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Original Contribution

Thioredoxin rod-derived cone viability factor protects against photooxidative retinal damage

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ABSTRACT

Rod-derived cone viability factor (RdCVF) is a trophic factor of the thioredoxins family that promotes the survival of cone photoreceptors. It is encoded by the nucleoredoxin-like gene 1 *Nxnl1* which also encodes by alternative splicing a long form of RdCVF (RdCVFL), a thioredoxin enzyme that interacts with TAU. The known role of thioredoxins in the defense mechanism against oxidative damage led us to examine the retinal phenotype of the *Nxnl1*$^{-/-}$ mice exposed to photooxidative stress. Here we found that, in contrast to wild-type mice, the rod photoreceptors of *Nxnl1*$^{-/-}$ mice are more sensitive to light after exposure to 1700 or 2500 lx. The delivery of RdCVF by AAV to mice deficient of *Nxnl1*$^{-/-}$ protects rod photoreceptors from light damage. Interestingly, the RdCVF2L protein, encoded by the paralog gene *Nxnl2*, is able to reduce TAU phosphorylation, as does RdCVFL, but does not protect the rod from light damage. Our result shows that the *Nxnl1* gene, through the thioredoxin RdCVFL, is part of an endogenous defense mechanism against photooxidative stress that is likely of great importance for human vision.

Introduction

The rate of oxygen metabolism is inversely correlated to life span [1,2]. During the life of aerobic organisms, reactive oxygen species (ROS), which are formed *in situ* in response to oxygen metabolism, can cause various forms of tissue damage. In order to reduce the damaging effect of ROS on cellular macromolecules, the redox potential of the cell is carefully regulated. Contributing to such homeostasis, aerobic organisms have developed a number of antioxidant systems, and among these are those mediated by superoxide dismutase, catalase, and the thioredoxin proteins [3]. Thioredoxin 1 (TXN), the founder of the thioredoxin family of proteins, is a 12 kDa protein with a redox active disulfide/dithiol group within its conserved active-site sequence CGPC. Reduced TXN catalyzes the reduction of disulfide bounds in many target proteins, and oxidized TXN is reversibly reduced by the action of thioredoxin reductase and NADPH [4].

Retinal photoreceptor (PR) cells are particularly prone to oxidative damage because of their high consumption of oxygen, their high proportion of polyunsaturated fatty acids, and the capture of energetic photons from visible light by opsin molecules encased in their outer segment (OS): rhodopsin in rods and cone opsins in cones [5]. TXNs have been implicated in promoting PR health and function in this highly oxidizing environment. Transgenic overexpression of TXN protects PRs against photooxidative damage [6] and up-regulation of TXN via the nuclear factor erythroid 2-related factor 2 (NRF2)-antioxidant response elicits an endogenous neuroprotective mechanism [7]. TXN plays an important role in maintaining a reducing environment in living cells and prevents apoptosis by sequestering the MAP kinase ASK1 under normoxic conditions [8,9].

Abbreviations: AAV, adeno-associated viral vector; AMD, age-related macular degeneration; ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; GO, Gene Ontology; ONH, optic nerve head; ONL, outer nuclear layer; OS, outer segments; PR, photoreceptor; RdCVF, rod-derived cone viability factor; RPE, retinal pigmented epithelium; ROS, reactive oxygen species; RP, retinitis pigmentosa; SEM, standard error of the mean; TXN, thioredoxin; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling.

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Nxnl1 was identified in a high content screen for secreted factors promoting cone survival [10]. *Nxnl1* encodes two protein products by alternative splicing [11]. One, rod-derived cone viability factor (RdCVF) which results from intron retention, is a truncated thioredoxin protein that lacks thioredoxin enzymatic activity. RdCVF slows down secondary cone degeneration in two genetically distinct rodent models of the inherited retinal degeneration retinitis pigmentosa (RP) [12,13]. The other, RdCVFL, generated by splicing of intron 1, exhibits thioredoxin enzymatic activity [14]. The retina of the *Nxnl1*$^{-/-}$ mice displays evidence of increased oxidative damage under normoxic conditions, and cone function is altered when the animals are raised in 75% oxygen [15]. As demonstrated for its paralogue *Nxnl2*, *Nxnl1* is probably bifunctional, with one product being a trophic factor and the second, a thioredoxin protein that increases the length of photoreceptor outer segments of the *Nxnl2*$^{-/-}$ mouse [16]. *In vitro*, RdCVFL protects cones against UV damage [17,18]. We found that RdCVFL interacts with the microtubule-associated protein TAU and protects it from oxidative damage [19]. The aggregation of TAU in the *Nxnl1*$^{-/-}$ retina is most likely linked to the progressive rod degeneration in that model [15].

In the retina, ROS are produced by light exposure and their concentration is increased under conditions of elevated blood oxygen [20,21]. Light-induced damage primarily affects rods [22]. These neurons discern dim light (scotopic vision) and are very sensitive to light within the maximum absorption spectra of their visual pigment, rhodopsin [23]. Rod degeneration in the first phase of RP is triggered by mutations in more than 50 different genes [24] and results in an increase in oxygen tension, which in turn affects the cones [25,26]. Taken together, these results suggest that RdCVFL is involved in the defense mechanism against light-induced oxidative injury on photoreceptors, rods, and cones, while RdCVF protects cones.

We show here that the rods of the *Nxnl1*$^{-/-}$ mouse are more susceptible to photooxidative damage. We also demonstrate that virally mediated reexpression of thioredoxin RdCVFL in the *Nxnl1* null mouse protects rods against light-induced damage.

Materials and methods

Animals

All procedures adhered to the ARVO statement for the use of animals in ophthalmic and vision research and have been approval by the Darwin ethic committee (Ce5/2010/008). Animals, males and females in equal proportion, were raised under 12 h, 50 lux (lx)/12 h, dark cycle and given ad libitum access to food and water. Mice used were 11–16 weeks of age. *Nxnl1*$^{-/-}$ and *Nxnl2*$^{-/-}$ mice were generated on a pure BALB/c background [15,16].

Light illumination

Mice were first dark-adapted 16 h prior to light exposure. They were exposed to a regulated amount of light, generated by cool, white fluorescent lamps, with animals placed in aluminum foil-wrapped polycarbonate cages (one mouse per cage) for 1 h. The mice then recovered for 24 h in the dark. The temperature was monitored not to be above 25 °C. A 0 lx corresponds to animals left in the dark while their littermates were exposed. Beyond 24 h, mice were returned to 12 h light/dark cycles of 50 lx.

Outer nuclear layer measurement

Animals were anesthetized 10 days after exposure with ketamine (160 mg/kg)/xylazine (32 mg/kg) and immediately perfused with 2.5% glutaraldehyde and 2% formaldehyde in phosphate-buffered saline (PBS). Eyes were enucleated and incubated in fixative overnight. Lenses were removed and eye cups washed 5 times in 5% sucrose, fixed for 1 h in 2% osmium tetroxide, and embedded in epoxy resin. Sections of 1 μm thick were made along the sagittal axis at the optic nerve level and stained with 1% toluidine blue. Measurements of the outer nuclear layer (ONL) thickness were made on one section from each mouse as described [27]. Briefly, in each of the dorsal (superior) and ventral (inferior) hemispheres, ONL thickness was measured in nine sets of three measurements each (27 measurements for each hemisphere). The first sets were centered 250 μm from the optic nerve head (ONH) and subsequent sets were located more peripherally. Within each 250 μm position, three measurements were made equidistant to each other by 50 μm. Those 54 measurements cover the entire retinal surface. Data were plotted as spidergrams from 6 animals for each condition.

Quantitative RT-PCR

QRT-PCR was performed according to Léveillard et al. [10]. RdCVF forward 5′-ATGGCATCTCTCTTCTCTGGACG-3′ and reverse 3′-CCTCACCTCCTCAGTTCATC ATGG-5′; RdCVFL forward 5′-GCAACAGGACCTCTTCCTCA-3′ and reverse 3′-CCAGACGCTGGATCTCCTC-5′; *Rps6* forward 5′-AAGCTCCGCACCTTCTATA-3′ and reverse 3′-ACTCTGCCATGGGTCAGAAC-5′. The expression of *Rps6* (ribosomal protein S6) was used for normalization.

Production and delivery of the AAV constructs to the Nxnl1$^{-/-}$ *mouse retina*

The adeno-associated viral vector (AAV)2/8-RdCVFL, AAV2/8-RdCVF2L, and AAV2/8-GFP were generated as described in Jaillard et al. [16]. Animals were anesthetized, and subretinal injections were performed with 1 μl of AAV2/8-RdCVFL, AAV2/8-RdCVF2L, or AAV2/8-GFP at 3×10^{12} genome copies (gc/ml) into the right eye at 2 months old.

Optical coherence tomography (OCT)

Treated mice at the age of 3 months were anesthetized and pupils were dilated as described above. Eye dehydration was prevented by regular instillation of sodium chloride drops. OCT images were recorded for both eyes using a spectral domain ophthalmic imaging system (spectral domain optical coherence tomography, Bioptigen 840 nm HHP, Bioptigen, NC, USA). We performed rectangular scans consisting of a 1.4 mm by 1.4 mm perimeter with 1000A-scans per B-scan with a total B-scan amount of 100. Scans were obtained first while centered on the optic nerve, and then with the nerve displaced superiorly/inferiorly. OCT scans were exported from InVivoVue as AVI files. These files were loaded into ImageJ (version 1.47, National Institutes of Health, Bethesda, MD) where they were registered using the Stackreg plug-in. Outer nuclear layer thickness was measured in the whole eye area every 100 μm and the thicknes of each point was averaged for each treated, AAV2/8-GFP, AAV2/8-RdCVFL, and AAV2/8-RdCVF2L ($n=6$).

Transmission electron microscopy

Animals were sacrificed 24 h after light exposure. The eye cups were fixed in 2.5% glutaraldehyde at room temperature for 2 h, washed overnight, and postfixed in osmium tetraoxide 1% for 1 h at room temperature. Samples were washed in Ringer-Krebs buffer (140 mM NaCl; 4.5 mM KCl, 2.2 mM $CaCl_2$, 12 mM $MgSO_4$, 12 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 5.55 mM glucose, pH 7.4) followed by dehydration in graded ethanol and acetone. Samples were embedded in epoxy resin and ultrathin sections (~500 nm) were cut and stained with uranyl acetate and Pb–citrate and observed using electron microscopy (Met Zeiss 912, at 80 kV).