BEFORE THE PUBLIC UTILITIES COMMISSION

Investigation to examine the benefits of re-starting the residential lighting program to promote the installation of high-efficiency lighting (LED) in Nevada.

Docket No.: 17-02011

NTEF'S COMMENTS FROM APRIL 21, 2017 WORKSHOP

On April 21, 2017, Joe Reynolds emphatically stated that 'he was a prosecutor and he knows science'. Predicated on that assertion, I will NOT be blondizing the research. As he should be able to easily understand the 'science' as it relates to the effects of the blue light that emanates from the LED light bulbs upon human health (retina, circadian rhythms, mitochondria etc.), that he is shoving upon the ratepayers via this investigatory docket.

That he seems to place an inordinate amount of weight upon Dr. Geller's degree in 'energy', thus, his degree should have incorporated the effects of light upon the human system, in the scientific field known as Photobiology. So we shall see if he has the curiosity to see if he is in fact right…that these bulbs are all he purports them to be.

Of course, the balance of the named parties are going to ignore the SCIENCE 1) they are afraid they will be shown to be wrong; 2) its way over their heads to understand.

HARVARD HEALTH! Is that good enough for you Reynolds?

"But not all colors of light have the same effect. Blue wavelengths—which are beneficial during daylight hours because they boost attention, reaction times, and mood—seem to be the most disruptive at night. And the proliferation of electronics with screens, as well as energy-efficient lighting, is increasing our exposure to blue wavelengths, especially after sundown.

While light of any kind can suppress the secretion of melatonin, blue light at night does so more powerfully. Harvard researchers and their colleagues conducted an experiment comparing the effects of 6.5 hours of exposure to blue light to exposure to green light of comparable brightness. The blue light suppressed melatonin for about twice as long as the green light and shifted circadian rhythms by twice as much (3 hours vs. 1.5 hours).

In another study of blue light, researchers at the University of Toronto compared the melatonin levels of people exposed to bright indoor light who were wearing blue-light–blocking goggles to people exposed to regular dim light without wearing goggles. The fact that the levels of the hormone were about the same in the two groups strengthens

the hypothesis that blue light is a potent suppressor of melatonin. It also suggests that shift workers and night owls could perhaps protect themselves if they wore eyewear that blocks blue light. Inexpensive sunglasses with orange-tinted lenses block blue light, but they also block other colors, so they're not suitable for use indoors at night.

If blue light does have adverse health effects, then environmental concerns, and the quest for energy-efficient lighting, could be at odds with personal health. *Those curlicue compact fluorescent lightbulbs and LED lights are much more energy-efficient than the old-fashioned incandescent lightbulbs we grew up with.* ***But they also tend to produce more blue light****.*

The physics of fluorescent lights can't be changed, but coatings inside the bulbs can be so they produce a warmer, less blue light. LED lights are more efficient than fluorescent lights, but they also produce a fair amount of light in the blue spectrum. Richard Hansler, a light researcher at John Carroll University in Cleveland, notes that ordinary incandescent lights also produce some blue light, although less than most fluorescent lightbulbs." http://www.health.harvard.edu/staying-healthy/blue-light-has-a-dark-side

I am sure almost everyone is going to DOUBT the following-so just call any A&P, cell bio/phys or histo prof at UNR/UNLV and they will CONFIRM that these are rooted in fact…as its part of ALL physiology/histology medical science classes.

A little histophysiology of the retina. All it takes is a single photon to trigger the production of a receptor potential in a rod

As light 'acts' to 'bleach', a photochemical process, upon the retina's visual pigment that amplifies mechanisms that result in a local response production, that winds up being transmitted to the CNS.

The light sensitive portions of the photoreceptors lies deep to other retinal neurons transparent enough for light to pass thru to reach these receptors. The nerve impulses relayed via the retina pass from its outermost to innermost layer OPPOSITE direction to light.

Rhodopsin (visual purple) is the visual pigment of the rods. Which is simply composed of an –CHO of retinal, that's bound to opsin proteins. Which is synthesized in the inner segment of the rods. That are sensitive to low intensity light, produce visual images perceived in shades of gray and subserve night vision. Basically it means that it is manifested as hyperpolarization-light absorption via visual pigments leading to receptor potentials.

This bleaching of the visual pigment in the membrane disks increases the calcium conductance of the disks membrane which in turn promotes a diffusion of the calcium to the INTRAcellular space of the OUTER segment of the photoreceptor. The calcium acts on the cell membrane REDUCING its permeability to sodium ions and PROMOTING cell hyperpolarization! Basically the DECREASED levels of GMP cause CLOSURE of

the sodium channels. As the INREASING light intensity INCREASES the degree of hyperpolarization.

When light hits the rhodopsin it has an isomerization from an 11-*cis* to all-*trans* form, this change has the dissociation of the retinal from opsin aka bleaching! As there is a series intermediates that are formed, one of which is metarhodopsin II. Vitamin A is necessary for the regeneration of rhodopsin.

REMEMBER: the rod disks have a steady turnover because new disks are formed every day! As they are formed and replaced at the bottom and displaced by NEWER ones until they reach the top, which they than are phagocytosed by the adjacent retinal pigment epithelium. See how easy that was!

STILL WITH ME? Now for the RESEARCH on this. **Exhibit A-** Procedure for Research #1, **Exhibit B** Abstract etc., Research #2. **Exhibit 3-** Basic Search PubMed- PubMed (pubmed.gov) is a free resource developed and maintained by the National Center for Biotechnology Information (NCBI) at the National Library of Medicine® (NLM). (is this good enough for the 'prosecutor?) THESE ARE PEER-REVIEWED JOURNALS/PUBLICATIONS!!

Science trumps propaganda!!!!!!!

REMEMBER YOUR STATISTICS: The value/creditability claims regarding the 'p-value'!

Blue light has been found to penetrate deeper into the eye than other wavelengths of light, and thus has the potential to cause changes in retinal tissues, including the macula.

Reduced blue light transmission to the retina, which results from aging and yellowing of the lens, strongly correlates with sleep disturbance, a recent Danish study suggests. Results revealed that every 1% increase in blue light lens transmission reduced the odds of sleep disturbance by 5%. Those odds also increased significantly with the extent of autofluorescence, a measure of lens transmission and yellowing.
[Kessel L, Siganos G, Jørgensen T, Larsen M. Sleep disturbances are related to decreased transmission of blue light to the retina caused by lens yellowing. Sleep. 2011 Sep 1;34(9):1215-9.]

Students are being impacted by these light bulbs. While this may be good for school budgets, it is a detriment to children's vision. LED-based lighting, even the LED lights that emit a whiter rather than blue light, emit a significantly greater amount of blue light than traditional fluorescent lights used in classrooms. The change to LED-based lighting, coupled with the frequent use of computers by our students in the classroom, is cause for significant concern. Blue light has been shown to cause significant overaccommodation in students. Researchers found that when exposed to wavelengths below 430nm, rather than having the typical 0.3D lag of accommodation when focusing on a near target, students experience 1.0D of overaccommodation on average, or 1.3D

sum total accommodative change from the normal posture for focusing at near. Additionally, this overaccomodation can cause distance blur as well. ***[Graef K and Schaeffel, F. Control of accommodation by longitudinal chromatic aberration and blue cones. J of Vis. 2012;12(1):14.]***

some lighting specialists are promoting the use of LED lights in the classroom to help reset student circadian rhythms for early start times and to improve student alertness, only lighting in the 444nm to 486nm wavelengths has been shown to impact the circadian rhythm, with peak sensitivity at 459nm to 464nm. [***Hanifin J, Brainard G. Photoreception for circadian, neuroendocrine, and neurobehavioral regulation. J Phys Anthro. 2007;26(2);87-94.]*** By switching school lighting systems to LED lights, students are being exposed to all of the blue wavelengths of light for the entire school day, including the problematic wavelengths below 455nm. If schools wish to improve student alertness with lighting, research needs to be conducted to determine the appropriate duration and time of day students should be exposed to the 455nm to 486nm bandwidth, before such measures are put into place. A better idea: resolve to set later start times for middle and high schools to naturally align with student circadian rhythms—as the American Academy of Pediatrics has recently proposed. ***[American Academy of Pediatrics. Policy statement: school start times for adolescents. Pediatrics. Sept 2014;134(3).]***

In 2004, researchers found evidence suggesting the impact of blue light exposure on the risk of macular degeneration in the retina. [***Karbownik M, Garcia J, Lewinski A, Reiter R. Carcinogen-induced, free radical-mediated reduction in microsomal membrane fluidity: reversal by indole-3-propionic acid. J. Bioenerg. Biomembr. 2001;33:73–8.]*** This led to subsequent studies on the long-term effects of visible light on the eye. In 2013, researchers identified the most damaging visible wavelengths to be in the blue-violet range of 415nm to 455nm. These wavelengths were found to be the most harmful to cells in the eye, as they can penetrate deeper into the eye and harm the retina, particularly the retinal pigment epithelium, causing the development of a toxic, apoptosis-causing molecule called N-retinlyidene-N-retinylethanolamine (A2E) to be produced within the RPE cells, causing cell viability loss. ***[Dillon J, Zheng L, Merriam JC, Gaillard ER. Transmission of light to the aging human retina: possible implications for age related macular degeneration. Exp Eye Res. 2004 Dec;79(6):753-9.] [Arnault E, Barrau C, Nanteau C, et al. Phototoxic action spectrum on a retinal pigment epithelium model of age-related macular degeneration exposed to sunlight normalized conditions. PLoS ONE. 2013;8(8):1-12.]***

While the blue wavelengths of 415nm to 455nm were found to be damaging to the retina, the wavelengths between 450nm to 550nm provide the strongest stimulation of circadian and neuroendocrine responses. [***Smick K et al. Blue light hazard: new knowledge, new approaches to maintaining ocular health. Report of roundtable sponsored by Essilor of America in NYC, NY. 2013, March 16.] [Hanifin J, Brainard G. Photoreception for circadian, neuroendocrine, and neurobehavioral regulation. J Phys Anthro. 2007 26(2):87-94.]***

Many of these digital devices have been shown to emit high levels of short-wavelength blue light, which research shows has a negative impact on melatonin production. Repeated, prolonged exposure to blue light can result in altered circadian rhythms and the possibility of increased general fatigue which, in turn, impacts the frequency and amount of eye fatigue. ***[Gringras P, Middleton B, Skene DJ, et al. Bigger, Brighter, Bluer-Better? Current Light-Emitting Devices – Adverse Sleep Properties and Preventative Strategies. Frontiers in Public Health. 2015;3:233.] [Chang AM, Aeschbach D, Duffy JF, et al. Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness. Proc Nat Acad Sci. 2015;112(4):1232-7.]***

RESEARCH STUDY 1

Kuse Y, Ogawa K, Tsuruma K, Shimazawa M, Hara H., Damage of photoreceptor-derived cells in culture induced by light emitting diode-derived blue light Scientific Reports 4, Article number: 5223 (2014) doi:10.1038/srep05223

Age-related macular degeneration (AMD), a retinal degenerative disease, affects more than 30% of the people at or over 75 years of age. [***Bok, D.*** **New insights and new approaches toward the study of age-related macular degeneration.** ***Proc Natl Acad Sci U S A 99, 14619–21 (2002).]***

The pathogenesis of AMD usually advances with retinal photic injury caused by excessive light exposure and consequent oxidative stress. ***[Shahinfar, S., Edward, D. P. & Tso, M. O.*** **A pathologic study of photoreceptor cell death in retinal photic injury.** ***Curr Eye Res 10, 47–59 (1991). Beatty, S., Koh, H., Phil, M., Henson, D. & Boulton, M.*** **The role of oxidative stress in the pathogenesis of age-related macular degeneration.** ***Surv Ophthalmol 45, 115–34 (2000).]***

The retina contains much chromophores which can lead to the photochemical damage when excited at the each wavelength light, and age-related decrease of antioxidants such as superoxide dismutase (SOD) and increase of ROS following light exposure can progress to the pathology of AMD. ***[Jarrett, S. G. & Boulton, M. E.*** **Consequences of oxidative stress in age-related macular degeneration.** ***Mol Aspects Med 33, 399–417 (2012).]***

The loss of vision is the major symptom of retinal diseases such as AMD, and the early pathogenesis involves degeneration of retinal pigment epithelial (RPE) cells. ***[Liang, F. Q. & Godley, B. F.*** **Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration.** ***Exp Eye Res 76, 397–403 (2003).]***

It is reported that the accumulation of lipofuscin and the formation of drusen in the Bruch's membrane cause apoptosis of RPE cells. ***[Ishibashi, T., Sorgente, N., Patterson, R. & Ryan, S. J.*** **Pathogenesis of drusen in the primate.** ***Invest Ophthalmol Vis Sci 27, 184–93 (1986); Wassell, J., Davies, S., Bardsley, W. & Boulton, M.*** **The photoreactivity of the retinal age pigment lipofuscin.** ***J Biol Chem 274, 23828–32 (1999); Suter, M. et al.*** **Age-related macular degeneration. The lipofusion component N-retinyl-N-retinylidene ethanolamine detaches proapoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cells.** ***J Biol Chem 275, 39625–30 (2000); Crabb, J. W. et al.*** **Drusen proteome analysis: an approach to the etiology of age-related macular degeneration.** ***Proc Natl Acad Sci U S A 99, 14682–7 (2002)]***

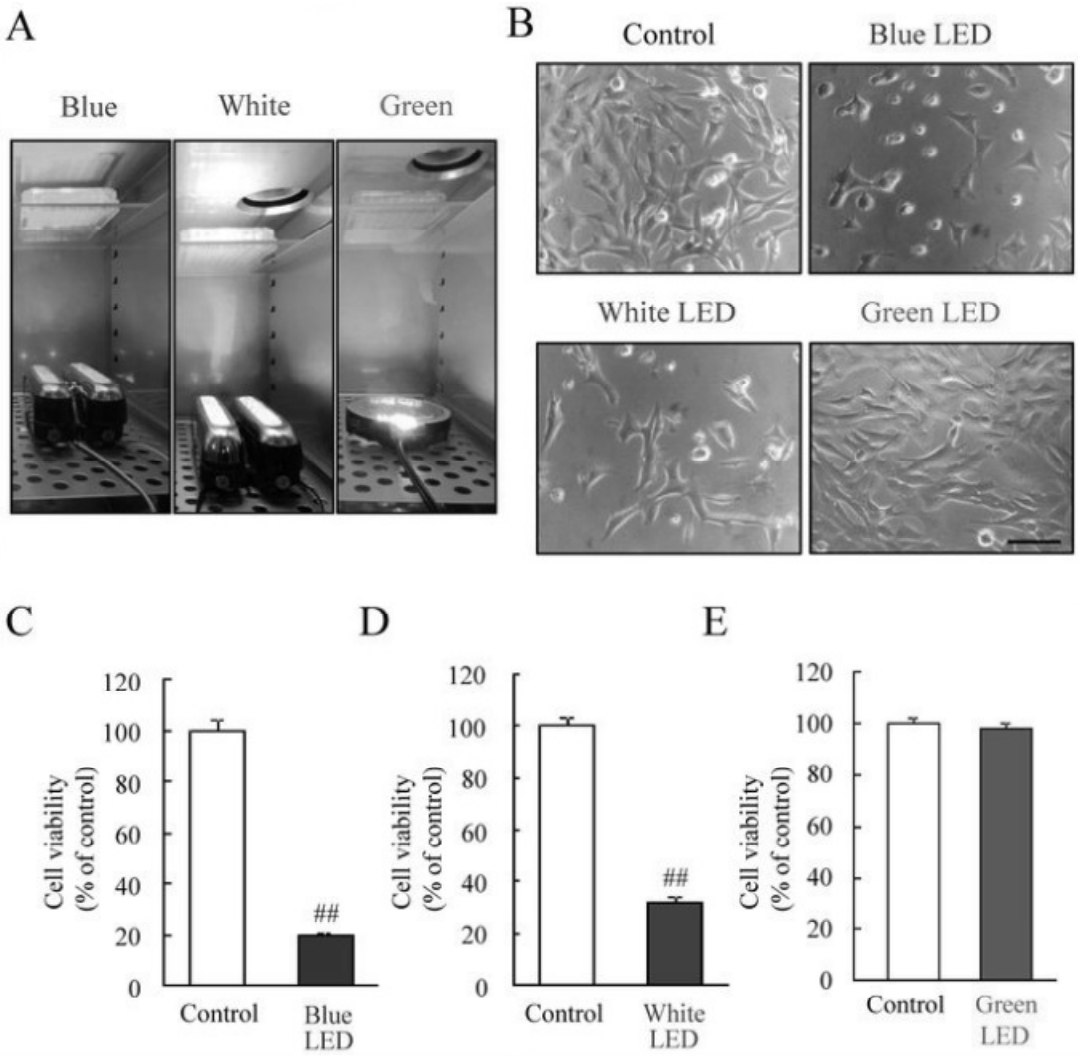
These are considered as the initial stages that lead to AMD. Subsequently, photoreceptor cell degeneration occurs after RPE cell death and can lead to vision loss. ***[Liang, F. Q. & Godley, B. F.*** **Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration.** ***Exp Eye Res 76, 397–403 (2003).]*** Furthermore, it is known that the photoreceptor cell death is facilitated by oxidative stress induced the generation of reactive oxygen species (ROS) such as superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) ***[Dunaief, J. L., Dentchev, T., Ying, G. S. & Milam, A. H.*** **The role of apoptosis in age-related macular degeneration.** ***Arch Ophthalmol 120, 1435–42 (2002)]*** In addition to RPE cell death, the oxidative stress due to ROS generation causes photoreceptor cell death. **[Luthra, S. et al.** ***Activation of caspase-8 and caspase-12 pathways by 7-ketocholesterol in human retinal pigment epithelial cells.*** **Invest Ophthalmol Vis Sci 47, 5569–75 (2006).]**

Blue light (from 450 to 495 nm) has a short wavelength, and it is a part of the high-energy visible light spectrum unlike several other colors. Previous reports suggested that the blue light more severe damaged retinal photoreceptor cells than green light in rats. [**Grimm, C. *et al. Rhodopsin-mediated blue-light damage to the rat retina: effect of photoreversal of bleaching. Invest Ophthalmol Vis Sci* 42, 497–505 (2001).]**

Following exposure to excessive light the regeneration could occur very rapidly through the process of photoreversal, and therefore rhodopsin can bleach several times in a short period *in vivo* [**Grimm, C., Reme, C. E., Rol, P. O. & Williams, T. P. *Blue light's effects on rhodopsin: photoreversal of bleaching in living rat eyes*. Invest Ophthalmol Vis Sci 41, 3984–90 (2000).]** While, the aggregation of short-wavelength opsins (S-opsin) can cause rapid cone degeneration. It is reported medium wavelength opsins (M-opsin) are easily degraded, but S-opsin is not easily degraded by proteasome degradation **Zhang, T., Zhang, N., Baehr, W. & Fu, Y. *Cone opsin determines the time course of cone photoreceptor degeneration in Leber congenital amaurosis. Proc Natl Acad Sci U S A* 108, 8879–84 (2011).]** We have reported that the excessive light exposure induced the aggregation of S-opsin, and leading to endoplasmic reticulum (ER) stress in the cone photoreceptor-derived cell line, 661 W **[Nakanishi, T. *et al. Role of endoplasmic reticulum stress in light-induced photoreceptor degeneration in mice. J Neurochem* 125, 111–24 (2013).]**

Blue LED light damaged most severely compared to white and green LED light
We first examined the relationship between the photoreceptor-derived cell damage and the difference in the color of the LED lights, under the same illuminance of 2,500 lux. Our results suggested that the blue LED light damaged the photoreceptor-derived cells more severely than white and green LED light Next, we investigated the effects of LED light on the cells under unified energy (0.38 mW/cm^2). This energy equals to 2,500 lux of green LED light. The photograph illustrates 96 well plates exposed to each LED light (Figure 1A). A representative photomicrograph of cell morphology was taken using bright field microscopy. The photoreceptor-derived cells were changed by blue and white LED light (Figure 1B). Green LED light did not change the cells. Quantitative data showed blue and white LED lights significantly reduced cell viability, but green LED light did not affect it (Figure 1C–E).

Figure 1: The effects of blue, white, and green LED lights on the cell viability.

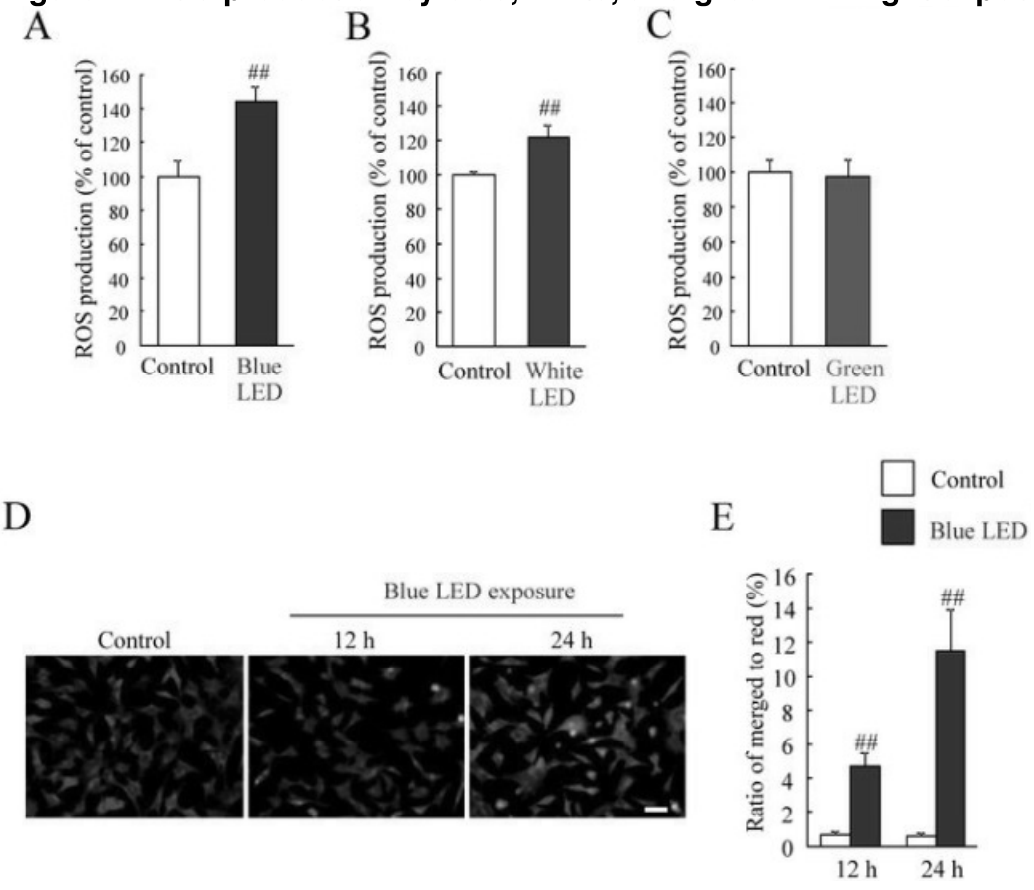


(A) The exposure of blue, white, and green LED light to cells cultured in a 96-well plate. (B) The observation of cell morphology using bright field microscopy, showing blue LED light caused the morphological changes compared with the control. Green LED light did not change the cells. (C–E) The quantitative evaluation of cell viability by the CCK-8 assay. This result is consistent with the observed change in cell morphology. Cell viability was reduced by blue and white LED light exposure, but not green LED light. The scale bar represents 50 μm. Data are expressed as mean ± SEM (n = 6). ## indicates $p < 0.01$ vs. control (ANOVA).

Blue LED light exposure increased ROS generation compared to white and green LED lights First, we evaluated the relationship between ROS generation and exposure to three different colored LED lights for 24 h at 2,500 lux. We found that blue LED light induced a higher ROS production than white and green LED lights Then, we investigated these changes under unified LED energy (0.38 mW/cm^2). Blue LED light induced ROS increase White and green LED lights also increased the ROS generation but at lower levels compared to blue LED light The direct comparison in each LED exposed groups showed blue LED light-induced ROS production was most severely

than the other LED light-induced ROS production Moreover, we examined whether the LED light exposure for 6 h induced ROS production. Blue LED light exposure for 6 h induced 1.4-fold ROS increase, and white LED light exposure for 6 h induced 1.2-fold ROS increase (Figure 2A, B). Green LED light exposure for 6 h did not induce ROS increase (Figure 2C). The photoreceptor cell death is promoted by oxidative stress induced the generation of ROS, [**Dunaief, J. L., Dentchev, T., Ying, G. S. & Milam, A. H.** ***The role of apoptosis in age-related macular degeneration. Arch Ophthalmol*** **120, 1435–42 (2002)]** and it is confirmed that the damage induced by light exposure reduces the mitochondrial membrane potential *in vivo* light-induced retinal degeneration model [**Donovan, M., Carmody, R. J. & Cotter, T. G.** ***Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent.*** **J Biol Chem 276, 23000–8 (2001).]** Therefore, we evaluated the mitochondrial membrane potential. The healthy cells were detected with mainly JC-1 J-aggregates (red) and apoptotic or unhealthy cells with mainly JC-1 monomers (green). Merged cells (yellow) were considered to be pre-apoptotic (early or middle state of transition to cell death) cells [**Yang, L. P., Zhu, X. A. & Tso, M. O.** ***Role of NF-kappaB and MAPKs in light-induced photoreceptor apoptosis. Invest Ophthalmol Vis Sci*** **48, 4766–76 (2007).**] Control cells were almost stained with red (Figure 2D). Blue LED light increased the pro-apoptotic cells (yellow) in time dependent manner (Figure 2E). The ratio of merged cells to red stained cells was significantly increased by blue LED exposure for 12 or 24 h (Figure 2E).

Figure 2: ROS production by blue, white, and green LED light exposure.

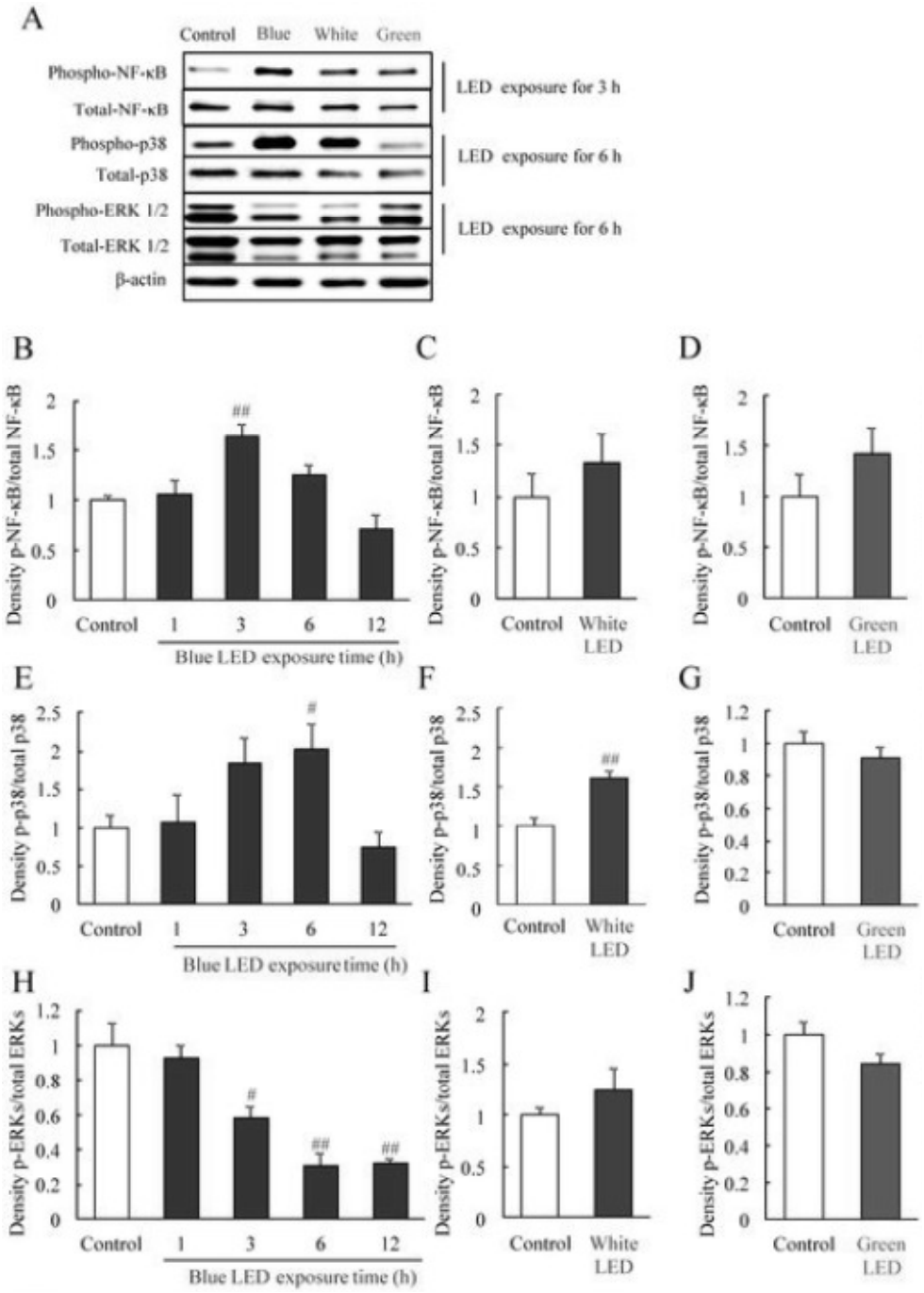


(A–C) Blue LED light and white LED light exposure increased each 1.4-fold and 1.2-fold ROS production, and green LED light did not increase ROS level. Data are expressed as mean ± SEM (n = 6). ## indicates $p < 0.01$ vs. control (ANOVA). (D) Representative images show JC-1 stained cells. The healthy cells with mainly JC-1 J-aggregates (red) and apoptotic or unhealthy cells with mainly JC-1 monomers (green). Merged cells (yellow) were considered to be pre-apoptotic (early or middle state of transition to cell death) cells. Scale bar represents 50 μm. (E) The number of cells with red or yellow color were counted. The ratio of merged cells to red color cells was increased by blue LED light exposure for 12 h or 24 h. Data are expressed as mean ± SEM (n = 6). ## indicates $p < 0.01$ vs. control (ANOVA).

Blue LED light altered the levels of activated-NF-κB, phosphorylated-p38 MAPK, and phosphorylated-ERK

ROS generation induces MAPK activation, and MAPK modulates inflammation, cell death and so on [**Junttila, M. R., Li, S. P. & Westermarck, J. *Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. Faseb J* 22, 954–65 (2008).**]. p38 MAPK is activated by light exposure [**Yang, L. P., Zhu, X. A. & Tso, M. O. *Role of NF-kappaB and MAPKs in light-induced photoreceptor apoptosis. Invest Ophthalmol Vis Sci* 48, 4766–76 (2007**).] [**Sun, M. H. *et al. Photoreceptor protection against light damage by AAV-mediated overexpression of heme oxygenase-1. Invest Ophthalmol Vis Sci* 48, 5699–707 (2007).**] Western blotting was used to investigate the mechanism of photoreceptor-derived cell damage by LED light exposure at 2,500 lux. The protein expression of NF-κB, p38, and ERK were detected after LED exposure (Figure 3A–J). The level of activated NF-κB significantly increased at 3 h after blue LED light (Figure 3B), but not white or green LED light exposure (Figure 3C and 3D). The level of phosphorylated p38 MAPK significantly increased at 3 h, peaked at 6 h, and then declined to control levels at 12 h after blue LED light exposure and similarly increased at 6 h after white LED exposure (Figure 3E and 3F). In contrast, green LED light did not alter the levels of phosphorylated p38 MAPK (Figure 3G). Moreover, blue LED light reduced the levels of phosphorylated ERK after LED light exposure in a time-dependent manner (Figure 3H). White or green LED light did not alter the levels of phosphorylated ERK (Figure 3I and 3J).

Figure 3: Changes in protein levels induced by blue LED light exposure.

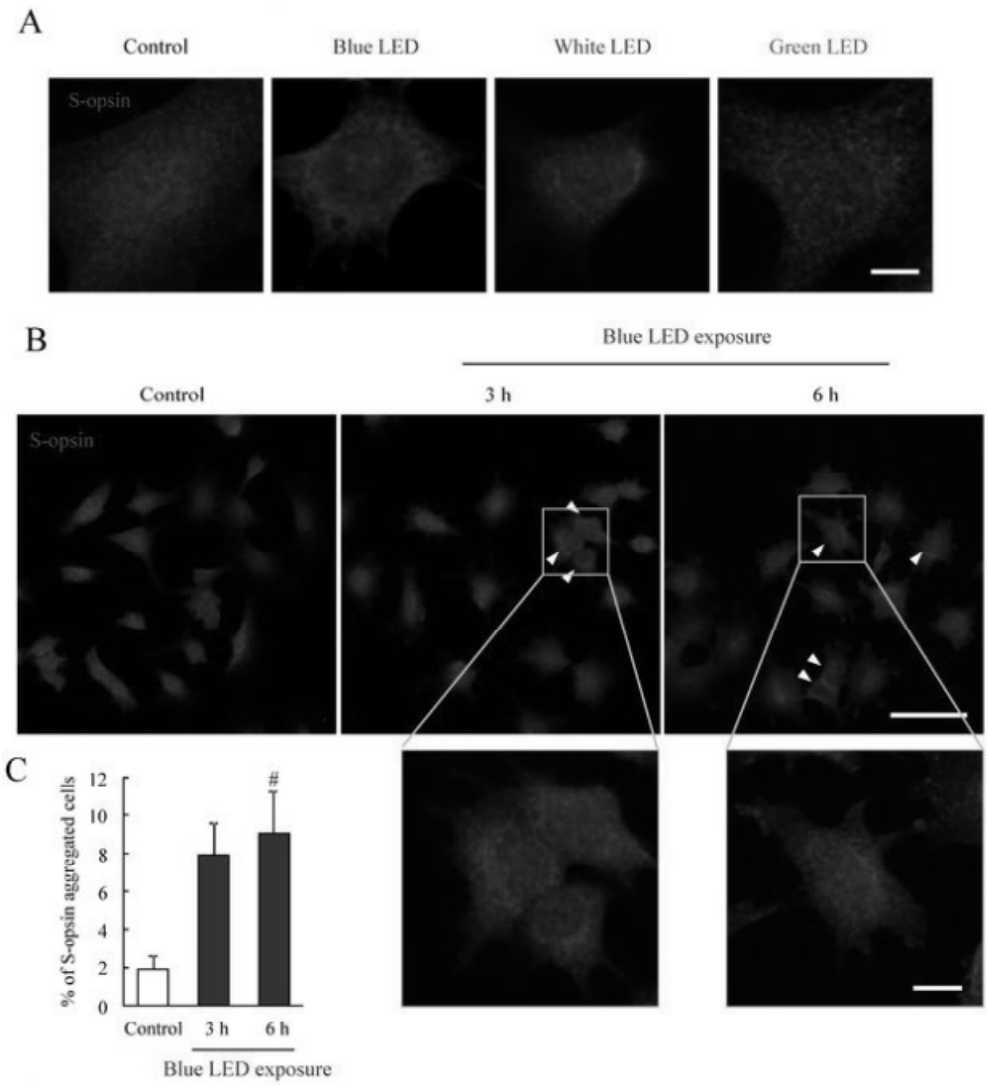


(A) Western blotting showed changes in the levels of phosphorylated NF-κB, p38, and ERK (p-NF-κB, p-p38, and p-ERK). The bands indicate protein expression levels at each 3 h (NF-κB), 6 h (p38), and 6 h (ERK) after LED light exposure. (B–D) Quantitative analysis of protein levels. Quantitative data of the groups of white LED and green LED exposure indicates 3 h (NF-κB), 6 h (p38), and 6 h (ERK) results. The phosphorylated NF-κB level is increased 3 h after exposure to blue LED light. The phosphorylated p38 level is increased 6 h after blue and white LED light exposure. The phosphorylated ERK level is decreased 6 h after blue LED light exposure. These changes were not observed

after green LED light exposure. Data are expressed as mean ± SEM (n = 3 to 6). # indicates ***p* < 0.05,** ## indicates ***p* < 0.01** vs. control (B, E, H; one-way ANOVA followed by Dunnett's test, F; ANOVA).

The aggregation of S-opsin induced by blue and white LED light was observed When compared to 661 W and NB1-RGB cell damage, 661 W cells were more damaged than NB1-RGB cells by blue LED light exposure. This difference may be due to the exisitence of cone photoreceptor specific protein, S-opsin. S-opsin did not observe in NB1-RGB cells. It has been reported that S-opsin is present in 661 W cells **[Tang, P. H., Buhusi, M. C., Ma, J. X. & Crouch, R. K. *RPE65 is present in human green/red cones and promotes photopigment regeneration in an in vitro cone cell model*. *J Neurosci* 31, 18618–26 (2011).]** We evaluated whether LED light exposure caused the aggregation of S-opsin in 661 W cells by immunostaining in LED light exposure for 24 h. The perinuclear aggregation of S-opsin as observed in blue and white LED light exposed cells (Figure 4A). Green LED light did not cause the aggregation (Figure 4A). Next, we investigated whether blue LED light-induced the S-opsin aggregation during early stage. When the cells were exposed by blue LED light for 3 h or 6 h, S-opsin aggregated cells (arrowhead) were observed (Figure 4B). The graph shows the ratio of S-opsin aggregated cells to total cell numbers was increased by blue LED light exposure for 3 or 6 h (Figure 4C).

Figure 4: The aggregation of S-opsin induced by blue LED light exposure.



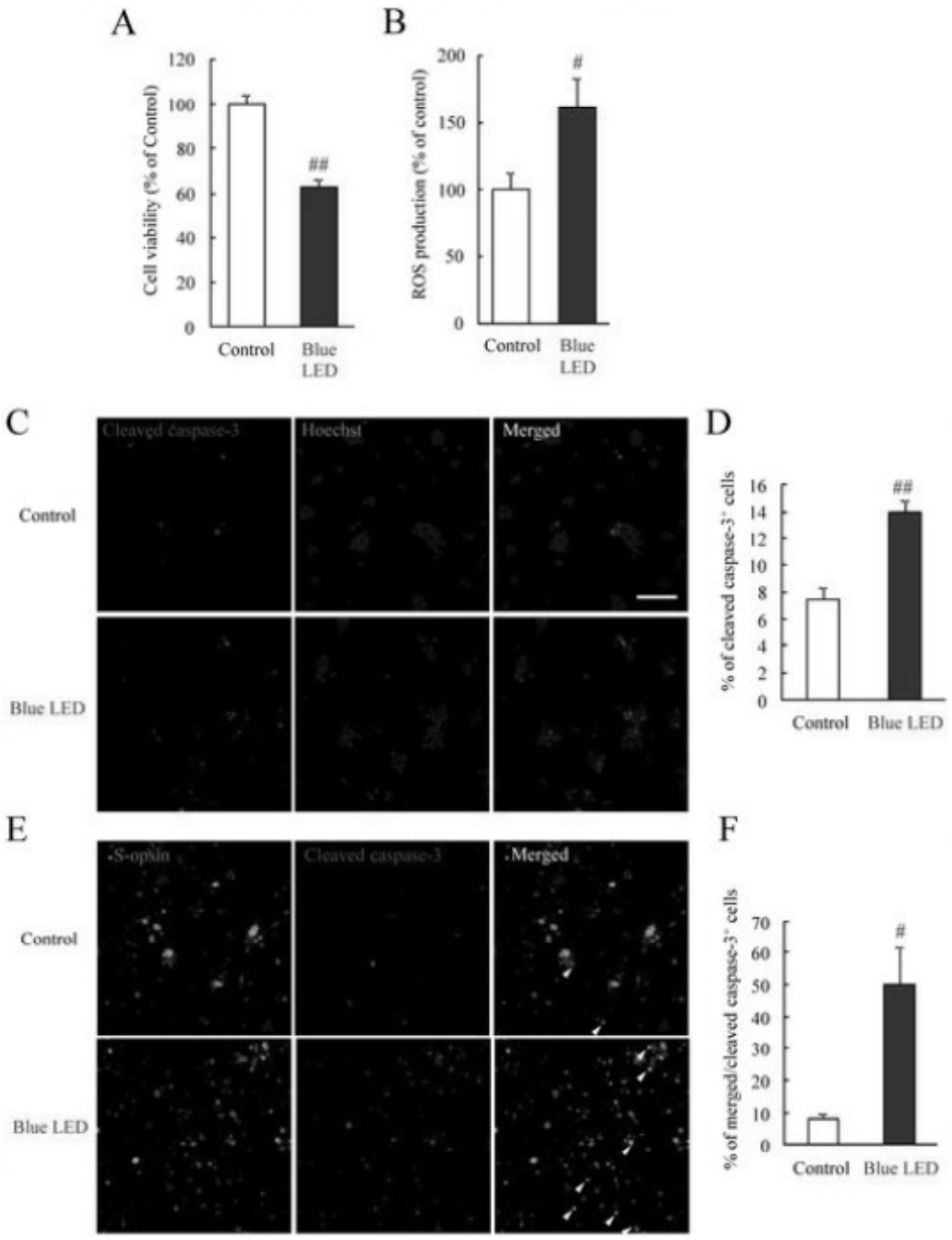
(A) Representative immunostaining images of S-opsin after LED light exposure for 24 h. Blue and white LED light-induced the perinuclear aggregation of S-opsin compared to control and green LED light. n = 4. (B) Representative immunostaining images of S-opsin shows that the S-opsin aggregated cells after blue LED light exposure for 3 or 6 h (arrowhead). (C) Quantitative analysis of immunostaining images. The ratio of the S-opsin aggregated cells was increased by blue LED light exposure for 3 or 6 h. Data are expressed as mean ± SEM (n = 3 or 4). $^{\#}$ indicates $\boldsymbol{p < 0.05}$ vs. control (one-way ANOVA followed by Dunnett's test). The scale bars represent 5 μm (A), 50 μm and 10 μm (B).

Blue LED light induced the photoreceptor cell specific damage

To consider the blue LED light-induced retinal photoreceptor cell damage in detail, we used the primary retinal cells. In this protocol of primary cell culture, the rod photoreceptor cells were obtained about 60% of total cells in our previous report [**Tsuruma, K. et al.** ***Role of oxidative stress in retinal photoreceptor cell death in N-methyl-N-nitrosourea-***

***treated mice*. J Pharmacol Sci 118, 351–62 (2012).].** In this study, we confirmed the ratio of S-opsin positive cells was about 15%. Blue LED light decreased the primary retinal cell viability (Figure 5A). Blue LED light increased the ROS level in primary retinal cells (Figure 5B). Next, we performed the experiment to confirm that the cell damage induced by blue LED light exposure is the definite event in photoreceptor cells. Blue LED light increased the cleaved caspase-3 positive cells by immunostaining (Figure 5C, D). Cleaved caspase-3 implicates active caspase-3. Then, we performed double immunostaining for S-opsin and cleaved caspase-3. Blue LED light increased the S-opsin and cleaved caspase-3 double positive cells (Figure 5E, F).

Figure 5: Blue LED light caused the primary retinal cell damage.

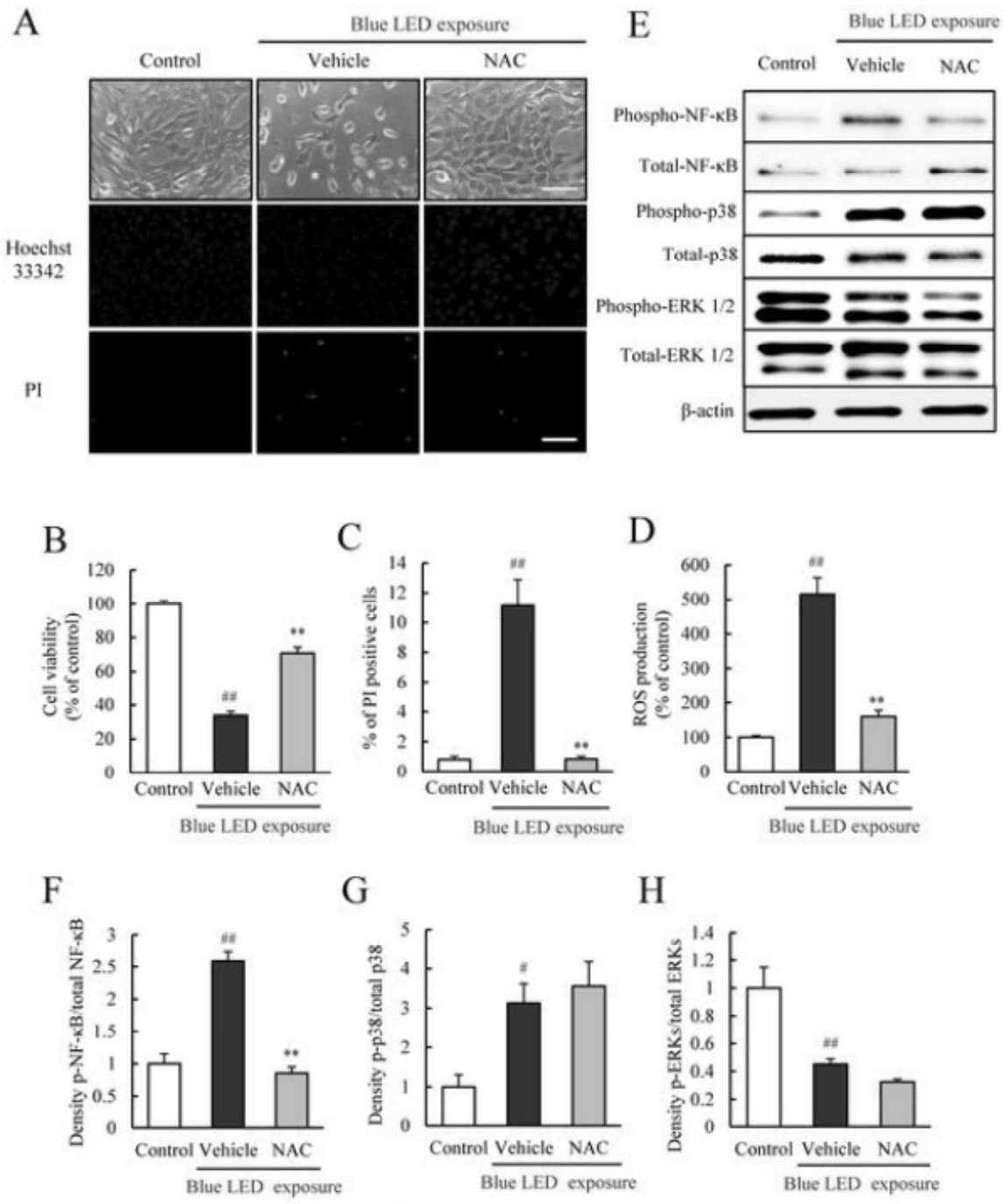


(A) Primary retinal cells were exposed to blue LED light for 24 h. The cell viability was evaluated by the CCK-8 assay. Blue LED light decreased the primary retinal cell viability. (B) Blue LED light increased the ROS level in primary retinal cells. (C, D) Immunostaining of cleaved caspase-3. Blue LED light increased the cleaved caspase-3 positive cells compared to control. (E, F) Double immunostaining for S-opsin and cleaved caspase-3. Blue LED light increased the S-opsin and cleaved caspase-3 double positive cells (arrowhead). Data are expressed as mean ± SEM (n = 3 or 4). $^{\#}$ indicates $p < 0.05$, $^{\#\#}$ indicates $p < 0.01$ vs. control (ANOVA). The scale bar represents 50 μm.

The antioxidant NAC, inhibited the blue LED light-induced cellular damage, ROS generation, and NF-κB activation

Our findings suggested that blue LED light damaged photoreceptor-derived cell most severely compared to the other LED lights. Then, we investigated the protective effects of an antioxidant against blue LED light-induced photoreceptor-derived cell damage. Representative photomicrographs of cell morphology, with Hoechst 33342 and PI staining shows the control, as well as cells treated with vehicle and NAC at 1 mM (Figure 6A). The number of dead cells (PI-positive) was increased in the vehicle-treated group. The treatment with NAC at 1 mM increased the number of viable cells and inhibited photoreceptor-derived cell death (Figure 6A). Furthermore, NAC improved cell viability, and markedly suppressed cell death and ROS production (Figure 6B–D). NAC at 1 mM suppressed the activation of NF-κB caused by blue LED light exposure, but did not affect the blue light-induced phosphorylation of p38 MAPK or ERK (Figure 6E–H).

Figure 6: NAC suppressed the blue LED light-induced damage and inhibited NF-κB activation.



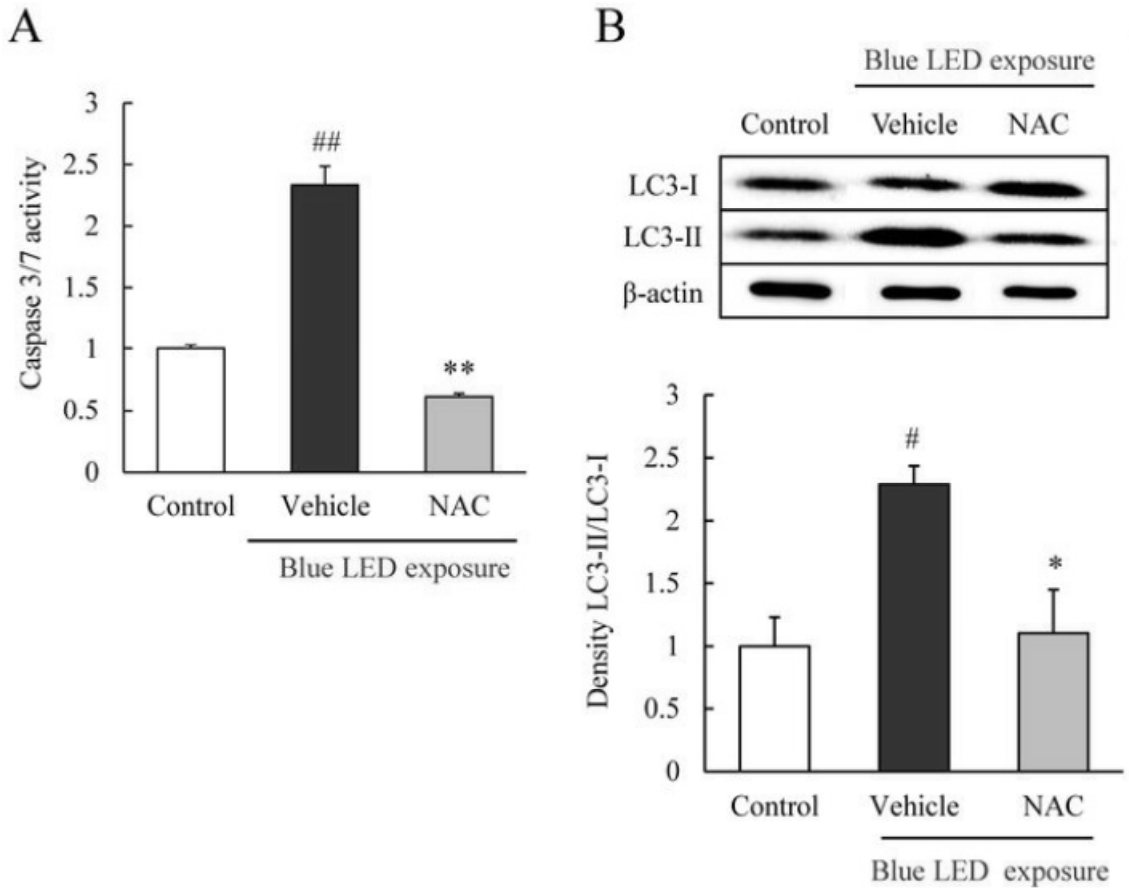
(A–C) Evaluation of the cell viability by the CCK-8assay and the rate of cell death by Hoechst and PI staining. The rate of cell death indicates by the ratio of PI (red) stained cells per Hoechst (blue) stained cells. NAC at 1 mM significantly improved the cell viability reduced by blue LED light. (D) NAC reduced the ROS level elevated by blue LED light. (E–H) The effect of NAC against blue LED light-induced changes in protein expression was assessed by Western blots. NAC suppressed the blue LED light-induced increase in activated NF-κB levels, but did not suppress activated p38. NAC did not alter the reduced ERK level. Data are expressed as mean ± SEM (n = 5 or 6). **

indicates ***p* < 0.01** vs. vehicle; ## indicates ***p* < 0.01** vs. control (one-way ANOVA followed by Tukey's test). The scale bar represents 50 μm. The cropped blots are used in this Figure.

NAC inhibited caspase-3/7 activity and autophagic cell death induced by blue LED light exposure

We evaluated the caspase-3/7 activity by using Caspase-Glo® 3/7 Assay kit. NAC significantly inhibited the increase in caspase-3/7 activity at 12 h after blue LED light exposure (Figure 7A). Moreover, blue LED light-induced changes in the expression of an autophagosome marker, LC3, were evaluated by western blotting. Activation of autophagy is indicated by the conversion of LC3-I into LC3-II. LC3-II was markedly upregulated by blue LED light exposure; however, the conversion of LC3 was significantly decreased by treatment with 1 mM NAC (Figure 7B).

Figure 7: NAC suppressed blue LED light-induced caspase-3/7 activation and autophagy activation.



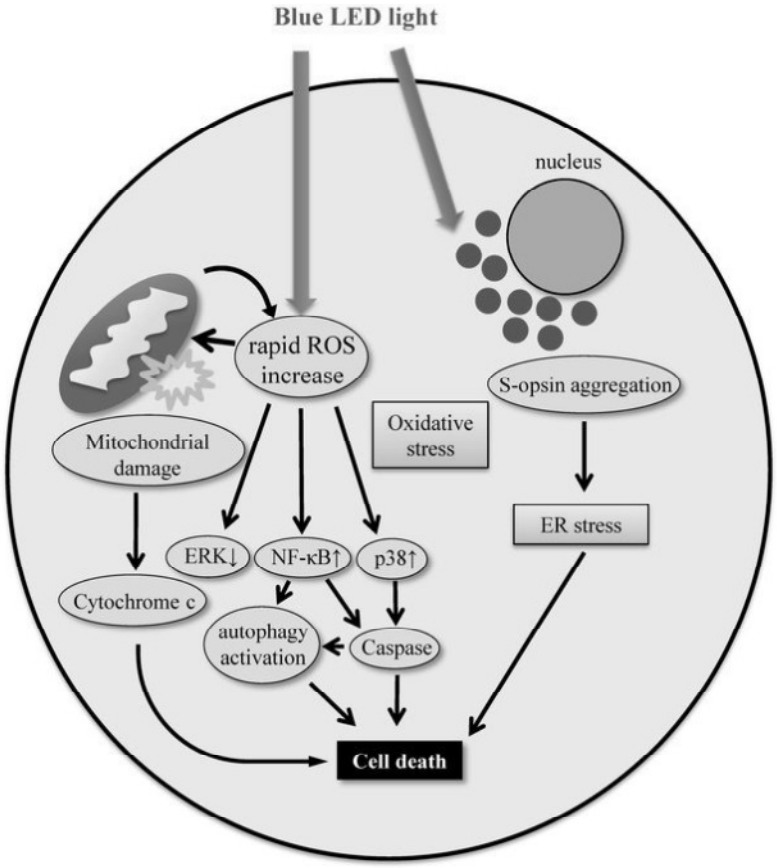
(A) Measurement of caspase-3/7 activity by Caspase-Glo® 3/7 Assay kit. Activation of caspase-3/7 was observed after blue LED light exposure. NAC treatment significantly inhibited the activation. Data are expressed as mean ± SEM (n = 3 or 4). ** indicates *p*

< 0.01 vs. vehicle; ## indicates ***p* < 0.01** vs. control (one-way ANOVA followed by Tukey's test). (B) Western blots of LC3-II/LC3-I indicated an increase in the expression level after blue LED light exposure. NAC treatment significantly reduced the expression. Data are expressed as mean ± SEM (n = 6). * indicates ***p* < 0.05** vs. vehicle; ## indicates ***p* < 0.01** vs. control (one-way ANOVA followed by Tukey's test). The cropped blots are used in this Figure.

Discussion

In the present study, we demonstrated that the *in vitro* exposure to blue LED light damaged the 661 W cells more severely compared to white or green LED light. In primary retinal cell culture, blue LED light damaged retinal photoreceptor cells. Blue LED light-induced 661 W cell damage was associated with rapid ROS increase, NF-κB activation, p38 activation, ERK 1/2 inactivation, S-opsin aggregation, and activated caspase-3/7 and autophagy (Figure 8).

Figure 8: The putative pathway of blue LED light-induced retinal photoreceptor-derived cell damage.



In 661 W cells, blue LED light induces ROS production and S-opsin aggregation. The rapid ROS increase leads to mitochondrial damage and the MAPK activation or the nuclear translocation of NF-κB. Activated MAPK and NF-κB induces the activation of

caspase and leads to apoptotic cell death. Active NF-κB also activates autophagy, and excessive autophagy leads to cell death. While, S-opsin aggregation causes endoplasmic reticulum (ER) stress. Blue LED light-induced retinal photoreceptor-derived cell death may be associated with both oxidative stress and ER stress.

Although the effect of blue LED light in altering the circadian rhythm has been reported [**Wright, H. R., Lack, L. C. & Partridge, K. J.** ***Light emitting diodes can be used to phase delay the melatonin rhythm.*** **J Pineal Res 31, 350–5 (2001).**] the retinal photoreceptor cell damage induced by blue LED light is not fully understood. Compared to three types of colored LED light, blue LED light damaged the photoreceptor-derived cells the more severely than white and green LED lights. It is known that blue light has a shorter wavelength, while green light has a longer wavelength. It has been reported that ROS levels are more increased by shorter wavelength light than by longer wavelength light exposure [**Rozanowska, M. et al.** ***Blue light-induced singlet oxygen generation by retinal lipofuscin in non-polar media.*** **Free Radic Biol Med 24, 1107–12 (1998).]**

Furthermore, in RPE, the association between blue light-induced ROS increase and mitochondria has been reported [**Godley, B. F. et al.** ***Blue light induces mitochondrial DNA damage and free radical production in epithelial cells.*** **J Biol Chem 280, 21061–6 (2005).**] This is thought that mitochondria include blue light-sensitive chromophore. In addition to this significance, photoreceptor cells possess S-opsin and S-opsin absorbs the short wavelength light. We observed the S-opsin aggregation by blue LED light exposure (Figure 4A–C). The S-opsin aggregates could cause the ROS increase. The photoreceptor cell death may be associated with the oxidative stress induced by ROS generation. In the present study, ROS production by blue LED light exposure for 24 h was greater than that induced by white and green LED lights exposure for 24 h. Also, green LED light did not induce the cell damage, although green LED light increased the ROS level in 24 h exposure. This may be due to the mild increase of ROS production over 24 h of green LED light exposure. ROS production by blue, white and green LED light exposure for 6 h increased each 1.4-fold, 1.2-fold and 1.0-fold (not changed) compared to control (Figure 2A–C). These findings indicate that 661 W cell damage is induced by the ROS generated by short wavelength LED light. Moreover, it was suggested that rapid ROS increase caused the decrease of the mitochondrial membrane potential. Although this result is consistent with in an *in vivo* light-induced retinal degeneration model [**Donovan, M., Carmody, R. J. & Cotter, T. G.** ***Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent.*** **J Biol Chem 276, 23000–8 (2001).**] it is considerable because this result is obtained *in vitro* study. The ratio of mitochondrial membrane potential decreased cells was about 12% and it seemed to be not correlated with the result of cell viability (Figure 2E, 1C).

Moreover, we observed the aggregation of S-opsin by blue and white LED light exposure. The perinuclear aggregates lead to the photoreceptor cell death, and the cell death is associated with ER stress [**Zhang, T., Zhang, N., Baehr, W. & Fu, Y.** ***Cone opsin determines the time course of cone photoreceptor degeneration in Leber congenital amaurosis. Proc Natl Acad Sci U S A*** **108, 8879–84 (2011).**] [**Nakanishi, T.** ***et al. Role of endoplasmic reticulum stress in light-induced photoreceptor degeneration in mice. J Neurochem*** **125, 111–24 (2013).**] The percent of S-opsin aggregated cells was about 9%, and it was thought that this ratio was correlated with 10% of PI positive cells in 12 h after blue LED light exposure (Figure 6C). These

findings indicate that the short wavelength LED light can cause the cone photoreceptor-derived cell death by both oxidative stress induced by rapid ROS increase and ER stress induced by the aggregation of S-opsin. In present study, blue LED light exposure decreased by 60% of cell viability and induced 1.6-fold increase of ROS production in primary retinal cells (Figure 5A, B). This result was different from the result of 661 W cells. This difference was due to containing the cells except photoreceptor cells in the primary retinal cells, and it was thought that blue LED light damaged only photoreceptor cells. The consideration reflected the increase of cleaved caspase-3 positive cells in S-opsin positive cells. However, about half of cleaved caspase-3 positive cells were not stained S-opsin. It is thought that rhodopsin absorves approximately 500 nm wavelength light, and it was reported blue light-induced retinal damage was mediated rhodopsin[1]. Hence, cleaved caspase-3 positive cells but not positive S-opsin were supposed to be rod photoreceptor cells.

In the present study, the activation of NF-κB, p38 MAPK, and ERK preceded the photoreceptor cell damage by blue LED light. Tumor necrosis factor α (TNF-α) and phorbol-12-myristate 13-acetate (PMA) induced NF-κB activation occur through ROS generation, and NAC suppressed this activation [**Staal, F. J., Roederer, M., Herzenberg, L. A. & Herzenberg, L. A.** ***Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus.*** ***Proc Natl Acad Sci U S A*** **87, 9943–7 (1990).**] The nuclear translocation of NF-κB promotes apoptosis [**Khandelwal, N.** ***et al. Nucleolar NF-kappaB/RelA mediates apoptosis by causing cytoplasmic relocalization of nucleophosmin.*** ***Cell Death Differ*** **18, 1889–903 (2011).**]Thus, it is presumed that the blue LED light-induced ROS production promotes NF-κB phosphorylation and subsequent the nuclear translocation of NF-κB, leading to photoreceptor cell death. Similar results were obtained in another study [**Tanito, M. et al.** ***Change of redox status and modulation by thiol replenishment in retinal photooxidative damage.*** **Invest Ophthalmol Vis Sci 43, 2392–400 (2002).**] [**Wu, T., Chen, Y., Chiang, S. K. & Tso, M. O.** ***NF-kappaB activation in light-induced retinal degeneration in a mouse model.*** ***Invest Ophthalmol Vis Sci*** **43, 2834–40 (2002).**] In this study, NAC suppressed the NF-κB phosphorylation and the ROS generation induced by blue LED light in 661 W photoreceptor cells. Taken together, it can be suggested that NAC inhibited the blue light-induced photoreceptor damage through suppression of NF-κB phosphorylation and nuclear translocation and ROS generation. p38 MAPK was also activated by blue LED light. This is considered to be an apoptotic factor, and in oxidative stress, its activation is mainly occurs by superoxide anion [**Aruoma, O. I., Halliwell, B., Hoey, B. M. & Butler, J.** ***The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid.*** ***Free Radic Biol Med*** **6, 593–7 (1989).**]

In the present study, blue LED light-induced cell death activated caspase-3/7 and autophagy. It has been reported that caspase-3/7 is involved in the photoreceptor cell death induced by light exposure [**Wu, J., Gorman, A., Zhou, X., Sandra, C. & Chen, E.** ***Involvement of caspase-3 in photoreceptor cell apoptosis induced by in vivo blue light exposure.*** ***Invest Ophthalmol Vis Sci*** **43, 3349–54 (2002).**]The damage induced by light exposure reduces the mitochondrial membrane potential [**Donovan, M., Carmody, R. J. & Cotter, T. G.** ***Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent.*** **J Biol Chem 276, 23000–8 (2001).**] [**van de Water, B., Zoeteweij, J. P., de Bont, H. J., Mulder, G. J. & Nagelkerke, J. F.** ***Role of mitochondrial Ca2+ in the oxidative stress-induced dissipation of the mitochondrial membrane potential. Studies in isolated proximal tubular cells using the nephrotoxin 1,2-dichlorovinyl-L-cysteine.*** ***J Biol Chem*** **269, 14546–52 (1994).**] We investigated the extent of cell damage using

CCK-8 assay, which reflects the mitochondrial function. Also, in our JC-1 study, we observed blue LED light caused the mitochondrial damage. Caspase-3 is activated via the release of cytochrome c from the mitochondria *[Li, Z. et al.* **Caspase-3 activation via mitochondria is required for long-term depression and AMPA receptor internalization***. Cell 141, 859–71 (2010).]* Thus, it is thought that blue LED light-induced caspase-3/7 activation is due to the disruption of the mitochondrial membrane potential.

On the other hand, autophagy is generally a self-clearance mechanism, that favors cell survival [***Levine, B. & Yuan, J.*** **Autophagy in cell death: an innocent convict?** ***J Clin Invest 115, 2679–88 (2005).]***while excessive autophagy can induce cell death *[**Pattingre, S. et al.** **Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy***. Cell 122, 927–39 (2005).] [**Yu, L., Strandberg, L. & Lenardo, M. J.** **The selectivity of autophagy and its role in cell death and survival***. Autophagy 4, 567–73 (2008).]* Moreover, NF-κB regulates the transcription of Beclin 1, which has a role in autophagy, and promotes this process *[**Copetti, T., Bertoli, C., Dalla, E., Demarchi, F. & Schneider, C.*** **p65/RelA modulates BECN1 transcription and autophagy***. Mol Cell Biol 29, 2594–608 (2009).]*Active caspase-3 facilitates in the cleavage of Beclin 1 and leads to autophagy *[**Kang, R., Zeh, H. J., Lotze, M. T. & Tang, D.** **The Beclin 1 network regulates autophagy and apoptosis***. Cell Death Differ 18, 571–80 (2011).] [**Tiwari, M., Lopez-Cruzan, M., Morgan, W. W. & Herman, B.** **Loss of caspase-2-dependent apoptosis induces autophagy after mitochondrial oxidative stress in primary cultures of young adult cortical neurons***. J Biol Chem 286, 8493–506 (2011).]* It has been reported that oxidative stress induces autophagy *[**Scherz-Shouval, R. et al.** **Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4***. Embo J 26, 1749–60 (2007).]* and LC3 conversion, from LC3-I into LC3-II, is influenced by light-induced photoreceptor cell damage *[**Kunchithapautham, K., Coughlin, B., Lemasters, J. J. & Rohrer, B.** **Differential effects of rapamycin on rods and cones during light-induced stress in albino mice***. Invest Ophthalmol Vis Sci 52, 2967–75 (2011).]*

These findings suggest that blue LED light activates caspases and autophagy, which are the downstream events of NF-κB activation.

In conclusion, the present findings suggest that the blue LED light can damage the retinal cone photoreceptor cells severely.

STUDY 2

Tosini G, Ferguson I, Tsubota K, *Effects of blue light on the circadian system and eye physiology*. Mol Vis. 2016 Jan 24;22:61-72. eCollection 2016.

The white-light LED (i.e., the most common type of LED) is essentially a bichromatic source that couples the emission from a blue LED (peak of emission around 450–470 nm with a full width at half max of 30–40 nm) *[**Nakamura S, Chichibu S. Introduction to Nitride Semiconductor Blue Lasers and Light Emitting Diodes. 2000; CRC Press; 1st 386 pages.**]* with a yellow phosphor (peak of emission around 580 nm with a full width at half max of 160 nm) that appears white to the eye when viewed directly' [Nakamura S. Present performance of InGaN-based blue/green/yellow LEDs. Light-Emitting Diodes: Research, Manufacturing, and Applications. Proc SPIE. 1997;xxx:26.] The specific pump wavelength of the phosphor in the range 450–470 nm depends critically on the absorption properties of the phosphor. Although the white-light LED can be considered the SSL analog of the fluorescent source, the power spectrum of the white-light LED is considerably different

from traditional, fluorescent, or incandescent white light sources [***Ferguson I, Melton A, Li N, Nicol D. Park, Tosini G. Imitating Broadband Diurnal Light Variations Using Solid State Light Sources. Journal of Light & Visual Environment. 2008;32:63–8.]*** (Figure 1).

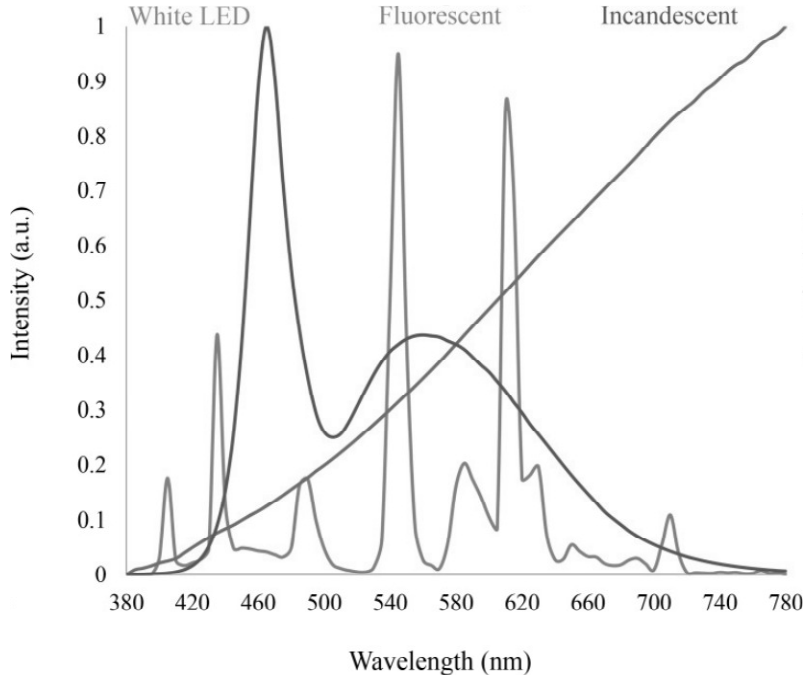


Figure 1
A comparison of the power spectrum of a standard white-light LED, a tricolor fluorescent lamp, and an incandescent source. The radically different power spectrums can look similar when viewed directly by the eye, irrespective of how much blue emission **...**
Early commercial devices lacked sophistication, adopting the currently available LED technology that was small, 350×350 mm^2, and operated at low drive currents, typically 20 mA, producing 1–16 mW of power. The last decade has seen the scaling of LEDs to larger areas, 1×1 μm^2, and higher drive currents of >350 mA with significantly increased power output >1,000 mW [***Pimputkar S, Speck J, DenBaars S, Nakamura S. Prospects for LED lighting. Nat Photonics. 2009;3:180–2.]***

In addition, white-light LEDs degrade over time primarily through bleaching of phosphors so that they no longer efficiently absorb blue light [***Brinkley S, Pfaff N, Denault K, Zhang Z, Hintzen H, Seshadri R, Nakamura S, DenBaars S. Robust thermal performance of $Sr_2SiN_8:Eu^{2+}$: An efficient red emitting phosphor for light emitting diode based white lighting. Appl Phys Lett. 2011;99:241106.]*** This shifts the color temperature of the device over time, with a corresponding change in the color-rendering index but, more importantly, an increasing blue emission from the device with time.

Non-image-forming photoreception
In mammals, photoreception occurs only in the retina ***[Yamazaki S, Goto M, Menaker M. No evidence for extraocular photoreceptors in the circadian system of the Syrian hamster. J Biol Rhythms. 1999;14:197–201.]*** by three types of photoreceptor: cones, rods, and the intrinsically photosensitive retinal ganglion cells (ipRGCs). The classical photoreceptors (e.g., rods

and cones) are mostly responsible for the image-forming vision, whereas the ipRGCs play a major role in non-image-forming photoreception, that is, the photoreceptive system that regulates circadian photic entrainment, pupillary light response, and other important biologic functions (Figure 2).

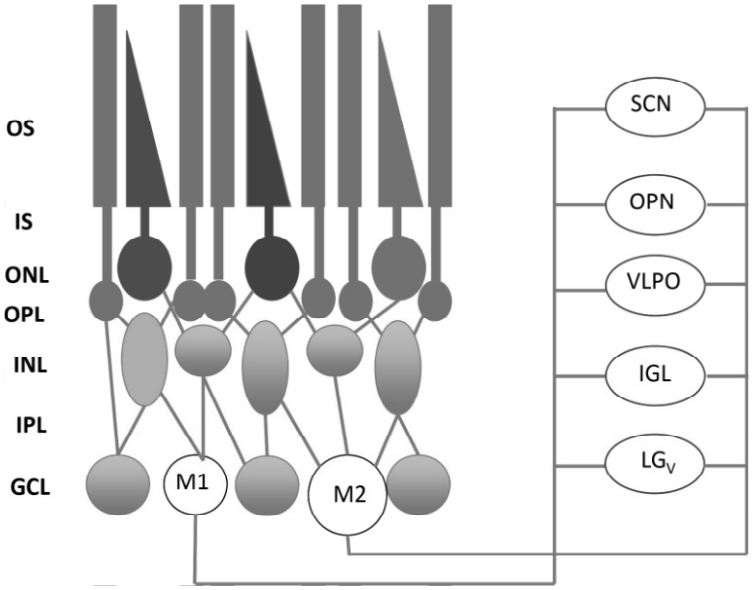


Figure 2

The most likely candidate to emerge as the circadian retinal photo pigment is a mammalian homolog of *Xenopus* melanopsin (*aka Opn4)* ***[Provencio I, Jiang G, DeGrip WJ, Hayes WP, Rollag MD. Melanopsin: An opsin in melanophores, brain, and eye. Proc Natl Acad Sci USA. 1998;95:340–5.] [Provencio I, Rodriguez IR, Jiang G, Hayes WP, Moreira EF, Rollag MD. A novel human opsin in the inner retina. J Neurosci. 2000;20:600–5.] [Bellingham J, Chaurasia SS, Melyan Z, Liu C, Cameron MA, Tarttelin EE, Iuvone PM, Hankins MW, Tosini G, Lucas RJ. Evolution of melanopsin photoreceptors: Discovery and characterization of a new melanopsin gene in non-mammalian vertebrates. PLoS Biol. 2006;4:e254.]*** In mammals, melanopsin mRNA (and protein) is expressed only in a small population (about 3–5%) of the RGCs **[*Provencio I, Rodriguez IR, Jiang G, Hayes WP, Moreira EF, Rollag MD. A novel human opsin in the inner retina. J Neurosci. 2000;20:600–5.] [Hattar S, Liao HW, Takao M, Berson DM, Yau KW. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science. 2002;295:1065–70.]*** that are directly photosensitive and have an absorption peak around 470–480 nm ***[Berson DM, Dunn FA, Takao M. Phototransduction by ganglion cells innervating the circadian pacemaker. Science. 2002;295:1070–3.] [Qiu X, Kumbalasiri T, Carlson SM, Wong KY, Krishna V, Provencio I, Berson DM. Induction of photosensitivity by heterologous expression of melanopsin. Nature. 2005;433:745–9.] [Bailes HJ, Lucas RJ. (2013. Human melanopsin forms a pigment maximally sensitive to blue light (λmax ≈ 479 nm. supporting activation of G(q/11. and G(i/o. signalling cascades. Proc Biol Sci. 2013;280:20122987.]*** These RGCs express pituitary adenylate cyclase-activating polypeptide (PACAP) **[*Hannibal J, Hindersson P, Knudsen SM, Georg B, Fahrenkrug J. The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. J Neurosci. 2002;22:RC191]*** and form the retinohypothalamic tract (RHT) ***[Hattar S, Liao HW, Takao M, Berson DM, Yau KW. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science. 2002;295:1065–70.] [Gooley JJ, Lu J, Chou TC, Scammell TE, Saper CB. Melanopsin in cells of origin of the retinohypothalamic tract. Nat Neurosci.*** 2001;4:1165.] . The RHT is responsible for conveying the light information from RGCs to the part of the brain that controls circadian rhythms within the whole body **[*Moore RY, Lenn NJ. A retinohypothalamic projection in the rat. J Comp Neurol. 1972;146:1–14.] [Johnson RF, Moore RY, Morin LP. Loss of***

entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract. Brain Res. 1988;460:297–313.] The RGCs that express melanopsin were named intrinsically photosensitive RGCs (ipRGCs), and these cells were no longer intrinsically photosensitive in melanopsin knockout (KO) mice, although the cell number, morphology, and projections remained unchanged [*Hattar S, Lucas RJ, Mrosovsky N, Thompson S, Douglas RH, Hankins MW, Lem J, Biel M, Hofmann F, Foster RG, Yau KW. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature. 2003;424:76–81.]*

Further studies have also shown that melanopsin-based photoreception is involved in the modulation of sleep **[***Lupi D, Oster H, Thompson S, Foster RG. The acute light-induction of sleep is mediated by OPN4-based photoreception. Nat Neurosci. 2008;11:1068–73.] [Altimus CM, Güler AD, Villa KL, McNeill DS, Legates TA, Hattar S. Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. Proc Natl Acad Sci USA. 2008;105:19998–20003.] [Tsai JW, Hannibal J, Hagiwara G, Colas D, Ruppert E, Ruby NF, Heller HC, Franken P, Bourgin P. Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4 (–/–. mice. PLoS Biol. 2009;7:e1000125.] [Muindi F, Zeitzer JM, Colas D, Heller HC. The acute effects of light on murine sleep during the dark phase: importance of melanopsin for maintenance of light-induced sleep. Eur J Neurosci.* **2013;37:1727–36.]** and mood and learning **[***LeGates TA, Altimus CM, Wang H, Lee HK, Yang S, Zhao H, Kirkwood A, Weber ET, Hattar S. Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. Nature. 2012;491:594–8.],* and recent data have also indicated that melanopsin-based photoreception may be involved in the regulation of metabolism *[Aytürk DG, Castrucci AM, Carr DE, Keller SR, Provencio I. Lack of Melanopsin Is Associated with Extreme Weight Loss in Mice upon Dietary Challenge. PLoS One. 2015;10:e0127031.].* Finally, it has been reported that loss of the melanopsin gene abolishes circadian control in some parameters of cone electroretinogram, causing significant attenuation of the diurnal variation in cone vision **[***Barnard AR, Hattar S, Hankins MW, Lucas RJ. Melanopsin regulates visual processing in the mouse retina. Curr Biol. 2006;16:389–95].* Melanopsin signaling may influence intraretinal signaling by acting on dopaminergic neurons *[Zhang DQ, Wong KY, Sollars PJ, Berson DM, Pickard GE, McMahon DG. Intraretinal signaling by ganglion cell photoreceptors to dopaminergic amacrine neurons. Proc Natl Acad Sci USA. 2008;105:14181–6.*]. Therefore, these data suggest melanopsin-dependent regulation of visual processing within the retina.

Melanopsin also plays an important role in mediating human circadian rhythms. Several studies have reported that in humans, the action spectra for melatonin suppression has a lambda max (λ_{max}) of around 460 nm, suggesting that melanopsin is a key player in the photic regulation of melatonin levels *[Brainard GC, Hanifin JP, Greeson JM, Byrne B, Glickman G, Gerner E, Rollag MD. Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. J Neurosci. 2001;21:6405–12.] [Thapan K, Arendt J, Skene DJ. An action spectrum for melatonin suppression: evidence for a novel non-rod, non-cone photoreceptor system in humans. J Physiol. 2001;535:261–7]. [Najjar RP, Chiquet C, Teikari P, Cornut PL, Claustrat B, Denis P, Cooper HM, Gronfier C. Aging of non-visual spectral sensitivity to light in humans: compensatory mechanisms? PLoS One. 2014;9:e85837.]* Additional studies have also shown that blue light in the range of 460–480 nm is more effective compared to monochromatic light of 555 nm in phase-shifting the human circadian clock *[Lockley SW, Brainard GC, Czeisler CA. High sensitivity of the human circadian melatonin rhythm to resetting by short wavelength light. J Clin Endocrinol Metab. 2003;88:4502–5.] [Rüger M, St Hilaire MA, Brainard GC, Khalsa SB, Kronauer RE, Czeisler CA, Lockley SW. Human phase response curve to a single 6.5 h pulse of short-wavelength light. J Physiol. 2013;591:353–63.]* Finally, a recent study expanded these previous results by showing that light in the 555 nm range may significantly affect the synchronization of the circadian system to light exposure of short duration or to low irradiance, whereas light in the 460 nm range is more effective in

phase-shifting the circadian system than exposure to light of longer duration and higher irradiance [***Gooley JJ, Rajaratnam SM, Brainard GC, Kronauer RE, Czeisler CA, Lockley SW. Spectral responses of the human circadian system depend on the irradiance and duration of exposure to light. Sci Transl Med. 2010;2:31ra33.].*** A recent study reported that exposure to light-emitting e-readers at bedtime may negatively affect sleep and the circadian system [***Chang AM, Aeschbach D, Duffy JF, Czeisler CA. Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness. Proc Natl Acad Sci USA. 2015;112:1232–7].***

With age, the lens becomes more yellowish, and thus, the spectrum of blue light transmission dramatically decreases through the years. It is suspected that one reason older individuals experience sleep problems is the lack of blue light during the daytime. Ayaki et al. ***[Ayaki M, Muramatsu M, Negishi K, Tsubota K. Improvements in sleep quality and gait speed after cataract surgery. Rejuvenation Res. 2013;16:35–42.]*** reported that after cataract extraction, sleep quality improved dramatically because more blue light could pass through the intraocular lens. In addition, there has been a discussion on whether a clear or yellow lens is preferable [***Mainster MA. Violet and blue light blocking intraocular lenses: photoreception versus photoreception. Br J Ophthalmol. 2006;90:784–92.]*** Of course, the yellow lens may protect the retina, but the clear lens provides more blue light during the day, providing better quality of sleep ***[Ayaki M, Negishi K, Suzukamo Y, Tsubota K. Color of intra-ocular lens and cataract type are prognostic determinants of health indices after visual and photoreceptive restoration by surgery. Rejuvenation Res. 2015;18:145–52.]*** Consistent with this result, Sletten et al. [***Sletten TL, Revell VL, Middleton B, Lederle KA, Skene DJ. Age-related changes in acute and phase-advancing responses to monochromatic light. J Biol Rhythms. 2009;24:73–84.]*** reported that in older people, acute exposure to blue light, but not to green light, significantly decreased their alertness and suppressed their sleep and melatonin production compared to young people.

Light-induced damage to the retina

Several investigations have shown that exposure to light of specific wavelengths or intensity may induce severe damage to the retina ***[Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. Prog Retin Eye Res. 2005;24:275–306.] [Organisciak DT, Vaughan DK. Retinal light damage: mechanisms and protection. Prog Retin Eye Res. 2010;29:113–34.]*** This type of damage is called light-induced damage. Light can induce damage via three mechanisms: photomechanical, photothermal, and photochemical. Photomechanical damage is due to a rapid increase in the amount of energy captured by the RPE, which may cause irreversible damage to the RPE and lead to photoreceptor damage. This type of retinal damage depends on the amount of energy absorbed and not on the spectral composition of the light. Photothermal damage occurs when the retina and the RPE are exposed to brief (100 ms to 10 s) but intense light that induce a significant increase in the temperature of these tissues **[*Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. Prog Retin Eye Res. 2005;24:275–306.] [Organisciak DT, Vaughan DK. Retinal light damage: mechanisms and protection. Prog Retin Eye Res. 2010;29:113–34.]***

A more common type of retinal/RPE damage is photochemical damage, which occurs when the eyes are exposed to light of high intensity in the visible range (390–600 nm). The current view suggests that there are two distinct types of photochemical damage. The first type is associated with short but intense exposure to light affecting the RPE, and the second type is associated with longer but less intense light exposure, affecting

the outer segment of the photoreceptors. Short (up to 12 h) exposure to blue light may induce damage in the RPE of the rhesus monkey [*Ham WT, Ruffolo JJ, Mueller HA, Clarke AM, Moon ME. Histologic analysis of photochemical lesions produced in rhesus retina by short-wave-length light. Invest Ophthalmol Vis Sci. 1978;17:1029–35.]* and a clear relationship has been found between the extent of the damage and the oxygen concentration [*[Ruffolo JJ, Ham WT, Mueller HA, Millen JE. Photochemical lesions in the primate retina under conditions of elevated blood-oxygen. Invest Ophthalmol Vis Sci. 1984;25:893–8.] [Jaffe GJ, Irvine AR, Wood IS, Wood IS, Severinghaus JW, Pino GR, Haugen C. Retinal phototoxicity from the operating microscope: the role of inspired oxygen. Ophthalmology. 1988;95:1130–41.]* The fact that many different antioxidants can reduce the damage suggests that this type of damage is associated with oxidative processes *[[Dillon J. The photophysics and photobiology of the eye. J Photochem Photobiol B. 1991;10:23–40.] [Organisciak DT, Winkler BS. Retinal light damage: practical and theoretical considerations. Prog Retin Eye Res. 1994;13:1–29.]* Experimental data suggest that lipofuscin is the chromophore involved in the mediation of light-induced retinal damage following the exposure to blue light *[Sparrow JR, Nakanishi K, Parish CA. The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. Invest Ophthalmol Vis Sci. 2000;41:1981–9.] [Rózanowska M, Jarvis-Evans J, Korytowski W, Boulton ME, Burke JM, Sarna T. Blue light-induced reactivity of retinal age pigment. In vitro generation of oxygen-reactive species. J Biol Chem. 1995;270:18825–30.] [Rózanowska M, Wessels J, Boulton M, Burke JM, Rodgers MA, Truscott TG, Sarna T. Blue light-induced singlet oxygen generation by retinal lipofuscin in non-polar media. Free Radic Biol Med. 1998;24:1107–12.] [Pawlak A, Rozanowska M, Zareba M, Lamb LE, Simon JD, Sarna T. Action spectra for the photoconsumption of oxygen by ocular lipofuscin and lipofuscin extracts. Arch Biochem Biophys. 2002;403:59–62.]*

The second type of light-induced photochemical damage occurs with longer (12–48 h) but less intense light exposure. This type of damage was initially observed in albino rats *[Noell WK, Walker VS, Kang BS, Berman S. Retinal damage by light in rats. Invest Ophthalmol Vis Sci. 1966;5:450–73.]* but has also been observed in other species. The cones seem to be more vulnerable compared to the rods [*Sykes SM, Robison WG, Waxler M. Kuwabara T. Damage to the monkey retina by broad-spectrum fluorescent light. Invest Ophthalmol Vis Sci. 1981;20:425–34.]* Several lines of evidence suggest that the visual photo pigments (e.g., rhodopsin and cone opsins) are involved in this type of damage. Early studies *[Noell WK, Albrecht R. Irreversible effects on visible light on the retina: role of vitamin A. Science. 1971;172:76–9.] [Organisciak DT, Noell WK. The rod outer segment phospholipid/opsin ratio of rats maintained in darkness or cyclic light. Invest Ophthalmol Vis Sci. 1997;16:188–90.] [Williams TP, Howell WL. Action spectrum of retinal light-damage in albino rats. Invest Ophthalmol Vis Sci. 1983;24:285–7.]* also provided evidence that the action spectrum for light-induced photoreceptor damage is similar to the absorption spectrum of rhodopsin, but later studies indicated that blue light (400–440 nm) might be more damaging *[Ham WT, Mueller HA, Ruffolo JJ, Clarke AM. Sensitivity of the retina to radiation damage as a function of wavelength. Photochem Photobiol. 1979;29:735–43.] [Van Norren D, Schellekens P. Blue light hazard in rat. Vision Res. 1990;30:1517–20.] [Rapp LM, Smith SC. Evidence against melanin as the mediator of retinal phototoxicity by short-wavelength light. Exp Eye Res. 1992;54:55–62.]* Grimm et al. [*Grimm C, Wenzel A, Williams TP, Rol P, Hafezi F, Remé C. Rhopopsin-mediated blue-light damage to the rat retina: Effect of photoreversal of bleaching. Invest Ophthalmol Vis Sci. 2001;42:497–505].* provided an explanation for this phenomenon, demonstrating that in vivo bleached rhodopsin may be regenerated not only via a metabolic pathway (e.g., via the visual cycle) but also via a photochemical reaction called photoreversal of bleaching *[Williams TP. Photoreversal of rhopospin bleaching. J Gen Physiol. 1964;47:679–89.]* that is most effective with blue light. Photoreversal of bleaching augments the capability of rhodopsin molecules to absorb photons by several orders of magnitude, thus allowing the molecules to reach the critical number of photons required to induce damage in the retinal cells [*Keller C, Grimm C, Wenzel A, Hafezi F, Remé C. Protective effect of halothane anesthesia on retinal light damage: inhibition of metabolic rhodopsin regeneration. Invest Ophthalmol Vis Sci. 2001;42:476–80.]*

This process can further increase the potential production of reactive oxygen species (ROS); thus, the oxidative damage can lead to the accumulation and build-up of lipofuscin in the RPE. The build-up of lipofuscin in the RPE can affect the ability of the RPE to provide nutrients to the photoreceptors, affecting photoreceptor viability ***[Steinberg RH. Survival factors in retinal degenerations. Curr Opin Neurobiol. 1994;4:515–24.]*** Moreover, when lipofuscin absorbs blue light, the material becomes phototoxic, which can lead to further damage in the RPE and in the photoreceptors ***[Sparrow JR, Nakanishi K, Parish CA. The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. Invest Ophthalmol Vis Sci. 2000;41:1981–9.].***The data from our laboratory indicate that in albino rats, exposure to blue light (λ_{max} 474 nm, 1×10^{-1} μW/cm^2) acutely suppressed melatonin levels **[*Ferguson I, Melton A, Li N, Nicol D. Park, Tosini G. Imitating Broadband Diurnal Light Variations Using Solid State Light Sources. Journal of Light & Visual Environment. 2008;32:63–8.]*** while exposure to blue light for 4 h/day for 30 days did not produce significant effects on photoreceptor viability (Figure 3). However, this treatment produced a small (10–20%) but significant reduction in the levels of melanopsin and short wavelength opsin mRNAs in rats exposed to white or green (λ_{max}513 nm) light (Figure 4).

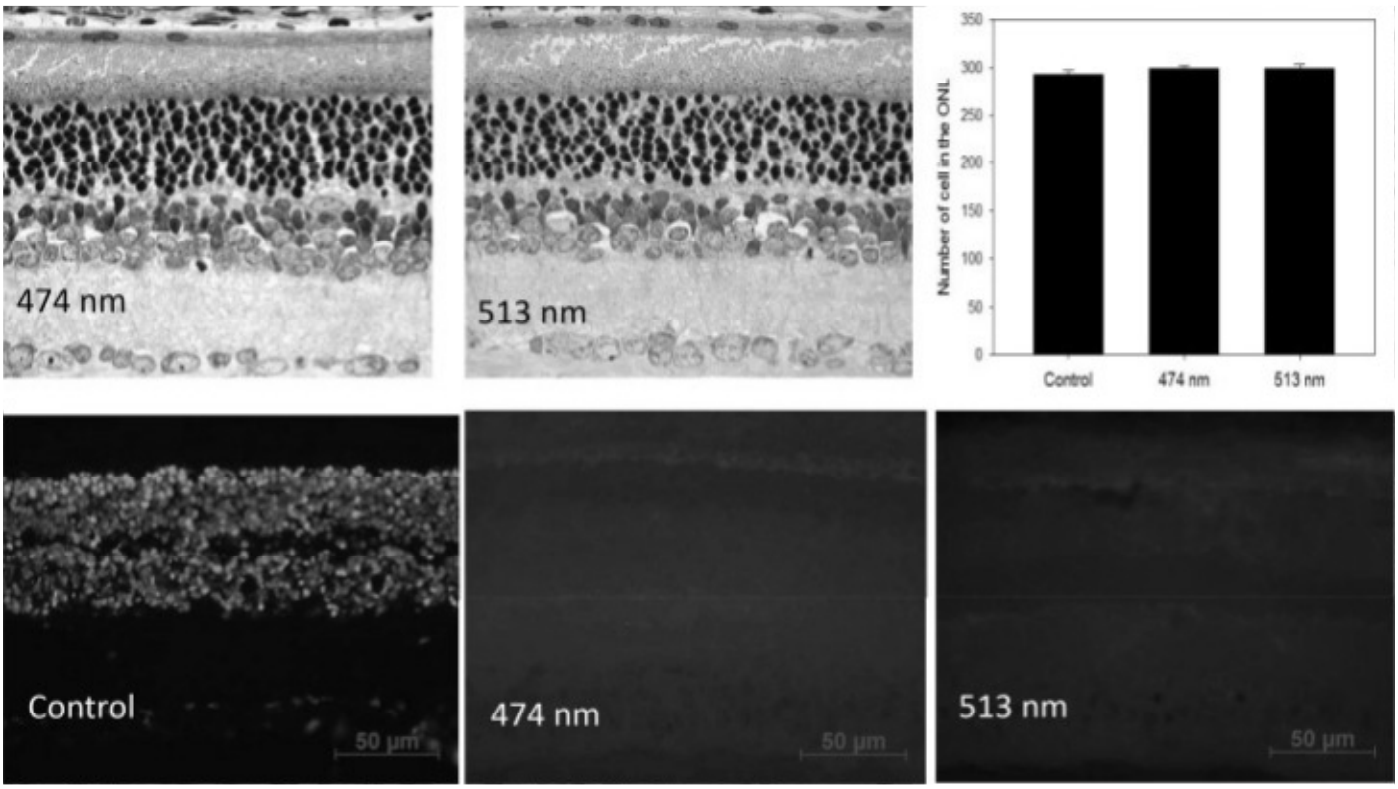


Figure 3
Top panels. The exposure to blue light (λ_{max} 474), green light (λ_{max} 513), or fluorescent light at the intensity of 1×10^{-1} μW/cm^2 for 4 h/day for 30 days did not produce a significant change in the number of cells **...**

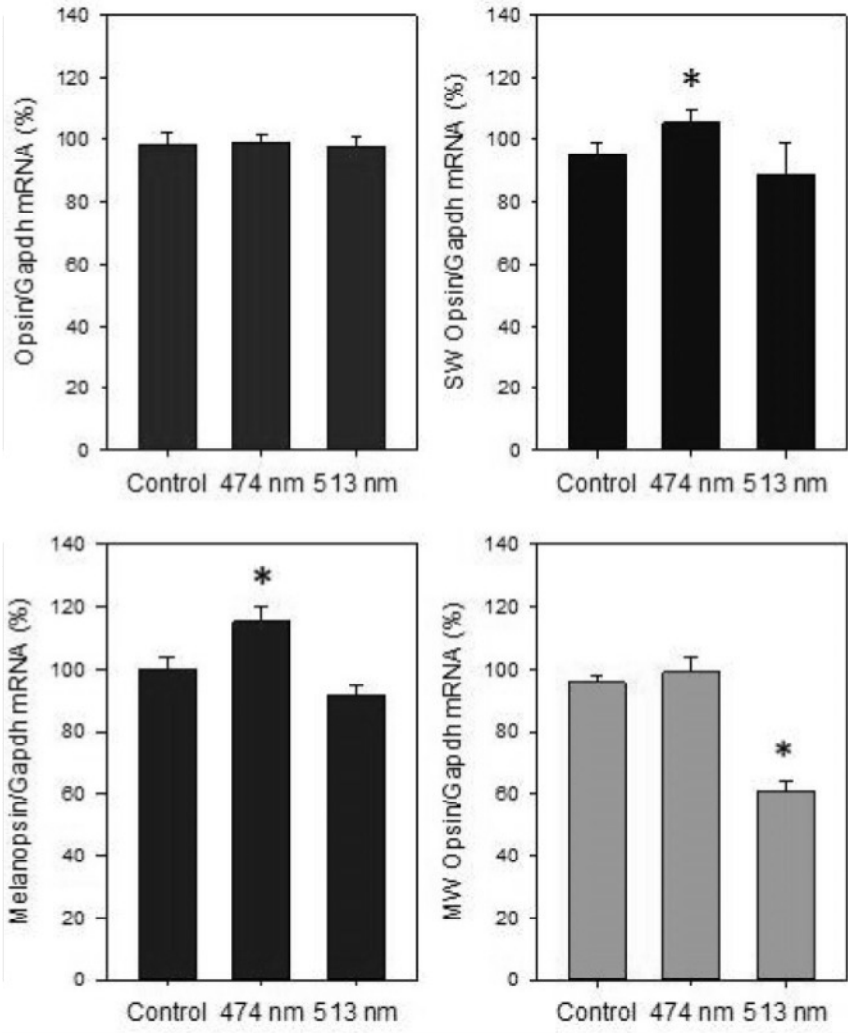


Figure 4
Different light treatments did not affect rhodopsin mRNA levels (one-way ANOVA, $p>0.1$). Exposure to blue light (λ_{max} 474) at the intensity of 1×10^{-1} μW/cm^2 for 4 h/day for 30 days produced significant changes

In this context, two recent studies on the effect of blue light exposure on the RPE and cone-like cells (661W, murine photoreceptor-derived cells [***Tan E, Ding XQ, Saadi A, Agarwal N, Naash MI, Al-Ubaidi MR. Expression of cone-photoreceptor-specific antigens in a cell line derived from retinal tumors in transgenic mice. Invest Ophthalmol Vis Sci. 2004;45:764–8]).*** should be mentioned. In the first study, Arnault et al. ***[Arnault E, Barrau C, Nanteau C, Gondouin P, Bigot K, Viénot F, Gutman E, Fontaine V, Villette T, Cohen-Tannoudji D, Sahel JA, Picaud S. Phototoxic action spectrum on a retinal pigment epithelium model of age-related macular degeneration exposed to sunlight normalized conditions. PLoS One.*** **2013;8:e71398.]** reported that in the primary porcine RPE, exposure to light with irradiance similar to that of natural sunlight, that is, light in the range of 415–455 nm, was the most effective in reducing cell viability.

In the second study, Kuse et al. ***[Kuse Y, Ogawa K, Tsuruma K, Shimazawa M, Hara H. Damage of photoreceptor-derived cells in culture induced by light emitting diode-derived blue light. Sci Rep. 2014;4:5223.]*** reported that 661W cells are more sensitive to light-induced damage when

exposed to light emitted by blue (464 nm) LEDs than when exposed to green (522 nm) or white LEDs (wavelength peak at 456 and 553 nm) of the same intensity (0.38 mW/cm^2). The exposure to blue light, unlike the exposure to white and green LEDs, also produced a significant increase in ROS and induced cell damage. Similar results were also observed in primary retinal cells [***Kuse Y, Ogawa K, Tsuruma K, Shimazawa M, Hara H. Damage of photoreceptor-derived cells in culture induced by light emitting diode-derived blue light. Sci Rep. 2014;4:5223.]*** These data support the idea that exposure to blue light in the range of 400–470 nm (even at low levels) may damage photoreceptors and retinal pigment epithelium cells.

Although most studies have focused on the acute effect of light exposure, several have also investigated the cumulative effect of light. For example, Noell ***[Noell WK. Effects of environmental lighting and dietary vitamin A on the vulnerability of the retina to light damage. Photochem Photobiol. 1979;29:717–23.]*** reported that a single 5 min exposure to light did not induce significant damage in photoreceptor cells, whereas a series of 5 min exposures led to significant photoreceptor damage. Furthermore, the time between exposures affects the cumulative effect of light ***[[Lawwill T. Effects of prolonged exposure of rabbit retina to low-intensity light. Invest Ophthalmol. 1973;12:45–51.] [Lawwill T, Crockett S, Currier G. Retinal damage secondary to chronic light exposure, thresholds and mechanisms. Doc Ophthalmol. 1977;44:379–402.] [Griess GA, Blankenstein MF. Additivity and repair of actinic retinal lesions. Invest Ophthalmol Vis Sci. 1981;20:803–7.]*** In some cases, intermittent light exposure may produce even more pronounced damage than an equivalent amount of light in a single exposure ***[Organisciak DT, Jiang YL, Wang HM, Pickford M, Blanks JC. Retinal light damage in rats exposed to intermittent light. Comparison with continuous light exposure. Invest Ophthalmol Vis Sci. 1989;30:795–805.]***

With age, superoxide dismutase 1 (SOD1) and protective enzymes do not function as well due to zinc deficiency. SOD1 does not function well because the enzyme activity is controlled by zinc. Imamura et al. have shown that even with normal light that contains some blue light, fluorescent light damaged the retina tremendously in the SOD1 knockout mouse, which is similar to an aging mouse ***[Imamura Y, Noda S, Hashizume K, Shinoda K, Yamaguchi M, Uchiyama S, Shimizu T, Mizushima Y, Shirasawa T, Tsubota K. Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration. Proc Natl Acad Sci USA. 2006;103:11282–7.]***

Finally, the severity of light-induced retinal damage changes with the time of the day ***[Duncan TE, O'Steen WK. The diurnal susceptibility of rat retinal photoreceptors to light induced damage. Exp Eye Res. 1985;41:497–507.] [White MP, Fisher LJ. Degree of light damage to the retina varies with time of day of bright light exposure. Physiol Behav. 1987;39:607–13.] [Organisciak DT, Darrow RM, Barsalou L, Kutty RK, Wiggert B. Circadian-dependent retinal light damage in rats. Invest Ophthalmol Vis Sci. 2000;41:3694–701.] [Vaughan DK, Nemke JL, Fliesler SJ, Darrow RM, Organisciak DT. Evidence for a circadian rhythm of susceptibility to retinal light damage. Photochem Photobiol. 2002;75:547–53.] [Wong P, Organisciak DT, Ziesel AC, Chrenek MA, Patterson ML. (Circadian effects on retinal light damage. In: The Retina and Circadian Rhythms 2014; (Eds G. Tosini, P.M. Iuvone, D.G. McMahon and S.P Collin. pp131–170. Springer.]*** For example, rats are three to four times more susceptible to light damage at night (01:00) than during the day (09:00 and 17:00). The circadian dependency of light-induced photoreceptor damage appears to involve mechanisms that control cAMP and c-fos levels both of which are under the control of the retinal circadian clock [***Fukuhara C, Liu C, Ivanova TN, Chan GC, Storm DR, Iuvone PM, Tosini G. Gating of the cAMP signaling cascade and melatonin synthesis by the circadian clock in mammalian retina. J Neurosci. 2004;24:1803–11.] [Humphries A, Carter DA. Circadian dependency of nocturnal immediate-early protein induction in rat retina. Biochem Biophys Res Commun. 2004;320:551–6.]***

Exposure to blue light during the night might have more negative effects compared to the same exposure during the daytime. However, in this case, this assumption is based on the experimental data obtained from nocturnal rodents. Thus, it is difficult to determine whether light-induced retinal damage has a daily rhythm in humans, and further studies on diurnal animal models (e.g., non-human primates) are required to address this important point.

Experimental evidence indicates that wavelengths in the blue part of the spectrum (400–490 nm) can induce damage in the retina, and although the initial damage following exposure to blue light may be confined to the RPE, a damaged RPE eventually leads to photoreceptor death. Although most studies on the effects of blue light have focused on the mechanisms responsible for the damage to the photoreceptors following an acute exposure to high intensity light, some studies have reported that sub-threshold exposure to blue light can also induce damage in photoreceptors [***Andley UP, Chylack LT. Recent Studies on photodamage to the eye with special reference to clinical and therapeutic procedures. Photodermatol Photoimmunol Photomed. 1990;7:98–105.***]. [***Boulton M, Rozanowska M, Rozanowski B. Retinal Photodamage. J Photochem Photobiol B. 2001;64:144–61***]. [***Chu R. Blue light irradiation inhibits the production of HGF by human retinal pigment epithelium cells in vitro. Photochem Photobiol. 2006;82:1247–50.***] In addition, several authors have proposed that the amount of blue light received during an individual's entire lifespan can be an important factor in the development of age-related macular degeneration (AMD).

Lipofuscin accumulates in the RPE in the form of granules located in the lysosomes of the RPE. The formation of lipofuscin begins in photoreceptors' outer segments as a byproduct of the degradation of rod photoreceptor discs [***Andley UP, Chylack LT. Recent Studies on photodamage to the eye with special reference to clinical and therapeutic procedures. Photodermatol Photoimmunol Photomed. 1990;7:98–105.***]. When lipofuscin absorbs blue light, ROS are produced, and these free radicals are responsible for the oxidative damage that occurs in the retina. The number of reactive oxygen species produced by lipofuscin is directly related to the spectral composition of the light, and it steadily decreases from 400 to 490 nm [***Pawlak A, Rozanowska M, Zareba M, Lamb LE, Simon JD, Sarna T. Action spectra for the photoconsumption of oxygen by ocular lipofuscin and lipofuscin extracts. Arch Biochem Biophys. 2002;403:59–62***] The accumulation of lipofuscin in the RPE, particularly in the macula, has been linked to photoreceptor death and to AMD [***Wolf G. Lipofuscin and macular degeneration. Nutr Rev. 2003;61:342–6.***] Furthermore, the amount of lipofuscin present in the RPE increases with age (i.e., the amount of lipofuscin is low in young and high in old animals); thus, the potential for blue light to damage the retina may increase with age [***Delori FC, Goger DC, Dorey CK. Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. Invest Ophthalmol Vis Sci. 2001;42:1855–66***]. Finally, it has been reported that chronic exposure to blue light may accelerate photoreceptor degeneration in an animal model in the study of retinal degeneration [***Thomas BB, Seiler MJ, Aramant RB, Samant D, Qiu G, Vyas N, Arai S, Chen Z, Sadda SR. Visual functional effects of constant blue light in a retinal degenerate rat model. Photochem Photobiol. 2007;83:759–65***]

Thus, experimental evidence obtained from different experimental models indicates that exposure to blue light in the 470–490 nm range may be less damaging to the eye compared to blue light in the 400–460 nm range. We believe that the development of LEDs with a peak emission of around 470–490 nm may represent an important

advancement in the safety of LEDs for ocular health *[Gaston KJ, Bennie J, Davies TW, Hopkins J. The ecological impacts of nighttime light pollution: a mechanistic appraisal. Biol Rev Camb Philos Soc 2013; 88: 912–927.]* (Figure 3).

Light exposure and age-related macular degeneration in humans
A series of studies in many animal models have shown that exposure to blue light may represent a risk for the development of AMD or other retinal pathologies *[Cruickshanks KJ, Klein R, Klein BEK. Sunlight and age-related macular degeneration—the Beaver Dam Eye Study. Arch Ophthalmol. 1993;111:514–8.] [Klein R, Klein BEK, Jensen SC, Cruickshanks KJ. The relationship of ocular factors to the incidence and progression of age-related maculopathy. Arch Ophthalmol. 1998;116:506–13.]* However, the real risk from artificial light (white or blue) exposure in humans is difficult to assess, since light therapy has been in use for only a few years and in a small number of individuals. In addition, individual susceptibility to blue light damage varies significantly among individuals, making the assessment of the risk associated with repeated exposure to blue light in the etiology of AMD difficult.

Previous epidemiological studies have indicated that chronic exposure to visible and blue light may be a cofactor in the development of AMD *[Algvere PV, Marshall J, Seregard S. Age-related maculopathy and the impact of blue light hazard. Acta Ophthalmol Scand. 2006;84:4–15] [Taylor HR, Muñoz B, West S, Bressler NM, Bressler SB, Rosenthal FS. Visible light and risk of age-related macular degeneration. Trans Am Ophthalmol Soc. 1990;88:163–73.] [Taylor HR, West S, Munoz B, Rosenthal FS, Bressler SB, Bressler NM. The long-term effects of visible-light on the eye. Arch Ophthalmol. 1992;110:99–104.]*

Conclusions
The use of blue light is becoming increasingly prominent in our society, and a large segment of the world population is now subjected to daily exposure (from a few minutes to several hours) of artificial light at an unusual time of the day (night). Because light has a cumulative effect and many different characteristics (e.g., wavelength, intensity, duration of the exposure, time of day), it is important to consider the spectral output of the light source to minimize the danger that may be associated with blue light exposure. Thus, LEDs with an emission peak of around 470–480 nm should be preferred to LEDs that have an emission peak below 450 nm.

DATED and DONE this 5th day of May, 2017.

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EXHIBIT A

Abstract

Our eyes are increasingly exposed to light from the emitting diode (LED) light of video display terminals (VDT) which contain much blue light. VDTs are equipped with televisions, personal computers, and smart phones. The present study aims to clarify the mechanism underlying blue LED light-induced photoreceptor cell damage. Murine cone photoreceptor-derived cells (661 W) were exposed to blue, white, or green LED light (0.38 mW/cm^2). In the present study, blue LED light increased reactive oxygen species (ROS) production, altered the protein expression level, induced the aggregation of short-wavelength opsins (S-opsin), resulting in severe cell damage. While, blue LED light damaged the primary retinal cells and the damage was photoreceptor specific. *N*-Acetylcysteine (NAC), an antioxidant, protected against the cellular damage induced by blue LED light. Overall, the LED light induced cell damage was wavelength-, but not energy-dependent and may cause more severe retinal photoreceptor cell damage than the other LED light.

Introduction

Humans spend increasing amounts of time in the presence of video display terminals (VDT) equipped with a liquid crystal display, such as televisions, personal computers, and smart phones. In addition to these VDT, we are constantly exposed to various other types of light that shine around us. Light emitting diodes (LED) light are emerging as an important source of light replacing conventional lights. It is widely used for illumination, especially in liquid crystal displays, car lights and so on. VDT emit a large amount of blue light, and blue light has been reported to be harmful to the retina

Age-related macular degeneration (AMD), a retinal degenerative disease, affects more than 30% of the people at or over 75 years of age. The pathogenesis of AMD usually advances with retinal photic injury caused by excessive light exposure and consequent oxidative stress. The retina contains much chromophores which can lead to the photochemical damage when excited at the each wavelength light, and age-related decrease of antioxidants such as superoxide dismutase (SOD) and increase of ROS following light exposure can progress to the pathology of AMD. The loss of vision is the major symptom of retinal diseases such as AMD, and the early pathogenesis involves degeneration of retinal pigment epithelial (RPE) cells. It is reported that the accumulation of lipofuscin and the formation of drusen in the Bruch's membrane cause apoptosis of RPE cells. These are considered as the initial stages that lead to AMD. Subsequently, photoreceptor cell degeneration occurs after RPE cell death and can lead to vision loss. Furthermore, it is known that the photoreceptor cell death is facilitated by oxidative stress induced the generation of reactive oxygen species (ROS) such as superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2). In

addition to RPE cell death, the oxidative stress due to ROS generation causes photoreceptor cell death.

Blue light (from 450 to 495 nm) has a short wavelength, and it is a part of the high- energy visible light spectrum unlike several other colors. Previous reports suggested that the blue light more severe damaged retinal photoreceptor cells than green light in rats[1]. The short wavelength (blue) light usually recover rhodopsin by photoreversal of bleaching in rod photoreceptor cells.. However, following exposure to excessive light the regeneration could occur very rapidly through the process of photoreversal, and therefore rhodopsin can bleach several times in a short period *in vivo*. While, the aggregation of short-wavelength opsins (S-opsin) can cause rapid cone degeneration. It is reported medium wavelength opsins (M-opsin) are easily degraded, but S-opsin is not easily degraded by proteasome degradation. We have reported that the excessive light exposure induced the aggregation of S-opsin, and leading to endoplasmic reticulum (ER) stress in the cone photoreceptor-derived cell line, 661 W.

In some groups, the mouse-derived 661 W cells have been used as a light-induced retinal damage model in vitro. In this study, we investigated how the *in vitro* exposure to blue LED lights affects 661 W cells and primary retinal cells. Furthermore, we evaluated the effects of an antioxidant, *N*-acetylcysteine (NAC), against the blue LED light-induced photoreceptor-derived cell damage.

Methods

Cell culture

Murine photoreceptor-derived 661 W cells were kindly gifted by Dr. Muayyad R. Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque Inc, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 µg/mL streptomycin (Meiji Seika) under a humidified atmosphere of 5% CO_2 at 37℃. These cells were passaged by trypsinization every 3 to 4 days.

LED light-induced cell death in 661 W cell cultures

The 661 W cells were seeded at a density of 3×10^3 cells per well into 96-well plates, and then incubated for 24 h under a humidified atmosphere of 5% CO_2 at 37℃. After 661 W cells were treated *N*-acetylcystein (NAC) (Wako, Osaka, Japan) or vehicle (1% FBS, DMEM), the cells were incubated for 1 h. Then, the cells were exposed to 0.38 mW/cm^2 [equivalent to 450 lux for blue LED light (464 nm); 1,600 lux for white LED light (the wavelength peak is 456 nm and 553 nm); and 2,500 lux for green LED light (522 nm)] or altermately, to 2,500 lux of blue, white, or green LED light from below the 96-well plates for 24 h. Subsequently, they were incubated for 12 h. Control cells incubated in the dark and light-irradiated 661 W cells were obtained from the same stock, thereby eliminating

any preexisting bias (such as light and temperature) as previously described by Kanan et al. (Kanan et al., 2007). The energy was measured by 1916-R Handheld Optical Power Meter (Newport, Osaka, Japan).

Cell viability assay

We examined the change in the fluorescence intensity after the cellular mitochondrial reduction of WST-8 to formazan. The 661 W cells were seeded at a density of 3×10^3 cells per well into 96-well plates, and then incubated for 24 h under a humidified atmosphere of 5% CO_2 at 37℃. After the addition of NAC at 1 mM or vehicle (1% FBS, DMEM), the cells were incubated for 1 h. Then, the cells were exposed to 0.38 mW/cm^2 or 2,500 lux of blue, white, or green LED light for 24 h. Cell viability was measured by culturing the cells in a culture medium containing 10% WST-8 (Cell Counting Kit-8; Dojin Kagaku, Kumamoto, Japan) for 2 h at 37℃ and then by scanning using with a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, USA).

Mitochondrial membrane potential assay

Mitochondrial membrane potential assay was performed after blue LED light exposure for 24 h in 661 W cells. The mitochondrial membrane potential was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol. The images were captured using a BZ-9000 Biorevo all-in-one fluorescence microscope (Keyence, Osaka, Japan), which detects healthy cells with mainly JC-1 J-aggregates (excitation/emission = 540/605 nm) and apoptotic or unhealthy cells with mainly JC-1 monomers (excitation/emission = 480/510 nm). Merged cells were considered to be pre-apoptotic (early or middle state of transition to cell death) cells. The number of cells (red or yellow stained cells) was counted in a blind manner with image-processing software (Image-J).

Measurement of cellular ROS production

The 661 W cells were seeded at a density of 3×10^3 cells per well in 96-well plates, and then incubated for 24 h under a humidified atmosphere of 5% CO_2 at 37℃. After the addition of NAC 1 mM or vehicle (1% FBS, DMEM), the cells were incubated for 1 h and then exposed to 0.38 mW/cm^2 or 2,500 lux of blue, white, or green LED light for 6 h or 24 h. Then 10 μM of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Invitrogen, Carlsbad, CA, USA), a free radical probe, was added to the cell culture after LED light exposure and incubation was continued for 1 h at 37℃. The radical probe was converted to 2′,7′-dichlorodihydrofluorescein (DCFH) by the action of intracellular esterase. Intracellular DCFH (nonfluorescent) was oxidized to 2′, 7′-dichlorfluorescein (DCF, fluorescent) by intracellular ROS. Fluorescence was measured by a Varioskan Flash 2.4 microplate reader (Thermo Fisher Scientific) at 485 nm (excitation) and 535 nm (emission).

Western blotting analysis

The 661 W cells were seeded at a density of 3×10^4 cells per well in 12-well plates, and then incubated for 24 h under a humidified atmosphere of 5% CO_2 at 37℃. After treatment with 1 mM NAC or vehicle (1% FBS, DMEM), the cells were incubated for 1 h. The cells were exposed to 2,500 lux of blue, white, or green LED light for 24 h. Then, the cells were washed with PBS, lysed in RIPA buffer (Sigma-Aldrich) containing 1% protease inhibitor cocktail and 1% of the phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich), and harvested. Lysates were centrifuged at 12,000 *g* for 15 min at 4℃. Protein concentrations were measured by using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as a standard. Thereafter, an equal volume of protein sample and sample buffer was mixed, and the samples were boiled for 5 min at 100℃. The protein samples were separated by 5–20% SDS-PAGE gradient electrophoresis and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). For immunoblotting, the following primary antibodies were used: rabbit anti-phospho NF-κB (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-NF-κB (Cell Signaling Technology), rabbit anti-p38 antibody (Cell Signaling Technology), rabbit anti-phospho p38 (Cell Signaling Technology), rabbit anti-phospho ERK (Cell Signaling Technology), rabbit anti-ERK (Cell Signaling Technology), rabbit anti-LC3-I and II (Cell Signaling Technology) and mouse anti-β-actin mouse monoclonal (Sigma-Aldrich) antibodies. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Pierce Biotechnology, Rockford, IL, USA) and an HRP-conjugated goat anti mouse antibody were used as secondary antibodies. Immunoreactive bands were visualized using Immunostar-LD (Wako) and a LAS-4000 luminescent image analyzer (Fuji Film Co., Ltd., Tokyo, Japan). β-actin was used as the loading control. The membrane was stripped by stripping buffer (Thermo Fisher Scientific) after observing phosphorylated-proteins, and then observed total-proteins.

Immunostaining

The 661 W cells were seeded at a density of 1.5×10^4 cells per well into glass chamber slides (Laboratory-Tek;Life Technologies, Gaithersburg, MD, USA), and incubated for 24 h. The medium was changed by 1% FBS, DMEM and incubated for 1 h. Then, the cells were exposed to 0.38 mW/cm^2 of blue, white, or green LED light for 24 h or blue LED light for 3 or 6 h. Thereafter, the cells were fixed with 4% paraformaldehyde for 15 minutes, blocked in 3% horse serum for 30 minutes, and incubated overnight at 4℃ with primary antibodies [anti-S-opsin rabbit polyclonal antibody (Chemicon, Temecula, CA,USA)]. After being washed, the cells were incubated for 1 h with secondary antibodies [Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen)]. Then, being washed, and counter-stained with Hoechst 33342 (Invitrogen). Images were taken using a confocal fluorescence microscope (Olympus). After taking images, the perinuclear S-opsin aggregated cells were counted in the 212 μm area with Image-J.

Cell death analysis
The cell death rate was calculated by double staining with two fluorescent dyes: Hoechst 33342 (Invitrogen) and propidium iodide (PI; Invitrogen). Hoechst 33342 stains the nuclei of all cells, whereas PI stains only dead cells. At the end of the culture period, Hoechst 33342 and PI were added to the culture medium for 15 min at final concentrations of 8.1 μM and 1.5 μM, respectively. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). The total number of cells was counted in a blind manner and the percentage of PI-positive cells was calculated.

Caspase 3/7 activation assay
Activation of caspase 3/7 was assayed after blue LED light exposure for 24 h in 661 W cells. Caspase 3/7 was measured by using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. After LED light exposure, caspase-Glo 3/7 reagent was added with at 1:1 ratio to the sample volume, and the cells were incubated for 1 h at 37℃. The luminescence of each sample was measured using a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, USA).

Animals
Female ddY pregnant mice and the neonatal mice (Japan SLC, Hamamatsu) were maintained under controlled lighting environment (12 h:12 h light/dark cycle). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Primary retinal culture
Retinas from P8 ddY mice were dissected without choroidal vessels and dissociated by activated papain for 30 min at 37℃, using the protocol of Tsuruma et al. Neurobasal medium (Invitrogen) including ovomucoid (Sigma-Aldrich) plus DNase (Invitrogen) was added, and the cells were centrifuged at 800 rpm for 8 min at room temperature. The pellet was suspended in neurobasal medium including ovomucoid without DNase, and recentrifuged. Then, the cells were resuspended in neurobasal medium containing L-glutamine, B27 (Invitrogen), and antibiotics. Cells were plated onto poly-D-lysine/laminin–coated 96-well dishes at 2.0×10^5 cells/well and glass chamber slides at 1.0×10^6 cells/well. After incubation for 20 h, medium was changed and blue LED light exposure started. After blue LED light exposure for 24 h, WST-8 assay and ROS measurement were performed. For immunostaining, the cells were fixed with 4% paraformaldehyde and subsequently same protocol as described above was applied. Following antibodies were used as primary antibodies [anti-S-opsin goat polyclonal antibody (Santa Cruz, CA, USA) and anti-cleaved caspase-3 rabbit polyclonal antibody (Cell Signaling Technology)] and as secondary antibodies [Alexa Fluor® 488 donkey anti-goat IgG (Invitrogen) and Fluor® 546 donkey anti-rabbit IgG (Invitrogen)]. Images were taken using a confocal fluorescence

microscope (Olympus). After taking images, the S-opsin and cleaved caspase-3 positive cells were counted in the 212 μm area with Image-J.

Statistical analysis

Data are presented as the means ± S.E.M. Statistical comparisons were conducted using ANOVA or one-way ANOVA followed by Bonferroni's test, Dunnett's test, Tukey's test [STAT VIEW version 5.0 (SAS Institute, Cary, NC, USA)]. $p < 0.05$ was considered as statistically significant.

EXHIBIT B

Abstract

Light-emitting diodes (LEDs) have been used to provide illumination in industrial and commercial environments. LEDs are also used in TVs, computers, smart phones, and tablets. Although the light emitted by most LEDs appears white, LEDs have peak emission in the blue light range (400–490 nm). The accumulating experimental evidence has indicated that exposure to blue light can affect many physiologic functions, and it can be used to treat circadian and sleep dysfunctions. However, blue light can also induce photoreceptor damage. Thus, it is important to consider the spectral output of LED-based light sources to minimize the danger that may be associated with blue light exposure. In this review, we summarize the current knowledge of the effects of blue light on the regulation of physiologic functions and the possible effects of blue light exposure on ocular health.

Introduction

Lighting sources and technology have experienced a revolution in the last 15–20 years. Lighting sources and technology, especially in non-commercial or industrial illumination applications, have traditionally been slow to change. In most homes, the incandescent bulb and Edison socket have been omnipresent. In the past 10 years, we have seen significant use of other technologies, such as compact fluorescent lamps (CFLs), replacing incandescent sources. However, this transition has often been driven by legislation, which has focused on energy-efficient sources instead of consumer desire for different light sources. The general user quickly noted the difference in the quality of CFL source but not necessarily in the specifics of its power spectrum. Simultaneously, the development and performance of high brightness light-emitting diodes (LEDs) have experienced tremendous advances. The coupling of a blue-light LED with a phosphor has also been used to produce a white light source, the white-light LED. This solid-state fluorescent analog has become known as solid-state lighting (SSL). This approach is now considered the next generation of

illumination due to the many inherent and potential advantages over current technologies.

In addition to use for general illumination, LEDs quickly became the choice for mobile devices, such as smart phones. The small size of LEDs and the limited screen size make them ideal for these applications. The potential for the use of LEDs for backlighted liquid crystal displays (LCDs) in laptop computers was also quickly realized. This transition was driven by the fragility of the microfluorescent lamps used for illumination and consumer desire for thinner screens. LEDs have now become the dominant technology for backlighted tablet displays, such as iPads and e-readers, and large LCD television sets. This now means that blue light prevails in red, green, and blue (RGB) and SSL illumination systems that did not exist a decade ago. The ways in which people read have also changed. Light is now being used directly for illumination in smart phones, tablets, and readers instead of for reflection, which is typical for reading from paper.

EXHIBIT C

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