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Research article

Effect of pharmacologically induced retinal degeneration on retinal autofluorescence lifetimes in mice

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ABSTRACT

Fluorescence lifetime imaging ophthalmoscopy (FLIO) was used to investigate retinal autofluorescence lifetimes in mouse models of pharmacologically induced retinal degeneration over time. Sodium iodate ($NaIO_3$, 35 mg/kg intravenously) was used to induce retinal pigment epithelium (RPE) degeneration with subsequent loss of photoreceptors (PR) whereas N-methyl-N-nitrosourea (MNU, 45 mg/kg intraperitoneally) was employed for degeneration of the photoreceptor cell layer alone. All mice were measured at day 3, 7, 14, and 28 after the respective injection of $NaIO_3$, MNU or NaCl (control). Fluorescence lifetime imaging was performed using a fluorescence lifetime imaging ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany). Fluorescence was excited at 473 nm and fluorescence lifetimes were measured in a short and a long spectral channel (498–560 nm and 560–720 nm). Corresponding optical coherence tomography (OCT) images were consecutively acquired and histology was performed at the end of the experiments. Segmentation of OCT images and histology verified the cell type-specific degeneration process over time. Retinal autofluorescence lifetimes increased from day 3 to day 28 in mice after $NaIO_3$ treatment. Finally, at day 28, fluorescence lifetimes were prolonged by 8% in the short and 61% in the long spectral channel compared to control animals ($p = 0.21$ and $p = 0.004$, respectively). In mice after MNU treatment, the mean retinal autofluorescence lifetimes were already decreased at day 3 and retinal lifetimes were finally shortened by 27% in the short and 51% in the long spectral channel at day 28 ($p = 0.0028$). In conclusion, degeneration of the RPE with subsequent photoreceptor degeneration by $NaIO_3$ lead to longer mean fluorescence lifetimes of the retina compared to control mice, whereas during specific degeneration of the photoreceptor layer induced by MNU shorter lifetimes were measured. Therefore, short retinal fluorescence lifetimes may originate from the RPE and may be modified by the overlaying retinal layers.

1. Introduction

Fluorescence lifetime imaging in ophthalmology (FLIO) recently emerged as an opportunity for detailed analysis of structural as well as metabolic changes within the retina. In principle, natural retinal fluorophores are excited using blue laser light and their electrons reach a higher energy level. This energy is then partly released in form of photons with longer wavelength and detected by retinal autofluorescence imaging. The measured autofluorescence can be quantified by its intensity as well as by the fluorescence lifetimes. Fluorescence lifetimes represent the time a molecule spends in its excited state before returning to its ground state. These lifetimes are specific for each fluorophore and can be modified by the fluorophore' s microenvironment namely the temperature, pH and oxygenation status (Schweitzer et al., 2007). Therefore, *in vivo* measurement of fluorescence lifetime within the retina might be used for investigation of metabolic changes before irreversible structural changes occur. In previous reports, fluorescence lifetimes have been characterized in mice (Dysli et al. 2014a) and the healthy human retina (Dysli et al., 2014b) as well as in specific retinal diseases like retinal artery occlusion, Stargardt disease and age related macular degeneration (Dysli et al., 2014b; Dysli et al. 2015b; Dysli et al. 2016a, Dysli et al. 2016b).

The main origin of fundus autofluorescence is the retinal pigment epithelium (RPE), containing a complex mixture of

Abbreviations: FAF, fundus autofluorescence; FLIO, fluorescence lifetime imaging ophthalmoscopy; OCT, optical coherence tomography; $NaIO_3$, sodium iodate; MNU, N-methyl-N-nitrosourea; RPE, retinal pigment epithelium.

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bisretinoid fluorophores summarized as lipofuscin (Sparrow et al., 2012); (Delori et al., 1995). Recently, the short autofluorescence lifetimes within the macular center were attributed to xanthophyll, showing a good correlation to the distribution of macular pigment within the fovea (Sauer et al., 2015). However, *in vivo* FLIO measurements from the fundus are assumed to comprise a sum of signals from different retinal layers as well as partly also from the crystalline lens. Differentiation of the contribution of individual layers to the measured mean fluorescence lifetime is not possible in the presence of intact retina. Therefore, a method to selectively address specific retinal layers is needed.

Previously, we established the FLIO technique for measurements in mouse models and characterized fluorescence lifetimes in different mouse strains (Dysli et al., 2014a). In the current study we aim to analyze the contribution of individual outer retinal layers to the measured fluorescence lifetime signal *in vivo* using two different models of pharmacologically induced retinal degeneration in C57Bl/6 mice: sodium iodate ($NaIO_3$) and N-methyl-N-nitrosourea (MNU). Thereby, time dependent changes of retina autofluorescence lifetimes are investigated.

$NaIO_3$ induces rapid degeneration of the RPE and is used as a model for RPE atrophy as seen in age-related macular degeneration (AMD) (Franco et al., 2009; Enzmann et al., 2006). There are different interaction modes described in literature for the toxic effect of $NaIO_3$ on the RPE: basal plasma membrane destruction (Sen et al., 1992), cross-reaction with melanin, leading to cytotoxic metabolites (Baich and Ziegler, 1992), inhibition of crucial enzymes (Korte et al., 1991), and altered adhesion between the neurosensory retina and the RPE layer (Yoon and Marmor, 1993). Subsequently, time- and concentration-dependent, the RPE atrophy and dysfunction leads to degeneration of the overlaying photoreceptor (PR) layer resulting in a decrease of the outer nuclear layer (ONL) (Yang et al., 2014). Additionally, direct toxic effects on the PR cells are also described recently (Wang et al., 2014).

In a second model, MNU was used, which specifically damages PRs in a concentration dependent manner (Tsubura et al., 2010). MNU acts through DNA adduct formation (Yoshizawa et al., 1999) and/or endoplasmatic reticulum stress (Reisenhofer et al., 2015). This leads to apoptosis within the PR resulting in a decrease of the thickness of the ONL. Therefore, MNU can serve as a model displaying general features of retinitis pigmentosa.

2. Material and methods

2.1. Mice

Wild type C57BL/6 mice at the age of 6–8 weeks were obtained from the breeding facility of the Department of Clinical Research, University of Bern, Bern, Switzerland. During experimentation, all mice were housed under a standard 12-h:12-h light–dark cycle with food and water available ad libitum. All procedures were performed after governmental approval according to the Swiss Federal Regulations on Animal Welfare and were in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All experiments are registered under the animal experimentation number BE127/12.

2.2. Study protocol

26 mice were investigated with six animals in the control group and ten animals in each degeneration group. Data was acquired in two independent experimental series. Both eyes of all animals were investigated at day 3, 7, 14 and 28 after injection by the same experienced investigators. Every examination included sequential acquisition of fluorescence lifetime images (FLIO) and optical coherence tomography (OCT) scans.

2.3. Pharmacological induction of retinal degeneration

Retinal degeneration was induced according to previously published protocols (Franco et al., 2009). Sodium iodate ($NaIO_3$; Sigma-Aldrich, Switzerland) was dissolved in sterile 0.9% NaCl (B. Braun Medical AG, Sempach, Switzerland) and injected intravenously at a concentration of 35 mg/kg bodyweight (BW). N-methyl-N-nitrosourea (MNU; Sigma-Aldrich, Buchs, Switzerland) was dissolved in sterile 0.9% NaCl containing 0.05% acetic acid (Fresenius Kabi, Oberdorf, Switzerland) and injected intraperitoneally at a concentration of 45 mg/kg BW. In the control group, animals were injected with 100 μl NaCl intravenously or intraperitoneally, respectively.

2.4. Animal preparation for imaging procedures

For image acquisition, all mice were anaesthetized by subcutaneous (s.c.) injection of 0.75 mg/kg medetomidine (Domitor, 1 mg/mL; Orion Pharma, Provet, Zug, Switzerland) and 45 mg/kg ketamine (Ketalar 50 mg/mL; Parke-Davis, Pfizer, Zürich, Switzerland). Not before 30 min after injection, anesthesia was reversed with 0.75 mg/kg atipamezole s.c. (Antisedan 5 mg/mL; Pfizer). Additionally, 400 μL of 0.9% NaCl was injected s.c. to prevent dehydration. Pupils were maximally dilated using tropicamide 0.5% and phenylephrine HCl 2.5% eye drops (ISPI, Bern, Switzerland). To prevent corneal desiccation during image acquisition, methylcellulose (Methocel 2%; OmniVision, Neuhausen, Switzerland) diluted 1:1 with balanced salt solution (Alcon, Schaffhausen, Switzerland) was applied onto the cornea.

2.5. Fluorescence lifetime imaging ophthalmoscope

Fluorescence lifetimes were measured using a fluorescence lifetime imaging ophthalmoscope, based on a Heidelberg Engineering Spectralis system (Heidelberg Engineering, Heidelberg, Germany). The technique of fluorescence lifetime imaging ophthalmoscopy (FLIO) has been previously described in detail (Dysli et al., 2014a,b). The following section will provide a short summary of the technical background.

A 25-diopter lens ($f = 40/+25$ diopters; Heidelberg Engineering) was added in front of the FLIO device in order to adapt to the short axial length of the murine eye (3.37 mm, (Remtulla and Hallett, 1985)). A confocal system with a 473 nm pulsed laser was used for exciting the endogenous retinal autofluorescence of the central retina. The emitted fluorescence photons were detected by two highly sensitive hybrid photon-counting detectors (HPM-100-40, Becker & Hickl, Berlin, Germany) in a short (498–560 nm) and in a long (560–720 nm) spectral channel and registered by time-correlated single-photon counting (TCSPC) modules (SPC-150; Becker & Hickl). Data was individually recorded for every single pixel within a 256 × 256 pixel grid. An infrared reflectance image was used for tracking of eye movements. This ensured that acquired fluorescence intensity and lifetime data were recorded at the correct spatial location and allowed for data accumulation over the scan duration of about 2–3 min that was required for the FLIO measurement (minimum of 700–1000 photons per pixel). Thereby, an effect of photo pigment bleaching can be excluded because it occurs within the first 20 s and FLIO results are averaged over a much longer acquisition period (Delori et al., 2011). The recorded lifetime data was bi-exponentially fitted by Becker & Hickl software (SPCImage 4.6) using a binning factor of one. An incomplete multiexponential decay model was applied and tail fit was started at the peak of fluorescence. In both, the short and the long spectral