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Quantitative analysis of pupillary light reflex by real-time autofluorescent imaging in a diabetic mouse model

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ABSTRACT

Here we (i) introduce a novel laser-based quantitative method of measuring pupillary light reflex (PLR) and applied it for the *in vivo* PLR monitoring of early diabetic retinopathy (DR) in a mouse model, (ii) investigate if melanopsin-expressing retinal ganglion cells (mRGCs) are implicated in the early progression of DR and, if so, is there an impact on PLR and (iii) determine if changes in PLR precede the onset of retinal hypoperfusion. A base-line PLR measurement is captured from C57BL/6J wild type mice followed by a single intraperitoneal administration of 200 mg/kg streptozotocin (STZ) and citrate buffer (vehicle) for the "diabetic" ($n = 5$) and "control" ($n = 5$) mice respectively, the very next day. PLR measurements are repeated once a week for four weeks. The PLR data comprises retinal autofluorescence intensity (AFI) values sampled over a 5-min period under confocal excitation with 488 nm high intensity blue laser light. AFI is used here as an indirect measure of pupil size since the amount of excitation light entering and emission light leaving the eye is proportional to the pupil area. Immunohistochemistry (IHC) staining of mRGCs and RT-PCR of melanopsin mRNA are performed at the end-point. The vascular calibre of both control and STZ-treated diabetic mice is assessed via *in vivo* fluorescein angiography (FA) on day 0 (base-line), 1/2, 1 and 4 months post-STZ treatment. The PLR profile shows a more rapid pupil constriction and slower dilation in diabetic mice compared to the control. Changes in PLR coincide or even precede the onset of retinal hypoperfusion. Extensive dendritic network of the mRGCs in retinal whole-mounts and increased melanopsin mRNA from the whole eye are also observed in diabetic mice. These pathological changes to mRGCs during early DR may in turn contribute towards changes in PLR. We present here a quantitative method of measuring PLR which enables an early detection of DR with potential application in the clinical setting. In contrast to conventional measurements of PLR, we are able to calibrate the amount of light reaching the retina which is a crucial parameter in longitudinal studies.

1. Introduction

An early detection and preventive treatment of diabetic retinopathy (DR) would significantly minimize complications such as permanent vision loss due to the late-stage treatment by laser photocoagulation. But this requires a paradigm shift in our thinking where we should see beyond the late-stage glucose-induced vascular disease of the retina that DR has been associated with for decades and instead study the early pathogenesis of DR where a wide spectrum of retinal neurons are implicated (Antonetti et al., 2006).

Pupillary light reflex (PLR) is an effective non-invasive tool for characterizing the early effects of diabetes on retinal neurons since PLR is mediated by a subtype of neurons called photoreceptive neurons i.e. rods, cones and the more recently discovered melanopsin-expressing ganglion cells (mRGCs). mRGCs, which are intrinsically photosensitive, have been found to play a complementary role with rod–cone photoreceptors in mediating PLR (Hattar et al., 2002; Hattar et al., 2003; Lucas et al., 2001; Lucas et al., 2003). The mRGC pathway is reportedly less sensitive and more sluggish compared to the rods/cones (Lucas et al., 2001) and pupil constriction is incomplete at high irradiances when the melanopsin gene is ablated (Lucas et al., 2003). To date, Wang et al. reported a loss of mRGCs in a rat glaucoma model (Wang et al., 2008) whereas Gastinger et al. reported morphological changes in the mRGCs of an $Ins2^{Akita/+}$ diabetic mouse model (Gastinger et al., 2008). However, the effects on PLR due to the pathological changes of mRGCs are still unknown.

Our aim here is to investigate if mRGCs are implicated in the early progression of DR and if so, is there an impact on PLR. However, the accuracy of conventional PLR measurement is limited by a wide range of confounding factors which could interfere with the PLR data such as ambient intensity, time of day of the

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experiment, etc. But most of these factors can be standardized with the exception of one – intensity of retinal illumination by the light stimulus.

Conventional pupillometer devices have two independent light sources: one which acts as a stimulus for PLR and another infra-red source which enables the acquisition of bright and contrasting pupil images so that changes in the pupil size during PLR can be computed. This is an important consideration especially for cases with poor pupil–iris contrast such as in some mouse strains. However, for quantitative and longitudinal studies, it is important that the amount of stimulus light reaching the retina is calibrated, but conventional pupillometers do not guarantee this. Moreover, the early effects of retinopathy can be more accurately and objectively assessed if light is focused, both laterally and longitudinally, onto the site of interest i.e. retina, instead of using the conventional PLR setup where stimulus light radiates in all directions.

Here, we propose a method for measuring PLR which is accurate, quantitative and repeatable as it allows us to determine and calibrate the initial amount of light illuminating the retina. It involves the monitoring of retinal autofluorescence (AF) emitted from the retina over a 5 min period in response to a laser light excitation directed at the inner retina. The total autofluorescence intensity (AFI) emitted is a function of pupil size where a larger pupil size enables more light to reach the retina and hence more AF is collected by the photodetector via the pupil. The method employs confocal scanning laser technology where the laser light can be accurately directed laterally and longitudinally, via optical sectioning, at the inner retinal layer where the mRGCs are located. Our focus is on ascertaining the link between early DR and mRGC-mediated PLR. We therefore use an excitation laser of 488 nm blue light operated at a high intensity of 770 $\mu W/cm^2$ since the mRGC-mediated pathway is most sensitive at this setting (Grozdanic et al., 2007; Lucas et al., 2001; Lucas et al., 2003).

2. Materials and methods

All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC).

2.1. Animal husbandry

We used 10-week old male C57BL/6J mice for the present study. Animals were housed at the Biological Resource Center in a controlled environment (room temperature at 21 °C and a 12 h light/dark cycle with lights switched off from 7 pm to 7 am) with free access to food and water in Biopolis, Singapore.

2.2. Induction of diabetes

Diabetes was induced using the streptozotocin (STZ) pharmacological model. The mice were administered intraperitoneally with a single dose of streptozotocin (STZ), dissolved in sodium citrate buffer (0.1 M, pH 4.5), at 200 mg/kg body weight. The mice developed diabetes within three days after administration. In the STZ-treated group ($n = 5$), a base-line retinal AF imaging is performed prior to the STZ treatment. This is followed by STZ treatment the very next day after which retinal AF imaging was performed once a week for a total of 4 weeks. Citrate buffer was used as a vehicle for the control group ($n = 5$). Care and use of animals adhered to the institutional guidelines for humane treatment of animals.

2.3. Preparation of animals

The pupils of the mice were dilated with a drop of 0.5% Cyclogyl® sterile ophthalmic solution (cyclopentolate hydrochloride, Alcon®, Puurs, Belgium) and the mice were dark adapted for approximately 1 h prior to PLR quantification. Mice were anaesthetized by intraperitoneal (i.p.) injections with 0.15 ml/10 g body weight of Avertin (1.5% 2,2,2-tribromoethanol; T48402) purchased from Sigma–Aldrich (St. Louis, MO, USA). Custom-made PMMA hard contact lenses (Cantor & Nissel, Northamptonshire, UK) were used to avoid dehydration of the cornea and minimize spherical aberration of the mouse eye which could compromise the retinal imaging procedure. Careful eye examination ruled out the presence of any corneal or lens opacities. The pupils were dilated for 15 min before the procedure.

2.4. PLR acquisition via confocal retinal AF imaging (blue light stimulus)

A commercially available confocal scanning laser ophthalmoscope (cSLO), the Heidelberg Retina Angiograph 2, HRA 2 (Heidelberg Engineering, Dossenheim, Germany) (Leung et al., 2008; Maass et al., 2007), was employed for the retinal imaging procedure on the mice. A 55° wide angle objective lens was used so that more light could enter the small mouse pupil. The 100% argon laser power ensures maximum excitation intensity at the desired wavelength of 488 nm (and emission at $\geq$500 nm). Light was consistently focused on the ganglion cell layers since this is the focal section where the mRGCs are located. This focal section is located by first operating the cSLO in the infra-red (IR) reflectance mode (exc.: 820 nm, em.: all pass) and ensuring that the resultant IR brightness is saturated all around the optic disc with the laser power and photodetector sensitivity fixed at 50% and 65% respectively. The cSLO was subsequently switched back to the fluorescence mode before acquiring a series of time-lapse AF images at 5 s intervals for a period of 5 min. Pupillary constriction was measured indirectly based on the amount of AF emitted from the focal section by a photodetector fixed at 93% sensitivity.

PLR measurements were taken in the morning from 9 am to 11 am for all 10 mice (5 mice per group i.e. control and diabetic) over two consecutive days (5 mice per day) for five weeks. The sequence in which the animals were subjected to PLR measurement was maintained across different experimental time points.

2.5. PLR quantification

We obtained an intensity profile of AF for a period of 5 min by computing the average pixel intensity of the corresponding AF image at each 5 s interval. The area under the 'intensity vs. time' curve (AUC) was then computed and used here as a measure of PLR. The AUC for a particular mouse on any given day and eye was normalized with respect to its day 0 (base-line) AUC. The quantified AUC values for a specific time point and experimental group, i.e. control or diabetic, were expressed as $\overline{AUC} \pm SEM$, where $\overline{AUC}$ denotes the average AUC value across all mice at a specific time point and experimental group whereas SEM denotes the corresponding standard error of the average AUC. The results were statistically tested using Student's two-tailed *t*-test assuming unequal sample variance.

2.6. In vivo fluorescein angiography (FA)

The vascular health of both control and STZ-treated diabetic mice were assessed via *in vivo* FA on day 0 (base-line), 1/2, 1 and 4 months post-STZ treatment. The anaesthetized mice were injected intraperitoneally with 10% Fluorescite® (Fluorescein, Alcon Laboratories Inc., TX, USA) at a dose of 0.01 ml per 5 g body weight. Taking into account the variable rates of intraperitoneal absorption, FA images were acquired at fixed intervals of 30 s for 10 min,