Molecular Pharmacology Fast Forward. Published on July 17, 2018 as DOI: 10.1124/mol.118.112581
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Mol. Pharma # 112581

TITLE PAGE

Protective effect of a locked retinal chromophore analogue against light-induced retinal degeneration

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Mol. Pharma # 112581

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RUNNING TITLE: Therapeutic effects of 11-*cis*-6-membered ring-retinal

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MANUSCRIPT STATISTICS

Text Pages: 35

Tables: 1

Figures: 8

References: 56

Abstract Length: 232 words

Introduction Length: 585 words

Discussion Length: 1174 words

Molecular Pharmacology Fast Forward. Published on July 17, 2018 as DOI: 10.1124/mol.118.112581
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ABBREVIATIONS: ABCA4, ATP-binding cassette subfamily A, member 4; ad, autosomal dominant; AF, autofluorescence; BTP, *bis*-tris propane; bw(s), body weight(s); 11-*cis*-6mr-retinal, 11-*cis*-6-membered ring-retinal; DAPI, 4'6'-diamidino-2-phenyl-indole; DHE, dihydroethidium; DDM, n-dodecyl-β-D-maltoside; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERG, electroretinography; GPCR, G protein–coupled receptor; H&E, hematoxylin and eosin; HPLC, high-performance liquid chromatography; INL, inner nuclear layer; i.p., intraperitoneal; IP_3, inositol-3-phosphate; LRAT, lecithin retinol acyltransferase; LC-MS, liquid chromatography-mass spectrometry; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; ONL, outer nuclear layer; PBS, phosphate-buffered saline; PLC, phospholipase C; PNA, peanut agglutinin; RDH8, retinol dehydrogenase 8; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SD-OCT, spectral domain-optical coherence tomography; SLO, scanning laser ophthalmoscopy; TPM, two-photon microscopy; WT, wild type.

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ABSTRACT

Continuous regeneration of the 11-*cis*-retinal visual chromophore from all-*trans*-retinal is critical for vision. Insufficiency of 11-*cis*-retinal arising from the dysfunction of key proteins involved in its regeneration can impair retinal health, ultimately leading to loss of human sight. Delayed recovery of visual sensitivity and night blindness caused by inadequate regeneration of the visual pigment rhodopsin, are typical early signs of this condition. Excessive concentrations of unliganded, constitutively active opsin and increased levels of all-*trans*-retinal, and its byproducts in photoreceptors also accelerate retinal degeneration following light exposure. Exogenous 9-*cis*-retinal iso-chromophore can reduce the toxicity of ligand-free opsin but fails to prevent the buildup of retinoid photoproducts when their clearance is defective in human retinopathies such as Stargardt disease or age-related macular degeneration. Here we evaluated the effect of a locked chromophore analogue, 11-*cis*-6-membered ring-retinal against bright light-induced retinal degeneration in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice. Using *in vivo* imaging techniques, optical coherence tomography, scanning laser ophthalmoscopy, and two-photon microscopy, along with *in vitro* histological analysis of retinal morphology, we found that treatment with 11-*cis*-6-membered ring-retinal prior to light stimulation prevented rod and cone photoreceptor degradation and preserved functional acuity in these mice. Moreover, additive accumulation of 11-*cis*-6-membered ring-retinal measured in the eyes of these mice by quantitative liquid chromatography-mass spectrometry indicated stable binding of this retinoid to opsin. Together, these results suggest that eliminating excess of unliganded opsin can prevent light-induced retinal degeneration in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice.

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INTRODUCTION

Visual perception starts when a photon stimulates an isomerization of the 11-*cis*-retinal chromophore of rhodopsin in the retina to its all-*trans* configuration. This change leads to conformational rearrangements of the protein's helical bundles, allowing binding of the heterotrimeric G protein transducin and the initiation of signal transduction (Jastrzebska B, 2013, Park PS et al., 2008). Eventually, the transiently formed complex between rhodopsin and G protein dissociates and all-*trans*-retinal leaves the binding pocket resulting in formation of free opsin and free all-*trans*-retinal. To sustain vertebrate vision and preserve retinal health, continuous regeneration of visual pigment is essential (Palczewski K, 2006, Rando RR, 1996). The supply of 11-*cis*-retinal results either from the dietary ingredients like β-carotene or through the regeneration of released all-*trans*-retinal back to its 11-*cis* form (Kiser PD et al., 2012, Kiser PD et al., 2014, Maeda T et al., 2011, von Lintig J, 2012). The latter is achieved by the so-called visual (retinoid) cycle. The proper function of proteins involved in retinoid renewal is critical for continuous regeneration of 11-*cis*-retinal needed to form rhodopsin and other visual pigments. Excessive light-induced release of all-*trans*-retinal from the visual pigment can be detrimental to photoreceptors (Chen Y et al., 2012, Maeda A et al., 2008).

Thus, clearance of all-*trans*-retinal and its toxic byproducts generated by photoreceptor cells is vital for retinal health (Travis GH et al., 2007). Functional impairment of the retinoid cycle resulting in insufficient regeneration of 11-*cis*-retinal and excess of unliganded, constitutively active opsin often results in progressive retinopathies and eventual loss of vision (Fan J et al., 2005, Travis GH et al., 2007, Woodruff ML et al., 2003). Limitations of current medical interventions for retinal degenerative diseases emphasize the need to establish therapies that are more effective. Pharmacological supplementation with the iso-chromophore 9-*cis*-retinal that eliminates the constitutive activity of ligand-free opsin can dramatically improve visual function in mouse models of Leber congenital amaurosis (LCA) and Stargardt disease (Batten ML et al.,

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2004, Maeda T et al., 2009, Maeda T et al., 2009, Palczewski K, 2010). Despite this positive finding, lengthy treatment with 9-*cis*-retinal can enhance toxicity if pigment regeneration is delayed or clearance of photoproducts is defective (Fan J et al., 2005, Woodruff ML et al., 2003). Thus, treatment with an 11-*cis*-6-membered ring-retinal (11-*cis*-6mr-retinal) chromophore analogue could constitute a viable complementary and/or alternative approach. This retinal contains a ring between C^{10}-C^{13} instead of the double bond between C^{11}=C^{12}, which prevents its isomerization from the 11-*cis* to all-*trans* configuration and locks this retinal analogue in the chromophore binding pocket (Bhattacharya S et al., 1992). Thus, therapy with 11-*cis*-6mr-retinal could bypass the excessive accumulation of all-*trans*-retinal in the retina released from the activated receptors under bright light conditions without abridging phototransduction because rod opsin bound to 11-*cis*-6mr-retinal exhibits residual sensitivity to light *in vivo* and *in vitro* (Fan G et al., 2002, Gulati S et al., 2017, Kuksa V et al., 2002). Moreover, this retinal analogue does not bind to cone opsin efficiently, eliminating the risk of fast deactivation of cone pigment signaling (Alexander NS et al., 2017).

In this work, we investigated the effect of 11-*cis*-6mr-retinal on acute light-induced retinal degeneration in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice, a model that resembles many features of human Stargardt disease (Chen Y et al., 2012, Fujinami K et al., 2015, Maeda A et al., 2009, Maeda A et al., 2008), in which exposure to bright light causes prominent photoreceptor cell death due to buildup of all-*trans*-retinal released from photobleached rhodopsin with subsequent activation of oxidative stress (Chen Y et al., 2012).

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MATERIALS AND METHODS

Chemicals and reagents

Dodecyl-β-D-maltopyranoside (DDM) (Affymetrix Inc., Maumee, OH) was used to solubilize opsin from mouse retinas. 4'6'-Diamidino-2-phenyl-indole (DAPI) and Alexa594-conjugated goat anti-mouse secondary antibody were purchased from Life Technologies (Grand Island, NY) for nuclear staining and immunostaining, respectively. DMSO and 9-*cis*-retinal were obtained from Sigma-Aldrich Corp. (St. Louis, MO). D5-all-*trans*-retinal was purchased from Toronto Research Chemicals (Toronto, Canada). 11-*cis*-6mr-retinal was synthesized as described previously and obtained from Novartis (Cambridge, MA) (Alexander NS et al., 2017, Gulati S et al., 2017). Mouse monoclonal 1D4 anti-rhodopsin antibodies were purified from hybridoma cells also as described previously (Adamus G et al., 1988, Adamus G et al., 1991, Hodges RS et al., 1988).

Animals: care and treatment

Abca4$^{-/-}$*Rdh8*$^{-/-}$ mice (Maeda A et al., 2008) with a pigmented C57BL/6 or C57BL/6 and 129SV mixed background were used for light-induced retinal degeneration assays and evaluation of treatment with 11-*cis*-6mr-retinal. *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice were genotyped to confirm that their lack of the *Rd8* mutation. Only mice with the Leu variation at amino acid 450 of RPE65 were used (Kim SR et al., 2004). BALB/c mice (Jackson Laboratory, Bar Harbor, ME) at 4-6 weeks of age were also employed to test the effects of 11-*cis*-6mr-retinal on bright light-induced retinal damage. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used to test the effect of 11-*cis*-6mr-retinal on the retinoid cycle and the accumulation of retinoids in the eye. Both male and female mice were used in all experiments. 11-*cis*-6mr-retinal was dissolved in DMSO and administered to mice by i.p. injection at 4-20 mg/kg bw. All mice were housed in the Animal Resource Center at the School of Medicine, Case Western Reserve University (CWRU) and maintained in a 12 h light (≤10,000 lux)/12 h dark cycle. Manipulations in the dark were performed under dim red light.

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All animal procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee at CWRU and conformed to recommendations of both the American Veterinary Medical Association Panel on Euthanasia and the Association for Research in Vision and Ophthalmology.

Bright light-induced retinal degeneration

Retinal degeneration was initiated by exposing dark-adapted *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ or BALB/c mice to 10,000 or 12,000 lux white light, respectively delivered from a 150-W spiral lamp (Hampton Bay, Home Depot, Atlanta, GA) for 30 or 60 min, as previously described (Chen Y et al., 2013). Before light exposure, pupils of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice were dilated with 1% tropicamide, but this was not needed for BALB/c mice. 11-*cis*-6mr-Retinal or DMSO was administered i.p. 30 min before exposure to bright light. Effects of 11-*cis*-6mr-retinal were tested at 4, 8, 12 and 20 mg/kg bw. Each injection volume was 70 µl. Retinal morphology and function were analyzed *in vivo* by spectral domain optical coherence tomography (SD-OCT) and electroretinography (ERG) seven days after bright light exposure. Mice then were euthanized, and their eyes were subjected to staining with Hematoxylin and Eosin (H&E), immunohistochemistry, and imaging.

Spectral Domain-Optical Coherence Tomography (SD-OCT)

To evaluate the effect of 11-*cis*-6mr-retinal treatment on *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice or BALB/c mice following bright light-induced retinal damage, we performed *in vivo* imaging of mouse retinas with ultrahigh-resolution SD-OCT (Bioptigen, Morrisville, NC) (Chen Y et al., 2013). Briefly, pupils of mice were first dilated with 1% tropicamide. Then, mice were anesthetized by i.p. injection of a cocktail containing ketamine (20 mg/ml) and xylazine (1.75 mg/ml) at a dose of 4 µl/g bw. The A scan/B scan ratio was set at 1200 lines. Four frames of OCT images scanned at 0^{o} and 90^{o} were acquired in the B-mode, averaged, and saved as PDF files. To evaluate changes in the retinas of mice exposed to bright light and assess the effect of 11-*cis*-6mr-retinal on retinal protection, the outer nuclear layer (ONL) thickness was measured 500 µm from the optic nerve head. Each

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treatment group contained 10 mice and the experiment was repeated three times. Values of ONL thickness were plotted using means and standard deviations (SD).

Scanning laser ophthalmoscopy (SLO) imaging

SLO (Heidelberg Engineering, Franklin MA) was performed for *in vivo* whole fundus imaging of mouse retinas (Huber G et al., 2009). Mice were anesthetized, and their pupils were dilated with 1% tropicamide prior to imaging. SLO was acquired in the autofluorescence mode and the number of autofluorescent spots was counted and subjected to statistical analysis.

Retinal histology

The structural morphology of mouse retinas subjected to bright light and either pretreated with 11-*cis*-6mr-retinal or DMSO as a control were assessed *in vitro* using H&E staining of paraffin sections. Mice were euthanized, their eyes were removed and fixed in 4% paraformaldehyde and 1% glutaraldehyde followed by paraffin sectioning. Sections (5 µm thick) were stained with H&E and imaged by light microscopy (Leica, Wetzlar, Germany).

Immunohistochemistry

Eyes collected from mice exposed to bright light were fixed in cryoembedding medium and processed for cryosectioning. Morphology of the retinas was assessed by immunostaining of 12 µm thick cryosections with monoclonal 1D4 anti-rhodopsin primary antibody and Alexa Fluor 594-conjugated goat anti mouse immunoglobulin G (IgG) used as a secondary antibody to detect rod photoreceptors. To detect cone photoreceptors biotinylated peanut agglutinin (PNA) and Alexa Fluor 488-conjugated streptavidin were employed. Nuclear staining was achieved with DAPI.

Two-photon microscope (TPM) imaging

Three days after light-induced retinal degeneration, *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ albino mice on a C57BL/6 and 129SV background treated either with DMSO or 11-*cis*-6mr-retinal 30 min prior to

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light stimulation for 60 min were subjected to TPM imaging as described previously (Maeda A et al., 2014). Briefly, a Leica TCS SP5 upright confocal microscope equipped with a 1.0-NA water immersion objective and tunable laser Vision S (Coherent) delivering 75-fs laser pulses at an 80-MHz pulse repetition frequency was used to image freshly enucleated intact mouse eyes. Before eye enucleation, mice were anesthetized by i.p. injection of a cocktail containing ketamine (6 mg/ml) and xylazine (0.44 mg/ml) at a dose of 10 µg/g bw and then euthanized.

Detection of reactive oxygen species (ROS) *in vivo*

Detection and quantification of ROS generated *in vivo* in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice after their exposure to bright light was performed as described previously (Chen Y et al., 2013). Dihydroethidium (DHE), a ROS probe, was administered to mice by i.p. injection at a dose of 20 mg/kg bw delivered in 50 µl of DMSO vehicle 1 day after illumination with 10,000 lux white light. Thirty min before light exposure, these mice were also treated with 11-*cis*-6mr-retinal or vehicle. One hour after DHE administration, eyes were collected and fixed in cryoembedding medium. Cryosections (12 µm thick) were used for microscopic evaluation of ROS fluorescence in the retina. Quantification of ROS fluorescence was performed with ImageJ software (NIH).

Electroretinography (ERG) analyses

To evaluate the protective effect of 11-*cis*-6mr-retinal on retinal function, ERG recordings were obtained for *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice or BALB/c mice at 4-5 weeks of age 7 to 14 days after light-induced damage. These mice were divided into an unbleached group, a DMSO-treated control group and an 11-*cis*-6mr-retinal-treated group. Ten *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ and 5 BALB/c mice were used in each group. The mice were given a single dose of 11-*cis*-6mr-retinal at 20 mg/kg by i.p. injection 30 min prior to light exposure. Before ERG recording, dark-adapted mice were anesthetized with 20 mg/ml ketamine and 1.75 mg/ml xylazine in PBS at a dose of 0.1-0.13 ml per 25 g bw, and pupils were dilated with 1% tropicamide. Contact lens electrodes were placed on the eyes, and a reference electrode was positioned between two ears, while a ground electrode

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was placed on the tail. Scotopic and photopic ERGs were recorded for both eyes of each mouse using a UTAS E-3000 universal testing and ERG system (LKC Technologies, Inc., Gaithersburg, MD). ERG data represent the means and SDs of both a-wave and b-wave amplitudes.

To evaluate the effect of 11-*cis*-6mr-retinal on the retinoid cycle, WT C57BL/6J mice were placed in a dark room one day before ERG measurements. DMSO, 11-*cis*-6mr-retinal at a dose of 20 mg/kg bw or retinylamine (a potent inhibitor of the visual cycle (Golczak M et al., 2005)) at a dose of 4 mg/kg bw were administrated by ip injection 30 min before light exposure. Mice were treated with 1% tropicamide eye drops for pupil dilation, exposed to 2,000 lux of illumination for 5 min and returned to the dark. Mice were anesthetized after bleaching for scotopic ERG recordings as described previously (Maeda A et al., 2005). Briefly, every 5 min a single-flash scotopic ERG at stimulating intensity of 1.6 $cd \cdot s \cdot m^{-2}$ was recorded within 1 h after light exposure. A-wave and b-wave amplitudes of each ERG recording were measured, averaged from five animals, and plotted as a function of time using Sigma Plot software version 11.

Purification of retinal pigment

Eyes were collected from three *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ or WT C57BL/6J mice, which were either dark adapted or treated with 11-*cis*-6mr-retinal at a dose of 20 mg/kg bw 30 min prior to bright light exposure for 30 min followed by housing in the dark for 24 h. This treatment was repeated 3 times. Additionally, eyes were collected from lecithin retinol aclyltransferase knockout (*Lrat*$^{-/-}$) mice either untreated or treated with 11-*cis*-6mr-retinal at the same dose as *Abca4*$^{-/-}$*Rdh8*$^{-/-}$or WT C57BL/6J mice. Eyes were stored at -80 ^{o}C or used immediately. Eyes were homogenized gently with a glass-glass homogenizer in buffer composed of 50 mM *bis*-tris propane (BTP), pH 7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail in the dark followed by a 15-min centrifugation at 16,000*g* in a benchtop Eppendorff centrifuge at 4 ^{o}C. Supernatants were discarded, and pellets were solubilized in 20 mM BTP, pH 7.5, 100 mM NaCl, 20 mM n-dodecyl-β-D-maltoside (DDM) for 1 h at 4 ^{o}C. Solubilized membrane lysates were cleared by centrifugation

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at 16,000*g* for 30 min, and the supernatants were incubated with 1D4-immunoaffinity resin (6 mg of 1D4 anti-rhodopsin antibody/ml resin) equilibrated with 20 mM BTP, pH 7.5, 100 mM NaCl, 2 mM DDM for 1 h at 4 ^{o}C. After washing, rhodopsin and 11-*cis*-6mr-retinal-bound opsin were eluted by addition of 1D4 peptide (TETSQVAPA) to the above buffer and spectra were then measured with a UV-visible spectrophotometer.

Detection and quantification of 11-*cis*-6mr-retinal in mouse eye

To measure the amount of 11-*cis*-6mr-retinal in mouse eye following its systemic delivery, 11-*cis*-6mr-retinal was administered to *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice in DMSO vehicle by i.p. injection at a dose of 20 mg/kg bw. Then mice were kept in the dark for 24 h and either euthanized to collect their eyes for analysis or the treatment was repeated twice.

Eyes from one mouse under the same treatment were homogenized on ice in 1 ml of PBS:methanol (1:1, v/v) in the presence of 100 pmol of an internal standard (d5-all-*trans*-retinal). The homogenate was incubated with 50 mM hydroxylamine for 20 min at RT. Then 4 ml of hexane was added to the homogenized sample, and the mixture was vortexed for 15 s. The mixture was centrifuged at 3,220*g* for 5 min at 4 ^{o}C to separate the hexanes from the aqueous layer. From the top hexane layer, 3.5 ml was transferred to a glass vial. These samples were then dried in a Savant speedvac concentrator (Thermofisher, Waltham, Massachusetts, USA) and dissolved in 300 µl of hexane. Ten µl of dissolved samples were injected into an Agilent Technology 1100 HPLC system and retinoids were separated on an xBridge C18 column (3.5 µm, 2.1 x 100 mm; Waters, Milford, MA) by a linear gradient of acetonitrile in water (50% - 100%, 30 min, at at flow rate of 0.5 ml/min). All solvents contained 0.1% formic acid (v/v). MS-based detection and quantification of 11-*cis*-6mr-retinal was performed with an LTQ linear ion trap mass spectrometer (Thermo Scientific) equipped with an electrospray ionization (ESI) interface operated in the positive ionization mode. Parameters of ionization and detection were tuned with synthetic oximes of the locked retinal. 11-*cis*-6mr-Retinal oximes and d5-all-*trans*-retinal oximes were detected in

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the selected reaction monitoring (SRM) mode using m/z 312.3 → 295.2 and 305.3 → 247.2 transitions, respectively. Calibration curves were calculated based on the linear relationship between areas under SRM ion intensity peaks corresponding to the locked retinal oximes and the internal standard versus molar ratios of the compounds in a range of 20 to 500 pmol. The experiment was done in triplicate.

Statistical analyses

Values of ONL thickness derived from SD-OCT imaging, counts of enlarged photoreceptors from TPM imaging, and counts of AF spots from SLO imaging are expressed as means ± standard deviations. Five or ten mice per treatment group were used. For two-group comparisons, Student's *t*-test was used. For multiple comparisons, the one-way ANOVA (using *SigmaPlot 11.0* software) with the post-hoc student's *t*-test was employed. Differences were considered statistically significant at a *P* value of <0.05 (**P* <0.05, ***P*<0.01, ****P* <0.001).

RESULTS

11-*cis*-6mr-Retinal protects against bright light-induced retinal degeneration in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice

Abca4$^{-/-}$*Rdh8*$^{-/-}$ mice lack both the ATP-binding cassette transporter 4 (ABCA4) and the all-*trans*-retinol dehydrogenase 8 (RDH8) enzyme and exhibit impaired clearance of all-*trans*-retinal photoproducts (Molday RS, 2007). They also feature many phenotypic changes found in patients with human juvenile macular degeneration (Maeda A et al., 2012, Maeda A et al., 2008, Maeda A et al., 2014). These mice develop both, chronic retinal degeneration under ambient light resembling the human condition as well as intense light-induced acute retinal degeneration. Thus, they serve as a model of both rod and cone photoreceptor degeneration. In this study, we evaluated the protective effect of the locked chromophore analogue, 11-*cis*-6mr-retinal against

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bright light-induced retinal degeneration. To determine the therapeutic dose of 11-*cis*-6mr-retinal, various concentrations of this retinal analogue (4, 8, 12 or 20 mg/kg body weight (bw)) or a DMSO vehicle were administered to 4-6-week-old *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice by i.p. injection 30 min before exposure to 10,000 lux bright light for 30 min. These mice then were kept in the dark for 7-14 days to allow clearing of dead cells before their evaluation as schematically depicted in Fig. 1A. Analysis of retinal morphology by *in vivo* OCT imaging revealed that morphological damage induced by bright light was largely prevented by pretreatment with 11-*cis*-6mr-retinal at 20 mg/kg and retinal structures closely resembled those of mice not exposed to bright light (Fig. 1B and C, and Table 1). Partial protection was noted with 11-*cis*-6mr-retinal at 12 mg/kg (Table 1). In contrast, control mice treated with DMSO vehicle exhibited disruption of photoreceptors manifested by severe changes in the thickness of the ONL (Fig. 1B and C).

Increased autofluorescence (AF) in the photoreceptor outer segments and retinal pigment epithelium (RPE) associated with bright light illumination was previously observed (Maeda A et al., 2011, Maeda A et al., 2008). Thus, we quantified AF spots in the eyes of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice pretreated with 11-*cis*-6mr-retinal or DMSO vehicle, followed by bright light exposure and compared these values to untreated and unilluminated mice. SLO imaging was performed to detect AF 7 days after treatment (Fig. 1A). The number of AF spots was greatly increased in DMSO-treated mice, while it was decidedly decreased in mice pretreated with 11-*cis*-6mr-retinal at 20 mg/kg 30 min before exposure to bright light (Fig. 1D and E). A partial decrease of AF spots was noted with 11-*cis*-6mr-retinal at 12 mg/kg (Table 1). Thus, the results obtained from retinal SLO imaging correlated with the retinal degeneration detected with OCT imaging.

To confirm the results obtained from *in vivo* analyses, we performed a histological examination of plastic sections after H&E staining from areas like those used for OCT imaging (Fig. 1F). The diminished ONL in DMSO-treated *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice exposed to bright light as well as the protective effect of treatment with 11-*cis*-6mr-retinal agreed with the findings from OCT imaging.

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Treatment with 11-*cis*-6mr-retinal preserves retinal morphology after exposure to bright light in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice

To assess light-induced damage to the retina in greater detail, we performed an immunohistochemical analysis of cryosections stained with an anti-rhodopsin antibody that labeled rod outer segments and peanut agglutinin (PNA) that labeled cone cells. DAPI staining was used to detect nuclei. As shown in Fig. 1G, *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice treated with DMSO vehicle showed severe changes in retinal morphology evidenced by a significantly decreased thickness of the ONL. Also, PNA staining was undetectable and only residual staining of rhodopsin remained. However, pretreatment with 11-*cis*-6mr-retinal before bright light exposure prevented light-induced damage and preserved the morphology of photoreceptors. Images of retinal morphology of 11-*cis*-6mr-retinal-treated mice closely resembled those images from mice unexposed to light as demonstrated by a similar thickness of the ONL and intense staining with anti-rhodopsin antibodies and PNA.

Treatment with 11-*cis*-6mr-retinal reduces photoreceptor death

One of the early manifestations of light-induced retinal degeneration is the swelling of the photoreceptor cells (Maeda A et al., 2014). We performed TPM imaging to assess and quantify changes in photoreceptor morphology in freshly enucleated mouse eyes 3 days after treatment with 11-*cis*-6mr-retinal and exposure to bright light. DMSO-treated *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ control mice showed a marked increase of enlarged photoreceptors, whereas pretreatment with 11-*cis*-6mr-retinal prior to light illumination significantly diminished the number of such photoreceptors (Fig. 2A, B and C).

Treatment with 11-*cis*-6mr-retinal preserves retinal function

ERG analysis of the visual response was used to assess retinal function. The results obtained were compared between three groups of mice: *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice either unexposed to light, DMSO-treated and exposed to bright light, or 11-*cis*-6mr-retinal-treated and exposed to bright light. Both scotopic a- and b-waves and photopic b-waves were nearly eradicated in

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Abca4$^{-/-}$*Rdh8*$^{-/-}$ mice treated with DMSO vehicle followed by 10,000 lux bright light illumination (Fig. 3A and B). Importantly, pretreatment with 11-*cis*-6mr-retinal prior to light exposure almost completely preserved visual acuity in these mice. Both scotopic a- and b-waves and photopic b-waves of 11-*cis*-6mr-retinal-treated mice were very similar to those of mice unexposed to bright light.

11-*cis*-6mr-retinal prevents light-induced retinal damage and preserves retinal function in WT mice

To determine if 11-*cis*-6mr-retinal protects against light-induced retinopathy in WT mice, we used 6-8-week-old albino BALB/c mice susceptible to light-induced retinal degeneration (LaVail MM et al., 1987, LaVail MM et al., 1987). As evidenced by OCT images, pretreatment with 11-*cis*-6mr-retinal 30 min prior to bright light exposure prevented retinal damage noted in DMSO-treated control mice (Fig. 4A and B). Seven days after bright light illumination, DMSO-treated WT mice featured severe disorganization of retinal structures and thinning of the ONL, just like *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice. In agreement with OCT images, SLO imaging revealed a significant reduction in the number of AF spots associated with light-induced retinal pathology in those animals treated with 11-*cis*-6mr-retinal (Fig. 4C and D). Histological examination of retinas from WT mice further confirmed the protective effect of 11-*cis*-6mr-retinal (Fig. 4E). Immunohistochemical analysis of cryosections stained with an anti-rhodopsin antibody to label rods and PNA to label cones also confirmed morphological changes in the retina, as evidenced by a decreased thickness of the ONL in mice treated with DMSO prior to light exposure. Notably, pretreatment with 11-*cis*-6mr-retinal significantly preserved photoreceptor morphology. The ONL was much thicker than in DMSO-treated mice and closely resembled the ONL thickness in mice unexposed to light (Fig. 4F). As revealed by ERG responses, consistent with the morphological changes observed in these mice, visual function that diminished after the exposure to bright light was almost completely preserved after pretreatment with 11-*cis*-6mr-retinal (Fig. 4G).

Treatment with 11-*cis*-6mr-retinal does not inhibit the retinoid cycle

Molecular Pharmacology Fast Forward. Published on July 17, 2018 as DOI: 10.1124/mol.118.112581
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To determine if 11-*cis*-6mr-retinal affects the retinoid cycle we measured the ERG response recovery in WT C57BL/6J mice within 1 h after illumination with white light at 2,000 lux for 5 min. Prior to illumination, these mice were either treated with DMSO vehicle, 11-*cis*-6mr-retinal or retinylamine; the last agent is a potent inhibitor of the visual cycle (Golczak M et al., 2005). Immediately after exposure to light, scotopic ERG responses were recorded every 5 min for a total of 1 h. As shown in Fig. 5, recovery of both a-wave (Fig. 5A) and b-wave (Fig. 5B) amplitudes after illumination in mice treated either with DMSO or 11-*cis*-6mr-retinal was evident, but not in mice treated with retinylamine. Thus, 11-*cis*-6mr-retinal did not inhibit the conversion of all-*trans*-retinal to its 11-*cis* configuration.

Treatment with 11-*cis*-6mr-retinal inhibits reactive oxygen species (ROS) generation in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice after bright light exposure

Oxidative stress that causes overproduction of ROS is considered the major mechanism of photoreceptor cell death induced by acute light exposure in model animals (Donovan M et al., 2001, Organisciak DT et al., 1992). Excessive production of ROS in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice from bright light illumination has been documented (Chen Y et al., 2013). Maintaining low levels of ROS therefore is extremely important for the viability of photoreceptor cells. Here, we tested if treatment with 11-*cis*-6mr-retinal could reduce the generation of ROS in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice exposed to bright light. Dark-adapted mice were injected with the fluorescent probe dihydroethidium (DHE), used to detect superoxide radicals, one day after exposure to 10,000 lux light for 30 min. ROS signals detected in the ONL were then compared between three groups of mice: those unexposed to light, DMSO-treated controls, and mice pretreated with 11-*cis*-6mr-retinal. The highest production of ROS was detected in vehicle-treated mice, whereas the ROS signal in the ONL was substantially decreased in mice treated with 11-*cis*-6mr-retinal to a level similar to that observed in dark-adapted mice (Fig. 6A and B). Together these data indicate that ROS generation contributing to light-induced retinopathy in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice is mitigated by treatment with 11-*cis*-6mr-retinal, thereby protecting photoreceptor cells.

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Accumulation of 11-*cis*-6mr-retinal in the eye

The constitutive activity of ligand-free opsin accelerates the degeneration of photoreceptors (Fan J et al., 2005, Woodruff ML et al., 2003). Coupling of a locked retinal analogue, 11-*cis*-6mr-retinal, to unliganded opsin would not only mitigate unwanted opsin activity, but it also would prevent its release from the binding pocket upon illumination and thus could contribute to the protective mechanism against light-induced retinal pathology. The 11-*cis*-6mr-retinal analogue binds to rod opsin resulting in formation of a pigment with a different absorption maximum (Gulati S et al., 2017). This property was used to determine whether the formation of 11-*cis*-6mr-retinal-bound pigment could be detected in the mix of rhodopsin and 11-*cis*-6mr-retinal-bound opsin after their purification from mouse eyes. *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ and WT mice were used. A difference in absorption maxima between samples purified from non-treated mice and mice treated with 11-*cis*-6mr-retinal was difficult to detect in normal dark spectra, likely due to the high amount of rhodopsin overlapping with 11-*cis*-6mr-retinal-bound opsin. However, a small shift of the absorption peak towards longer wavelengths with a maximum at ~505 nm was noted in the difference spectra, when spectra obtained after illumination were subtracted from the dark spectra (Fig. 7A and B). Thus, these results indicate that 11-*cis*-6mr-retinal-bound opsin was formed *in vivo* in the eyes of mice treated with 11-*cis*-6mr-retinal before illumination. As an additional control for binding of 11-*cis*-6mr-retinal to rod opsin we used *Lrat*$^{-/-}$ mice in which regeneration of rhodopsin is impaired and opsin accumulates in rod outer segments (Batten ML et al., 2004). After being kept in the dark, these mice were injected with a single dose of 11-*cis*-6mr-retinal for three consecutive days. UV-visible spectra of the protein purified from the eyes of these mice revealed that opsin regeneration with 11-*cis*-6mr-retinal had occurred and the pigment had an absorption peak maximum at 508 nm (Fig. 7C), further supporting the hypothesis that silencing the constitutive activity of opsin could serve as a protective mechanism against light-induced retinal damage in mouse eyes.

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To further confirm and to quantify the accumulation of 11-*cis*-6mr-retinal in the mouse eye, liquid chromatography-mass spectrometry (LC-MS)-based analyses were performed. Retinoids were extracted from eyes of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice treated with either a single i.p. injection or three consecutive injections of 11-*cis*-6mr-retinal. An internal standard (d5-all-*trans*-retinal) was added to the eye homogenate prior to retinoid extraction to enable MS-based quantification of 11-*cis*-6mr-retinal retained in the eye. The results obtained from LS-MS analyses confirmed accumulation of 11-*cis*-6mr-retinal in the eyes of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice (Fig. 8A, B and C). Quantification revealed the presence of the locked retinal analogue in the eyes of these mice after a single administration with a concentration in the low picomolar range (~6 pmoles) and its additive accumulation after three injections (~12 pmoles) (Fig. 8C). Twenty-four hours post administration most drugs would likely be eliminated unless they remained bound to a targeted molecule. Thus, detection of 11-*cis*-6mr-retinal one day after its administration and additive accumulation four days later after multiple administrations indicates that the retinoid analogue penetrates and persists in the eye by stably binding to opsin.

DISCUSSION

Excessive light can be detrimental to retinal health (Chen Y et al., 2012, Maeda A et al., 2008, Organisciak DT et al., 1998). Overstimulation of rhodopsin with bright light triggers photoreceptor cell damage and ultimately blindness (German OL et al., 2015, Grimm C et al., 2000, Rozanowska M & Sarna T, 2005). Interestingly, rhodopsin knockout mice are completely protected from such light-induced retinal damage suggesting that rhodopsin is required for phototoxicity to occur (Grimm C et al., 2000, Hao W et al., 2002, Jacobson SG & McInnes RR, 2002). The elevated concentrations of all-*trans*-retinal and its byproducts, which accumulate in photoreceptors following stimulation by bright light, are a leading cause of retinopathy. These

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materials are especially harmful when they over-accumulate and pigment regeneration is defective (Chen Y et al., 2012). Stargardt disease, an inherited juvenile form of age-related macular degeneration, typifies these situations (Weng J et al., 1999). Patients with Stargardt disease experience progressive rod-cone dystrophy associated with an over-accumulation of lipofuscin and drusen, thickening of Bruch's membrane, and choroidal neovascularization (Maeda A et al., 2012, Maeda A et al., 2008, Maeda A et al., 2014). A mutation in the ABCA4 transporter found in these patients interferes with the transport of released all-*trans*-retinal from the inner space of the rod outer segment discs to the cytoplasm, resulting in buildup of this retinoid and its metabolite, the bis-retinoid N-retinylidene-N-retinylethanolamine (A2E) (Conley SM et al., 2012, Molday RS, 2007, Wu Y et al., 2009). Further, the insufficient conversion of all-*trans*-retinal back to its 11-*cis* form in such a condition also results in delayed regeneration of visual pigment. In fact, loss of rod and cone sensitivity appears in these patients before anatomical changes to the retina are detectable (Jackson GR et al., 2014, Salvatore S et al., 2014).

Decreasing levels of all-*trans*-retinal either with inhibitors of the retinoid cycle such as retinylamine (Golczak M et al., 2005, Golczak M et al., 2005) and the retinylamine-derived potent inhibitor, emixustat (Kubota R et al., 2014, Zhang J et al., 2015) or FDA-approved drugs containing amines that sequester accumulated free aldehyde (Maeda A et al., 2011) can significantly improve overall retinal health in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice, corroborating that all-*trans*-retinal is the toxic photo-metabolite.

High levels of rhodopsin (~5 mM) are expressed in rod photoreceptor cells (Palczewski K, 2006). Thus, in patients with Stargardt disease illumination of only 10% of the visual pigment would result in the prolonged exposure of toxic concentrations of free all-*trans*-retinal of ~500 μM. Reversible conjugation of this highly cytotoxic retinoid with amino group-containing compounds has a protective effect against retinal damage (Maeda A et al., 2011). However, high doses of such compounds are necessary to achieve a therapeutic outcome.

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Inhibition of the visual cycle with specific inhibitors of enzymes involved in the regeneration of 11-*cis*-retinal decreases accumulation of all-*trans*-retinal and it byproducts by slowing the regeneration of rhodopsin. However, prolonged inhibition of pigment regeneration could lead to an increase of chromophore-free opsin in photoreceptor cells that also accelerates retinal degeneration (Fan J et al., 2005, Woodruff ML et al., 2003). In fact, administration of 9-*cis*-retinal acetate to *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice prior to stimulation with bright light limited retinal damage in these mice indicating that in fact delayed regeneration of 11-*cis*-retinal can result in the accumulation of ligand-free opsin. However, pharmacological supplementation with 9-*cis*-retinal does not address accumulation of all-*trans*-retinal and its byproducts after light illumination when its regeneration to 11-*cis*-retinal and clearance from photoreceptors is faulty (Maeda T et al., 2009, Palczewski K, 2010). Therefore, we aimed to evaluate the effect of a locked chromophore analogue, 11-*cis*-6mr-retinal, against bright light-induced retinal degeneration. The hypothesis was that 11-*cis*-6mr-retinal administered to *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice before exposure to light would bind to opsin, silencing not only its toxic constitutive activity but also avoid excessive accumulation of all-*trans*-retinal due to its locked conformation in the chromophore binding pocket. *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice, a model of bright light-induced rod and cone photoreceptor degeneration, were used to test this hypothesis. The results also were confirmed in BALB/c WT mice, which are susceptible to retinal damage with strong light (LaVail MM et al., 1987, LaVail MM et al., 1987). Both *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ and BALB/c WT mice developed severe retinal degeneration within 7 days after exposure to 10,000 or 12,000 lux light, respectively. However, the retinopathy did not appear when mice were treated with 11-*cis*-6mr-retinal 30 min before light stimulation. As demonstrated by multiple imaging techniques, retinal structure and specifically the morphology of rod and cone photoreceptors of mice pretreated with 11-*cis*-6mr-retinal were comparable to that of mice kept in the dark, suggesting that avoiding the accumulation of unliganded opsin is critical to prevent or delay retinal degeneration in mice susceptible to light-induced damage. In fact, 11-*cis*-6mr-retinal was detected in the eyes of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice 24 h after administration and its additive accrual

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was found after multiple consecutive doses as quantified by LC-MS, indicating its stable binding to opsin. Although partial protection against light damage was observed earlier upon administration of 9-*cis*-retinal-acetate (Maeda T et al., 2009), almost full protection was achieved with 11-*cis*-6mr-retinal treatment in this study. Moreover, pretreatment with 11-*cis*-6mr-retinal before bright light insult prevented not only morphological damage of the retina but also preserved visual acuity in the treated mice. Furthermore, 11-*cis*-6mr-retinal did not impair the visual, retinoid cycle. As observed, the conversion of all-*trans*-retinal to 11-*cis*-retinal was not inhibited in mice administered with 11-*cis*-6mr-retinal. This is not a surprising result because as shown before 11-*cis*-locked-retinals are poor substrates for the visual cycle enzymes, including 11-*cis*-retinal dehydrogenase (11-*cis*-RDH) and lecithin retinol acyltransferase (LRAT) (Kuksa V et al., 2002).

Excessive concentrations of all-*trans*-retinal that induce oxidative stress through the activation of the phospholipase C (PLC) → inositol-3-phosphate (IP_3) → Ca^{2+} pathway and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase resulting in photoreceptor death can be prevented with antioxidants (Chen Y et al., 2012, Chen Y et al., 2016, Chen Y et al., 2013). Such oxidative stress could also be reduced by *cis*-retinoids administered systematically at low levels in a mouse model of diabetic retinopathy (Berkowitz BA et al., 2015). Significantly, production of ROS in the photoreceptors of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice was completely inhibited by pretreatment with 11-*cis*-6mr-retinal, indicating its antioxidant properties at concentrations used in this study. Moreover, as evaluated by ERG recordings, photoreceptor function *in vivo* was fully preserved by 11-*cis*-6mr-retinal given to mice before light stimulation. This result demonstrated that 11-*cis*-6mr-retinal has few if any adverse effects on phototransduction in this rodent model. However long-term follow up studies would need to be performed to determine the toxicity profile for this compound.

In summary, this study demonstrates that 11-*cis*-6mr-retinal is a promising drug candidate against the development of light-induced retinopathy in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ and WT mice. *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ rodents are used as a model of human Stargardt disease, associated with

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delayed clearance of all-*trans*-retinal photoproducts and delayed pigment regeneration. Therefore, results presented here add to our understanding of the mechanisms underlying a debilitating blinding condition and may offer therapeutic opportunities in the future either alone or in combination with other treatments.

ACKNOWLEDGEMENTS

We thank Elizabeth Gutierrez for reading the manuscript. We are also grateful to Dr. Muneto Mogi (Novartis Inc.) for the 11-*cis*-6mr-retinal.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Jastrzebska, Gao, and Palczewski.

Conducted experiments: Jastrzebska, Gao, Parmar, Palczewska, Dong, and Golczak

Contributed new reagents: Golczak

Performed data analysis: Jastrzebska, Gao, Parmar, Palczewska, and Golczak

Wrote or contributed to the writing of the manuscript: Jastrzebska, Gao, Golczak, and Palczewski

Discussed the results and commented on the manuscript: Jastrzebska, Gao, Parmar, Palczewska, Dong, Golczak, and Palczewski

Coordinated and oversaw the research project: Jastrzebska

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Molecular Pharmacology Fast Forward. Published on July 17, 2018 as DOI: 10.1124/mol.118.112581
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FOOTNOTES

FUNDING

This research was supported by grants from the National Institutes of Health (NIH) [EY025214, EY027283, EY024864, EY009339, EY025451, EY023948 and P30EY011373].

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

FIGURE LEGENDS

Figure 1. Protective effect of 11-*cis*-6mr-retinal against bright light-induced retinal degeneration in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice. A, Scheme of mouse treatment. 11-*cis*-6mr-retinal or DMSO vehicle were administered to 4-6-week-old *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ or WT BALB/c mice by i.p. injection 30 min before exposure to bright light (10,000 lux) for 30 min for *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice and 12,000 lux for 60 min for WT BALB/c mice. Then, mice were kept in the dark for 7-14 days prior to their examination. The morphology of the retina was assessed by OCT and SLO in *vivo* imaging as well as by H&E staining and immunohistochemistry. Retinal function was assessed by ERG. TPM imaging was used to determine abnormalities in photoreceptor cells on the fourth day after treatment. **B**, Representative OCT images obtained 7 days after treatment of mice with 11-*cis*-6mr-retinal (20 mg/kg bw) 30 min before exposure to white light at 10,000 lux for 30 min. After light illumination, mice were kept in the dark until morphological examination. ONL, outer nuclear layer; INL, inner nuclear layer. Asterisk indicates severely disrupted photoreceptor structures in DMSO-treated control mice. **C**, Quantification of the ONL thickness obtained in 10 mice per treatment group. Error bars indicate SDs. Changes in the ONL thickness observed after treatment with 11-*cis*-6mr-retinal compared to DMSO-treated group were statistically significant (P<0.001); no significant difference in the ONL thickness was observed between animals unexposed to light and those treated with 11-*cis*-6mr-retinal (P=NS)**.** Statistical significance was calculated with the one-way ANOVA and post-hoc students *t-test*, unpaired two-tailed. **D**, Representative SLO images show autofluorescent (AF) spots in the retina of a mouse exposed to light after pretreatment with DMSO (center). Mice unexposed to bright light (left) or exposed to bright light after pretreatment with 11-*cis*-6mr-retinal (right) exhibited much fewer spots. **E**, Quantification of AF spots performed in 10 mice per treatment group. Error bars indicate SDs. Changes in the number of AF spots after treatment with 11-*cis*-6mr-retinal compared to the DMSO-treated group were statistically significant (P<0.001). No significant difference was

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observed between animals unexposed to light and those exposed to light after treatment with 11-*cis*-6mr-retinal (*P*=NS). Statistical significance was calculated with one-way ANOVA and post-hoc the students *t-test*, unpaired two-tailed. **F**, Examination of retinal morphology after staining with H&E in paraffin sections prepared from eyes collected from *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice either unexposed to light or exposed to bright light after the indicated treatments. Asterisk indicates severely disrupted photoreceptor structures in DMSO-treated control mice. **G**, Examination of retinal morphology by IHC in cryosections prepared from the eyes collected from *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice either unexposed to light or exposed to bright light after the indicated treatments. Sections were stained with an anti-rhodopsin C-terminus specific antibody (red) which indicates the structural organization of rod photoreceptors, PNA staining (green) which indicates the health of cone photoreceptors, and DAPI staining of nuclei (blue). Asterisk indicates severely disrupted photoreceptor structures in DMSO-treated control mice. Scale bar, 50 µm.

Figure 2. Treatment with 11-*cis*-6mr-retinal protects photoreceptor cells from light induced degeneration. *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ albino mice on a C57B6/J and 129SV background were treated with 11-*cis*-6mr-retinal (20 mg/kg bw) or DMSO vehicle 30 min before bright light exposure for 60 min and then kept in the dark. On day 3 after the light exposure, TPM imaging was performed in intact mouse eyes immediately after enucleation to quantify enlarged photoreceptors and assess the effect of 11-*cis*-6mr-retinal. **A**, 3-D views of the photoreceptor-RPE interface. Enlarged photoreceptors are visible as fluorescent pillars with a diameter over 2 µm (the diameter of unaffected photoreceptors was ~ 1.2 µm). The center of the RPE layer was set at z = 0 µm, and the section through the photoreceptor cell layer is shown 10 µm below. **B**, Representative, large field images of the retina and RPE are shown. The RPE is visible at the edges of the images as the curvature of the intact eye ball brings it in focus. **C**, Quantification of the enlarged photoreceptors. The number of enlarged photoreceptors caused by illumination with bright light

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was significantly decreased (*P*=0.02) in mice treated with 11-*cis*-6mr-retinal prior to light exposure. Error bars represent SDs. Statistical significance was calculated with the students *t-test*, unpaired two-tailed.

Figure 3. Effect of 11-*cis*-6mr-retinal pretreatment on visual function in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice exposed to bright light. **A**, Single flash ERG responses to increasing light intensity obtained under dark conditions in mice either unexposed to light or exposed to bright light after treatment with either DMSO or 11-*cis*-6mr-retinal at a dose of 20 mg/kg bw. **B**, ERG responses compared between treatment groups revealed significant protective effects in 11-*cis*-6mr-retinal-treated mice before acute light illumination as compared to DMSO-treated mice in both scotopic a- and b-waves and in photopic b-waves (**C**). ERG measurements were carried out in 10 mice per group.

Figure 4. 11-*cis*-6mr-retinal prevents bright light-induced retinal degeneration in WT mice. BALB/c mice were i.p. injected with 11-*cis*-6mr-retinal (20 mg/kg bw) 30 min before exposure to white light at 12,000 lux for 1 h and then kept in the dark for 7 days before examination of retinal morphology and function. **A**, Representative OCT images show substantial protection of the ONL in mice pretreated with 11-*cis*-6mr-retinal as compared to DMSO-treated control mice. Asterisk indicates severely disrupted photoreceptor structures in DMSO-treated control mice. Scale bar, 50 µm. **B**, Quantification of the ONL thickness in mice unexposed to light or in mice treated either with DMSO vehicle or 11-*cis*-6mr-retinal prior to bright light exposure in 5 mice per each treatment group. Error bars indicate SDs. Statistical significance was calculated with one-way ANOVA and post-hoc students *t-test*, unpaired two-tailed. **C**, Representative SLO images show AF spots in the retina of mice unexposed to light or exposed to bright light after pretreatment either with DMSO vehicle or 11-*cis*-6mr-retinal. Scale bar, 50 µm. **D**, Quantification of AF spots in 5 mice per

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treatment group. Error bars indicate SDs. Changes in the number of AF spots after treatment with 11-*cis*-6mr-retinal compared to DMSO-treated group were statistically significant (*P*<0.001). No significant difference was observed between mice unexposed to light and those treated with 11-*cis*-6mr-retinal (*P*=NS)**.** Statistical significance was calculated with one-way ANOVA and post-hoc students *t-test*, unpaired two-tailed. **E**, Examination of gross retinal morphology after H&E staining of paraffin sections from eyes collected from WT mice either unexposed to light or exposed to bright light after indicated treatments. Asterisk indicates severely disrupted photoreceptor structures in DMSO-treated control mice. Scale bar, 50 µm. **F**, Examination of retinal morphology by IHC in cryosections prepared from eyes of BALB/c mice either unexposed to light or exposed to bright light after indicated treatments. Sections were stained with an anti-rhodopsin C-terminus specific antibody (red) that indicates the structural organization of rod photoreceptors, PNA staining (green) denotes the health of cone photoreceptors and DAPI staining (blue) reveals the nuclei. Asterisk indicates severely disrupted photoreceptor structures in DMSO-treated control mice. Scale bar, 50 µm. **G**, Visual function determined by ERG responses. ERG recordings revealed significant protective effects in 11-*cis*-6mr-retinal-treated mice before acute light illumination as compared to DMSO-treated mice in both scotopic a- and b-waves and in photopic b-waves. ERG measurements were carried out in 5 mice per group.

Figure 5. Effect of 11-*cis*-6mr-retinal on the retinoid cycle. WT C57BL/6J mice were i.p. injected with either DMSO vehicle, 11-*cis*-6mr-retinal at a dose of 20 mg/kg bw) or retinylamine (a potent inhibitor of the visual cycle (Golczak M et al., 2005)) at a dose of 4 mg/kg bw, 30 min prior to illumination with white light at 2,000 lux for 5 min. Scotopic ERG responses were recorded immediately after exposure to light and every 5 min thereafter for a total of 60 min. Increasing amplitudes of both a-waves (**A**) and b-waves (**B**) after light illumination in mice treated either with DMSO or 11-*cis*-6mr-retinal were noted but were much slower in mice treated with retinylamine. Five mice per group were used for these ERG measurements. Error bars represent SDs.

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Figure 6. Effect of 11-*cis*-6mr-retinal on photoreceptor generation of ROS in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice exposed to bright light. A, Dark-adapted *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice were injected i.p. with the ROS probe DHE 1 day after illumination with 10,000 lux white light for 30 min. These mice were treated with either DMSO or 11-*cis*-6mr-retinal (20 mg/kg bw) 30 min before light exposure. Mice unexposed to bright light also were treated with the DHE probe. Eyes were harvested 1 h later, fixed and processed for cryosectioning. The ROS signal detected on sections from different experimental groups, was obtained with a fluorescence microscope. DAPI staining was used to visualize nuclei and the retinal layers. Scale bar, 50 µm. **B**, Quantification of fluorescence intensity in arbitrary units obtained from various regions of the photoreceptor cell layers (mean ± S.D.). Changes in fluorescence intensity observed in the photoreceptor layers after treatment with 11-*cis*-6mr-retinal compared to the DMSO-treated group were statistically significant (P<0.001). No significant difference was observed between mice unexposed to light and those treated with 11-*cis*-6mr-retinal (P=NS). Statistical significance was calculated with the one-way ANOVA and post-hoc students *t-test*, unpaired two-tailed.

Figure 7. Detection of 11-*cis*-6mr-retinal-bound pigment in mouse eyes. 11-*cis*-6mr-retinal (20 mg/kg bw) was administered to *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ or WT mice 30 min before their exposure to bright light for 30 min. After illumination, mice were kept in the dark. This procedure was repeated 3 times. On the fourth day (24 h after third treatment), eyes were harvested, and pigment was purified by 1D4 anti-rhodopsin immunoaffinity chromatography. Additionally, *Lrat*$^{-/-}$ mice were injected i.p. with 11-*cis*-6mr-retinal for three consecutive days and 24 h after the last injection eyes were collected and used for pigment purification. UV-visible spectra of the rhodopsin or rhodopsin/11-*cis*-6mr-retinal-bound opsin mixture purified from *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice (**A**), WT mice (**B**) or *Lrat*$^{-/-}$ mice (**C**), that were either untreated (black spectrum) or treated with 11-*cis*-6mr-retinal (green spectrum); left panel. Difference spectra were obtained by subtracting the UV-

visible spectrum of the light-illuminated sample from the spectrum recorded in the dark; right panel.

Figure 8. Detection and quantification of 11-*cis*-6mr-retinal (20 mg/kg bw) in mouse eyes. **A**, Elution profiles for 11-*cis*-6mr-retinal oximes (solid line) and d5-all-*trans*-retinal oximes (dashed line). Chromatograms represent ion intensities for m/z = 312.3 $[M+H]^+$ and m/z = 305.3 $[M+H]^+$, respectively. The MS spectrum averaged between 12 and 13 min of elution indicates ions corresponding to the oximes of endogenous retinal (a) (m/z = 300.3 $[M+H]^+$), d5-all-*trans*-retinal (b), and 11-*cis*-6mr-retinal (c). Fragmentation patterns for 11-*cis*-6mr-retinal oximes and d5-all-*trans*-retinal oximes are shown in panels **B** and **C**, respectively. Characteristic fragmentation profiles for these compounds were used to design the selected reaction monitoring-based detection and quantification method. **D**, Dose-dependent accumulation of 11-*cis*-6mr-retinal retinal in mouse eyes of *Abca4*$^{-/-}$*Rdh8*$^{-/-}$. The observed accumulation of 11-*cis*-6mr-retinal in mouse eyes was statistical significant (P<0.05). Statistical significance was calculated with the students *t-test*, unpaired two-tailed.

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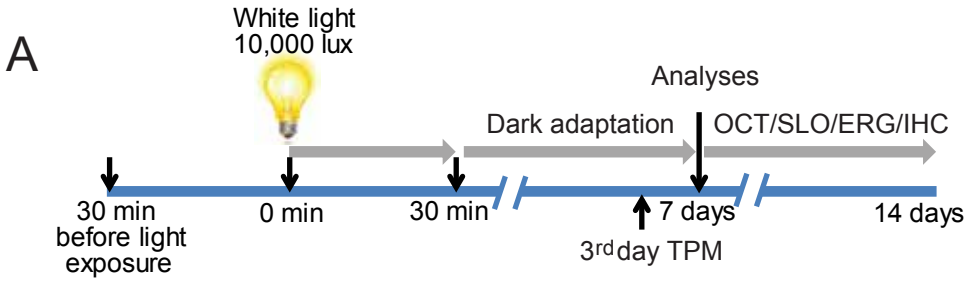
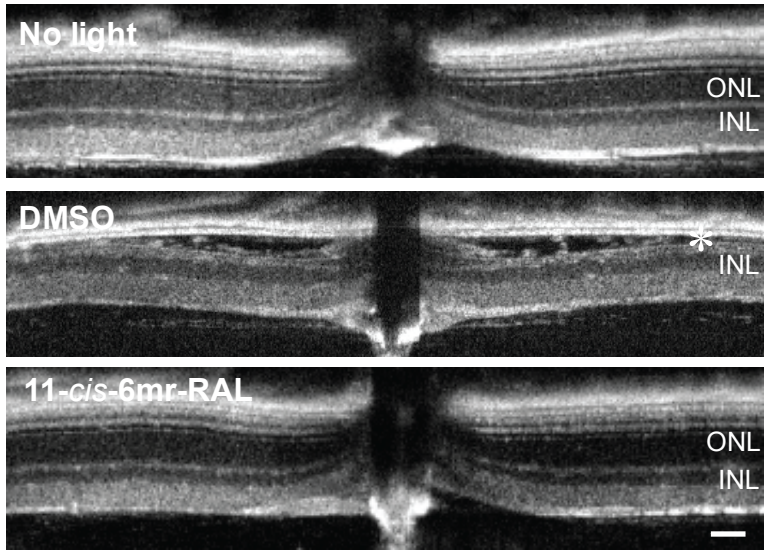
Table 1. Dose-dependent effect of 11-*cis*-6mr retinal on retinal health in *Abca4*$^{-/-}$*Rdh8*$^{-/-}$mice.

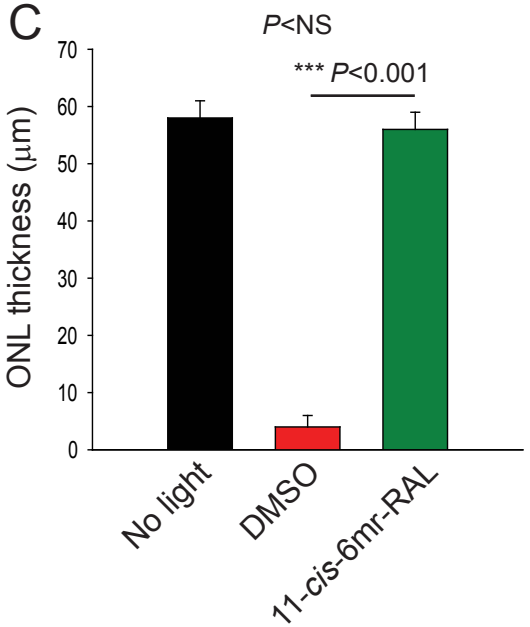
Treatment	OCT ONL thickness (µm)	Statistical significance (*p*)	SLO AF, spot counts	Statistical significance (*p*)
No light	58±3	NA	4±2	NA
DMSO	4±2	NA	1200±100	NA
11-*cis*-6mr-retinal (4 mg/kg bw)	5±3	NS	1100±200	NS
11-*cis*-6mr-retinal (8 mg/kg bw)	15±10	<0.05	1000±200	<0.05
11-*cis*-6mr-retinal (12 mg/kg bw)	27±18	<0.001	400±100	<0.001
11-*cis*-6mr-retinal (20 mg/kg bw)	58±3	<0.001	4±3	<0.001

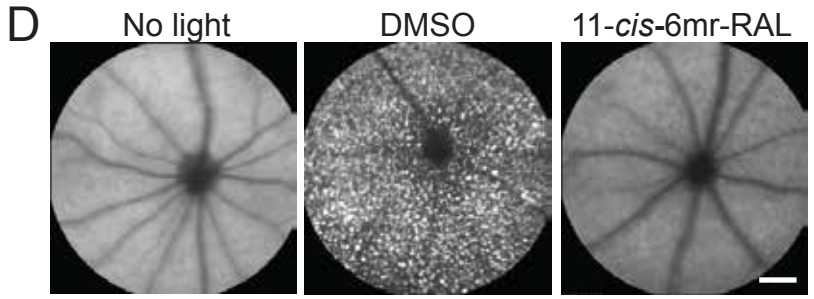
The thickness of the ONL and the number of AF spot were evaluated in OCT or SLO images respectively obtained from *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mouse eyes under different treatment. Mice were either kept in the dark, treated with DMSO or with different doses (4 mg/kg, 8 mg/kg, 12 mg/kg or 20 mg/kg) of 11-*cis*-6mr-retinal 30 min prior to bright light illumination. Morphological changes induced by bright light were largely prevented by pretreatment with 11-*cis*-6mr-retinal at 20 mg/kg; the ONL thickness and AF spots count closely resembled those of mice not exposed to bright light. Partial protection was achieved with 11-*cis*-6mr-retinal at 12 mg/kg. NA, not applicable. NS, not statistically significant. Statistical significance was calculated with the students *t-test*, unpaired two-tailed for two group comparison and one-way ANOVA test (*SigmaPlot 11.0* software) for multiple group comparison.

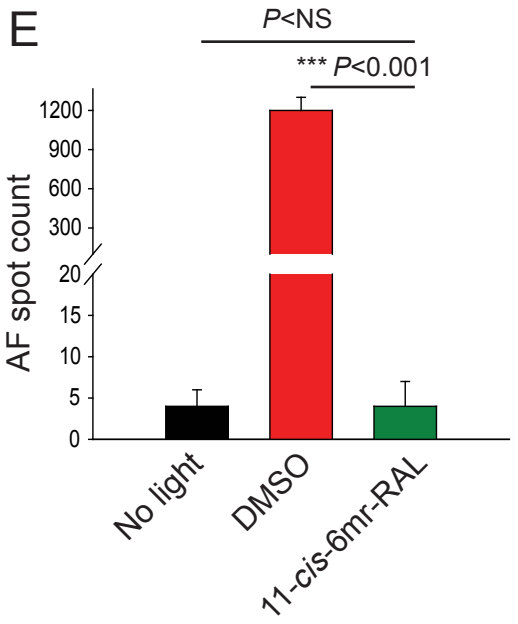
Figure 1

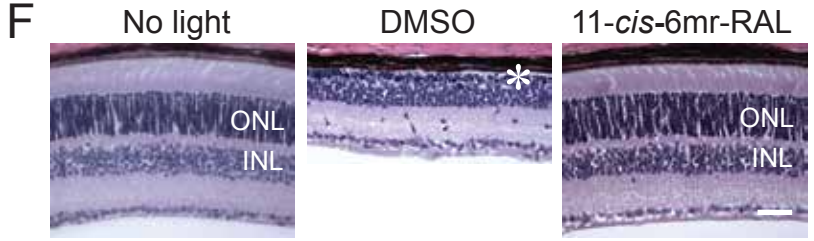












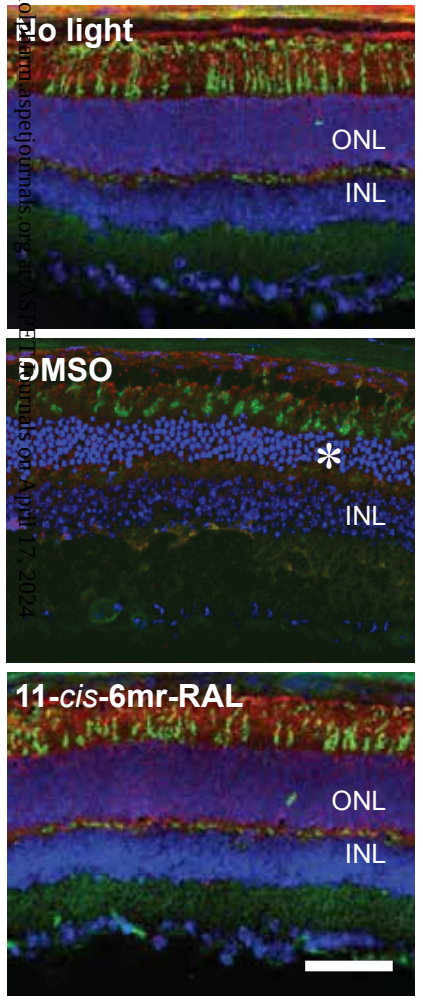


Figure 2

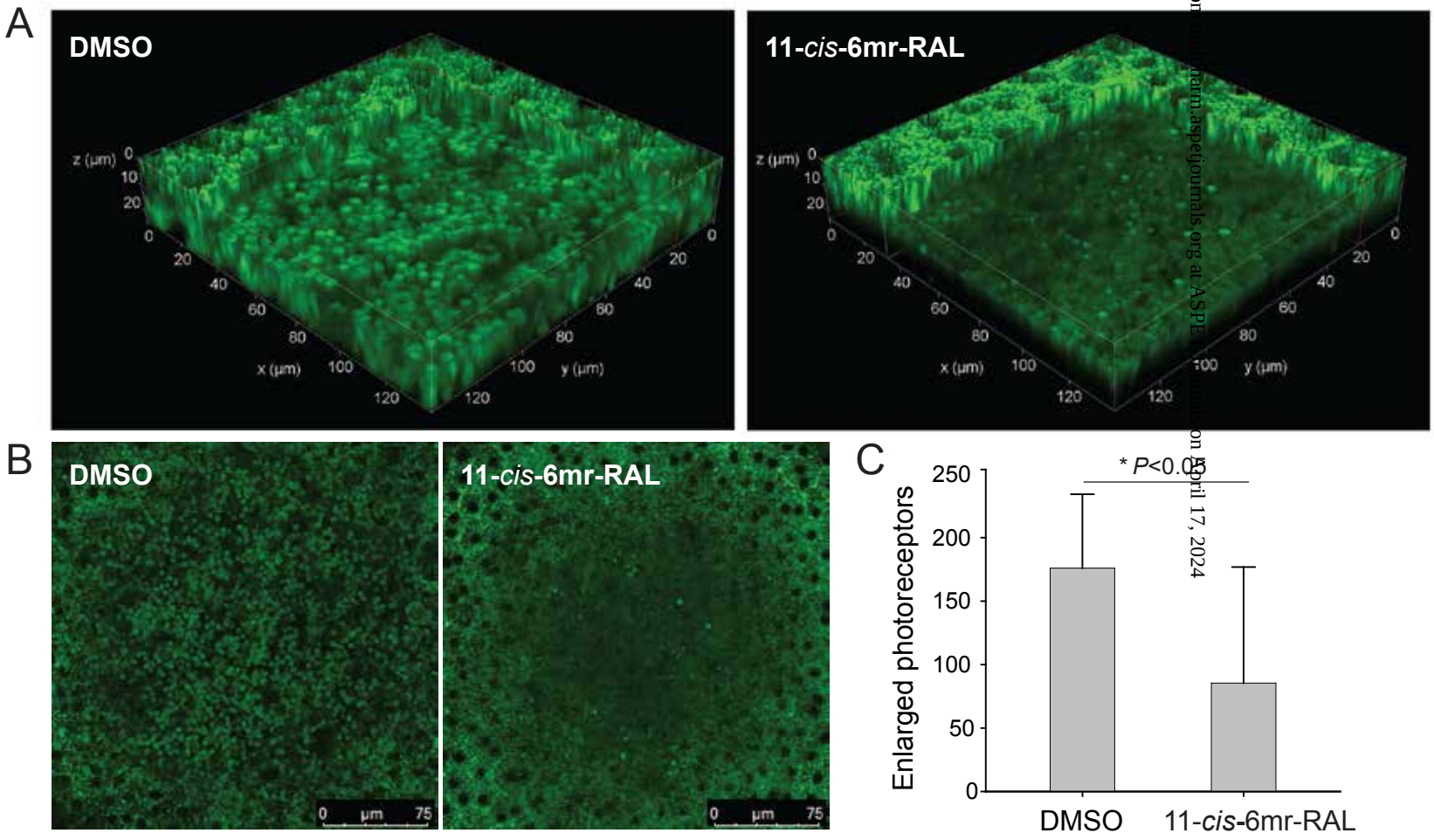


Figure 3

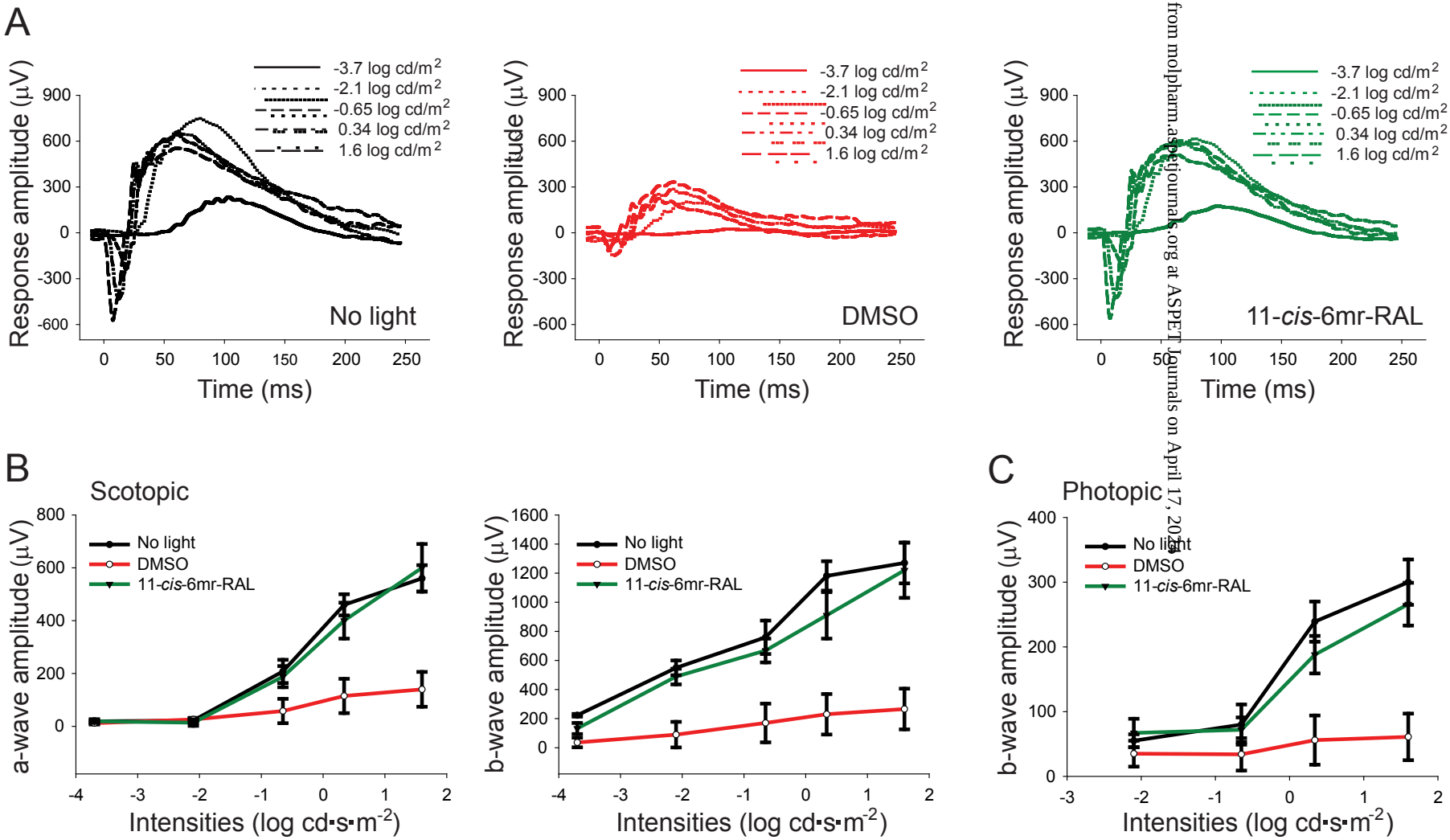


Figure 4

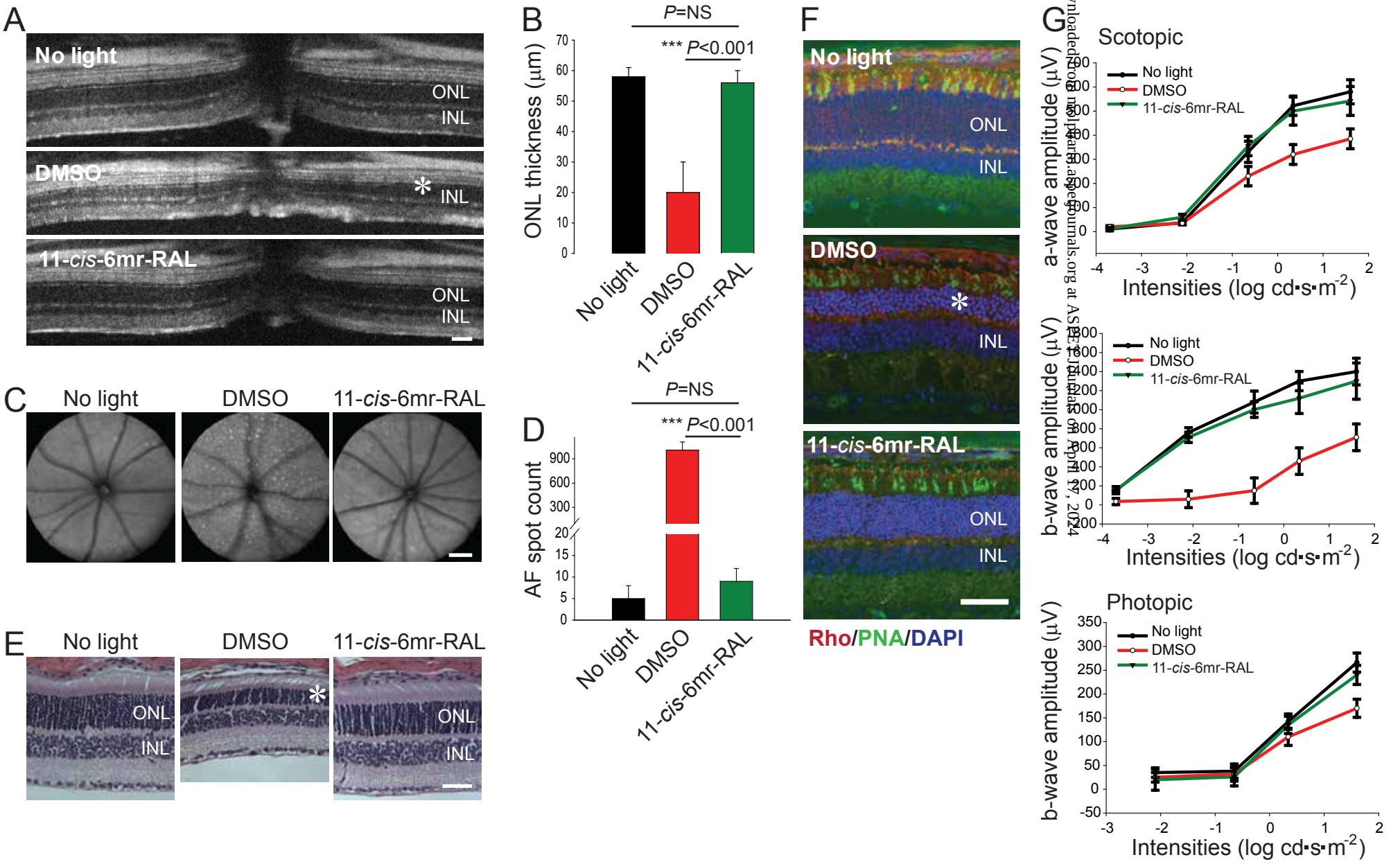


Figure 5

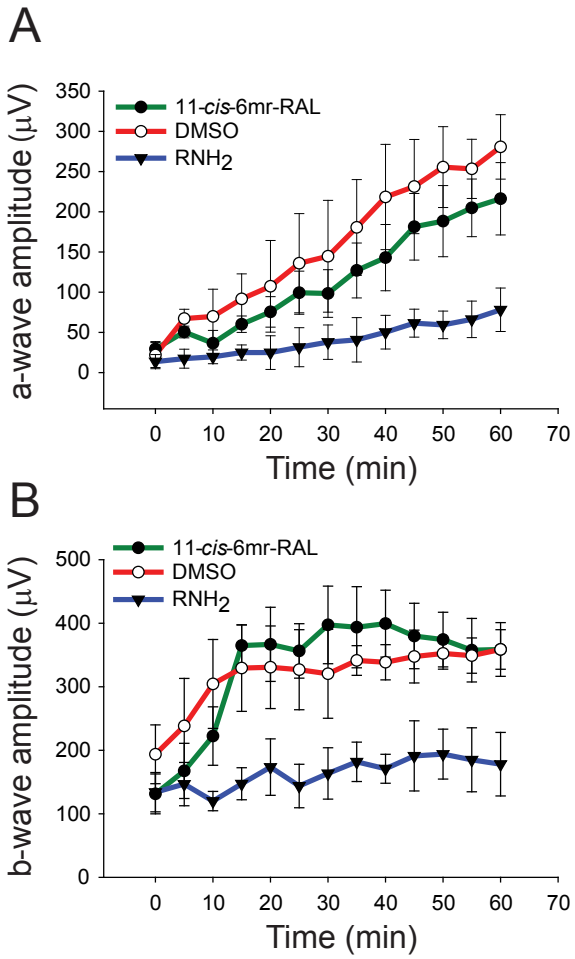
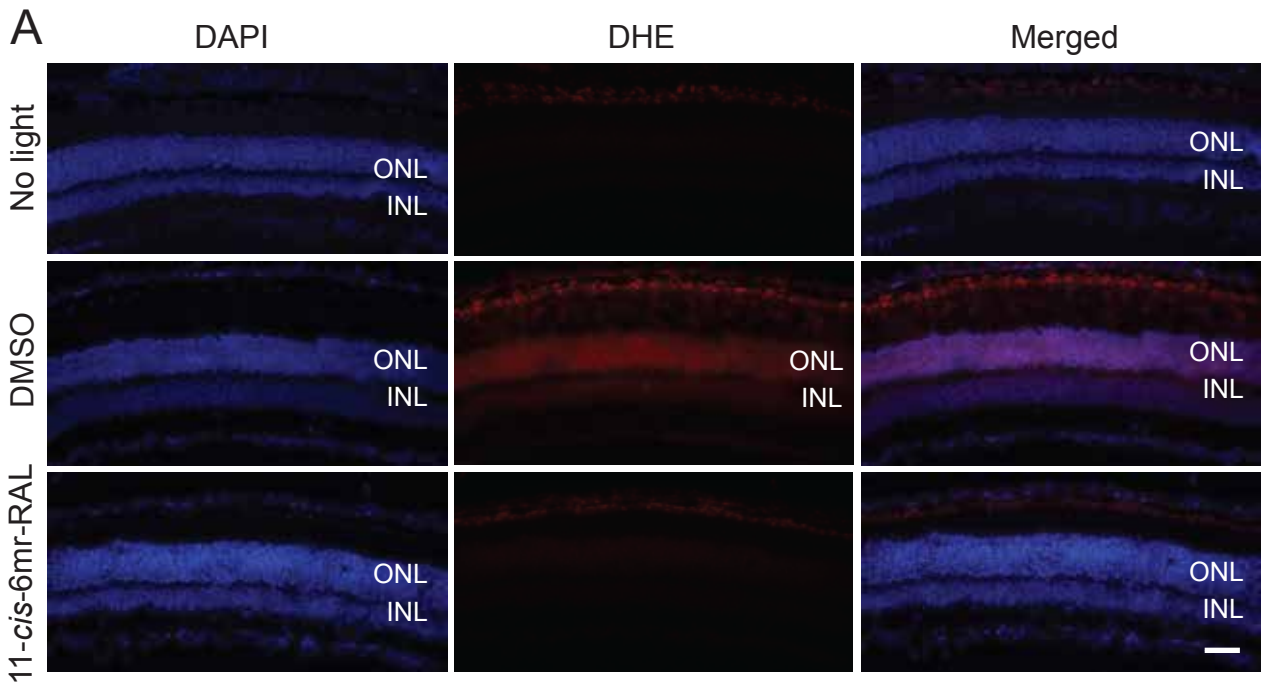


Figure 6



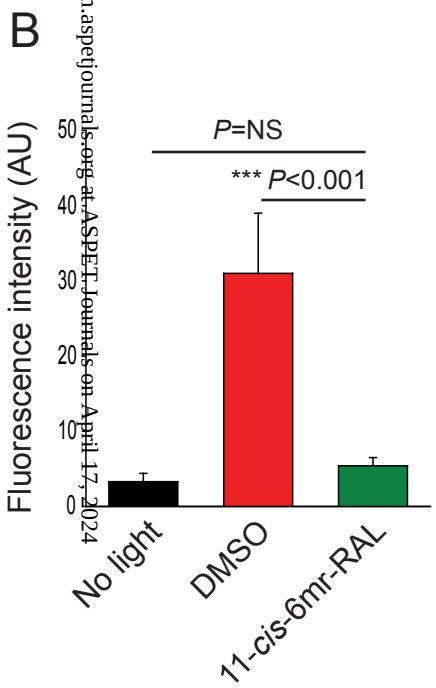


Figure 7

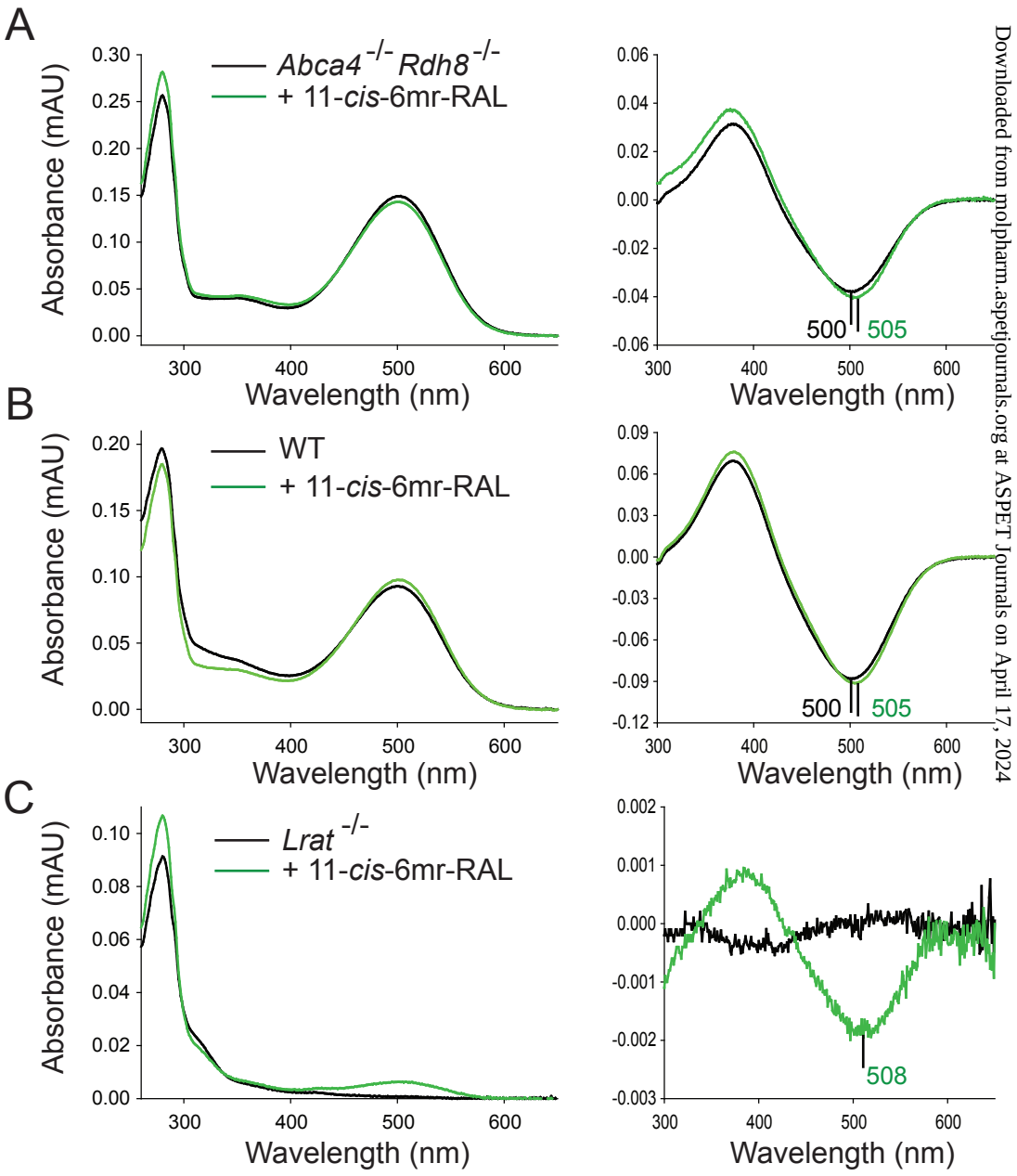


Figure 8