones (Brunette & Lafond 1983; Li et al. 1992; van der Torren & Mulder 1993).

Effect of baseline pupil size and total retinal illumination

The amount of light reaching the retina depends on the BPD, and previous studies have shown that a smaller pupil results into decreased melanopsin-mediated PIPR (Nissen et al. 2011; Ba-Ali et al. 2017b). In the current study, we did not find any significant difference in BPD between healthy controls and diabetics. Neither, we found any significant difference in BPD among the diabetic subgroups. In addition, p-values for pupillometric outcomes were adjusted for BPD in our ANOVA model. Furthermore, we presented the light in Maxwellian view to account for the age-related miotic effect on retinal illumination (van de Kraats & van Norren 2007; Adhikari et al. 2015). The total retinal area illuminated in the current study was 705.76 mm^2, and given that the total retinal area is 1094 mm^2, the illumination covered 64% of the retina. (Kolb 1995) And since the density of ipRGCs is highest at 2 mm eccentricity from central fovea, we assume that our stimulus protocol evoked enough ipRGC-response.

Another factor which might affect the retinal illumination is the reduced light transmission through the lens due to cataract, especially in diabetic patients, in whom the process of cataract development is accelerated (van Best et al. 1985). We measured the transmission of blue light through lenses in 10 healthy controls and 10 diabetic patients without history of cataract surgery by using a commercially available autofluorescence-based transmittance technique (Fluorotron Master, Ocumetrics, Mountain View, CA, USA). For more details on the measurement and the calculation method, please see our previous work by Broendsted et al. (Broendsted et al. 2011). Due to small study sample, we performed an overall comparison in the transmittance between healthy controls ($n = 10$) and diabetics ($n = 10$) and found significantly reduced light transmission in the diabetic sample compared with the healthy controls ($p = 0.04$). On the other hand, the number of patients with IOL was eight in the diabetic group, whereas in the healthy control group, no one had undergone cataract surgery. Since the IOLs do not filter the blue light, we assume that the higher number of patients with IOL would compensate for the reduced light transmission due to the yellowing of the lenses in the diabetic patients without IOL.

Limitations

An important limitation is that pupillometry asses the afferent pathway of PLR, while diabetes also affects the efferent neuronal pathway of the iris. Thus, we did not evaluate the effect of autonomic nerve system on the iris dynamics in patients with diabetes, which is also known to affect both the sympathetic and the parasympathetic nerve system (Cahill et al. 2001; Pittasch et al. 2002).

We performed power calculation to determine the common sample size in each group. For one-way ANOVA, with 0.05 significant level, 80% power, using the between-group (0.01) and the within-group variance (0.04) of $PIPR_{Late}$ in the five groups of the current study, we determined the number in each of the five group to be 11. The number of T1DM patients without NPDR in the current study was 12 and slightly lower in the remaining groups. Hence, the reason that we did not find any significant difference in melanopsin-mediated $PIPR_{Late}$ between patients with T2DM without NPDR and healthy controls might not be due to low power.

Another limitation of the study was reduced light transmission due accelerated cataractous yellowing of the lens in the diabetic patients. Both the reduced maximal pupil constriction and the reduced PIPR in the diabetics with NPDR could be due to the decreased light transmission through the cataractous lens. However, since a higher number ($n_{surgery} = 8$) of diabetics with NPDR had previously undergone cataract surgery compared with healthy controls ($n_{surgery} = 0$), the higher light transmission through the IOL in the diabetic groups might compensate for the reduced transmittance in the remaining diabetic patients.

Furthermore, the stimulus retinal illumination is not matched for diabetic and control groups due to the smaller, although non-significant, pupil areas in the diabetic groups.

Conclusion

Melanopsin-mediated $PIPR_{Late}$ was not reduced, neither in T1DM patients nor T2DM patients with and without NPDR. The combined rod/cone and melanopsin-mediated maximal pupillary constriction and $PIPR_{Early}$ to both blue and red light were reduced in T1DM as well as in T2DM with NPDR, but not in diabetics without NPDR.

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Ba-Ali S., Brøndsted, A.E. Sander, B., Jennum, P. and Lund-Andersen H., None; Andersen, H. U. Owns stocks in Novo Nordisk A/S and works in advisory boards for Novo Nordisk A/S and Abbott Inc. We recruited our patients from Steno Diabetes Center, which is an outpatient Clinic and owned by Novo Nordisk A/S. However, our results do not affect the treatment of diabetes patients and our results were not affected by the company at all.