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Light stress affects cones and horizontal cells via rhodopsin-mediated mechanisms

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ABSTRACT

Retinal degenerations are a major cause of blindness in human patients. The identification of endogenous mechanisms involved in neurodegeneration or neuroprotection helps to understand the response of the retina to stress and provides essential information not only for basic retinal physiology but also for defining molecular targets for neuroprotective strategies. Here we used excessive light exposure as a model system to study mechanisms of photoreceptor degeneration in mice. Using one wild type and four genetically modified mouse strains, we demonstrate that light exposure resulted not only in the degeneration of rods but also in an early but transient repression of several cone-specific genes, in a reversible hyperreflectivity of the outer retina including the outer plexiform layer, and in the loss of horizontal cells. The effects on cones, horizontal cells and the inner retina depended on light absorption by rhodopsin and, at least partially, on leukemia inhibitory factor. This demonstrates the existence of intercellular communication routes that transduce rod stress to other cells, likely to provide support for photoreceptors and increase cell survival in the injured retina.

1. Introduction

Retinal degeneration is a major cause of visual impairment and blindness. Treatment options are sparse and involve subretinal injections of voretigene neparvovec-rzyl (Luxturna) as a gene augmentation therapy for the treatment of Leber's congenital amaurosis (LCA) caused by bi-allelic *RPE65* mutations (Ameri, 2018) and anti-vascular endothelial growth factor (VEGF) therapies for the treatment of neovascular age-related macular degeneration (nAMD) (Villegas et al., 2017). The lack of a larger palette of therapeutic options is partly based on an incomplete understanding of the molecular mechanisms that are involved in intra- or intercellular signaling and communication during retinal degenerative processes.

Many inherited and induced model systems are available to study molecular mechanisms, identify potential target molecules for treatment and test therapeutic approaches for retinal degenerations (Samardzija et al., 2010). One of the most frequently used models of induced degeneration is the exposure of mice to high levels of white light. Since time, duration and intensity of exposure can be adapted to experimental needs, the model is frequently used to investigate molecular events during initiation, execution and/or clearance phases of

photoreceptor cell death (Wenzel et al., 2005). Using this model it has been shown that retinal degeneration induced by a short exposure to high intensity light depends on the presence of bleachable rhodopsin in photoreceptors (Grimm et al., 2000; Wenzel et al., 2001). Examinations have also revealed that cones are less susceptible to light damage than rods (Okano et al., 2012) but that cones may also die with a delay as a result of a bystander effect (Krebs et al., 2009) that may include impaired choroidal circulation (Tanito et al., 2007) and/or reduced metabolic support (Ait-Ali et al., 2015).

Studies suggest that retinal degeneration not only affects photoreceptor cells but also neurons of the inner retina including bipolar and horizontal cells (HCs), which remodel their synaptic connections and may even sprout into the ONL (reviewed in (Strettoi, 2015; Kalloniatis et al., 2016)). In addition, Müller glia cells respond to photoreceptor injury by the production of stress, survival and/or inflammatory factors (Bringmann and Reichenbach, 2001). Among others, Müller cell derived cytokines of the interleukin-6 family of proteins, especially leukemia inhibitory factor (LIF) are prominent proteins that support photoreceptor survival (Joly et al., 2008). Other cells receiving signals from stressed or damaged photoreceptors are microglia residing in the inner layers of the retina. They get activated, leave their quiescent state

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Table 1
Primers used for real-time PCR.

Gene	Forward 5' – 3'	Reverse 5' – 3'	Product [bp]
<i>Actb</i>	CAACGGCTCCGGCATGTGC	CTCTTGCTCTGGGCTCG	153
<i>Arr3</i>	CTGGCACTGGATGGCAAAC	CCCCATAGGACACCACCAAG	139
<i>Cngb3</i>	TGATCCAGCCAAGACTCCAAT	ATGCCACATCCATCCGAAAC	108
<i>Gnat1</i>	GAGGATGCTGAGAAGGATGC	TGAATGTTGAGCGTGGTCAT	209
<i>Gnat2</i>	GCATCAGTGCTGAGGACAAA	CTAGGCACTCTTCGGGTGAG	192
<i>Lif</i>	AATGCCACCTGTGGCATAACG	CAACTTGGTCTTCTGTGCCG	216
<i>Marc1</i>	TCCTCCAGTGCAGAGTGCAT	AGGCCACAGGACTGCATCTT	103
<i>Opn1sw</i>	TGTACATGGTCAACAATCGGA	ACACCATCTCCAGAATGCAAG	153
<i>Pou4f1</i>	CGCCGCTGCAGAGCAACCTCTT	TGGTACGTGGCGTCCGGCTT	130
<i>Rho</i>	CTTCACCTGGATCATGGCGTT	TTCGTTGTTGACCTCAGGCTTG	130
<i>Vsx2</i>	CCAGAAGACAGGATACAGGTG	GGCTCCATAGAGACCATACT	111

and start to migrate towards the injured site to help resolving tissue damage (Rashid et al., 2018). The inflammatory response also involves production of monocyte attractant protein 1 (MCP1) (Chen et al., 2004) and infiltration of the retina by blood-born monocytes (Joly et al., 2009). Although this shows that photoreceptor damage affects retinal cell types in addition to rods, signaling mechanisms between photoreceptors and these cells are largely unknown.

Here we addressed the early retinal response to acute light exposure, investigated effects of excessive light absorption by rhodopsin on inner retinal structures, horizontal cell viability and gene expression in cones, and analyzed a potential contribution of LIF to these events.

2. Material and methods

2.1. Mice and light exposure

All animal experimentations were conducted in accordance to the ARVO Statement for the use of animals in ophthalmic and vision research and the regulations of the veterinary authorities of Kanton Zürich. 129S6 (Taconic, Ejby, Denmark), *Rpe65*^{-/-} (Redmond et al., 1998), *Nrl*^{-/-} (Mears et al., 2001), *Rpe65*^{R91W}; *Nrl*^{-/-} (Samardzija et al., 2014) and *Lif*^{+/-} (Escary et al., 1993) mice were kept at the Laboratory Animal Services Center (LASC) of the University of Zürich in a 14 h: 10 h light-dark cycle (lights on at 6 a.m., lights off at 8 p.m.) and had access to food and water *ad libitum*. *Rpe65*^{R91W}; *Nrl*^{-/-} and *Lif*^{+/-} mice were on a mixed 129S6/BL/6, and *Rpe65*^{-/-} and *Nrl*^{-/-} mice on a BL/6 background. *Lif*^{+/-} mice were generated by breeding *Lif*^{+/-} heterozygous mice. Note that all *Lif*^{+/-} mice carried the *Rpe65*^{450Leu/Leu} variant and expressed a GFP transgene under control of the *Rlbp1* promoter (Vázquez-Chona et al., 2009), except for two mice which were *Rpe65*^{450Leu/Met} and did not carry the GFP transgene. These two mice were used as dark controls in the light damage experiment.

After dark adaptation over night, pupils of mice were dilated with 1% Cyclogyl (Alcon, Cham, Switzerland) and 5% Phenylephrine (Ciba Vision, Niederwangen, Switzerland) 45 min prior to exposure to 2 h of 13'000 lux of white light (Grimm and Remé, 2013). Light exposure was from 9 a.m. to 11 a.m. for analyses at 2 d and 10 d after exposure and from 7 p.m. to 9 p.m. for the 12 h time point. Retina isolations were done at 9 a.m. for all post-exposure time points and dark controls to avoid variations in RNA levels due to potential circadian regulation of gene expression. After exposure, mice were returned to their normal cages, kept in darkness for 12 h (h) and then returned to the normal cyclic light conditions.

2.2. Morphology

To analyze retinal morphology, eyes were enucleated and fixed in glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.3) over night. Eyes were cut in a dorsal and a ventral part through the optic nerve head, washed in cacodylate buffer, treated with 1% osmium tetroxide for 1 h and dehydrated using increasing ethanol concentrations. After

embedding in Epon 812, 0.5 µm thick sections were cut and counter-stained with toluidin blue. Images were taken from the ventral retina close to the optic nerve head.

2.3. Electroretinogram (ERG), fundus imaging, optical coherence tomography (OCT)

ERG traces, fundus images and OCT scans were recorded from dark-adapted and anesthetized mice with dilated pupils (see above) essentially as described (Barben et al., 2018a). Briefly, anesthesia was induced by a subcutaneous injection of ketamine (85 mg/kg, Parke-Davis, Berlin, Germany) and xylazine (4 mg/kg, Bayer AG, Leverkusen, Germany). ERG responses were recorded using an LKC UTAS Bigshot unit (LKC Technologies, Inc. Gaithersburg, MD, USA) using flash intensities ranging from -40 db (-3.7 log[cd*s/m²]) to 15 db (1.89 log[cd*s/m²]) for scotopic and from -10 db (-0.64 log[cd*s/m²]) to 25 db (2.86 log[cd*s/m²]) for photopic responses. Before photopic responses were recorded, mice were light-adapted for 5 min. Ten recordings were averaged per light intensity. Fundus images and OCT scans were recorded with the Micron IV system (Phoenix Research Labs, Pleasanton, CA, USA) as described (Geiger et al., 2015). Significance was tested using 2-way ANOVA with Holm-Sidak multiple comparisons test (Prism 8; Graphpad software, La Jolla, CA, USA).

2.4. RNA isolation and real-time PCR

Total RNA was prepared from isolated retinas using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) including a DNase treatment to remove traces of genomic DNA. RNA concentrations were determined using a Nanodrop spectrophotometer (NanoDrop, Wilmington DE, USA) and adjusted to 50 ng/µl with RNase-free water. 1 µg of total RNA was reverse transcribed using oligo(dT) primers and M-MLV reverse transcriptase (Promega, Dübendorf, Switzerland). cDNA from 10 ng RNA was used per real-time PCR reaction.

Amplifications were done in duplicates using appropriate primer pairs (Table 1) either in a Light-Cycler instrument 480 (Roche Diagnostics AG, Basel, Switzerland) or in a QuantStudio 3 system (Thermo Fisher Scientific, Reinach, Switzerland). RNA levels were normalized to β-actin (*Actb*) and relative expression was calculated using the comparative threshold cycle method (ΔΔC_T). Significance was tested using a one-way ANOVA with Dunnett's multiple comparisons test to compare transcript levels after light exposure to dark controls (Figs. 2 and 4), or with a Students t-test to compare mRNA levels between *Lif*^{+/-} and *Lif*^{-/-} mice after light exposure (Fig. 6; Prism 8; Graphpad software, La Jolla, CA, USA).

2.5. Immunofluorescence

Immunofluorescence was done according to published protocols (Barben et al., 2018bb). Briefly, eyes were marked dorsally and fixed in 4% paraformaldehyde (PFA) in phosphate buffer for 1 h at 4 °C. After

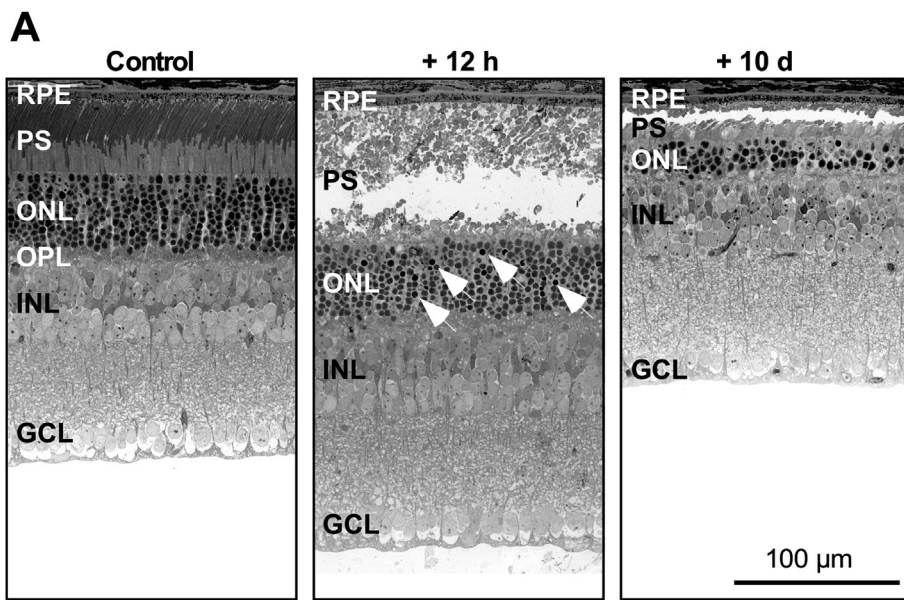
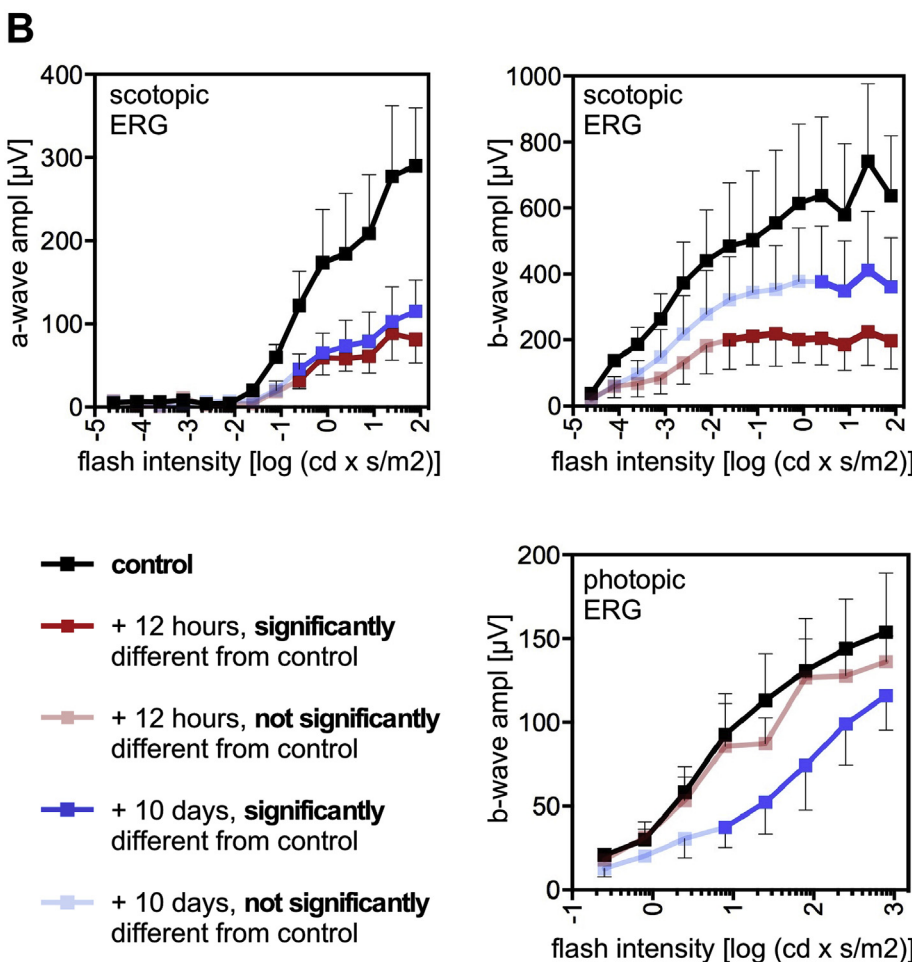


Fig. 1. Consequence of light exposure for retinal morphology and function. A) Morphology of wild type 129S6 mice was analyzed before (control), and at 12 h (+12 h) and 10 d (+10 d) after light exposure. Shown are representative images of the most affected areas. White arrows point to pyknotic nuclei. $N = 3$. B) Scotopic a- and b-wave amplitudes and photopic b-wave amplitudes of 129S6 mice before (control, black line), at 12 h (+12 h, red line) and at 10 d (+10 d, blue line) after light exposure are presented as a function of flash intensities. Wave amplitudes significantly different from dark control are shown in full color, wave amplitudes not significantly different from dark control are shown in opaque colors. Shown are mean values \pm SD of $N = 3$ mice (5–6 eyes). Two-way ANOVA with Holm-Sidak multiple comparisons test was used to compare wave amplitudes of all conditions. Amplitudes are presented in μ V (y-axis) and flash intensities as log ($\text{cd} \times \text{s}/\text{m}^2$) (x-axis). RPE, retinal pigment epithelium; PS, photoreceptor segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 100 μ m.



removal of cornea and lens eyecups were post-fixed for additional 2 h in 4% PFA, immersed in 30% sucrose (in PBS 0.1 M), embedded in tissue freezing medium (O.C.T., Leica Biosystems Nussloch GmbH, Nussloch, Germany) and stored at -80°C . Cryosections (12 μ m) were blocked in 3% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA), 0.3% Triton X-100 (Sigma) and incubated with anti-calbindin (1:250,

214005, SynapticSystems, Goettingen, Germany) and/or anti-oncut1 (1:50, sc-13050, Santa Cruz, Dallas, Texas, United States) primary antibodies overnight at 4°C . Sections were washed with PBS, incubated with Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) for 1 h at room temperature (RT), counterstained with DAPI (4',6-Diamidino-2'-

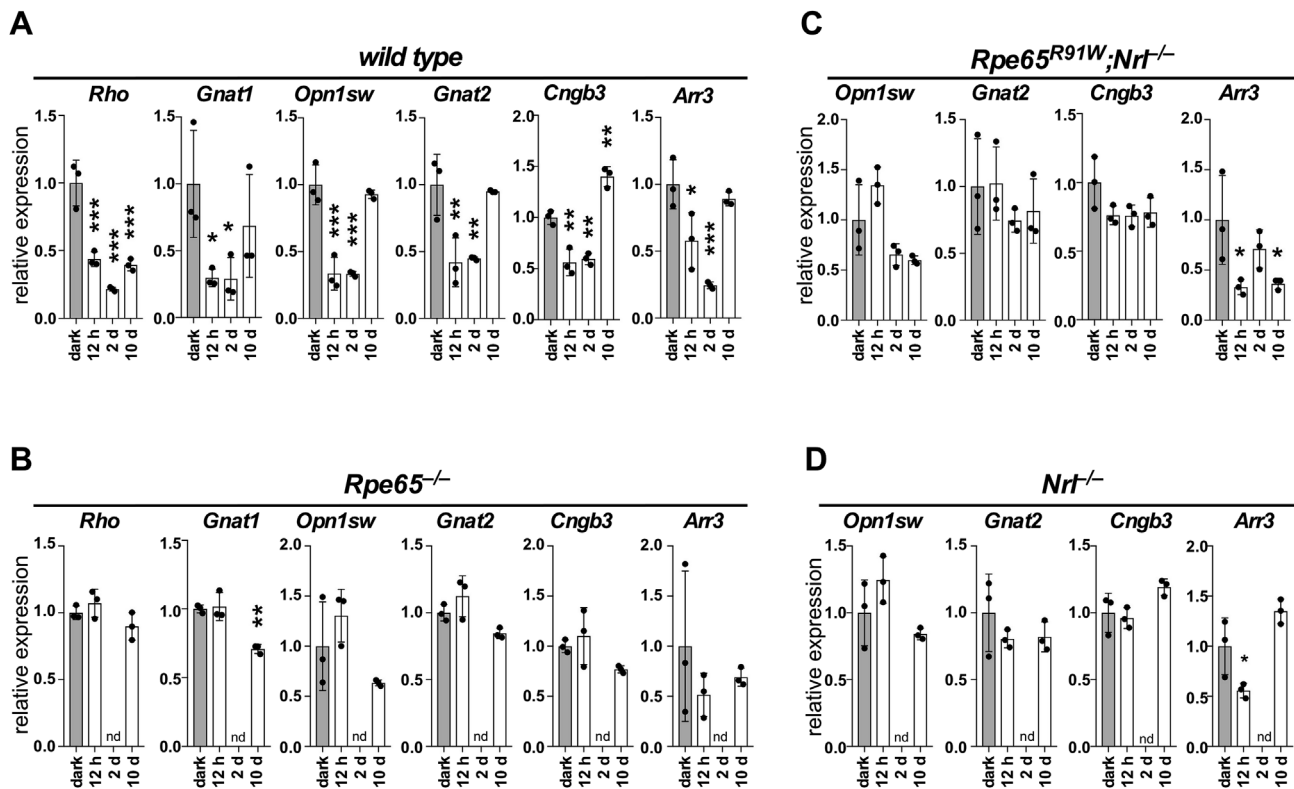


Fig. 2. Expression of rod- and cone-specific genes after light exposure. Gene expression was analyzed by real-time PCR in controls not exposed to light (dark) and at 12 h, 2 d and 10 d after light offset. Gene expression was evaluated in 129S6 wild type (A), *Rpe65*^{-/-} (B), *Rpe65*^{R91W};*Nrl*^{-/-} (C) and *Nrl*^{-/-} (D) mice as indicated. Values were normalized to *Actb* and expressed relative to dark controls which were set to 1. Shown are individual values and means \pm SD of $N = 3$. One-way ANOVA with Dunnett's multiple comparison test was used to compare each mean to dark control. Black bar: dark control. Grey bars: light-exposed. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. nd: not determined.

phenylindole dihydrochloride, Roche, Basel, Switzerland), and analyzed by fluorescence microscopy (Axioplan, Zeiss, Oberkochen, Germany).

3. Results

3.1. Partial recovery of scotopic retinal function after light exposure

Exposure to high levels of white light induces photoreceptor degeneration in mice (Wenzel et al., 2001). To test early and late effects of light exposure on retinal function, we correlated retinal morphology with ERG recordings at 12 h and 10 days (10 d) after light exposure (Fig. 1). 129S6 wild type mice showed disintegration of photoreceptor segments with some pyknotic nuclei in the most affected retinal region already at 12 h after exposure (Fig. 1A). At 10 d after exposure, most photoreceptors had died and been cleared from the retina in the damaged region. Although damage was widespread it was stronger in the central retina leaving peripheral photoreceptors intact (not shown). Light microscopy did not reveal any obvious changes to the morphology of the inner retinal layers (Fig. 1A).

Degeneration of photoreceptors was accompanied by compromised retinal function in wild type 129S6 mice. Scotopic a-wave amplitudes were significantly different from dark controls for the 6 highest flash intensities at both post-exposure time points. Scotopic b-wave amplitudes, however, were significantly different from controls for the 8 highest flash intensities at the 12 h time point but only for the 4 highest flash intensities at the 10 d time point (Fig. 1B; Table S1). This indicates a partial recovery of the scotopic b-wave but not a-wave amplitudes late after light exposure. Since the a-wave is generated by photoreceptors and the b-wave by 2nd order neurons in the inner nuclear layer (INL) (Weymouth and Vingrys, 2008), acute light exposure may have affected

also cells of the inner retina, in addition to photoreceptors. Some of the effects on the inner retina may have been transient as suggested by the partial recovery of the b-wave amplitudes at 10 d after exposure. Despite the strong deterioration of the photoreceptor segments (Fig. 1A, middle panel), the photopic ERG was not reduced at 12 h after light exposure (Fig. 1B, lower panel), indicating that cones were still intact and functional at this time point. At the late time point, however, the photopic response was slightly but significantly reduced, indicating a delayed and rather mild effect on cones (Fig. 1B; Table S2).

3.2. Light-induced repression of cone-specific genes

Although function of rods but not of cones was strongly reduced at 12 h after light exposure (Fig. 1), expression not only of rod but also of cone-specific genes was repressed at this time point. mRNAs for rhodopsin (*Rho*) and G protein subunit alpha transducin 1 (*Gnat1*), both specific for rods, were detected at roughly 60%–70% lower levels already at 12 h after exposure (Fig. 2A), probably because rods were heavily damaged by the exposure to light. Surprisingly, expression of short wavelength cone opsin (*Opn1sw*), *Gnat2*, cyclic nucleotide gated channel beta 3 (*Cngb3* (Gerstner et al., 2000),) and cone arrestin (*Arr3*), all of which are cone-specific genes, was also reduced by 40%–60% at the 12 h time point, even though ERG recordings indicated normal photopic function and did not suggest strong damage to cones (Fig. 1B). Expression of these cone-specific genes remained significantly reduced up to 2 d but recovered to normal levels at the 10 d time point. This indicates that cones reacted to acute light exposure by downregulating expression of some genes despite their low susceptibility to light-induced degeneration. In contrast to the RNA, the proteins encoded by the downregulated genes may still have been present and sufficiently active to sustain photopic function at least up to 12 h after light

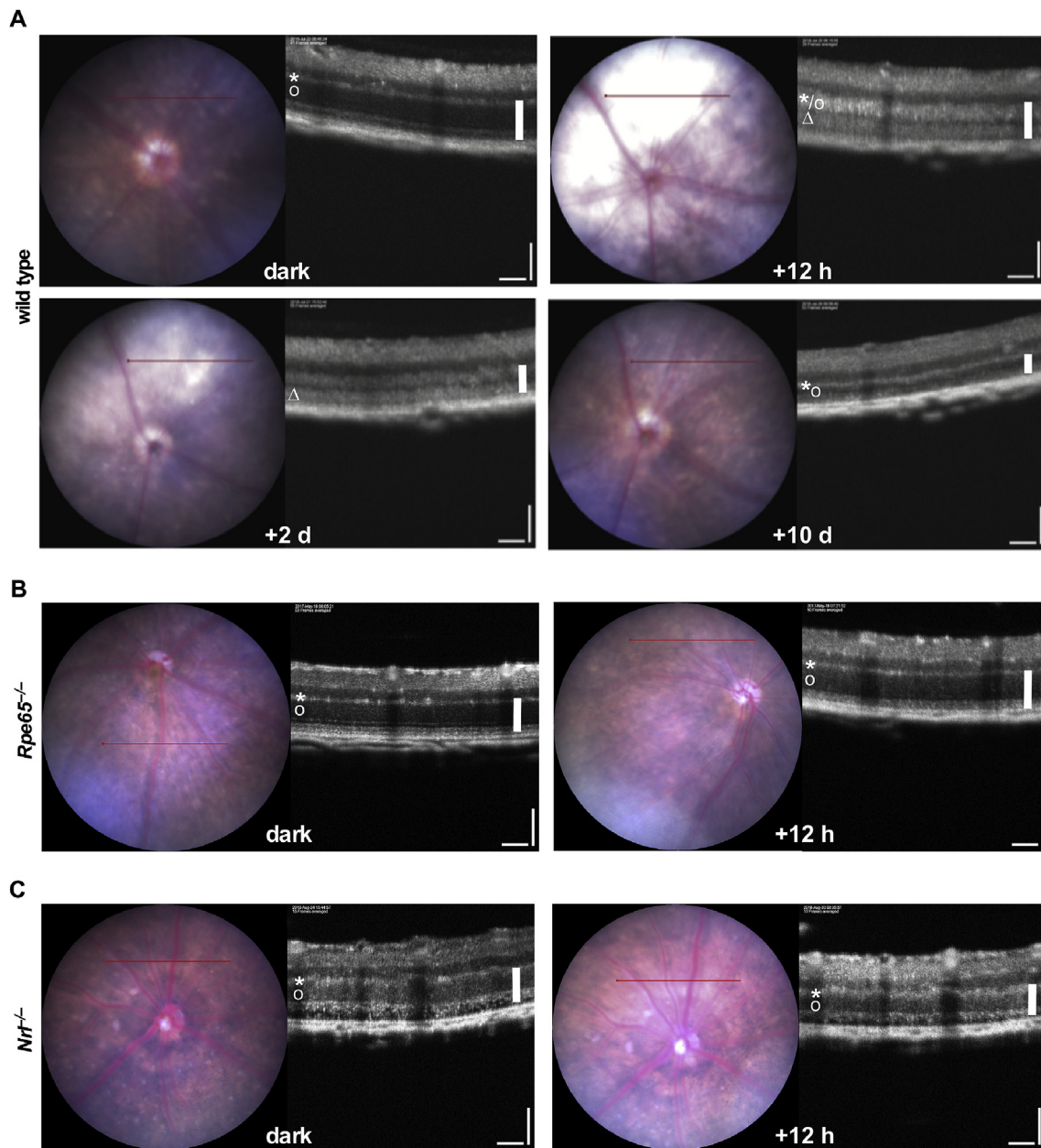


Fig. 3. Fundus and OCT imaging after light exposure. Fundus and OCT images were acquired from 129S6 wild type (A), *Rpe65*^{-/-} (B) and *Nrl*^{-/-} (C) mice before (dark) and at 12 h after light exposure (+12 h). 129S6 mice were also examined at 2 d and 10 d after light exposure. Fundus images are shown on the left with the corresponding (red line) OCT scan on the right. *: OPL. o: ONL. Δ: reflective band after light exposure. White bar: distance OPL – RPE. Scale bars: 100 μm.

exposure.

It has been shown that light damage depends on bleachable rhodopsin (Grimm et al., 2000; Wenzel et al., 2001) and that *Rpe65*^{-/-} mice lacking 11-*cis*-retinal are resistant to light-induced retinal degeneration (Grimm et al., 2000). We tested whether the reduced expression of cone-specific genes after exposure to damaging light also depended on light absorption by visual pigments or whether downregulation was a direct effect of light and possibly oxidative stress. Light exposure did not affect expression of any of the tested rod- and cone-specific genes in *Rpe65*^{-/-} mice with the exception of *Gnat1* at the 10 d time point (Fig. 2B). This indicated that reduced gene expression in cones of wild type mice was a result of light absorption by rhodopsin and/or cone visual pigments. To investigate whether cone opsins contributed to light-induced repression of cone-specific genes, we performed the same experiment with *Rpe65*^{R91W}; *Nrl*^{-/-} mice that are characterized by a functional all-cone retina (Samardzija et al., 2014). In these mice light

exposure did not reduce expression of cone-specific genes, with the exception of *Arr3* (Fig. 2C). To exclude the possibility that the reduced 11-*cis*-retinal levels in *Rpe65*^{R91W}; *Nrl*^{-/-} mice (Samardzija et al., 2014) caused this effect, we additionally exposed *Nrl*^{-/-} mice (Mears et al., 2001), which have a 5-fold higher content of the retinal chromophore (Samardzija et al., 2014). Similarly to *Rpe65*^{R91W}; *Nrl*^{-/-} mice, light-exposed *Nrl*^{-/-} mice did not reduce expression of cone-specific genes, again with the exception of *Arr3*. This suggests that downregulation of cone-specific genes by exposure to high levels of white light was not mediated through light-absorption by cone pigments, but required the presence of rods and absorption of light by rhodopsin. Since *Arr3* mRNA levels were not only reduced in light-exposed wild type and cone-only (*Rpe65*^{R91W}; *Nrl*^{-/-} and *Nrl*^{-/-}) but also in *Rpe65*^{-/-} mice (by roughly 50%, without reaching significance) that lack bleachable photopigments, we conclude that expression of *Arr3* may be directly affected by the oxidative stress generated by light exposure. Downregulation of

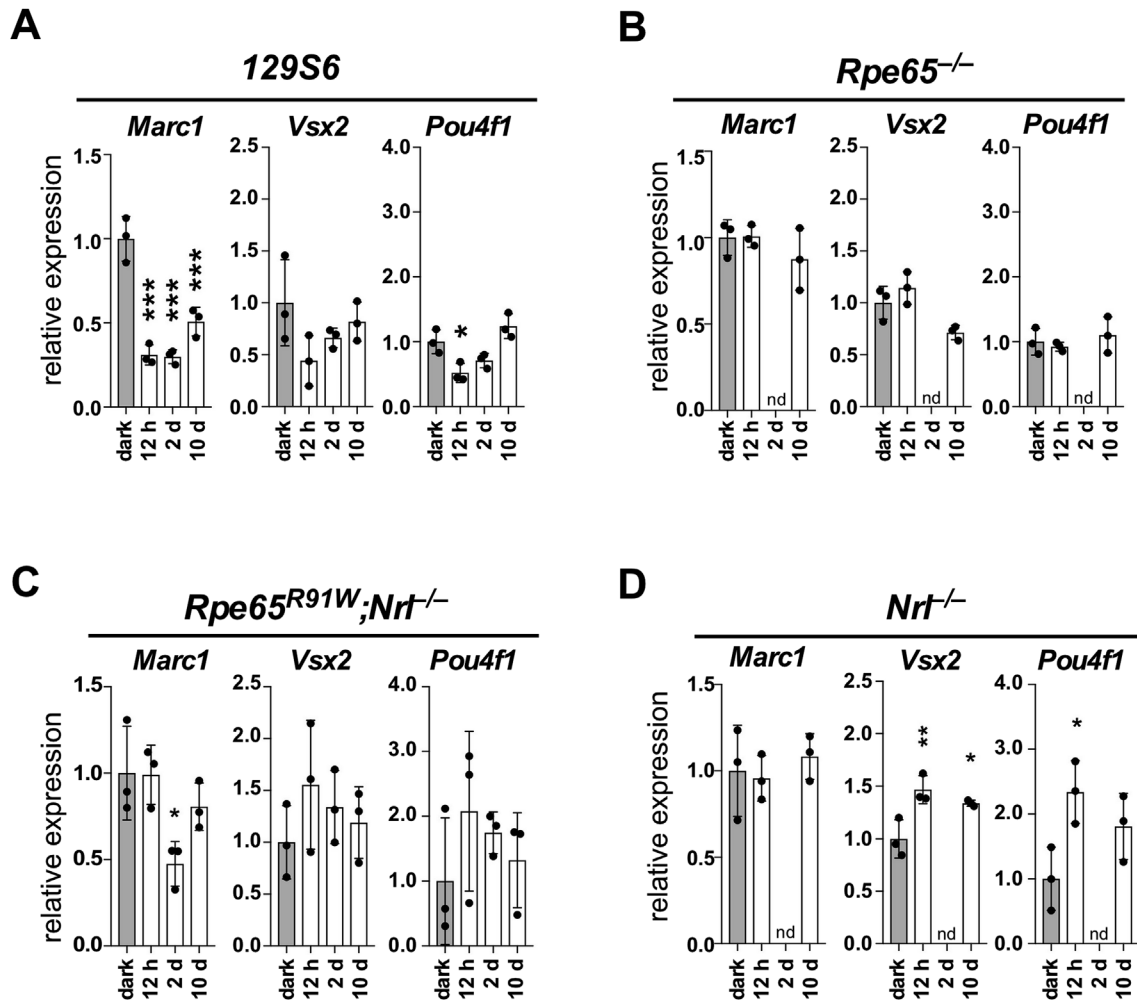


Fig. 4. Expression of marker genes of inner retinal cells after light exposure. Expression levels of *Marc1* (horizontal cells), *Vsx2* (bipolar cells) and *Pou4f1* (ganglion cells) were analyzed by real-time PCR in 129S6 wild type mice (A), *Rpe65*^{-/-} (B), *Rpe65*^{R91W};*Nrl*^{-/-} (C) and *Nrl*^{-/-} (D) mice before (dark) and at 12 h, 2 d and 10 d after offset of light exposure. Values were normalized to *Actb* and expressed relative to dark controls, which were set to 1. Shown are individual values and means \pm SD of $N = 3$. One-way ANOVA with Dunnett's multiple comparison test was used to compare each mean to dark control. Black bar: dark control. Grey bars: light exposed. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. nd: not determined.

other cone-specific genes (*Opn1Sw*, *Gnat2* and *Cngb2*), however, depended on the presence of bleachable rhodopsin in rods. Even though mice were on different genetic backgrounds (see Materials and Methods) which may influence their response to light exposure, our data imply that repression of gene expression in cones after light exposure may involve some form of rod-to-cone communication.

3.3. Effects of high levels of white light on the inner retina

The time-dependent and partial recovery of the scotopic ERG b-wave (Fig. 1B) suggests that not only photoreceptors but also cells and/or structures of the inner retina were affected by light exposure. This is supported by optical coherence tomography (OCT) scans of wild type retinas at 12 h after light exposure (Fig. 3A). At this time point, the outer retina including the outer plexiform layer (OPL) was hyperreflective, OPL (*) and ONL (o) were no longer well separated and a thick reflective band (Δ), likely representing debris of photoreceptor segments (see Fig. 1A), was prominent in the outer retina (Fig. 3A, +12 h). The overall thickness of the retina was not affected. These changes resolved gradually until 10 d post-exposure when OPL and ONL were clearly separated again. Also, the reflective band in the outer retina had largely disappeared at this time point reinforcing our interpretation that it may have been generated by photoreceptor debris which was

now cleared from the subretinal space (compare to Fig. 1A). The ONL appeared much thinner resulting in a reduced overall thickness of the retina and supporting our morphological findings of strong photoreceptor death and clearance after light exposure (Fig. 1A). In summary, light exposure induced not only the expected changes in the outer retina, but also at the intersection of OPL and ONL. Although these latter changes were at least partially resolved with time this suggests that light exposure affected cells and/or structures adjacent to photoreceptor synapses.

In contrast to wild type mice, the ONL and OPL of light-resistant *Rpe65*^{-/-} mice remained unchanged and clearly separated in OCT scans at 12 h after light exposure (Fig. 3B) indicating that hyperreflectivity of the ONL and OPL depended directly or indirectly (through degeneration) on light absorption by bleachable visual pigments. Since light exposure of *Nrl*^{-/-} mice, which have functional cone opsins but not rhodopsin did also not affect the appearance of the retina in the OCT scans (Fig. 3C), our data suggest that alterations of the OPL and ONL depended on excessive light absorption by rhodopsin and not by cone opsins.

The recovery of the light-induced changes at the OPL/ONL border in wild type mice as well as the lack of changes in *Rpe65*^{-/-} and *Nrl*^{-/-} mice correlated with the time course and pattern of gene expression in cones of the respective mice. This may indicate that the OPL is involved

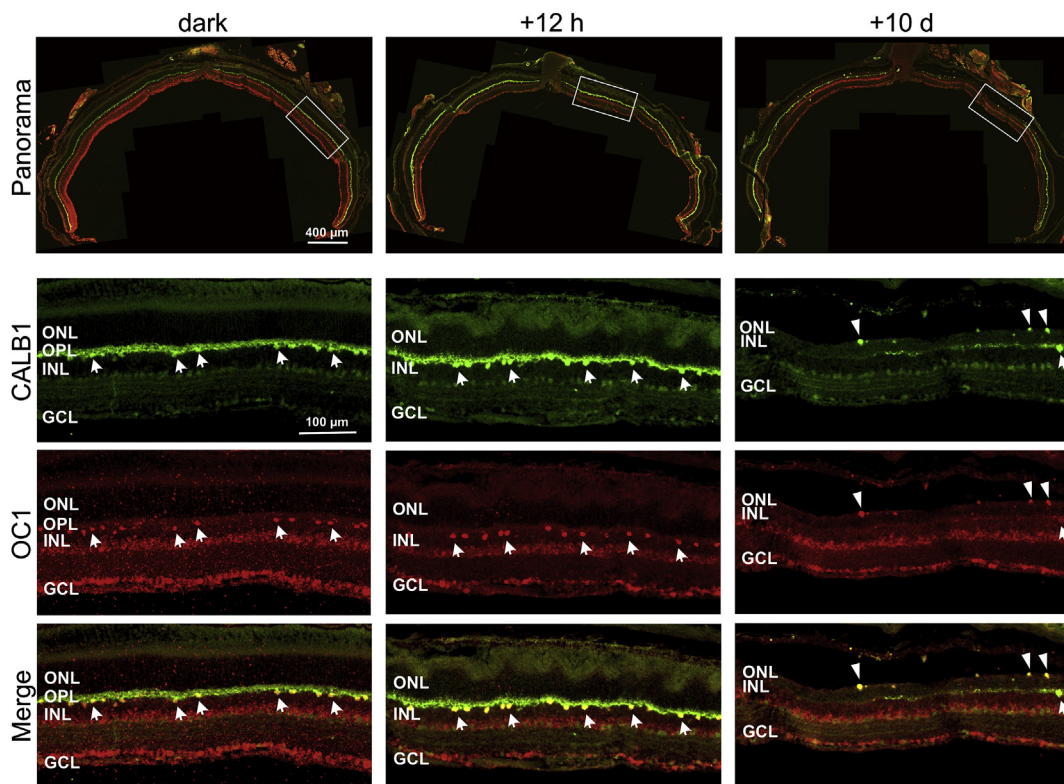


Fig. 5. Loss of horizontal cells after light exposure. Retinal sections of wild type mice before (dark) or at 12 h and 10 d after light exposure were stained with antibodies against CALB1 (green) and OC1 (red). Merged images indicate co-localization (yellow) of CALB1 and OC1. Top panels show full panoramas of merged images. Lower panels show higher magnifications of boxed areas indicated in the top panels. Shown are representative stainings of $N = 3$ mice. Arrows: horizontal cells. Arrow heads: autofluorescent material at the outer border of the remaining ONL. Abbreviations as in Fig. 1. Scale bar: 100 μm .

in the communication between rods and cones leading to repression of cone-specific genes after rhodopsin-mediated light absorption. Since HCs connect rods to cones through synaptic signal transmission in the OPL (reviewed in (Fain and Sampath, 2018)), they are good candidates to be involved in this communication.

The hypothesis that light absorption by bleachable rhodopsin affects cells of the inner retina including HCs is supported by gene expression studies of markers for HCs (mitochondrial amidoxime reducing component 1, *Marc1*), bipolar cells (visual system homeobox 2, *Vsx2*) and ganglion cells (POU class 4 homeobox 1, *Pou4f1*). Expression of all three marker genes appeared reduced by about 50% at the early time point in light-exposed wild type mice, although significance was only reached for *Marc1* and *Pou4f1* (Fig. 4A). Importantly, gene expression was only reduced in the presence of bleachable rhodopsin, but not in mice that express only cone opsins (*Rpe65^{R91W}*; *Nrl^{-/-}* and *Nrl^{-/-}*) or mice lacking the chromophore (*Rpe65^{-/-}*; Fig. 4B–D). This indicates that absorption of excessive light by rhodopsin affects gene expression in 2nd and 3rd order neurons.

3.4. Loss of horizontal cells

Our data suggest a strong but indirect (through bleaching of rhodopsin) reaction of HCs to light exposure already early after lights off (Fig. 4). Since expression of *Marc1* did only partially recover until 10 d post-illumination, HCs may have been permanently damaged in the light-exposed wild type retina. To test this, we used anti-calbindin (CALB1) and anti-onectin 1 (OC1) antibodies to specifically detect HCs (Haverkamp and Wässle, 2000; Wu et al., 2012) by immunofluorescence before and after light exposure (Fig. 5, arrows). Fewer CALB1- and OC1-positive signals were present at the outer border of the INL at the 10 d time point. In the most affected region, the OPL was even almost devoid of CALB1 and OC1-positive material suggesting that

light exposure led to loss of HCs. The green and red signals (Fig. 5, arrowheads) at the outer border of the remaining ONL at 10 d after light exposure was autofluorescent material that partially co-localized with IBA-1 positive phagocytic cells (not shown). This material may thus represent cellular debris that is being cleared from the subretinal space.

3.5. LIF may be involved in signaling to cones and horizontal cells

Developmental overexpression of LIF decreases expression of phototransduction genes in rods and cones (Graham et al., 2005) and application of recombinant LIF down-regulates the visual cycle by reducing RPE65 levels and activity in the RPE (Chucair-Elliott et al., 2012). Thus, we tested whether endogenous LIF may be involved in regulating cone-specific gene expression also after light exposure.

Light exposure strongly induced *Lif* expression (65-fold) within 12 h after lights off in *Lif^{+/-}* mice (Fig. 6A), as expected (Samardzija et al., 2006). Expression of rod-specific genes (*Rho*, *Gnat1*) was similarly reduced in light-exposed *Lif^{-/-}* and *Lif^{+/-}* control mice (Fig. 6B). However, expression of cone-specific genes was significantly less down-regulated in *Lif^{-/-}* mice (Fig. 6C), even though light-induced retinal degeneration in *Lif^{-/-}* mice is stronger than in wild type mice (Bürge et al., 2009). The effect was less pronounced for *Arr3*, which may be regulated by additional or other mechanisms such as oxidative stress, as suggested above. In addition to cone-specific genes, the horizontal cell marker *Marc1* was also less affected in *Lif^{-/-}* mice whereas markers for bipolar and ganglion cells were not differentially regulated in *Lif^{-/-}* and control mice (Fig. 6D). This indicates that endogenously produced LIF may indeed be involved in the repression of cone-specific genes and may also affect HCs after light exposure. However, additional factors are likely to contribute to this effect since lack of LIF did not completely abolish downregulation of the genes that were tested.

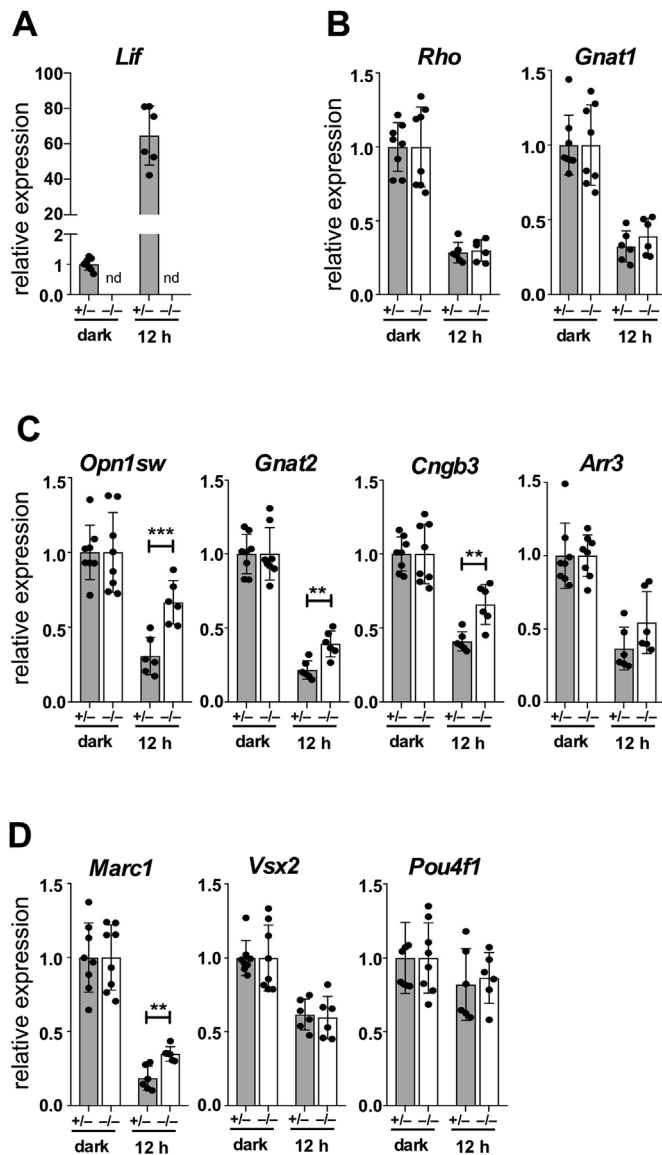


Fig. 6. Contribution of LIF to downregulation of photoreceptor-specific genes after light exposure. Gene expression in *Lif*^{+/+} (+/+) and *Lif*^{-/-} (-/-) mice was analyzed before (dark) and at 12 h after exposure. Dark control levels for each strain were set to 1. **A**) Expression of *Lif* after light exposure. **B-D**) Expression of rod-specific (**B**) and cone-specific (**C**) genes and of genes specific for horizontal cells (*Marc1*), bipolar cells (*Vsx2*) and ganglion cells (*Pou4f1*) (**D**). Shown are individual values and means \pm SD of *N* = 3 mice (6 eyes; *Lif*^{+/+}, + 12 h; *Lif*^{-/-}, + 12 h) or *N* = 4 mice (8 eyes; *Lif*^{+/+}, dark; *Lif*^{-/-}, dark). mRNA levels of light-exposed mice were compared using a Student's *t*-test. Black bars: *Lif*^{+/+}. Grey bars: *Lif*^{-/-}. **: *P* < 0.01; ***: *P* < 0.001. nd: not determined.

4. Discussion

Exposure to high levels of visible light induces degeneration of rod photoreceptors while survival of cones is not or much less affected (Yetemian et al., 2010; Okano et al., 2012) (Collier et al., 1989). Due to the predominance of rods in the mouse retina, only little is known about molecular effects of light exposure on cones and/or cells of the inner retina. Here we show that exposure to high levels of visible light induces degeneration not only of rods but also of HCs, and transiently affects gene expression in cones. These effects depend on light absorption by rhodopsin and may, at least partially, involve endogenous LIF.

4.1. Light exposure affects cones and the inner retina through photon absorption by rhodopsin

Exposure to high levels of white light caused severe retinal degeneration in wild type mice (Fig. 1). While loss of rods is thought to be a direct effect of rhodopsin-mediated excessive light absorption, degeneration of cones may be secondary to rod loss (Krebs et al., 2009). Such a delayed but mild degeneration of cones may be reflected by the reduced photopic b-wave at 10 d but not at 12 h after light exposure in wild type mice (Fig. 1B, Table S2). Alternatively, reduced cone function might be connected to loss of HCs at the 10 d time point, as it was shown that artificial ablation of HCs leads to both rod and cone dysfunction (Sonntag et al., 2012). Intriguingly, RNA levels of several cone-specific transcripts were significantly downregulated already early after light exposure but recovered with time, indicating that cones adapt their metabolism quickly but transiently in response to rod damage.

Not only photoreceptors but also the inner retina responded to light exposure (Fig. 3). Some of the observed effects including changes at the OPL/ONL intersection were transient, similar to the repression of gene expression in cones. Others, like the b-wave amplitude of the scotopic ERG, recovered only partially with time. Since the generation of the scotopic b-wave depends on input from rod photoreceptors, it is unavoidable that retinal degeneration leads to reduced amplitudes. However, since scotopic a-wave amplitudes were similar at 12 h and 10 d post exposure, 2nd order neurons received similar inputs from the ONL at both time points and thus, scotopic b-wave amplitudes should also have been similar if 2nd order neurons were not otherwise affected by light exposure. Therefore, increased b-wave amplitudes at the late time point indicate partial functional recovery of neurons in the inner retina, a phenomenon that parallels the re-appearance of the clear separation of OPL and ONL as detected in OCT scans (Fig. 3). Thus, our data indicate that reduced function of scotopic b-wave generating 2nd order neurons (Weymouth and Vingrys, 2008) was not solely due to photoreceptor degeneration, but was at least partially caused by a reduction of these cells to the strong light insult.

HCs appeared to be quickly and non-reversibly affected by light exposure. Expression of *Marc1* was reduced already at 12 h after light exposure and remained low at least until the 10 d time point. Most likely, affected HCs eventually degenerated as the number of CALB1- and OC1-positive cells was strongly reduced in the INL at the late time point. The light-induced effects in cones and cells of the inner retina depended on light absorption by the visual pigment in rods. Exposure of *Rpe65*^{-/-} mice, which lack bleachable visual pigments due to the absence of 11-*cis*-retinal (Redmond et al., 1998), or of *Nrl*^{-/-} mice, which lack rhodopsin but not bleachable cone opsin (Mears et al., 2001), did not result in changes of the OPL/ONL intersection in OCT scans, or in reduced expression of marker genes for cones and inner retinal neurons. Identifying the responsible mechanisms that originate from rods and signal to cones and cells of the inner retina will help to understand intercellular communication and network connections in the retina.

4.2. Signaling from rods to cones

Our data indicate that consequences of strong light exposure are not restricted to rod photoreceptors and argue for a communication between damaged rod cells and cones as well as cells of the inner retina, probably in an attempt to maintain tissue homeostasis under stress conditions. HCs are strong candidates for being involved in such a signaling mechanism. Not only are HCs strongly affected by light-induced retinal degeneration (Fig. 5) but they are also natural connector cells that contact rods with their axon and cones with their dendrites to provide inhibitory feedback signals for contrast inhibition (Peichl and González-Soriano, 1994; Szikra et al., 2014; Boije et al., 2016). These lateral connections make horizontal cells ideal not only for integrating light-evoked signals but also for transmitting information concerning the physiologic status of cells between photoreceptors in the ONL.

Other cells that are activated by damaged rods are Müller glia cells that react with the production of LIF as survival factor, which helps the retina to cope with light stress (Joly et al., 2008; Santiago et al., 2018). LIF is not only neuroprotective for photoreceptor cells (Joly et al., 2008; Bürgi et al., 2009; Santiago et al., 2018), but was also identified as factor that can downregulate expression of photoreceptor-specific genes. When overexpressed during retinal development, LIF prevented normal expression levels of *Rho* and cone opsins (Graham et al., 2005), and intravitreal injection of recombinant LIF in adult mice resulted in the downregulation of rhodopsin and rod transducin in wild type and rd1 mice, respectively (Chucair-Elliott et al., 2012) (Joly et al., 2008). Our data support similar activities also for endogenously produced LIF in the damaged retina and extend its influence to HCs as indicated by the reduced downregulation of *Marc1* in light exposed *Lif*^{-/-} mice (Fig. 6D), even though *Lif*^{-/-} mice are more susceptible to light damage than wild type mice (Bürgi et al., 2009). LIF, however, may not be the only factor participating in the downregulation of gene expression. Although cone-specific genes were significantly less repressed in light-exposed *Lif*^{-/-} mice, expression levels were still below the levels in control retinas (Fig. 6C). Thus, LIF may act in concert with HC-mediated and/or other signaling mechanisms to repress gene expression in cones of the light-exposed retina.

4.3. Hypothesis

Excessive light is absorbed by rhodopsin. This results in rod damage, which activates a subpopulation of Müller glia cells to produce LIF as a neuroprotective measure. In addition, absorption of excessive light by rhodopsin also affects horizontal cells, the 2nd order neurons in the inner retina that connect rods with cones for lateral inhibition. We hypothesize that HCs signal rod damage to cones causing them to repress gene expression early after light damage. This may support the activity of LIF and may help cones to survive, e.g. by channeling their energy into repair processes. Since Müller cells produce LIF only transiently (Joly et al., 2008) and HCs eventually die after light-induced photoreceptor degeneration, the putative repressing signals from Müller cells and HCs may disappear with time. This may allow cones to regain expression of their genes at a later time point after light exposure.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exer.2019.107719>.

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