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BLINDED BY THE LIGHT; RETINAL PHOTOTOXICITY IN THE CONTEXT OF SAFETY STUDIES

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albino rodents; CRO survey; light damage; retinal atrophy; retinal phototoxicity

In a seminal study published in 1966, Werner K. Noell and associates reported that the "retina of laboratory rats is affected irreversibly by intense light applied for less than an hour or for up to 2 days depending on the experimental conditions." (Noell *et al.*, 1966). They found this effect when the retinas of "normal unanesthetized and unrestrained rats were maintained continuously for 24 hours in an environment illuminated by ordinary fluorescent light bulbs". In an insightful introductory comment, the authors indicated that the "interest in this effect rests mainly on the assumption that any unusual vulnerability of the retina to physical or chemical agents may relate to the cellular abnormalities which lead to retinal degeneration on a hereditary basis". This ushered in an era of intense investigation of the interactions between light and photoreceptor degeneration that has helped unravel the mechanism(s) of this effect, identify potential consequences to human patients, and develop therapies that could be translated to the clinic (e.g., studies on neuroprotection from light damage by ciliary neurotrophic factor and its eventual use in human clinical trials: (LaVail *et al.*, 1992; Tao *et al.*, 2002; Sieving *et al.*, 2006)). From 1966 to August, 2012, over 184 papers have been published on retinal light damage in rats. In mice, where the same finding was first reported in 1971 (Aoyagi, 1971), there have been >134 publications (search: light damage/retina/rat and light damage/retina/mouse with manual review of the search results to eliminate references that are not pertinent).

Although light and light damage mechanisms have been extensively examined in basic retinal cell biology studies (see (Organisciak and Vaughan, 2010) for recent review), there seems to be a paucity of research and awareness, from the pharmaceutical industry perspective, on the damaging effects of light on the retina, particularly in albino rodents. A partial reason for this is that many of the experimental light damage paradigms use intense

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Declaration of Conflict of Interest

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light exposures for short duration, while, in an industrial setting, the exposures are generally lower, but longer, i.e. 4 and 13 weeks, 6, months, 1 and 2 years. Thus a common framework for relating experimental and industrial light exposures, and the means of assessing and ascertaining that the damage is light induced, are not readily available to many in industry. That is not to say that light damage has not been considered as a factor in the context of safety studies. In the early 1970's, Weisse and associates established a causal association between light intensity and retinal damage in albino rats receiving clonidine, and convincingly established that this effect was caused by the pupillary dilation that accompanied drug administration (Weisse *et al.*, 1971). Subsequently, they reported on the age- and light-dependent changes in the rat retina, and attempted to characterize and differentiate the changes that occur in each (Weisse *et al.*, 1974). Bellhorn further examined lighting in the animal environment, and characterized some of the light conditions that favor or prevent retinal degeneration in albino rodents (Bellhorn, 1980). These were incorporated in the recommendations included in the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996) and the 2010 revised edition (NRC, 2010).

Based on safety studies reviewed by one of the authors (GDA) in recent years, it appears that retinal degeneration, putatively caused by light, has been a confounding factor in studies using albino rodents, and this appears to have increased in frequency. Differentiation of light induced from test-article related retinal damage has become a costly and effort-intensive undertaking in order to ensure that the observed findings have limited or no potential adverse effects on human patients (Figure 1). Part of this increase could be attributed to new caging methods for laboratory rodents that increases light fluxes within the cage. Instead of the traditional individual cages with solid metal back and sides, and a mesh front, many of the new caging systems are clear plastic. If ambient illumination is not precisely controlled in the animal rooms, the higher light fluxes within the new enclosures enhances cumulative light exposures, and favors retinal light damage.

In this review, we discuss concepts and mechanisms of light damage, and indicate how these might relate to pre-clinical safety studies. As well, we provide recommendations for evaluating the retina, and developing protocols for evaluating a potential light damage mechanism in studies where retinal degeneration is observed.

Retinal light damage: general principles

The majority of light damage studies have been carried out in albino rodents because of their apparent "exquisite" sensitivity to light damage (O'Steen *et al.*, 1972; Organisciak *et al.*, 1985; Wenzel *et al.*, 2005; Organisciak and Vaughan, 2010). On a quantal absorption basis, however, photoreceptors in albino animals are as sensitive to light damage as those of pigmented animals (Rapp and Williams, 1980; Penn and Williams, 1986). Nonetheless, the pigment in the uveal tract (iris, ciliary body, choroid) and retinal pigment epithelium (RPE) of non-albino animals serves as a protective screen that limits light fluxes in the retina, and "prevents" light damage. This protection, however, can be abrogated in pigmented animals by pupillary dilatation (Williams *et al.*, 1985).

Although the rat has been used for most biochemical studies of light-induced retinal degeneration, it is the albino mouse that has been used to define the molecular mechanisms and pathways involved in light damage (Wenzel *et al.*, 2005). In general, damage is mediated by rhodopsin, and is prevented by absence of vitamin A chromophore (Grimm *et al.*, 2000). Based on studies in mice, there is a requirement for repetitive photon absorption to cause damage (Wenzel *et al.*, 2005), and this is facilitated by having a more active form of the *RPE65* gene, involved in the RPE retinoid cycle, to produce a faster turnover and

greater availability of vitamin A (11-cis retinaldehyde) to the photoreceptors (Wenzel *et al.*, 2001).

In addition to the above-described modifying effect of pigmentation and *RPE65* gene product activity, there are other parameters that can influence the damaging effect of light on the retina (Wenzel *et al.*, 2005) such as:

- **a.** Quality of light. It is assumed that fluorescent white light is more damaging as it has a spectrum similar to daylight. Broadband green and more narrow-spectrum blue and green lights also cause damage, and are used to more precisely examine the damaging effects of light and the wavelength specificity.
- **b.** Exposure duration and intensity. There is an interplay between duration, wavelength and light intensity that affects the outcome; as well, the outcome depends on whether the study is carried out in mice or rats. Additionally, cyclic illumination, e.g. 12 hrs on/12 hrs off, is less damaging to the retina than constant illumination, but that too is intensity dependent.
- **c.** Age of the animal and temperature. Juvenile rats are more resistant to light damage than adults (Joly *et al.*, 2006). Induced hyperthermia during light exposure accelerates damage (Organisciak *et al.*, 1995). However, the timing of the increased body temperature is critical as transient hyperthermia prior to light exposure reduces the damaging effects of light by increasing production of heat shock proteins (Barbe *et al.*, 1988).
- **d.** Gender. Based on comparisons between ovariectomized and intact female rats in terms of light damage susceptibility, it appears that ovariectomy protects against light damage, and estrogen supplementation overcomes the protective effect of ovariectomy, suggesting that intact female rats are more sensitive to the damaging effects of light (Olafson and O'Steen, 1976; O'Steen, 1977). Furthermore, the pituitary through direct prolactin effects on the retina, also modulates light damage, and hypophysectomized animals show protection from light damage which is abrogated by prolactin administration (O'Steen, 1980). However, with few exceptions, direct male to female comparisons in terms of light damage sensitivity have not been published.

Topography of retinal light damage

Prior studies have demonstrated that retinal light damage in albino rodents does not occur uniformly across the retina, at least in the early stages, but has a very specific topographic distribution. The superior retinal quadrants, particularly above and near the optic disc are preferentially damaged, while the inferior and peripheral regions are spared (Organisciak *et al.*, 1999) (Figure 2). The distribution of damage across the retina can be quantified by making maps of outer nuclear layer (ONL) thickness at different retinal positions; these are commonly referred to as "spidergraphs" (Rapp, 1980) (Figure 3A). The increased susceptibility to retinal light damage by the central retina has been proposed to result from increased rod outer segment lengths (Figure 3B) accompanied by increased rod opsin packing density in the central photoreceptors (Rapp *et al.*, 1985). Recent work now shows that it is the superior temporal region that shows the greatest sensitivity to light damage, and topographic maps of outer nuclear layer thickness clearly demonstrate these findings (Tanito *et al.*, 2008) (Figure 3C, D).

Differences in light damage susceptibility in albino rats

In mice, light damage susceptibility is strain dependent (LaVail *et al.*, 1987a; LaVail *et al.*, 1987b), and recent studies have shown several genetic loci that determine this susceptibility

(Danciger *et al.*, 2004), of which the *RPE65* locus appears to confer the most resistance (Wenzel *et al.*, 2001). In rats, a light damage study by Borges et al. has shown that Wistar and Fischer (F344) strains are equally sensitive, but more resistant than Buffalo and Lewis strains (Borges *et al.*, 1990). In addition, two other studies have compared albino rat strains, and these showed SD rats to be more resistant than Wag/Rij (O'Steen and Donnelly, 1982), and that Fischer (F344) rats are more resistant than Wag/Rij (LaVail *et al.*, 1987c).

It is important to note that these evaluations, and most experimental light damage studies, use "acute" exposures with high light intensities that last a few hours, or are constant for several days. These light intensities are far higher than routinely used in animal rooms during pre-clinical safety assessment, thus a direct comparison is not possible.

Age- and light-dependent retinal changes in albino rats

A study by Weisse and associates examined age- and light-dependent retinal changes in albino Wistar rats (Weisse *et al.*, 1974). Age-related changes showed that females were preferentially affected. Light-related changes were similar in both sexes, with light intensity showing a significant relationship to the incidence of retinal damage. A more recent study using only male SD rats also found that older rats reared in dim cyclic light were more susceptible to light damage than young animals, while dark reared animals were equally susceptible at all ages (Organisciak *et al.*, 1998).

Eye pigmentation protects from light damage

Constant light at a level that destroys almost all photoreceptors in albino rats and mice in 1 week has no effect in pigmented rat or mouse eyes exposed to light for almost a year. However, if the pupils are dilated in pigmented rat eyes, degeneration occurs. The protective effect of pigment is due to both RPE pigmentation as well as screening by melanin in the anterior and posterior uvea that limits light fluxes in the retina (Rapp, 1980; LaVail and Gorrin, 1987; Sanyal and Zeilmaker, 1988; Weisse, 1995; Wasowicz *et al.*, 2002).

RPE65 gene and differences between rats and mice in light damage susceptibility

Albino rats have been used most often to define the biochemical pathways involved in light damage because the experimental light damage paradigms result in more consistent outcomes that are subject to less variation than comparable studies in albino mice (Bicknell *et al.*, 2002; Ablonczy *et al.*, 2005; Tanito *et al.*, 2005; Duncan *et al.*, 2006). In contrast, the albino mouse has been the preferred species to dissect the molecular mechanisms and genetic determinants of light damage (Wenzel *et al.*, 2005), and these studies have provided details on strain differences in light damage susceptibility in mice.

LaVail and associates examined the sensitivity to light-induced photoreceptor degeneration in 7 different inbred albino mouse strains, and compared the findings to the BALB/cByJ (BALB/c) albino mice (sensitive to light damage), and to C57BL/6J-c2J albino mice (resistant to light damage) (LaVail *et al.*, 1987b). Mice of the A/J, AKR/J and NZW/LacJ strains were indistinguishable from BALB/c mice in light damage sensitivity. Mice of the Ma/MyJ and RF/J strains were somewhat more sensitive to light than BALB/c mice, and those of the RIIIs/J were far more sensitive than all of the other strains (LaVail *et al.*, 1987a).

Subsequent quantitative genetic studies in the C57BL/6J-c2J albino mice mapped the locus for resistance to light damage to a genomic region that included the *RPE65* gene, and this accounted for ~50% of the protective effect. At this locus, a leucine 450 methionine (Leu450Met) amino acid substitution was found to co-segregate with the increased resistance to light damage (Danciger *et al.*, 2000). This change has been examined in the 6J-

c2J and other mouse strains, and found to determine light damage resistance (Wenzel *et al.*, 2001). Light sensitive strains, e.g. BALB/c, B6CF1 and 129/Ola, have leucine at codon 450, and produce normal amounts of RPE65 protein. In contrast, the RPE65 450 methionine substitution found in light damage resistant strains, e.g. 6J-c2J and B6;129S(N)2, show markedly decreased amounts of RPE65 protein, and consequently lower amounts of rhodopsin following bleaching (less 11-cis retinaldehyde is produced).

In a safety context, there is little information about the light damage susceptibility of CD-1 outbred mice, or their status at the Leu450Met locus. As this is the most widely used mouse strain in chronic and carcinogenicity studies, the information is critical in order to separate adverse retinal changes secondary to the test article from light/age related findings; however, this information is not readily available.

To this end, we have sequenced the relevant portion of the *RPE65* gene in outbred CD-1 mice surrounding the M/L 450 single nucleotide polymorphism (SNP) using gene-specific primers and genomic DNA from six CD-1 outbred male mice (Charles River Laboratories, Germany) as template (Figure 4; Dr, U. Certa, F. Hoffmann-La-Roche Ltd.). A unique PCR product of 250 base pairs was obtained from all six animals which had the predicted size and is about 80 bases longer than the product obtained with cDNA because it includes intronic sequences (Figure 4A; Wenzel *et al.*, 2001). Following AluI digestion of the genomic fragment the expected products appeared (Figure 4A). Sequence analysis showed that all animals carried the Leucine450 RPE65 allele, indicating that these mice do not have the protective polymorphism at this locus (Figure 4B).

The genetic contribution to light damage susceptibility is still far from completely defined. As noted above, the *RPE65* locus only contributes ~50% of the heritable component to this trait (Danciger *et al.*, 2000). Even in mice that have the same *RPE65* protective genotype and are light damage "resistant", there are differences still in light damage susceptibility, and it takes 10-fold more photons to induce light damage in 6J-c2J than in B6;129S(N)2 (Wenzel *et al.*, 2001). Furthermore, additional quantitative trait loci have been found that modify the light sensitive leucine450 variant of *RPE65* (Danciger *et al.*, 2004).

In contrast to mice, changes in *RPE65* gene sequences have been excluded as modifiers of the light damage phenotype in rats (Iseli *et al.*, 2002). Thus this major modifier locus can be excluded when considering light damage susceptibility or resistance in different rat strains, and at present, pigmentation appears to be the main ((Rapp, 1980), but not the only light damage modifier.

Retinal Structure and light damage

Standard procedures for histopathological assessment of retinal findings in rodents typically involve evaluation of a cross section of each eye, pupil-lens-optic nerve, and, in general, the section plane is not considered. When retinal light damage is a concern, however, the lack of topographic information can be problematic in determining compound vs light damage findings.

Another issue that complicates assessment is that the threshold used to establish severity often favors overlooking significant abnormalities. Although grading scales may vary by pathologists, it is generally accepted to use a scale ranging from *normal->minimal->slight->moderate->marked->severe* (Figure 5). The less severe categories, i.e. *normal* through *slight*, generally assess the entire retina, and give considerable more weight to inner retinal preservation, and absence of retinal layer disorganization or rosette formation. These lesions tend to be localized rather than diffuse, while the more advanced stages tend to be more widely distributed. Thus, in one of the author's experience (GDA), so called *normal* retinas

can have a 20–25% decreases in the outer nuclear layer (ONL) thickness (number of vertical rows of photoreceptor nuclei), a significant loss, indeed, of photoreceptor cells. M*inimal* and *slight* grades can represent decreases of 65–90%, respectively. In general, such grading system appears to be common, if not the industry the standard, although we are not aware of publications that formally set out this classification.

Given that long-term safety studies, 13 weeks or longer, have an "acceptable" level of outer retinal pathology that can be considered a "normal" aging change, evaluation of photoreceptor damage can be underestimated. This is illustrated in Figures 6–8 which illustrate retinal findings from typical 2 year carcinogenicity albino rat and mouse studies. Not only are the characteristic peripheral retinal changes present, but, in addition, the images clearly show that the ONL is thinned to focally absent with loss of photoreceptors, and the inner nuclear layer (INL) becomes attenuated. When such extensive retinal damage that occurs in all treatment groups, it is not possible to assess whether or not the test article causes any incremental retinal damage.

Light and animal housing environment

There is an interplay between light in the animal housing environment, and the likelihood of retinal phototoxicity. Several factors come into play among which are the actual room illumination, reflective properties of the floor and walls, position of the cage in the rack, type of caging used, and, most importantly, the animal's sensitivity to light damage (e.g. albino vs pigmented, *RPE65* Leu450Met etc). Older metal cages, some of which had water bottles on the front of the cage and food pellets on the top and front of the cage lid, provided sufficient shielding to minimize the illumination inside the cage. In contrast, modern cages generally are clear, and constructed of products such as plastic polycarbonate Makrolon with a stainless steel wire lid that permit greater light fluxes within the cage. As well, individually ventilated cages (IVC) have been reported to reduced in cage illumination in comparison to open cages (Kemppinen *et al.*, 2008); however, as differences in the construction material and design can vary by manufacturer, i.e. polysulfone, polycarbonate, polyetherimide, this variation can affect the illumination inside the cages (M. Hargaden, Roche, Nutley, NJ-personal communication). Thus recommending an animal room light level that is safe in terms of avoiding retinal phototoxicity is difficult, and is greatly influence by multiple factors. For these reasons, the appropriate determinant is not so much the actual room illumination, but the light levels inside the cage (see below).

Bellhorn's 1980 study in albino indicated light levels of 323 lux measured at 0.91 meter above the floor in the center of the room did not cause clinical signs of phototoxic retinopathy (Bellhorn, 1980). Presumably this study was used to generate the 1996 and 2010 NRC recommendations which follow closely on these values (325 lux at ~ 1m above the floor surface (NRC, 2010). This illumination appears sufficient for animal care needs and study procedures, and presumably is below the light damage threshold. In this handbook, other items specific to lighting and animal care for rodents are also discussed (NRC, 2010).

The light fluxes occurring in standard rodent rooms/caging indicate that light intensity is variable, and depends on cage position in the rack, being lowest in the bottom rows, and higher close to the light fixtures (Rao, 1991). As well, light intensity at the sides of the rack and front of the cage also vary. Table 1 illustrates the differences in illumination measured when using standard plastic polycarbonate Makrolon cages, and overhead shaded fluorescent bulbs as illustrated in Figure 9. The table demonstrates that light measurements depend on placement and orientation of the light meter in relation to the light source. Such variability can underestimate the light intensity to which rodents are exposed and lead to inconsistent retinal findings in safety studies.

The light levels recommended by the NRC still may be too high for long-term studies in albino rodents, especially mice that do not have the protective *RPE65* SNP. In addition to maintaining rodent rooms in a cyclic light environment (12 hr dark:12 hr light), many research investigators that work on retinal light damage and neuroprotection recommend dim to very dim light levels during the light part of the cycle. To avoid confounding effects, they use light levels of 5 lux (Li *et al.*, 2003) to 20–30 lux (Organisciak *et al.*, 1995; Organisciak *et al.*, 1998). For Sprague Dawley rat strains, room light levels of no higher than 15–20 lux are recommended (R.E. Anderson, personal communication). While the NRC guidelines are important in minimizing retinal phototoxicity, it is critical to also be aware that light levels recommended will be influenced greatly by factors noted above, i.e. cage type, reflectivity of floor, walls and ceiling, cage position, etc. The illumination level that is most important, however, is the actual in cage illumination, and this should be measured, recorded and, if possible, kept in the 20 lux range or lower. Thus the discrepancy in the recommended light levels between those used in industry and in major retinal research laboratories highlights some of the potential confounding problems that can arise in safety studies.

Incidence of retinal phototoxicity-a selected industry survey

In our experience, the incidence of retinal degeneration encountered in control albino rodents in safety studies has increased in recent years, and this degeneration often confounds interpretation of test article-related effects. To address this issue, we have conducted a survey of Contract Research Organizations (CROs) located in North America, Europe and Asia using a survey questionnaire (Supplementary Table 1) that requested information on housing, illumination conditions and incidences of retinal atrophy in control animals. Information was obtained for different strains of mice and rats commonly used in toxicology testing (6-month and carcinogenicity studies). Responses to the questionnaire were provided by nine out of ten CROs contacted, and meaningful data obtained from eight: North America (2), Western Europe (2) and Japan (4). Details of the survey are provided in Supplementary Table 2 (mouse) and 3 (rats), and the results are summarized below.

General observations

Strains and housing conditions

- There is a great variation in caging, illumination levels and housing condition between different CROs.
- Mouse strains used are CD-1 and B6C3F1, and, infrequently, C57BL/6 or NMRI. B6C3F1 and C57BL/6 are pigmented strains. Rat albino strains used are mainly Wistar, Sprague Dawley and, to a lesser extent, Fischer.
- In principle the same cage type (material) is used for either rats or mice in 6-month chronic toxicity as well as in carcinogenicity studies within the individual CROs. Half of CROs are using clear polycarbonate (Makrolon) or polysulfone plastic cages with metal wire mesh covers, the other half use wire mesh cages with a metal cover. In all cases, a metal plate is used to cover the top of the racks reducing the illumination level for animals in the upper tier. All cages are suspended in movable racks.
- Rotation of racks and/or cages varies by CRO. Of eight CROs, only 3 rotate the cage racks on a monthly basis in the 6-month rodent studies. In the 2-year carcinogenicity studies, five of eight CROs rotate racks either monthly (3), every 2nd (1) or 6 weeks (1), respectively. In general, rotating cages vs racks may be preferred, but cage rotation is usually not done as this is more work and

logistically more complex. Lastly, in three CROs rotation is not performed for any studies using rats and mice.

- Animals are housed individually or in pairs (mostly rats) or in groups up to 5 animals per cage (mostly mice). Some CROs arrange housing conditions according to Sponsor's request. [MD1]

Illumination conditions

- All CROs use a similar 12 hour light-dark cycle (on: 5–7 a.m.; off: 5–7 p.m.).
- The range of illumination intensity varies between 150–650 lux (mean range of 210–490 lux) at a height range of 0.80–1.5 m above floor. In one CRO intensity was measured at 2 m and in another CRO directly underneath the ceiling lamp although illumination values were not provided. With one exception, no information was provided on the instruments or methods used, an indication that direct comparisons between CROs is not possible. At face value, some of these light levels appear to be slightly above those of the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996; NRC, 2010).

Historical control data on retinal atrophy (histopathology)

- A higher incidence of retinal atrophy was seen in 2-year vs. 6-month studies; these findings are considered light-related, although an age effect can not be excluded. The net contribution of age vs light on retinal atrophy in the 2-year studies can not be quantified as CROs generally do not differentiate between focal (i.e. light-induced in early stages) and diffuse retinal atrophy (mainly age-related although diffuse atrophy occurs in end-stage retinal light damage) (Weisse *et al.*, 1974). These results are summarized in Supplemental Tables 2 and 3).
    - -For 2 year studies in mice, the percent incidence (male/female) of retinal atrophy ranged from 0/0–56/65 in CD-1 albino mice to 0/0–0/1 in the B6C3F1 pigmented strain.
    - -In 2 year studies in rats the percent incidence (male/female) of retinal atrophy ranged from 5/5–24/51 in the Wistar strain to 0/3–21/38 in Sprague Dawley. In comparison, 6 month studies showed a lower incidence (Wistar: 0/0–2/4; SD: 0/0–6/9).
- Limited data from albino NMRI mice with a low incidence of retinal atrophy as well, point to strain differences as another contributor. The status of this strain in regards to *RPE65* Leu450Met is unknown.
- The Fischer strain was used infrequently, and only for carcinogenicity studies. Based on one study, one CRO did not report retinal atrophy while another CRO indicated incidences of retinal atrophy around 70%, and comparable between males and females in 16 studies.
- A possible difference in gender susceptibility to retinal atrophy was observed in studies with rats. In the majority of the studies, females tended to have a higher incidence of retinal atrophy than males.

How to address retinal phototoxicity issues in a safety study

It is important to establish *a priori* an algorithm to differentiate test article associated retinal damage from retinal phototoxicity, or to identify the possible interaction between the drug and light that results in retinal atrophy (Figure 1). While the first two possibilities are

straightforward, even though the mechanism of compound-associated retinal degeneration may be difficult to precisely define, the third possibility, i.e. interaction between the drug and light, can create obstacles in the path to drug development. Drugs or their metabolites can have a direct effect on photoreceptors that are light exposed (Izumi *et al.*, 2004), or alternatively, an indirect effect where the compound causes a change resulting in increased light damage susceptibility. For example, drugs that cause pupillary dilatation such a clonidine (Weisse *et al.*, 1971) result in higher incidence of retinal phototoxicity. As well, drugs that cause behavioral changes such as increased locomotion or position the animal closer to the front or sides of the cage have been found to cause a higher incidence of retinal light damage (Aguirre, unpublished). These possibilities can be assessed, respectively, by either pupillometry (Aleman *et al.*, 2004) or spontaneous activity measurements (Masuo *et al.*, 1997).

Mechanistic studies to specifically address light damage as a direct cause of retinal atrophy would take one of several forms. These would include studies with pigmented and albino animals receiving the test article, and maintained under standard housing conditions. As the pigmented retina is more resistant to light damage, presence of retinal degeneration in albino but not pigmented animals would be interpreted as evidence in support of phototoxicity. As well, studies in which the albino animals would be maintained under dim cyclic light illumination, e.g. ~ 20 lux at the front of the cage, without retinal damage would further support phototoxicity vs a direct compound effect on photoreceptors. In both cases, it is important ascertain that the length of the studies are comparable to the one where the initial finding was made as duration of light exposure is a critical component. Although having a low light intensity arm to such studies is of importance, we do not recommend that a high light intensity cohort be used. As more light results in more retinal degeneration, such a group would provide no additional mechanistic information.

A critical component of any study that aims to investigate a light damage mechanism is the morphologic assessment of the topography of retinal damage. In general, direct retinal damage by compounds tends to cause diffuse rather than more locally extensive outer retinal damage. On the other hand, the retinal pathology resulting from phototoxicity is more localized, and restricted to the superior-temporal regions above the optic disc at the stages when the damage is not too advanced (see Figures 2 and 3; ((Rapp, 1980; Organisciak *et al.*, 1999; Tanito *et al.*, 2008)). Consequently, it is important to critically maintain section orientation in order to establish the topography of the damage.

Suitable dissection and processing of the eye for histologic evaluation is essential for proper diagnosis of retinal atrophy and to establish whether it is light-induced. We recommend that the eyes be removed with the optic nerve and upper eyelid, and fixed in a fixative such as Davidson’s solution. Proper orientation of the eye during embedding and sectioning is important and the dorso-ventral and temporal-nasal landmarks of the globe need to be retained. The globes are oriented and trimmed in the dorso-ventral plane (Figure 3A; ((Rapp, 1980)), or, alternatively, in the supero-temporal to infero-nasal plane (Figure 3D; (Tanito *et al.*, 2008)). Technically, it is easier to orient the globes in the dorso-ventral plane. We recommend this as a routine practice as it is not possible to predict in most studies whether a retinal finding will occur that will require differentiation from retinal light damage.

The sections are then examined to record ONL thickness, preferably in terms of vertical rows of nuclei, at predetermined sites, usually in 0.5 mm intervals from the optic disc edge along the dorsal and ventral meridians, and the results graphically displayed (Figure 3A). Assessment is made at each location in the section, and the output represented as "spidergraphs". Typically, the early stage of retinal light damage is clearly represented by a marked decrease in ONL in an ~2 mm region above the edge of the optic disc (Figure 3A).

A point that was clearly brought out in the surveys (see Supplementary Tables 2 and 3), is the lack of consistent methodologies for measuring light intensities in animal rooms. While the NRC guidelines give suggested values and locations for measurements, the instruments and procedures used are not specified. Consequently, it was not possible to compare results across the different CROs included in the survey. For that reason, it would be important to develop a uniform industry-wide SOP for measuring and recording light levels in the animal room, and at the front and inside of the cages. This would specify the instrument, probes, and their position and orientation in the room and cages. In this manner, it would be possible to reliably determine retinal phototoxicity in future studies, and assess whether modifications in housing or husbandry ameliorate or worsen the incidence of this finding.

Summary and conclusions

Light damage is a frequent finding in long-term safety studies of rodents, and is a confounding effect in differentiating direct toxicity of the test article from environmental phototoxicity. The finding is mainly limited to albino strains, and is dependent on the intensity and exposure duration. Animal associated factors include age, gender, pigmentation, body temperature and strain. Genetic factors include a protective SNP at Leu450Met in *RPE65* where the methionine variant is protective as it markedly reduces the vitamin A cycle in the RPE. Environment associated factors include room illumination, type of caging, husbandry practices. A survey of 8 CROs in Western Europe, Asia and North America indicated variability in caging and husbandry practices; likewise, there was a broad range of incidence of retinal atrophy in control animals in the different CROs. The purpose of the survey was to examine for consistencies and differences in management practices, and not to establish a causal association between the incidence of retinal atrophy and specific housing or husbandry practices. The data advocate for careful monitoring of background incidences of retinal atrophy in repeated dose studies. Even though the data do not allow a precise conclusion on the role of light levels, it is expected that lower light levels should help reduce the incidence of phototoxic retinopathy without impact on study quality. Also evident from our survey is the need to develop a uniform industry-wide SOP for measuring and recording light levels in the animal room. As retinal light damage has characteristic morphology, topography, and can be prevented by decreased ambient light levels and/or pigmentation, the tools are readily available to interpret retinal findings in safety studies from the perspective of light damage unrelated to the test article.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CRO	contract research organization
INL	inner nuclear layer
ONL	outer nuclear layer
RPE	retinal pigment epithelium
SNP	single nucleotide polymorphism
SOP	standard operating procedure

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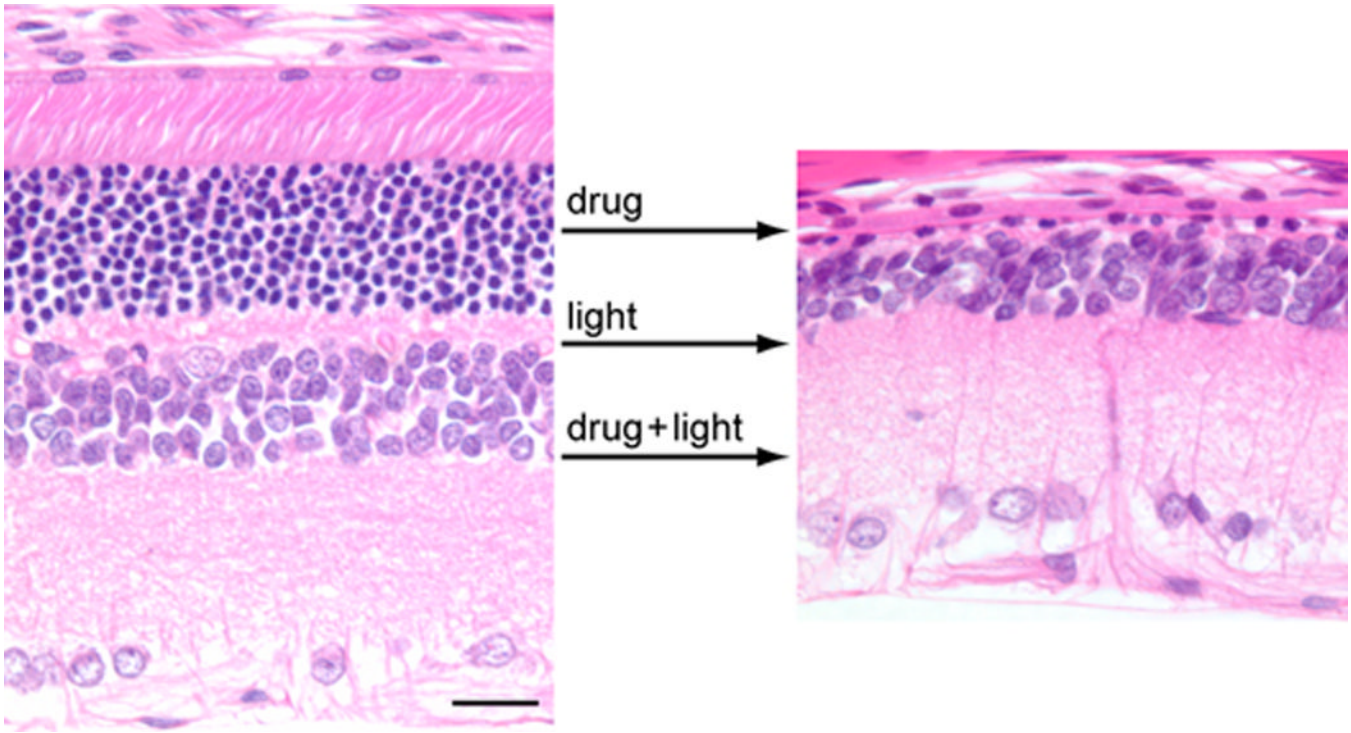


Figure 1.
Photomicrograph of normal albino Sprague-Dawley retina (left). In the context of safety studies, the change from normal to extensive photoreceptor degeneration (right) can result from the test article, phototoxic retinopathy or a combination of both. In this example, the damage, regardless of cause, results in nearly complete loss of photoreceptor cells. Calibration bar= 40 μm.

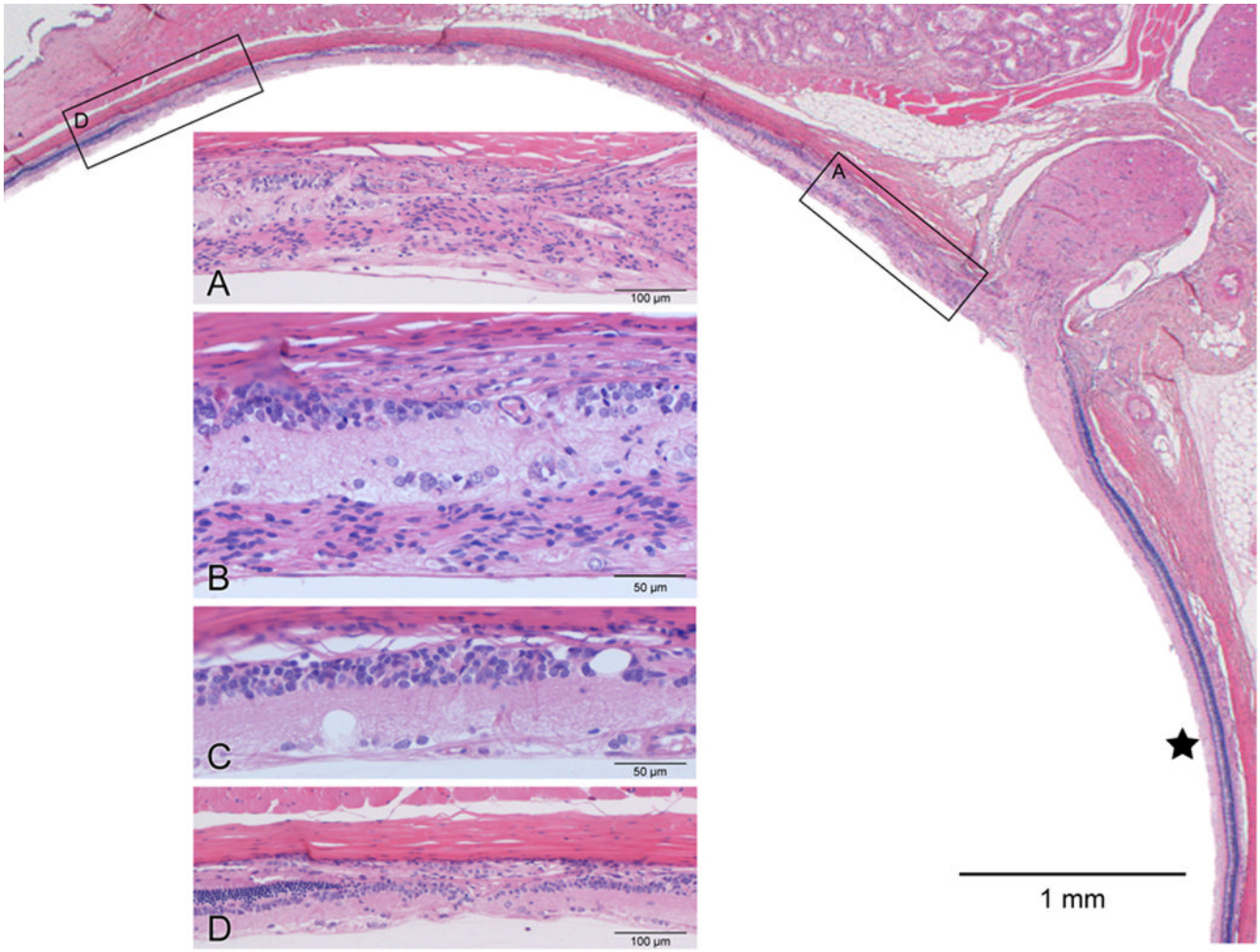


Figure 2.
Light-induced retinal degeneration in an albino rat from a 2-year carcinogenicity study. There is a distinct difference in thickness of the retina on either side of the optic disk. The ★ marks the relatively normal inferior retina. Approximately 50% of the superior retina is markedly reduced in thickness, and shows different degrees of retinal atrophy. These begin adjacent to the disc (boxed area-A, Figure A) and extend to the mid-periphery (boxed area-D, Figure D); intervening regions are illustrated in B and C. A. The retina nearest the optic disc shows full-thickness loss of retinal layer organization and fibrous connective tissue in the vitreal aspect of degenerating retina. B. The same tissue overlays the ganglion cell layer; the inner nuclear layer is moderately reduced, and abuts the choroid and sclera. The outer retinal layers and retinal pigment eipthelium are absent. C. Microcystoid vacuoles are present within remnant inner retinal layers. D. There is an abrupt transition into less affected retina with preservation of retinal pigment epithelium, outer nuclear and photoreceptor layers. H&E.

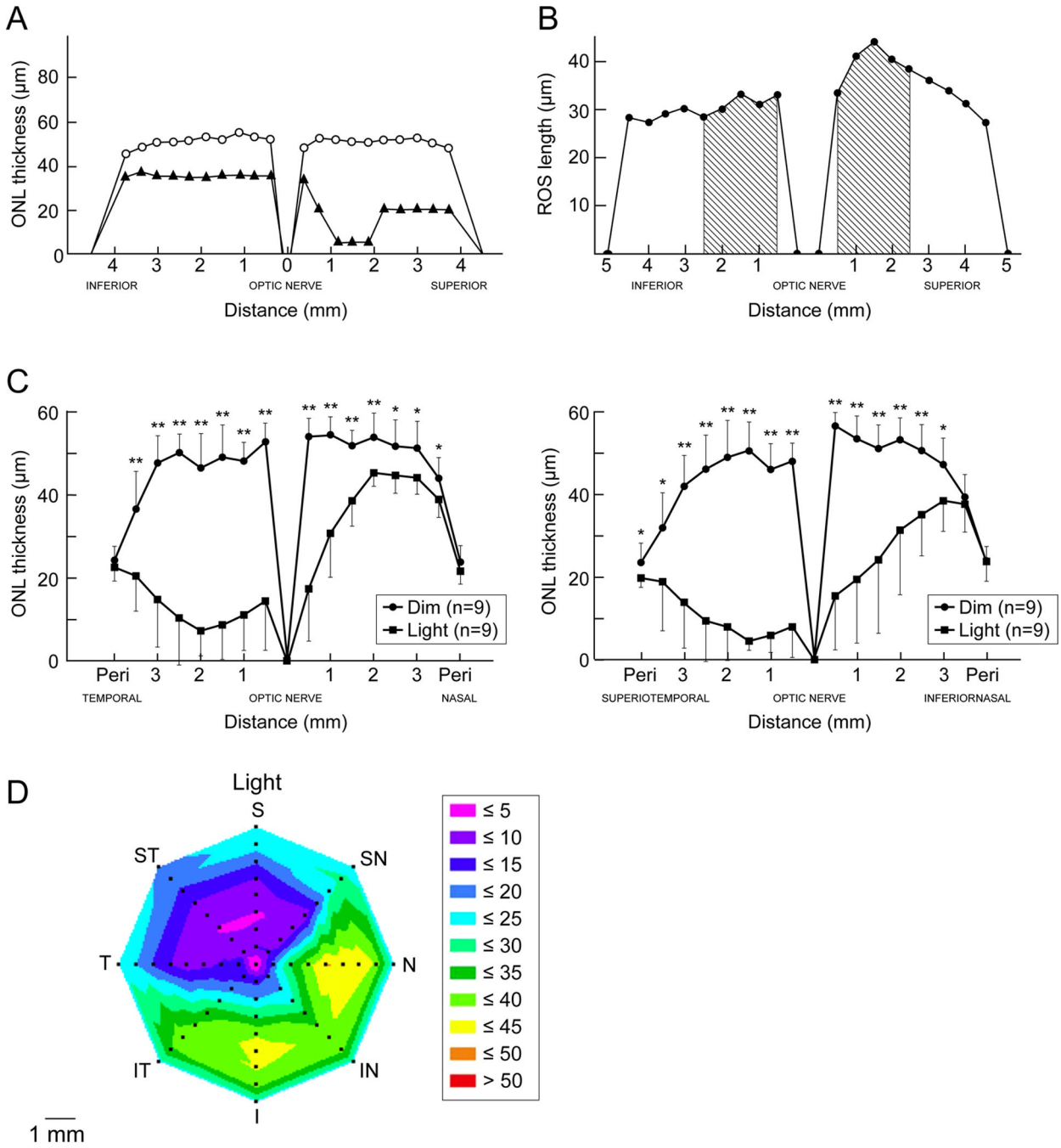
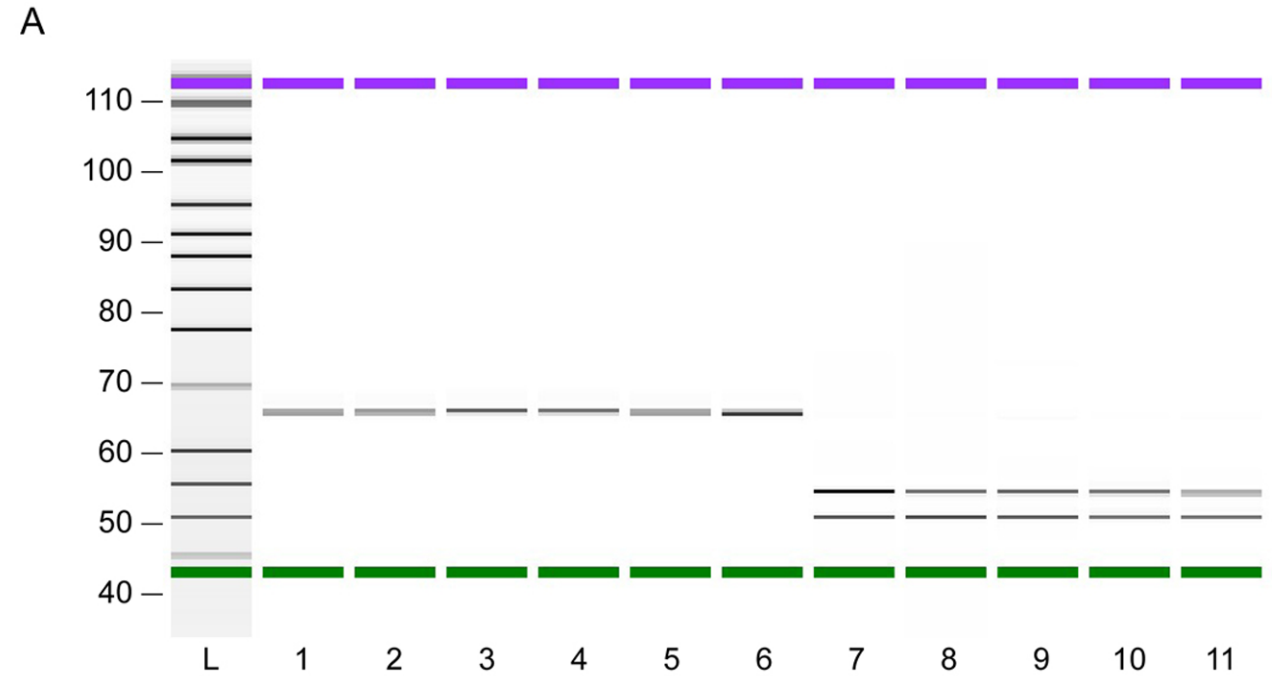


Figure 3.

Topography of retinal light damage. A. Spidergraphs illustrating the exquisite sensitivity of the superior retina to light damage in an albino Sprague-Dawley rat (black triangles) exposed to 4 days of continuous illumination at 40 lux compared to a cyclic light control (open circles). Loss of photoreceptor cells results in thinning of the outer nuclear layer (ONL) that is most severe above the optic nerve head. (Figure modified and reprinted with permission from Figure 2 of ((Rapp, 1980)). B. Schematic representation of rod outer segment (ROS) length at different locations in the superior and inferior quadrants of the rat retina. ROS are longer at the site where the retina is most susceptible to light damage. (Figure modified and reprinted with permission from Figure 2 of (Rapp *et al.*, 1985)). C.

Intense short-term light exposure (**Light**, 5000 lux for 6 hours) also results in a topographically distributed retinal lesion in comparison to **Dim** light control (5 lux cyclic light). When the damage is evaluated across different retinal regions, the superiotemporal and temporal quadrants are most sensitive. D. The damage is best illustrated in color-coded maps of ONL thickness which illustrate that the ONL thickness has decreased to <15 μm; scale in right correlates color with ONL thickness in μm. (Reprinted with permission (C=Figure 2A; D=Figure 3B) from (Tanito *et al.*, 2008)).



B

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                                                                                  480
sw:rpe_mouse       INYQKFGGKP YTYAYGLGLN HFVPDKLCKL NVKTKEIWMW QEPDSYPSEP IFVSQPDALE     480

userseq:infile_1   .......... ...AYGLGLN HFVPDKLCKL NVKTKEIWMW QEPDSYPSEP ..........      37
```

Figure 4.

RPE65 genotype of CD-1 outbred mice. A. The amplicons generated by Rpe65 gene-specific primers (Rpe65_forward: CTGACAAGCTCTGTAAG; Rpe65_reverse: CATTACCATCATCTTCTTCCA) from genomic DNA are displayed by agarose electrophoresis for six CD-1 outbred male mice. The primers generated a single 250bp band in all six mice (lanes 1–6 below) which is larger than that reported for cDNA in the literature because the amplicon included intronic sequences (Wenzel *et al.*, 2001). Sequencing indicated that the 80 bp intronic sequence was present just before the AluI site (lanes 7–11). B. Sequence alignment of the relevant *RPE65* coding region (Boulanger *et al.*, 2001) from all six CD-1 outbred mice indicated Leucine at codon 450 (**L**, boxed region**)** indicating that they do not have the protective polymorphism at this locus.

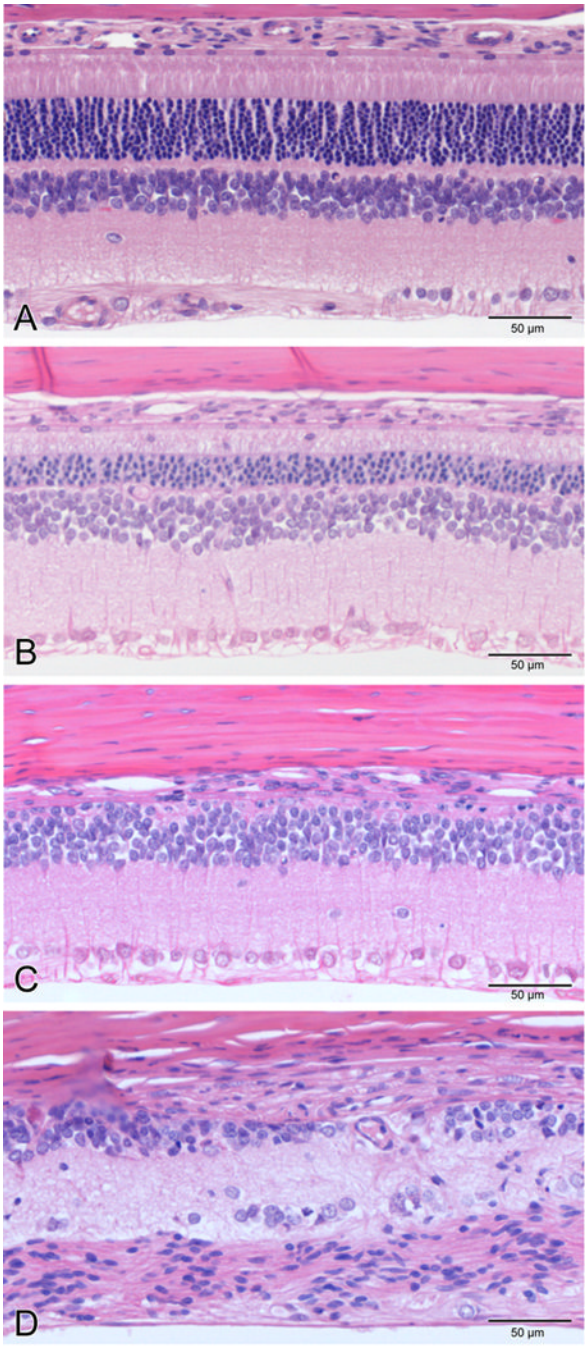

Figure 5.

Example of a generally accepted grading scale (*normal->minimal->slight->moderate->severe*) to evaluate retinal atrophy in rat toxicity studies. A. *Normal* retina: the different retinal layers are distinct, photoreceptor inner and outer segments are elongated, outer nuclear layer is intact. B. *Slight* retinal atrophy: different layers remain distinct, but the outer nuclear layer is reduced in thickness, and macrophages are present in the photoreceptor layer. C. *Moderate* retinal atrophy: the retinal pigment epithelium, photoreceptors and most of the outer nuclear layer are lost; the outer plexiform layer is not present. The inner nuclear layer remains relatively intact albeit irregular. D. *Severe* retinal atrophy: There is marked

disruption of the normal retinal architecture with loss of retinal layer organization. Remnants of inner nuclear and ganglion cells remain. H&E.

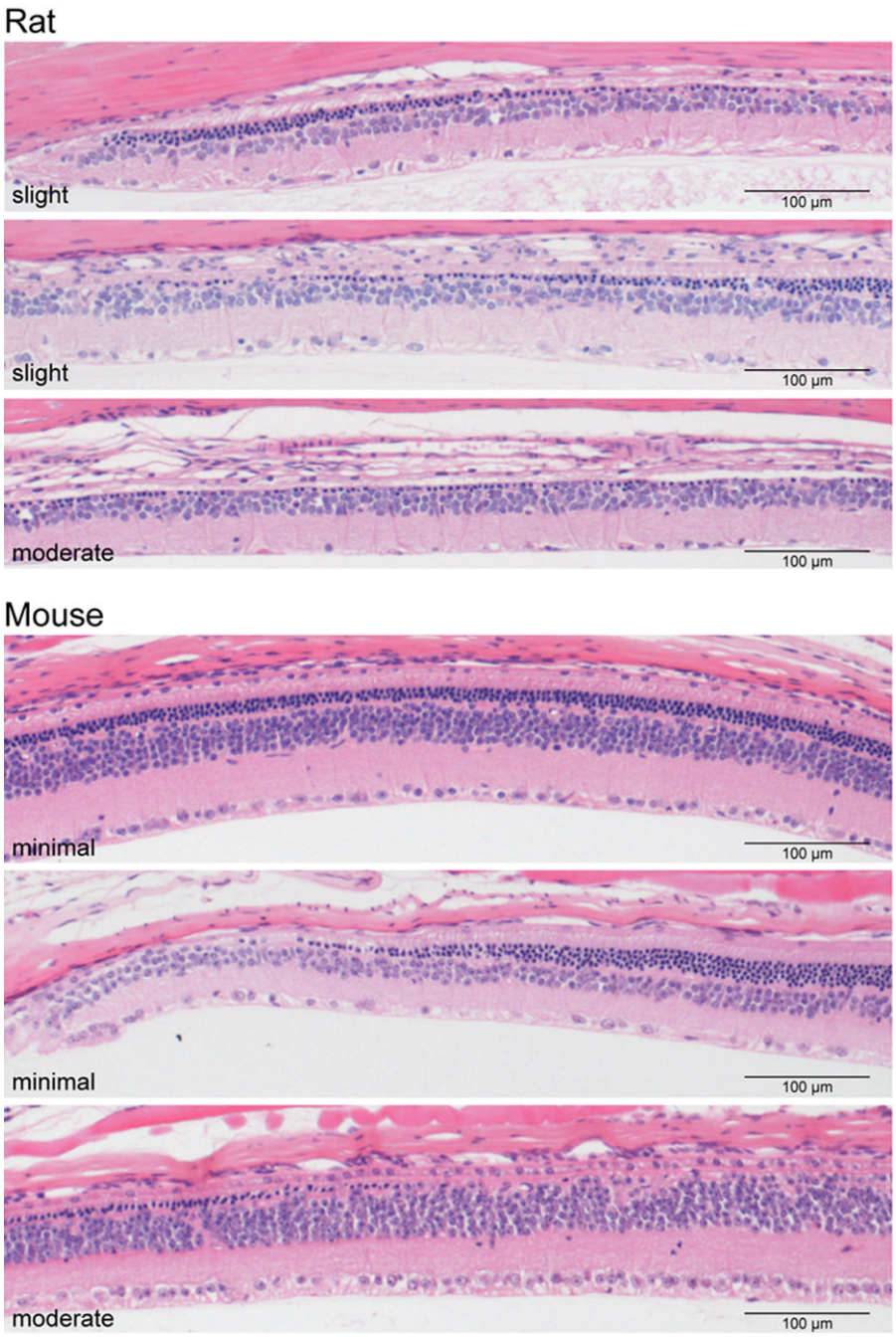

Figure 6.
Examples of retinal atrophy findings in rat and mouse carcinogenicity studies illustrating the grading classification currently used. Peripheral retinal atrophy in control Sprague-Dawley rats and CD-1 mice in two-year carcinogenicity studies. H&E.

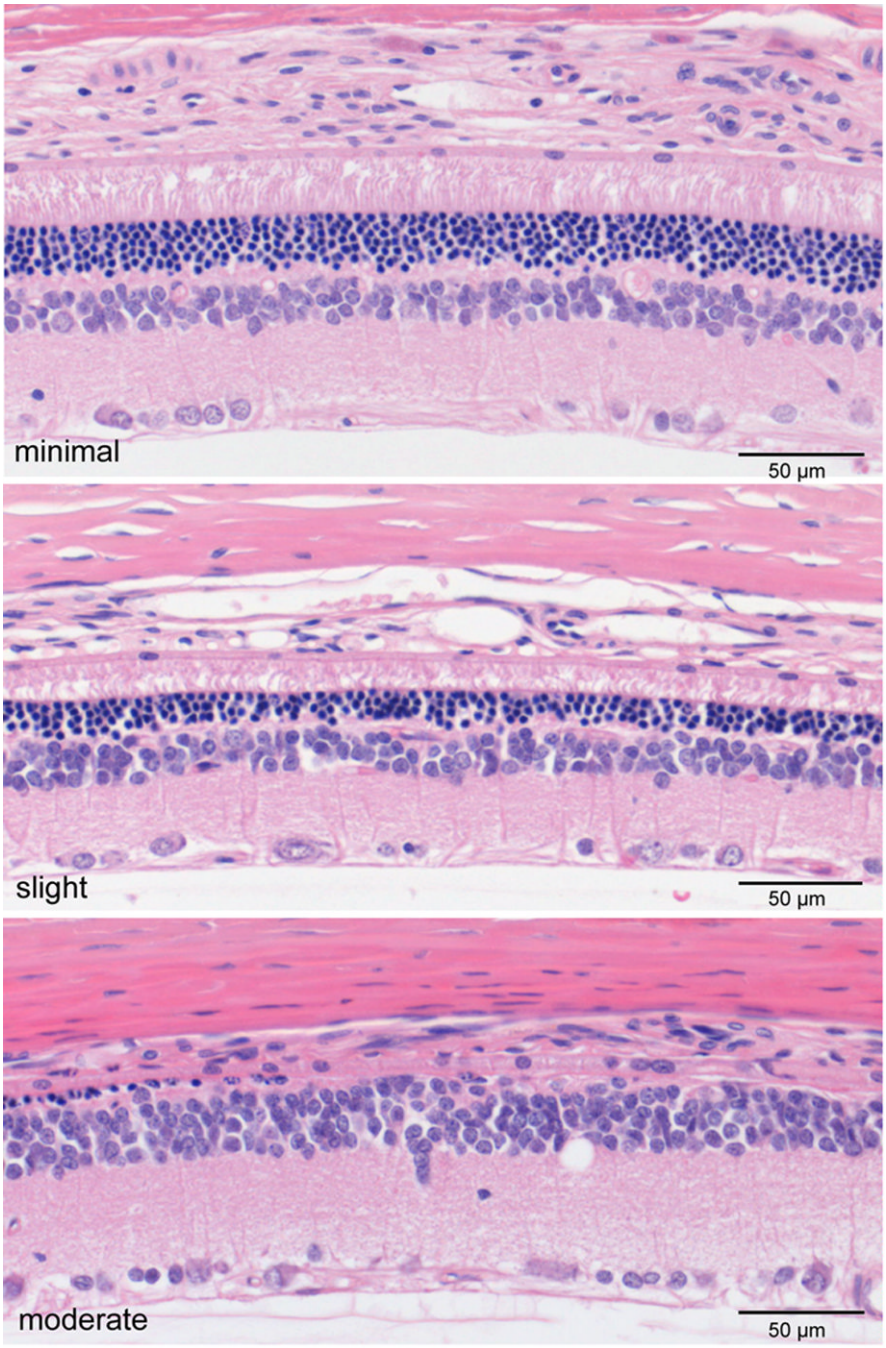

Figure 7.
Examples of retinal atrophy findings in control Wistar rats in two-year carcinogenicity studies. There is atrophy in the central and midpheripheral regions the retina. There is minimal to severe thinning of the outer nuclear layer, loss of photoreceptors, and occasional microcystoid vacuoles in the inner nuclear layer. Note that *slight* and *minimal* gradings still represent significant photoreceptor pathology. H&E.

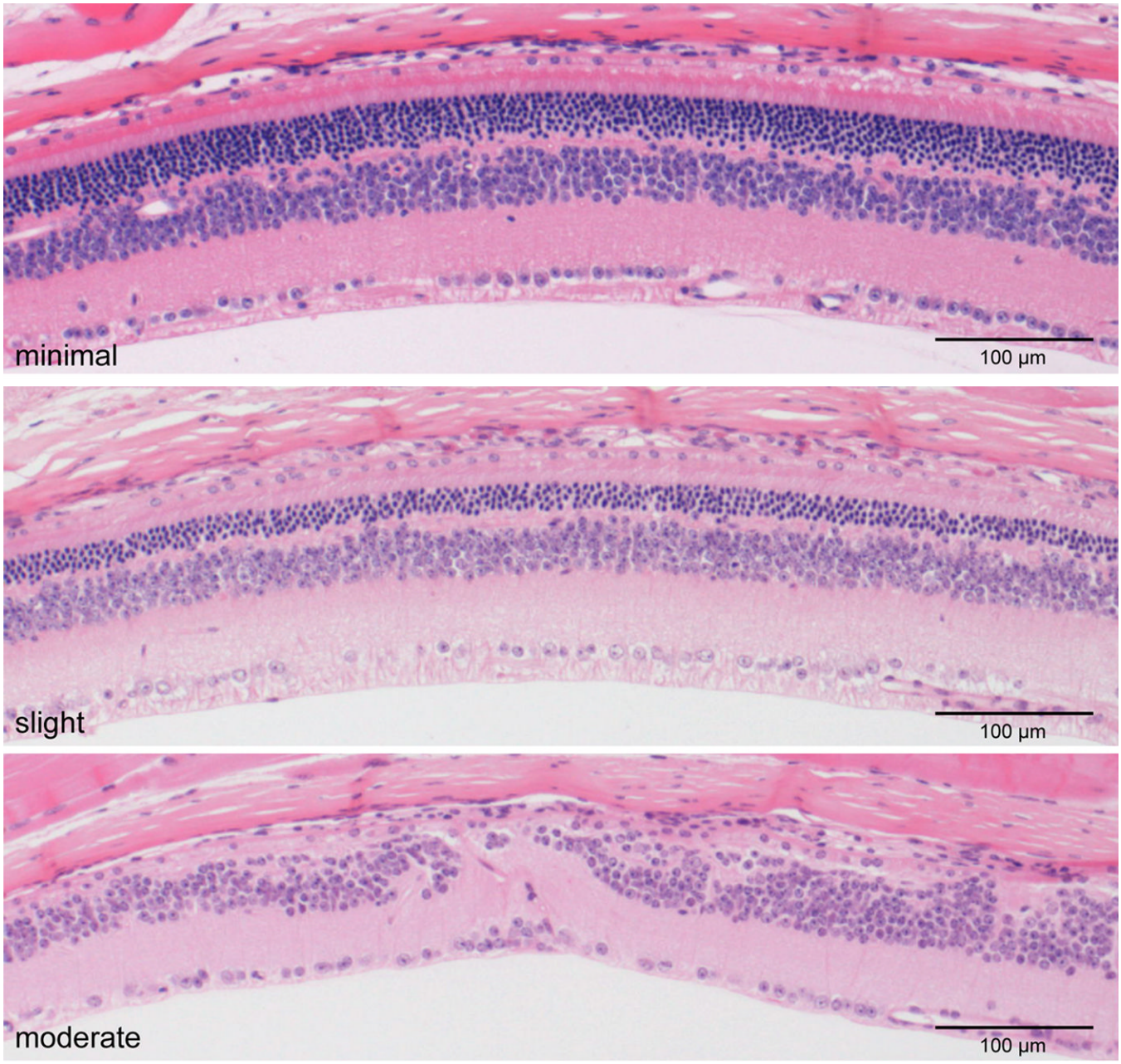

Figure 8.
Examples of retinal atrophy findings in CD-1 mice in two-year carcinogenicity studies. There is minimal to severe thinning of the outer nuclear layer, and loss of photoreceptors and outer nuclear layer. The inner nuclear layer is often attenuated and disorganized when outer retinal changes are extensive. H&E.

Figure 9.
Example of a typical rack holding 6 levels of 3 cages per level. Two rats are kept in each plastic polycarbonate Makrolon cage with a stainless steel wire lid. A stainless steel plate covers the uppermost shelf. Shaded fluorescent bulbs provide the light source.

Table I

Light levels (in lux) within or in front of polycarbonate cages at various locations in a typical rack used in toxicity studies (see Figure 9). Two methods of orienting the light meter (Voltcraft MS-1300) relative to the light source were used.

Method I: Light meter placed horizontally, facing the light source		
A. Light meter placed inside the cage.		
49.9	53.9	41.5
76.1	34.1	49.8
73.4	23.0	50.8
55.0	22.6	44.0
43.0	20.4	27.5
29.0	17.5	27.9

B. Light meter placed in front of the cage.				
	208	197	207	
207	677	746	724	305
244	548	592	599	333
239	464	483	460	267
208	353	374	350	224
174	325	341	329	187
148	273	288	277	158

Method II: Light meter placed vertical to the light source.		
A. Light meter placed inside the cage.		
81.9	72.0	73.3
79.3	59.7	64.8
59.8	49.7	56.5
58.2	47.3	51.5
48.9	38.4	43.6
41.8	30.0	32.1

B. Light meter placed in front of the cage				
	364	363	333	
244	390	419	411	302
246	241	300	234	253
202	202	224	218	180
136	165	181	180	151
113	188	151	147	128
102	147	144	146	101