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A Reduced Zinc Diet or Zinc Transporter 3 Knockout Attenuate Light Induced Zinc Accumulation and Retinal Degeneration△

Shi Bai[a], **Carolyn R. Sheline**[a,b], **Yongdong Zhou**[a], and **Christian T. Sheline**[a,c]

[a]Department of Ophthalmology and The Neuroscience Center of Excellence LSU Health Sciences Center 2020 Gravier Street, Suite D New Orleans, LA 70112
sbai@lsuhsc.eduyzhou@lsuhsc.edu

Abstract

Our previous study on retinal light exposure suggests the involvement of zinc (Zn^{2+}) toxicity in the death of RPE and photoreceptors (LD) which could be attenuated by pyruvate and nicotinamide, perhaps through restoration of NAD^+ levels. In the present study, we examined Zn^{2+} toxicity, and the effects of NAD^+ restoration in primary retinal cultures. We then reduced Zn^{2+} levels in rodents by reducing Zn^{2+} levels in the diet, or by genetics and measured LD. Sprague Dawley albino rats were fed 2, or 61 mg Zn^{2+}/kg of diet for 3 weeks, and exposed to 18 kLux of white light for 4h. We light exposed (70 kLux of white light for 50h) Zn^{2+} transporter 3 knockout (ZnT3-KO, no synaptic Zn^{2+}), or RPE65 knockout mice (RPE65-KO, lack rhodopsin cycling), or C57/BI6/J controls and determined light damage and Zn^{2+} staining. Retinal Zn^{2+} staining was examined at 1h and 4h after light exposure. Retinas were examined after 7d by optical coherence tomography and histology. After LD, rats fed the reduced Zn^{2+} diet showed less photoreceptor Zn^{2+} staining and degeneration compared to a normal Zn^{2+} diet. Similarly, ZnT3-KO and RPE65-KO mice showed less Zn^{2+} staining, NAD^+ loss, and RPE or photoreceptor death than C57/BI6/J control mice. Dietary or ZnT3-dependent Zn^{2+} stores, and intracellular Zn^{2+} release from rhodopsin recycling are suggested to be involved in light-induced retinal degeneration. These results implicate novel rhodopsin-mediated mechanisms and therapeutic targets for LD. Our companion manuscript demonstrates that pharmacologic, circadian, or genetic manipulations which maintain NAD^+ levels reduce LD.

Keywords

rat; mouse; rhodopsin; RPE65; ZnT3; photoreceptors

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[c]corresponding author, FAX: (504) 568-5801, telephone: (504) 599-0880 csheli@lsuhsc.edu.
[b]current address: University of California, Santa Barbara 93106; carolynsheline@gmail.com

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1. Introduction

Light-induced retinal damage can occur after acute or chronic sun exposure and surgery (Fuller et al., 1978; Zigman et al., 1979; Kuhn et al., 1991; Thanos et al., 2001; Codenotti et al., 2002; Jain et al., 2009; Vojnikovic et al., 2009). Light also accelerates disease progression and neurodegeneration in many retinal diseases in which light or oxidative stress (OS) are implicated (RP, glaucoma, macular degeneration) (Wang et al., 1997; Bicknell et al., 2002; Organisciak et al., 2003; Ranchon et al., 2003; Richards et al., 2006; Vaughan et al., 2006; Yang et al., 2007; Rodriguez and Fliesler, 2009) (Reviewed in (Organisciak and Vaughan, 2010)). Intense light damage is preferentially confined to the photoreceptors of the outer nuclear layer (ONL) in the superior central retina of rats and mice (Gordon et al., 2002; Cortina et al., 2003). This damage involves apoptotic rod cell death and necrotic cone cell death (reviewed in (Gordon et al., 2002; Organisciak and Vaughan, 2010)). Protection in inferior retina attributes to shorter outer segment and lower rhodopsin level than those in superior retina (Battelle and LaVail, 1978; Penn et al., 1987). Inferior retina also has a better intra-retinal circulation and neuroprotective factor synthesis in response to intense light exposure (Liu et al., 1998; Li et al., 2003). It was previously demonstrated that Zn^{2+} accumulation and toxicity play a role in retinal ischemia mediated cell death (Yoo et al., 2004). We have now demonstrated that photoreceptors stain for Zn^{2+} before dying after light exposure, and cyclic light, pyruvate, or nicotinamide attenuated LD (Sheline et al., 2010a).

The loosely bound or free Zn^{2+} is histochemically reactive and present physiologically in different layers of retina, and varies between dark and light. In ambient light, it notably exists in the rod inner and outer segments (RIS, ROS) of the ONL, the outer plexiform layer (OPL), and retinal pigment epithelial (RPE) cells. In the dark, however, this histochemically-reactive Zn^{2+} appears in photoreceptor perikarya of ONL (Wang et al., 2006). Zn^{2+} plays important roles in retinal functions including dark-light adaptation (reviewed in (Ugarte and Osborne, 2001)), modulating neurotransmission and regulating intracellular metabolism (Rosenstein and Chappell, 2003; Redenti et al., 2007; Chappell et al., 2008).

Zn^{2+} neurotoxicity is involved in many injuries and diseases including retinal ischemia (Yoo et al., 2004; Choi et al., 2006), global ischemia (Koh et al., 1996), trophic deprivation (Sheline et al., 2010b) and hypoglycemia mediated neuronal death (Suh et al., 2004; Suh et al., 2008). Excessive Zn^{2+} either from extracellular Zn^{2+} uptake through voltage gated calcium channels under depolarization, or release from intracellular Zn^{2+} binding proteins or organelles under oxidation is neurotoxic (Canzoniero et al., 1999; Sheline et al., 2010b). In neurons both in vitro and in vivo, excess Zn^{2+} triggers NAD^+ loss which in turn inhibits glycolysis. Pyruvate and nicotinamide restore NAD^+ levels and attenuate Zn^{2+} neurotoxicity in the central nervous system (Sheline et al., 2000; Lee et al., 2001; Suh et al., 2003; Cai et al., 2006; Sheline et al., 2010b). In this study we further investigated whether dietary or genetic reduction of Zn^{2+} levels could attenuate light-induced damage. We decreased the Zn^{2+} levels in the diet and genetically reduced Zn^{2+} levels by knocking out Zn^{2+} transporter 3 (ZnT3-KO, no synaptic Zn^{2+}) or RPE65 (RPE65-KO, no rhodopsin recycling). We propose a model in which ZnR or reduced Zn^{2+} release attenuates retinal Zn^{2+} toxicity and LD.

2. Research Design And Methods

2.1. Primary retinal culture

Primary retinal cultures were generated from retinas (16 retinas/plate) of P1 mouse pups. Retinas were isolated and mechanically dissociated into single cells by trituration with fire-

polished Pasteur pupettes. Then triturated retinas were plated in DMEM, 10% FBS, 1% glutamine, 0.1% P/S, 25mM KCL solution. Retinal cultures were grown in 5% CO_2, 95% humidity at 37°C. Toxic exposures were initia ted after 10 days of culturing. Cells were exposed to Zn^{2+} in the presence of pyruvate, nicotinamide or NAD^+. Cell viability was determined after 24h by adding in PI (5 μg/ml) for 30 min at 37°C and fluorescence measured (ex 530/em 645).

2.2. Live-cell imaging

FluoZin3 AM is a sensitive and selective fluorescent probe used to measure Zn^{2+} in living cells. FluoZin3 AM has extremely high affinity to Zn^{2+} (K_D=15nM) that is unperturbed by Ca^{2+} concentrations (Haugland, 2005). FluoZin3 AM increases in fluorescence more than 50 fold upon saturation with Zn^{2+} (Gee et al., 2002). PRC were washed and exposed to 0 or 150 μM Zn^{2+} in serum free medium for 4h (Figure 1). FluoZin3 AM (5 μM; Invitrogen/Life Technologies, Carlsbad, CA) was then loaded into washed cells for 30 min at 37°C, washed again and photomicrographs o f identical duration were taken. The specificity of increased $[Zn^{2+}]_I$ was demonstrated using a zinc chelator, TPEN. TPEN is cell permeable and selectively chelates intracellular heavy metal ions such as Zn^{2+}, Cu^{2+} and Fe^{2+} (Haugland, 2005). It has extremely high affinity to Zn^{2+} ($K_D=10^{-15.6}$, but no affinity for Ca^{2+}, or Mg^{2+}, and has been used as a Zn^{2+} specific chelator (Canzoniero et al., 2003). Adding TPEN to Zn^{2+}-, or H_2O_2- treated cell cultures completely blocked the increased signal detected by FluoZin3 (data not shown).

2.3. Zn^{2+} restricted and normal diets in the rat light damage model

Four wk old Sprague-Dawley albino rats (Charles River, Wilmington, MA) weighing 70g were fed a 2 mg/kg purified Zn^{2+} diet, and 61mg/kg purified Zn^{2+} diet for 3 wk as previously reported (Sheline, J. Nutrition, accepted Takeda et al., 2003; Suh et al., 2009). Zn^{2+} deficient diet contains ~1 mg/kg diet, which was supplemented with 1, or 60 mg Zn^{2+}/kg diet Zn^{2+} equivalent $ZnSO_4$ in 18 mega-Ohm purified drinking water(= 2 or 61 ppm). The animals were maintained in plastic cages with plastic water bottles to minimize Zn^{2+} contamination. Rats fed normal standard diet which contains 61 mg/kg Zn^{2+} were supplemented with 18 mega-Ohm purified drinking water (normal Zn^{2+} diet). The Zn^{2+} restricted diet was from Harlan Teklad (TD.85419, Madison WI), and the normal diet (contains 61 mg/kg Zn^{2+}) was also from Harlan Teklad (2019). The Zn^{2+} deficient diet is composed of: 200 g/kg of egg white solids, 634 g/kg of dextrose, 100 g/kg of corn oil, 30 g/kg of cellulose, 10 g/kg of vitamin mix, 0.004 g/kg of biotin, 25.7 g/kg of Zn^{2+} deficient mineral mix, 0.02 g/kg of chromium potassium dodecahydrate, and 0.02 g/kg of ethoxyquin. The 2019 normal diet is a standard extruded diet with 19% protein which is derived from ground wheat, ground corn, corn gluten meal, wheat middlings, and soybean oil with vitamins and minerals added. Each experiment is internally controlled as only Zn^{2+} deficient diet is used in varying the dietary Zn^{2+} (Figure 2), and only normal diet is used when the genotype is being varied (Figures 3–4). Animals consumed similar rates of diet and water with 1g food for 1ml water, and no developmental problems were observed.

2.4. Rat light damage model

Rats were dark adapted for 60h before light exposure. Pupils were dilated with 1% tropicamide ophthalmic solution USP in room light and exposed to bright cool white fluorescent light from 8× 20W-circular fluorescent bulbs (18,000 Lux) for 4h at <25C (starting at 8:30am) (Gordon et al., 2002). The animal chamber was ventilated and gently rotated during the light exposure to ensure temperature maintenance and that the animals were awake with their eyes open. This was followed by recovery in the dark for 24h. After this, they were returned to cyclic, dim (30 Lux) overhead fluorescent light environment for six days. All studies were conducted within guidelines established by the Institutional

Animal Care and Use Committee (Louisiana State University Health Sciences Center, New Orleans), and were in accordance with the PHS Guide for the Care and Use of Laboratory Animals, USDA Regulations, and the AVMA Panel on Euthanasia guidelines.

2.5. Genetically manipulated mice

ZnT3-KO, and RPE65-KO (originally prepared in a hybrid 129/Sv background) were backcrossed into C57/BI6/J for at least 13 generations. We used wild-type C57/BI6/J as the controls. The ZnT3-KO mice were kindly supplied by Dr. Palmiter (University of Washington). The RPE65-KO mice were from Jackson laboratories (Bar Harbor, ME) and contained the rd12 spontaneous mutant which was bred to C57/Bl6/J.

2.6. Mouse light damage model

C57/Bl6/J, ZnT3-KO, and RPE65-KO mice were all in the C57/Bl6/J background and were used at 7 weeks of age. They were acclimated for 5 days to a cyclic, dim overhead fluorescent light (30 Lux) followed by a 70h dark adaption. Pupils were dilated with 1% tropicamide ophthalmic solution USP under red light illumination and then exposed to white fluorescent light from 8× 17W fluorescent bulbs using a mirrored reflector (70 kLux) at <25C for 50 h (starting at 7:30pm). The animal chamber was ventilated and gently rotated during the light exposure as above. Diet gel (Clear H_2O; Portland, ME) was given during the light exposure to make sure the mice were fed and hydrated. After light exposure, mice were maintained totally in the dark for 7d until retinas were examined. This paradigm was necessary to induce substantial mid-superior photoreceptor loss in these light resistant mouse strains (Bai et. al., 2012, co-submission).

2.7. Optical Coherence Tomography (OCT)

OCT is an optical signal acquisition and processing method providing extremely high-quality, micrometer-resolution, three-dimensional images from within optical scattering media (Spectralis, Heidelberg Engineering; Heidelberg, Germany) (Knott et al., 2011). On the seventh day after light damage, rats and mice were anesthetized with ketamine and xylazine and OCT was performed to measure the thickness of the mid superior outer nuclear layer (ONL). A real time eye tracker was used to couple confocal Scanning Laser Ophthalmoscopy (cSLO) and spectral domain-OCT (SD-OCT) scanners to position and stabilize the OCT scan on the retina. Scaling X was 3.24–3.31 μm/pixel; scaling Z was 3.87 μm/pixel. The built-in scale bar was used when performing OCT analysis. The thickness of the ONL was measured from the bottom edge of the outer plexiform layer to the top edge of the RIS at 3 points of the mid superior region and averaged.

2.8. Retinal Histology

After OCT, rats and mice were sacrificed by CO_2 asphyxiation. Eyes were fixed in 2% formaldehyde/2% glutaraldehyde, and cut in half along a superior-to-inferior meridian through the center of the optic nerve. After a one-hour fixation period in 1% osmium tetroxide and sequential dehydration in ethanol, eyes were embedded in plastic resin (Electron Microscopy Systems; Hatfield, PA). Retinal sections of 1.5 microns were cut, mounted on glass slides and stained with 0.1% toluidine blue for 2 mins. The number of ONL nuclei was counted on sections from 8–12 different retinas at increasing distances from the optic nerve on the superior-to-inferior meridian. Pictures were taken in the area of mid-superior hemispheres.

2.9. Retinal Zn^{2+} staining

Eyes of Sprague Dawley rats, ZnT3-KO, RPE65-KO mice and C57/BI6/J control mice were collected 0–12h after light exposure under red light illumination. Zn^{2+} staining started to

appear from 0–4h after LD, and was reduced by 14–24h. The peak varies from albino rats to pigmented mice (1–12 hr, data not shown). Fresh frozen cryostat sections (10 microns) were prepared, dried, and stained with 5μM ZinPyr-1 (ZP1, TefLabs, Galveston, TX) for 2 min, washed with PBS, and photomicrographs were taken immediately using the exposure times indicated (ex: 480nm; em: 530nm). ZP1 has high specificity for releasable zinc in secretory granules (K_D=0.7nM). It is stably-lipophilic, and thus penetrates subcellular organelles such as the outer segments. It increases many fold in fluorescence when binding to Zn^{2+} (or other transition metals), but does not fluoresce in response to Mg^{2+}, or Ca^{2+} (Burdette et al., 2001; Chang et al., 2004; Giblin et al., 2006). There was no autofluorescence at this wavelength either basally or after light damage, and the ZP1 staining was prevented by pretreatment of the section with the Zn^{2+} specific chelator, N,N,N'N'-tetrakis(−)[2-pyridylmethyl]-ethylenediamine (TPEN) (data not shown).

2.10. NAD^+ measurements

The effect that Zn^{2+} had on NAD^+ levels in PRC cultures was determined at 3hrs of Zn^{2+} exposure. For pigmented $RPE65Met_{450}$ mice, it took 50hrs to light damage the retinas. The peak of Zn^{2+} was between 0 and 4 h post-LD. Therefore, the effect that LD had on NAD^+ levels in ZnT3- KO, and cytNMNAT1 Tg retinal tissues was determined at 12hrs which was prior to the onset of cell death. Curved forceps were gently inserted into back of the eye, by cutting the lens in front and simultaneously pulling upwards on the back of the eye, retinas were rapidly isolated. Retinas or PRC cultures were then immediately lysed in NaOH/EDTA lysis buffer. Aliquots were acidified and heated to destroy NADH. These lysates were then neutralized and stored at −80°C for up to 1 year. The lysates generated were used for enzymatic cycling determinations of NAD^+ using the malate dehydrogenase/ alcohol dehydrogenase cycling pair (Lin et al., 2001; Cai et al., 2006). The malate produced was then quantitatively converted to NADH, and measured fluorimetrically (excitation at 365nm, emission monitored at 460nm). The dynamic range of this cycling assay can be varied (10^{-15} to 10^{-9} moles) by the amount of cycling enzymes used and the duration of the cycling time. The results obtained are compared to a calibration curve, and normalized to protein content.

2.11. Data analysis and statistics

The numbers in each experiment were performed with an n=6–12 from 2–3 experiments. Means ± SEM were plotted and analyzed for significance using a one-way ANOVA followed by a Student t-test with significance achieved by $P< 0.05$.

2.12. Reagents

All materials were purchased from Sigma Chemical Co. (Saint Louis, MO) unless otherwise stated.

3. Results

3.1. Zn^{2+} induces an increase in $[Zn^{2+}]_i$, toxicity, and NAD^+ loss in PRC

PRC were exposed to Zn^{2+} in the presence or absence of pyruvate, nicotinamide, or NAD^+ and $[Zn^{2+}]_i$ (A), toxicity (B), and NAD^+ levels (C) were determined (Figure 1). $[Zn^{2+}]_i$ was increased by 150 μM Zn^{2+} in PRC; toxicity was induced which was significantly attenuated by pyruvate, nicotinamide and NAD^+. Only pyruvate and NAD^+ (not shown) significantly restored NAD^+ levels in PRC cultures.

3.2. Light induced Zn^{2+} staining was attenuated by ZnR

We have previously used this diet paradigm to depress Zn^{2+} levels in the brain and pancreas and have shown that it is reversible (Sheline et. al., 2012 J. Nutrition, accepted and Suh et al., 2009; Sheline et al., 2010b). LD was produced in rats fed the diets indicated for 3 wks prior. Retinas were analyzed for Zn^{2+} staining 1h after LD. There was an increase in Zn^{2+} staining in the cell bodies of photoreceptors after light exposure, which was attenuated by ZnR. These are the regions that are specifically sensitive to light exposure. The staining of the ROS is variable, and zinc deficiency was shown to induce ER stress in brain and paradoxically increase Zn^{2+} staining (Stoltenberg et al., 2007). The number of photoreceptors which stained for Zn^{2+} was significantly higher in the 61 mg/kg (ppm) diet compared to no LD controls, and was significantly reduced by ZnR (Figure 2A and Table 1).

3.3. Light induced retinal damage was attenuated by ZnR

The number of photoreceptors remaining was determined in male littermates 7 days after LD. ZnR attenuated damage to both superior and inferior retina, as shown by OCT and plastic sectioning (Figures 2B–E). Rats fed with 61 mg/kg purified Zn^{2+} diet showed complete loss in mid-superior retinas and severe loss in inferior retinas, whereas 2 mg/kg purified Zn^{2+} diet showed a less severe and smaller range of damage. Feeding 2 mg/kg Zn^{2+} for 2 wks followed by 1 wk of 61 mg/kg Zn^{2+} restored sensitivity to LD (data not shown). The numbers of photoreceptor nuclei at increasing distances superior or inferior to the optic nerve were plotted as a spider graph which is shown in figure 2C. Quantitations of mid retinal ONL thickness, measured as the distance between the RIS and the OPL were made by OCT (Figures 2D & 2E). In this study, 61 mg/kg purified Zn^{2+} diet rats were set as the controls for 2 mg/kg purified Zn^{2+} diet rats. We found that although rats fed a 61 mg/kg purified Zn^{2+} diet had similar photoreceptor Zn^{2+} staining as rats fed the normal Zn^{2+} diet (contains 61 mg/kg Zn^{2+}), they showed more severe damage than the normal Zn^{2+} diet rats (Figure 2, and (Sheline et al., 2010a)). This may be because the normal diet contains vitamin A, carotenoids (lutein and zeaxanthin) whereas the 2 mg/kg purifed Zn^{2+} diet does not. Those supplements serve as important precursors of all-trans-retinol for vision generation and antioxidants for irradiant protection.

3.4. Light induced Zn^{2+} accumulation and retinal damage were attenuated, and NAD^+ levels were not significantly reduced in ZnT3 KO mice

LD was performed on retinas from ZnT3 mice in a C57/Bl6/J $RPE65^{Met/Met}$ background and analyzed 4h (Zn^{2+}, Figure 3A), or 7 days (death, Figure 3B–C) after 50h of LD. ZnT3 KO mice showed little photoreceptor Zn^{2+} staining and damage compared to B6 control mice (quantified in Table 1). Photoreceptors were counted and plotted in figure 3C. NAD^+ levels in ZnT3-KO mice after LD were 17.9 ± 1.1 nmols/mg of protein which was not significantly different than ZnT3-KO no LD retinas, or C57/Bl6 no LD control mice (19.3 ± 0.7, and 22.8 ± 0.8, respectively, n=6). NAD^+ levels were significantly reduced in C57/Bl6 mice after LD (Bai et. al., 2012, co-submission).

3.5. LD induced Zn^{2+} accumulation was reduced in retina of RPE65 KO mice

RPE65 knockout mice can not reisomerize all trans-retinol resulting in a block in rhodopsin recycling. RPE65 KO mice are resistant to LD at 6–10 weeks of age, and develop spontaneous retinal degeneration starting at 10 weeks of age (Redmond et al., 1998; Grimm et al., 2000). LD was performed on 7 week old RPE65 KO mice and Zn^{2+} staining was analyzed 4h after 50h of LD. We showed a lack of Zn^{2+} staining in photoreceptors after 50h LD in 7 week old RPE65 KO mice compared to C57/Bl6/J control mice (Figure 4, and Table 1).

4. Discussion

We previously showed that intense light can induce early Zn^{2+} accumulation in rats, specifically in severely damaged superior retinal layers, including RPE cells, ROS, OPL and especially ONL. This early (before cell loss), preferential Zn^{2+} accumulation suggested a role for Zn^{2+} toxicity in light-induced damage (Sheline et al., 2010a). In the present study, we find that: 1) Zn^{2+} causes toxicity in primary retinal cultures dependent on Zn^{2+} accumulation and loss of NAD^+ levels which is attenuated by pyruvate, nicotinamide, and NAD^+. 2) a reduced Zn^{2+} diet (2 mg/kg) attenuated photoreceptor degeneration after LD in albino rats compared to normal Zn^{2+} diet (61 mg/kg). The number of photoreceptors staining for Zn^{2+} was reduced in the rats receiving 2 mg/kg diets 1h after LD. 3) ZnT3-KO mice showed less Zn^{2+} staining and death in photoreceptor and RPE cells than those of C57/BI6/J control mice after light exposure. 4) NAD^+ levels after the LD were not significantly reduced in ZnT3-KO mice. 5) RPE65-KO mice are resistant to light damage, and showed no RPE or photoreceptor Zn^{2+} staining after light exposure compared to C57/Bl6/J Zn^{2+} staining.

Zn^{2+} is involved in retinal health and the importance of Zn^{2+} is being demonstrated in an increasing number of disease conditions in both animals and humans. Excess Zn^{2+} accumulation has been shown to be toxic in retinal ischemia (Yoo et al., 2004; Choi et al., 2006), global ischemia (Koh et al., 1996), trophic deprivation (Sheline et al., 2010b) and hypoglycemia mediated neuronal death (Suh et al., 2004; Suh et al., 2008). Acute Zn^{2+} chelation or Zn^{2+} therapeutics were efficacious (Lee et al., 2001; Choi et al., 2006; Sheline et al., 2010b). Also, in Alzheimer disease (AD), Zn^{2+} increases the aggregation of beta-amyloid protein (Lee et al., 1999; Lynch et al., 2000; Lee et al., 2002). Knocking out Zn^{2+} transporter protein (ZnT3) was found to significantly decrease beta-amyloid plaque formation (Lee et al., 2002). The metal chelating agents desferrioxamine and clioquinol have been suggested as potential therapeutics for AD (Finefrock et al., 2003).

In the present study, we further examined the role of Zn^{2+} in light induced retinal damage. Previous studies showed that retinal cell-line cultures were susceptible to Zn^{2+}, and oxygen radicals causing Zn^{2+} accumulation, NAD^+ loss, glycolytic inhibition and death that was attenuated by pyruvate, nicotinamide, and NAD^+ (Sheline et al., 2010a) (accompanying NAD^+ paper). We now demonstrate that PRC cultures (Figure 1) are similarly affected by Zn^{2+}, though nicotinamide is less effective at restoring NAD^+. Retinal neurons require high metabolic energy (ATP) from glycolysis to survive (Winkler, 1981), and GAPDH has been shown to be enriched on retinal outer segment membranes (Hsu and Molday, 1990). We went on to examine the affects of a reduced Zn^{2+} diet in vivo. This diet can significantly decrease the total Zn^{2+} content in the brain, and switching back to a Zn^{2+} adequate diet can restore Zn^{2+} levels (Takeda et al., 2003; Suh et al., 2009). The Zn^{2+} transporters involved in organismal Zn^{2+} homeostasis have been studied, and their expression profiles in different cell types, cellular localization, and response to dietary Zn^{2+} have been studied. Vision deficits have not been reported due to this dietary restriction (reviewed in Lichten and Cousins, 2009). Here we showed a reduced Zn^{2+} diet (2 mg/kg) attenuated photoreceptor Zn^{2+} staining and death after light exposure in albino rats compared to a diet containing normal levels of Zn^{2+} (61 mg/kg). Feeding 2 mg/kg Zn^{2+} for 2 wks followed by 1 wk of 61 mg/kg Zn^{2+} restores sensitivity to LD (data not shown), suggesting specificity of these effects for Zn^{2+} over effects this diet could have on uptake of other metals. Our studies were performed on an acute dietary reduction of only 3 wks to minimize the potential complications such as defects in rhodopsin synthesis which requires a longer term Zn^{2+} deficiency (Dorea and Olson, 1986).

In addition to dietary manipulations to alter Zn^{2+} levels, we also tested strains of mice that have genetic manipulations which reduce retinal Zn^{2+} levels. Genetic strains that increase retinal Zn^{2+} levels are not available. Unlike rats, susceptibility of light damage in mouse is determined genetically by the expression levels of a retinal pigment epithelial protein RPE65 (Danciger et al., 2000; Wenzel et al., 2001; Iseli et al., 2002). RPE65 is an essential enzyme for the visual cycle, catalyzing the conversion of all-transretinyl esters to 11-cis retinal for rhodopsin regeneration. Substitution of leucine by methionine at position 450 of the RPE65 protein ($RPE65Met_{450}$) reduced RPE65 expression, and decreased the rate of rhodopsin regeneration after bleaching. This caused a lower susceptibility towards light exposure compared to $RPE65Leu_{450}$, demonstrating that rapid visual cycling increases LD (Wenzel et al., 2001). Similarly, RPE65 KO mice are resistant to LD if examined prior to spontaneous retinal degeneration which starts at 10 weeks of age (Redmond et al., 1998; Grimm et al., 2000). The C57/BI6/J control and ZnT3-KO mice in this study are all pigmented $RPE65Met_{450}$ mice. To achieve light damage in these resistant mice required modifications to the LD protocol (Bai et. al., 2012, co-submission).

Zn^{2+} is present physiologically in different layers of the retina. The expression and localization of Zn^{2+} transporters suggest that Zn^{2+} transporter 7, (ZnT7, slc30a7), and ZnT3 (slc30a3), as well as ZIP transporters (slc39a1–16) (Wang et al., 2006; Leung et al., 2008) could be important sources for physiologic or patho-physiologic Zn^{2+} in the eye (reviewed in Ugarte and Osborne, 2001). ZnT3 is expressed in specific layers in the retina. In inner and outer plexiform layers, it is associated with synaptic interactions. In photoreceptor inner segments and outer limiting membrane, it co-localizes with the Zn^{2+} staining pattern after light adaptation. ZnT3 has also been found to be expressed in Müller cells which transverse throughout all of the retinal layers and may be involved in Zn^{2+} homeostasis (Redenti and Chappell, 2004, 2007). ZnT3 KO mice have a 20% reduction in total Zn^{2+} in brain regions where histochemically reactive Zn^{2+} is usually detected, and were not reported to have vision deficits (Cole et al., 1999). ZnT3 KO mice have reduced neuronal injury after LD, rescuing more than half of the photoreceptors in the superior retina (Figure 3). Injury in ZnT3 KO mice is also reduced after global ischemia and hypoglycemia (Sheline and Wei, unpublished observation, and (Suh et al., 2008)). We also showed in this study that NAD^+ levels in ZnT3-KO mice were not significantly reduced after LD, as expected due to the lack of Zn^{2+} toxicity. This suggests that ZnT3-dependent Zn^{2+} is released and accumulates in photoreceptors contributing to their loss. This Zn^{2+} appears in perikaya of photoreceptors especially after a dark/depolarized period. This results in NAD^+ loss causing potentiation of metabolic inhibition and cell loss.

Rhodopsin regeneration is positively correlated to severity of light damage (Grimm et al., 2000). Zn^{2+} has been shown to bind to rhodopsin at a high affinity site comprised of Glu^{122} (at the end of transmembrane region 3, TM 3) and His^{211} (at the end of transmembrane region 5, TM 5) to stabilize the inactive rhodopsin structure in the dark (Shuster et al., 1992; Stojanovic et al., 2004). Additional Zn^{2+} ions (>1) binding at low affinity sites His^{100} and His^{195} are required for destabilizing the structure of Rho in RPE cells where $[Zn^{2+}]_i$ is high, potentially allowing its degradation (Gleim et al., 2009). Light induces changes in rhodopsin structure that moves TM 3 and TM 5 away from each other, increasing the tetrahedral binding distance for Zn^{2+} which is inconsistent with continued Zn^{2+} binding. We therefore predict rhodopsin could serve as a pool for Zn^{2+} release after injuries. A single episode of complete photobleaching of rhodopsin does not induce retinal degeneration, but block of rhodopsin recycling does prevent retinal degeneration. It is the repeated photobleaching and rhodopsin recycling that is required to induce retinal degeneration induced by light damage. Others have also shown that the visual cycle must be allowed to cycle in order to induce damage. Therefore, we postulate that it is the repeated cycling that is required to induce zinc accumulation in photoreceptors which contributes to their death (Figures 3 and 4). After

extensive bleaching, the initial rate of rhodopsin regeneration in control rats and zinc-deficient rats was the same, and no vision deficits were noted. The only difference was that the extent of rhodopsin regeneration in control rats kept in the dark for 2 hrs was higher than that in zinc-deficient rats. The explanation is that the rhodopsin concentration is lower in ROS in zinc-deficient rats (Dorea and Olson, 1986). Therefore, manipulating Zn^{2+} levels may not affect rhodopsin's capability to absorb photons or to regenerate. Zn^{2+} restriction may reduce the rhodopsin pool available to store zinc, which in turn reduces zinc release and cell death.

RPE65 protein is required for rhodopsin regeneration. RPE65-KO (no RPE65 protein) prevents the production of 11-cis-retinal and rhodopsin regeneration (Grimm et al., 2000). Therefore, in RPE65-KO mice less cycling occurs resulting in less Zn^{2+} release and staining, and less LD. Our findings that light exposure does not induce an increase in photoreceptor Zn^{2+} staining in RPE65-KO mice compared to wildtype mice supports this possibility, as shown in Figure 4 and Table 1. We postulate that ZnT3-, rhodopsin-, and oxidation-dependent release of zinc each contribute to the zinc accumulation, and light-induced damage.

These studies suggest: 1) Zn^{2+} toxicity in PRC may be mediated by reduced NAD^+ levels, consistent with previous studies in other neuronal culture, 2) reduced dietary Zn^{2+} reduces $[Zn^{2+}]_i$ leading to reduced light damage, 3) rhodospin recycling and release of bound Zn^{2+} is a possible source of excessive $[Zn^{2+}]_i$, as is ZnT3-dependent stores. Zn^{2+} toxicity is partially mediated by reducing NAD^+ levels in cortical and retinal neuronal cell death. We therefore have examined the efficacy of nicotinamide mononucleotide adenyl-transferase-1 (NMNAT1, an NAD^+ synthetic enzyme) overexpression on reducing LD and increasing NAD^+ levels in a companion manuscript (Bai et. al., 2012, co-submission).

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Abbreviations

(TPEN)	^{d}-N,N,N'N'-tetrakis(-)[2-pyridylmethyl]-ethylenediamine
(GCL)	ganglion cell layer
(LD)	light damage
(INL)	inner nuclear layer
(ONL)	outer nuclear layer
(IPL)	inner plexiform layer
(OPL)	outer plexiform layer
(ROS)	rod outer segments
($[Zn^{2+}]_i$)	intracellular zinc concentration
(OS)	oxidative stress
(ppm)	part per million = mg/kg
(ZnR)	reduced zinc diet
(ZnE)	supplemented zinc diet

(RP)	retinitis pigmentosa
(ZnT3)	Zinc transporter 3
(ZP1)	ZinPyr-1

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1. Zn^{2+} accrual and NAD^+ loss induce PRC toxicity which is reduced by P, N, and NAD^+.
2. A Zn^{2+} reduced diet reduced photoreceptor Zn^{2+} staining and degeneration after LD.
3. ZnT3-KO mice have less Zn^{2+} staining and photoreceptor and RPE cell death after LD.
4. NAD^+ levels after the LD were not significantly reduced in ZnT3-KO mice.
5. RPE65-KO mice are resistant to LD, and have no photoreceptor Zn^{2+} staining after LD.

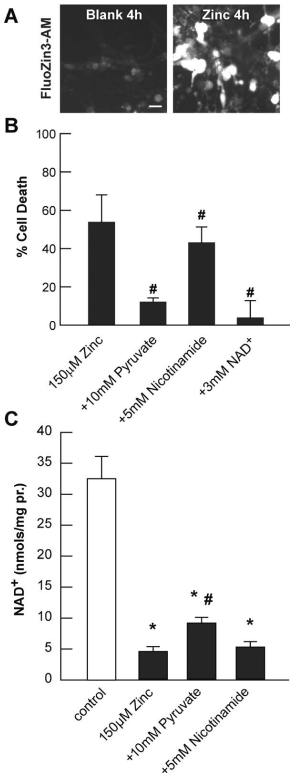

Fig. 1. Zn^{2+} exposure causes accumulation, toxicity, and NAD^+ loss in PRC cultures

A. PRC cultures were post-loaded with 5 μM FluoZin3-AM and exposed to 150 μM Zn^{2+} as indicated. Photomicrographs were taken at equivalent exposures. **B.** Cell death was measured in sister cultures after exposure for 24 h as indicated using propidium iodide staining. **C.** NAD^+ levels were measured in sister cultures after exposure for 3 h as indicated using enzyme-cycling reactions. * indicates difference from control, and # indicates difference from Zn^{2+} exposure at $P < 0.05$ by one-way ANOVA and a Student t-test, n>8.

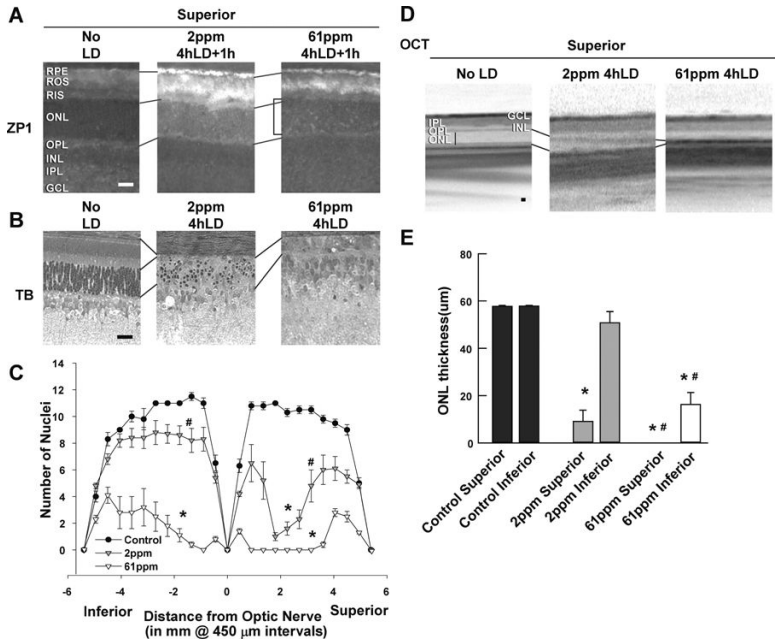

Fig. 2. Light induced Zn^{2+} staining and retinal damage were attenuated by ZnR

LD was performed on rats fed the diets indicated for 3 wks prior and retinas were analyzed for Zn^{2+} staining (**A**) 1h after sham exposure (No LD), or 1 h after 4h of LD. Zn^{2+} accumulation (white regions) was assessed by ZP1 staining (5 μM for 2 min) in fresh frozen, dried cryostat sections. Representative photomicrographs (n = 4) were taken of the mid superior regions of the retina at 0.2 second exposure. Notice the large increase in the number and intensity of Zn^{2+} stained cells in superior ONL (brackets) of 61 ppm animals. This is reduced in the 2 ppm animals. **B.** Retinas of male littermates mice were analyzed 7 days after LD by plastic sectioning cutting from eyes along a superior to inferior meridian encompassing the optic nerve and stained with 0.1% Toluidine Blue. Photomicrographs were taken of the mid superior regions of the retina. **C.** ONL nuclei counted at increasing distance from the optic nerve was averaged and plotted as a function of distance from the optic nerve for each of the experimental conditions above (n=6). **D.** Representative ONL images of central superior were taken from OCT; vertical lines show the thickness of the ONL layer. **E.** The mean thickness of OCT measurement in central superior and inferior hemispheres of the retina in microns is presented ± SEM (n=6). Lines align the RIS or OPL of different panels. Retinal layers are labeled, and the horizontal bars represent 25 microns. * indicates a significant difference from no LD, and # indicates a significant difference from light damage at $P < 0.05$ by one-way ANOVA and a Student t-test.

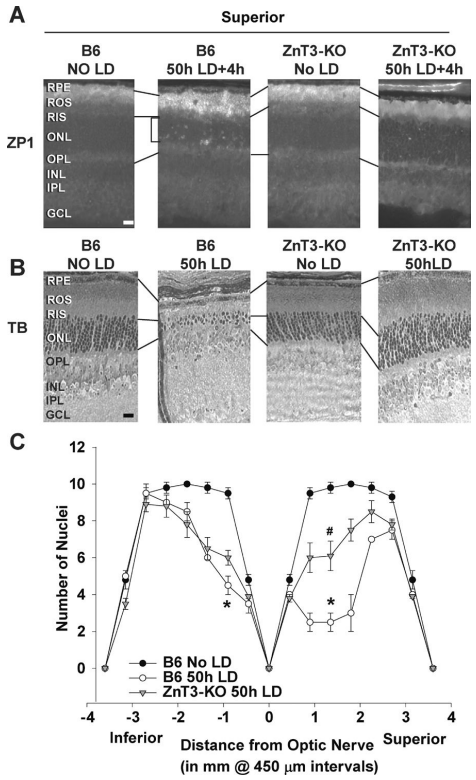

Fig. 3. Light induced Zn^{2+} accumulation and retinal damage were attenuated by ZnT3 KO

A. LD was performed on ZnT3 mice in a C57/Bl6 RPE65$^{Met/Met}$ background and retinas were analyzed 4h after 50h of LD. Zn^{2+} accumulation (white regions) was assessed by ZP1 staining. Representative photomicrographs (n = 4) were taken of the mid superior regions of the retina at 0.3 second exposure. Notice the decrease in number and intensity of Zn^{2+} staining in superior retina after LD exposure in ZnT3 KO retina. **B.** Male littermates retinas were analyzed 7 days after LD by plastic sectioning cutting from eyes along a superior to inferior meridian encompassing the optic nerve and stained with 0.1% Toluidine Blue. Photomicrographs were taken of the mid superior regions of the retina, and the dotted lines show the alignment of the retinal layers between the panels. **C.** ONL nuclei counted at increasing distance from the optic nerve were averaged and plotted as a function of distance from the optic nerve for each of the experimental conditions above (n=6). * indicates a significant difference from control, and # indicates a significant difference from light damage at $P < 0.05$ by one-way ANOVA and a Student t-test. Layers are as marked. Bar represents 25 microns.

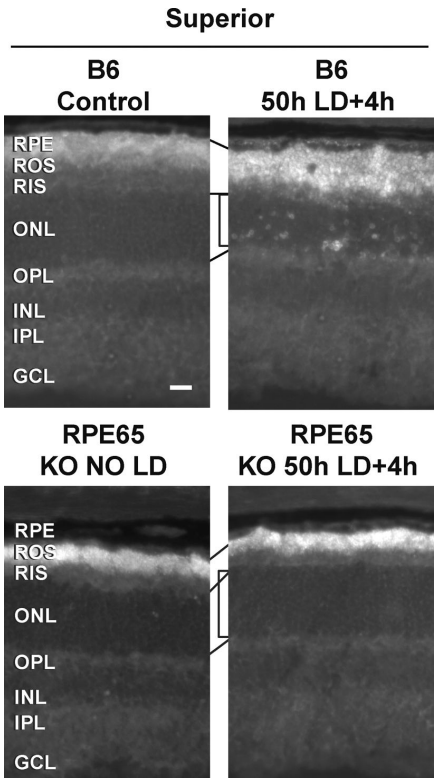

Fig. 4. Light induced Zn^{2+} accumulation was attenuated in retina of RPE65 KO mice

LD was performed on RPE65 KO mice in a C57/Bl6/J $RPE65^{Met/Met}$ background and retinas were analyzed 4h after 50h of LD. Zn^{2+} accumulation (white regions) was assessed by ZP1 staining. Representative photomicrographs (n = 4) were taken of the mid superior regions of the retina at 0.3 second exposure. Notice the decrease in number and intensity of Zn^{2+} staining in superior retina after LD exposure in RPE65 KO retina (bracket). Layers are as marked, and lines show alignment. Bar represents 25 microns.

Table 1

LD induced Zn^{2+} staining of photoreceptors which was attenuated by ZnT3 and RPE65 knockout, and 2 ppm Zn^{2+} diet[a].

Condition	Number of Zn^{2+} Stained Photoreceptors/Field
No LD Control +1h	0.2 ± 0.1
2ppm **Zn^{2+}** Diet-4hLD + 1h	25.3 ± 2.7 *#
61ppm **Zn^{2+}** Diet-4hLD +1h	58.1 ± 2.0 #
B6-basal (No LD)	0.3 ± 0.1
B6-50hLD +0h	85.0 ± 3.6 #
B6-50hLD +4h	58.3 ± 2.1 #
ZnT3-KO no LD	0.3 ± 0.2
ZnT3-KO-50hLD +4h	10.4 ± 2.6 *#
RPE65-KO no LD	1.5 ± 0.3
RPE65-KO 50hLD +4h	1.2 ± 0.5 *

[a] sections from the indicated animals were stained for Zn^{2+} using ZP1, and stained cells in the mid superior photoreceptor layer fields were counted (n=12).

# indicates a significant difference from no LD control at $P < 0.05$, n=12.

* indicates a significant difference from LD control at $P < 0.05$, n=12.