Reversal of functional loss in the P23H-3 rat retina by management of ambient light

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Abstract

The present experiments were undertaken to test recovery of function in the retina of the rhodopsin-mutant P23H-3 rat, in response to the management of ambient light. Observations were made in transgenic P23H-3 and non-degenerative Sprague–Dawley albino (SD) rats raised to young adulthood in scotopic cyclic light (12 h 5 lx 'daylight', 12 h dark). The brightness of the day part of the cycle was increased to 300 lx (low end of daylight range) for 1 week, and then reduced to 5 lx for up to 5 weeks. Retinas were assessed for the rate of photoreceptor death (using the TUNEL technique), photoreceptor survival (thickness of the outer nuclear layer), and structure and function of surviving photoreceptors (outer segment (OS) length, electroretinogram (ERG)). Exposure of dim-raised rats to 300 lx for 1 week accelerated photoreceptor death, shortened the OSs of surviving photoreceptors, and reduced the ERG a-wave, more severely in the P23H-3 transgenics. Returning 300 lx-exposed animals to 5 lx conditions decelerated photoreceptor death and allowed regrowth of OSs and recovery of the a-wave. Recovery was substantial in both strains, OS length in the P23H-3 retina increasing from 17% to 90%, and a-wave amplitude from 33% to 45% of control values. Thinning of the ONL over the 6 week period studied was minimal. The P23H-3 retina thus shows significant recovery of function and outer segment structure in response to a reduction in ambient light. Restriction of ambient light may benefit comparable human forms of retinal degeneration in two ways, by reducing the rate of photoreceptor death and by inducing functional recovery in surviving photoreceptors. © 2006 Elsevier Ltd. All rights reserved.

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1. Introduction

Increasing the ambient light experienced by the retina accelerates photoreceptor death, and reduces the outer segment length and visual responsiveness of the surviving photoreceptors. Even modest increases, for example from scotopic to mesopic conditions, produce measurable rises of photoreceptor death in the normal rat retina (Penn and Anderson, 1991; Walsh et al., 2004). In the normal retina, however, bright ambient light does not destabilize the retina; indeed the

light-experienced retina is more resistant to damage from very bright lights (Penn and Anderson, 1991), and in this sense is more stable than the less damaged, less light-exposed retina. Moreover, if ambient light levels are reduced, the shortening of the OS is reversed and the amplitude of the ERG is largely restored.

The present study concerns the P23H transgenic rat, in which the transgene is a mutant form of rhodopsin (a proline for histidine substitution at position 23), engineered to mimic a naturally occurring human mutation, which causes an autosomal dominant degeneration of photoreceptors. The transgene causes a comparable autosomal dominant degeneration in the P23H-3 rat (Machida et al., 2000), making it a valuable model of the human condition. Recently, we have shown that in this transgenic, modest (scotopic to mesopic) increases in

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ambient light levels have a more severe effect on photoreceptors (Walsh et al., 2004), and a similar hypersensitivity of photoreceptors to light has been described in rhodopsin-mutant dog model of retinal degeneration (Cideciyan et al., 2005). In the P23H-3 rat, photoreceptor death rates are sharply accelerated, and OS length and the ERG amplitude are severely reduced. This suggests that levels of light typical for everyday visual function may, in patients with comparable disease, accelerate photoreceptor death, and make surviving photoreceptors insensitive. This study tests the working hypothesis that part of the visual loss in the P23H-3 retina is due to damage to surviving photoreceptors, and may therefore be reversible.

Limitation (partial prevention) of photoreceptor damage in genetically driven degenerations of human retina can be inferred from evidence of sectoral variation of the severity of the degeneration (Heckenlively et al., 1991), and from evidence that light restriction slows retinal degeneration in rodent models of genetically induced degenerations (Dowling and Sidman, 1962; Kaitz, 1976; Naash et al., 1996; Walsh et al., 2004). Present results show that, when ambient light is reduced, outer segments re-grow, and the ERG shows significant recovery. The capacity of P23H photoreceptors to regrow outer segments when light is reduced had been suggested previously (Bicknell et al., 2002), on the basis of dark-induced increases in rhodopsin content. This regrowth in the P23H-3 rat suggests that, in humans suffering visual loss due to comparable genetic mutations, light management should improve retinal performance, as well as slowing the underlying photoreceptor degeneration. The idea was reviewed and tested by Berson (1971) who reported negative results, and was revisited by Pe'er and Meron (Stone et al., 1999), with a positive outcome.

2. Methods

2.1. Animal models

All experiments were in accord with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Observations were made in albino Sprague–Dawley (SD) and P23H Line 3 (P23H-3) transgenic rats aged from P (postnatal day) 93–185. The P23H-3 rats were heterozygotes, obtained by mating P23H-3 homozygotes (from breeding stock obtained from Beckman Vision Laboratories, San Francisco) with SD albinos.

2.2. Light exposure

Rats were born and reared in dim (<5 lx), cyclic light conditions (12 h 5 lx/12 h dark). Some were exposed to brighter cyclic light (12 h 300 lx/12 h dark) for 1 week, and then returned to dim cyclic light for 2–5 weeks.

2.3. Morphology of outer segments

Eyes were obtained from 5 or 6 animals for each time point. Eyes were immersion-fixed in a mixture of 2.5% glutaraldehyde and 2% formaldehyde in 1 M sodium cacodylate (pH

7.4) buffer, for at least 2 h. The cornea and lens were removed with a scalpel blade, fine forceps and scissors. The eyecup was then divided through the optic nerve head into nasal and temporal segments and then returned to the fixative and stored for up to 2 weeks. The segments of eyecups were dehydrated in ascending alcohols, followed by a 1:1 100% ethanol:propylene oxide mixture for 15 min step and 100% propylene oxide for 15 min, and embedded in araldite. Blocks were cut at 2 μ m. Sections were stained with toluidine blue and photographed with a 63 $\times$ objective. For each animal, 5 images were taken, usually from a single section. The images were taken at least 0.5 mm from the anterior or disc edge of the retina, to avoid edge-related changes in morphology (reviewed in Stone et al., 2005), and from sites spaced at approximately equal intervals between the two edges. Within each image, OS length was measured at three points. Measurements were averaged for each image, then for each animal, and then for each group of animals.

2.4. Estimates of dying and surviving photoreceptors

Eyes were obtained from 5 or 6 animals for each time point. Eyes were immersion-fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) buffer at pH 7.4 and 4 °C for 1–3 h. After three rinses in 0.1 M PBS the eyes were left overnight in a 15% sucrose solution to provide cryoprotection. Eyes were embedded in mounting medium by snap freezing in liquid nitrogen and were cryosectioned at 20 μ m.

To demonstrate cells dying, sections were labelled with the TUNEL technique (Gavrieli et al., 1992) to identify the fragmentation of DNA characteristic of dying cells, following protocols published previously (Maslim et al., 1997). To demonstrate cells surviving, sections were also labelled with the DNA-specific dye bisbenzamide (Calbiochem, La Jolla, CA), by incubating them for 2 min at room temperature in a 1:10,000 solution of bisbenzamide in 0.1 M PBS.

Counts of TUNEL+ profiles (apoptotic cells) were made using a calibrated 20 $\times$ objective and an eyepiece graticule. Each section was scanned from the superior to inferior edge in 500 μ m steps, and the number of TUNEL+ profiles was recorded for each 500 μ m length of the section. Sections cut adjacent to or through the optic nerve head were used, to minimise variations in retinal length and position. Counts were averaged from at least two sections per animal. Counts were recorded separately for the outer nuclear layer (ONL), inner nuclear layer (INL) and inner retina (layers internal to the INL). The counts from the several sections were then tallied and averaged to produce a result expressed in TUNEL+ profiles/mm retina. The thickness of the ONL was measured using an eyepiece graticule which, with a 20 $\times$ objective, delineated an area of 500 μ m $\times$ 500 μ m on the section, with subdivisions that marked 100 50 μ m $\times$ 50 μ m squares.

2.5. Electrophysiology

The dark-adapted flash-evoked electroretinogram (ERG) was recorded, as described previously (Walsh et al., 2004).

We measured the amplitude of the a-wave, as an index of the responsiveness of photoreceptors, principally (in the rat) of rods. In each animal, the ERG was recorded to flashes increased in 10 steps from 13.5 to 20×10^5 photoisomerisations/s. The response to the brightest flash was used to measure the a-wave, for each animal; this intensity was chosen because it produced a response which was near maximal, but not saturated.

2.6. Statistical analyses

The present experiments were designed to test the working hypothesis that a return to scotopic conditions after exposure of the retina to brighter (300 lx) ambient light will induce a slowing of photoreceptor degeneration, a regrowth of the outer segments and an increase in the amplitude of the a-wave. Since each prediction concerns the direction of change, we have used the one-tailed form of *t*-test.

3. Results

3.1. Photoreceptor death rate rises and falls with ambient light

The quality of TUNEL labelling of dying photoreceptors obtained in these experiments has been illustrated previously for SD and P23H-3 rats (Walsh et al., 2004; Yu et al., 2004). In 5-lx raised animals, the frequency of TUNEL+ profiles in the ONL was higher in the P23H-3 strain than in the non-degenerative SD strain (Fig. 1, $p < 0.0001$), confirming previous findings (Walsh et al., 2004; Wellard et al., 2005; Yu et al., 2004). When ambient light was increased from 5 lx to 300 lx for 1 week, a rise in the frequency of TUNEL+ profiles in the

Fig. 1. Frequency of TUNEL+ (dying) profiles in the ONL in SD and P23H-3 rats before and after exposure to bright (300 lx) ambient light for 1 week. Frequencies were consistently lower in the SD strain. In both strains, frequencies were increased by exposure to 300 lx ambient light, and fell to control levels over a period of 5 weeks back in 5 lx conditions. Group sizes were 5–9 animals. Error bars show ± 1 standard deviation.

ONL was apparent in both strains. The rise was smaller in the SD strain, but approached statistical significance ($p = 0.0535$). The rise was larger in the P23H-3 strain and highly significant ($p < 0.00001$). The greater size of the rise in the P23H-3 strain was also significant ($p < 0.007$).

When the animals were returned to 5 lx conditions for 2 and 5 weeks, the frequencies of TUNEL+ profiles in the ONL fell to control levels in both strains. TUNEL+ frequencies in the inner nuclear and ganglion cell layers showed no changes with ambient light (data not shown).

3.2. OSs shorten and lengthen as ambient light rises and falls

In animals raised in 5 lx, OS length was greater in SD than in P23H-3 rats, as reported previously (Walsh et al., 2004). Exposure to 300 lx for 1 week reduced OS length in both strains, and the reduction was markedly greater in the P23H-3 strain (compare Fig. 2A and C, and Fig. 3), confirming previous findings (Walsh et al., 2004). In addition, while SD OSs retained their organized structure after exposure to 300 lx (Fig. 2A), the OSs of the P23H-3 retina appeared damaged and disordered (Fig. 2C).

In P23H-3 animals returned to 5 lx for 2 and 5 weeks, substantial regrowth of OSs was apparent. After 2 weeks back in 5 lx, OSs were longer and appeared well organized (Fig. 2D). After 5 weeks back in 5 lx conditions (Fig. 2E), the OSs of the P23H-3 retina appeared mature and well organised, but still shorter than in the pre-exposure retina.

In SD rats exposed to the same pattern of ambient light, a similar trend was seen. Mean OSs was reduced after 1 week in 300 lx and increased towards control levels in animals returned to 5 lx conditions (Fig. 3). The changes were relatively small, however, only the difference between control and 1 week in 300 lx approached significance ($p = 0.093$) in our data. In the P23H-3 data, the reduction in OS length after 1 week in 300 lx was significant ($p < 0.003$), and the regrowth (1 week at 300 lx vs 2 weeks or 5 weeks re-exposure to 5 lx) were also significant ($p < 0.007$).

3.3. The a-wave of the ERG decreases and increases with ambient light

Exposure of the P23H-3 rat raised in 5 lx conditions to 300 lx cyclic light for 1 week reduced the a-wave markedly (Fig. 4A,B), confirming Walsh et al. (2004). Returning the animals to 5 lx conditions for 2–5 weeks was followed by an increase in the a-wave (Fig. 4C,D). When observations were made systematically in SD and P23H-3 animals, three trends emerged (Fig. 5). In animals raised in 5 lx conditions, the a-wave of the ERG was larger in the SD than in the P23H-3 strain, confirming previous reports (Walsh et al., 2004; Yu et al., 2004). Second, 300 lx-induced reduction was greater in the P23H-3 strain (amplitude fell to 33% of control in the P23H-3, as against 63% in the SD). Finally, the trend for the a-wave to decrease in 300 lx conditions, and to recover during

Fig. 2. Outer layers of P23H-3 retinas before and after they were exposed to 300 lx ambient light for 1 week, and returned to 5 lx conditions for 2 weeks and 5 weeks. The double-headed arrows show the length of the outer segments. o, outer nuclear layer; i, inner nuclear layer. (A) SD retina, after exposure to 300 lx for 1 week. (B) P23H-3 retina, reared in 5 lx conditions (control). (C) P23H-3 retina, reared in 5 lx, and exposed to 300 lx for 1 week. The OSs are severely shortened, and their membranes appear damaged and irregular. (D) P23H-3 retina, reared in 5 lx, exposed to 300 lx for 1 week, then returned to 5 lx conditions for 2 weeks. The OSs have re-grown quite robustly. (E) P23H-3 retina, reared in 5 lx, exposed to 300 lx for 1 week, then returned to 5 lx conditions for 5 weeks.

the 2–5 week period in 5 lx conditions was observed in all 5 SD and 9 P23H-3 animals examined (Fig. 5).

When the data were normalized to the amplitude at the end of the 1 week exposure to 300 lx (Fig. 6), it was apparent that the a-wave increased in the two strains by similar proportions. In the SD strain, the increase in a-wave amplitude (over the reduced level) was 40% at 2 weeks and 23% at 5 weeks ($p < 0.03$). In the P23H-3 strain, the increase was 27% at 2 weeks and 33% at 5 weeks ($p < 0.002$).

3.4. The thickness of the ONL decreases steadily, in low and high ambient light

In the young adults studied, the ONL was consistently thinner in the P23H-3 strain (Fig. 7). In both strains the thickness of the ONL decreased by a small but consistent amount, over the 6 weeks of the study. The declines did not reach statistical significance in our data. The slow thinning of the ONL with

age has been documented in both strains previously, over longer periods (Machida et al., 2000; Penn et al., 1992).

4. Discussion

4.1. Two causes of vision loss, two benefits of light restriction

The description by Walsh et al. (2004) of the severe effect of low levels of ambient light on P23H-3 photoreceptors had discouraging implications for the management of human retinal degenerations with similar causes. It suggested that the ability of such retinas to respond to light was reduced, not only by the underlying loss of photo receptors, but also by damage to surviving photoreceptors. Vision loss in these retinal degenerations thus had two causes, photoreceptor death and photoreceptor dysfunction.

The same finding raised the more hopeful possibility that the photoreceptor damage/dysfunction component of visual loss might be reversible, allowing improvement of vision in sufferers. The present results confirm this possibility. In the P23H-3 retina, light restriction was followed by regrowth of OSs and recovery of the a-wave, two key parameters of retinal performance. Since light restriction also slowed the underlying death of photoreceptors, the potential benefits of light restriction are dual.

4.2. Mechanisms

The underperformance of photoreceptors surviving in the P23H-3 retina may have several causes. Mutant rhodopsin may be less effective in triggering the phototransduction cascade or maintaining its function. A second likely cause is the severe reduction of OS length induced by ambient light; this shortening would be expected to reduce photoreceptor dark current and thereby the magnitude of the light-induced change in that current. A third possible cause is a reduction in ATP production by photoreceptor mitochondria, due to oxidative stress resulting from the rise in pO_2 in the outer retina which occurs in this strain (Yu et al., 2004). This would reduce

Fig. 3. Length of OSs in SD and P23H-3 strains, before and after exposure to 300 lx ambient conditions for 1 week. OSs were consistently longer in the SD strain. In both strains the lengths of OSs were reduced by exposure to 300 lx conditions, and re-grew when returned to 5 lx conditions. Error bars show ± 1 standard deviation.

Fig. 4. ERGs recorded from P23H-3 rats before and after exposure to 300 lx ambient light for 1 week. Each of (A)–(D) shows 9 responses to a series of flashes of increasing intensity, from 13.5 to 20×10^5 photoisomerisations/s. The amplitude of the response was greatly reduced by exposure to 300 lx conditions, and recovered over 2–5 weeks in 5 lx conditions.

the ATP available to enable the cell to maintain dark current (Demontis et al., 1997).

The present study provides evidence that light restriction allows significant functional recovery in P23H-3 retina. One mechanism for the recovery is the regulatory phenomenon called photostasis (Penn and Williams, 1986; Williams, 1998), the tendency of photoreceptors to match OS length and retinal rhodopsin content to ambient light, reducing both in bright ambient conditions, and increasing them in darker conditions. The light-restriction-induced regrowth of OSs noted here in both P23H-3 and SD strains could be understood as the operation of photostasis. A second possible mechanism lies in the effect of light on oxygen tension in the outer layers of the retina. A reduction in light levels increases the consumption of oxygen by photoreceptors, reducing outer retina pO_2 (Linsenmeier, 1986); this may counter the oxidative stress resulting from the chronically high levels of oxygen in the outer layers of the P23H-3 retina.

Fig. 5. Data showing a-wave amplitudes for 5 SD and 9 P23H-3 rats. Amplitudes for an individual animal are shown as a cluster of 4 bars. Each bar is the amplitude of the a-wave in the response to a 2×10^6 photoisomerisations/s flash. Each animal was examined 4 times, before 1 week exposure to 300 lx conditions, immediately after exposure and 2 weeks and 5 weeks after. Data are shown for SD (A) and P23H-3 (B) rats. Partial recovery of a-wave response was observed in all cases.

4.3. Discrepancy: a-wave recovery is less than OS regrowth

Over the 2–5 weeks during which we monitored recovery in dim light, the OSs of P23H-3 photoreceptors grew 5-fold from their length after 1 week of exposure to 300 lx conditions (Figs. 2 and 3). The a-wave of the ERG increased over the same period, but only by ~ 0.3 -fold (Figs. 4–6). Previous studies (Machida et al., 2000; Walsh et al., 2004), by contrast, reported an approximately linear relation between OS length and a-wave amplitude in the SD and P23H-3 retina. The present discrepancy is robust and unexplained, and requires further study. It suggests a potential for the a-wave of the P23H-3 retina to recover considerably more than observed here, if the right conditions can be established.

4.4. Is the capacity for reversal of visual loss specific or general?

The generality of the present results will require detailed testing. The P23H transgenic is not an exact model of human

Fig. 6. Normalised data for the 5 SD and 9 P23H-3 animals shown in Fig. 5. The amplitude of the a-wave in each case was normalized to its amplitude immediately after exposure to 300 lx conditions for 1 week. In the SD strain, a-wave amplitude recovered after 2 weeks in 5 lx conditions to 140% of its 300 lx-reduced value, and to 123% after 5 weeks recovery. In the P23H-3 strain, a-wave amplitude recovered to 127% and 133% over the same periods. For both strains, these rises were significant ($p < 0.03$). The error bars show ± 1 standard deviation. Group sizes were 5–9 animals.

rhodopsin-mutant retinal degenerations, for example, because its photoreceptors are producing both normal and mutant rhodopsin. Several lines of evidence suggest, however, that the finding will not be specific to this transgenic strain. Light-induced acceleration of photoreceptor death has been described in the RCS rat (Dowling and Sidman, 1962; Kaitz, 1976), in which the degeneration is caused by a mutation which does not involve rhodopsin (D'Cruz et al., 2000); and in a naturally occurring rhodopsin-mutant degeneration found in dogs (Cideciyan et al., 2005). Ambient light acceleration of retinal degeneration has also been suggested in humans, evident as sectoral degeneration (Heckenlively et al., 1991). In addition, light-induced degradation and light-restriction-induced recovery of the ERG are features of the normal retina, in the photostasis phenomenon (Penn and Williams, 1986).

Fig. 7. Thickness of the ONL, expressed as a ratio of the thickness of the ONL to the thickness of the retina, measured between the inner and outer limiting membranes. The ONL is consistently thicker in the SD rat. In both strains the ONL decreased modestly over the 6 weeks of observations. The error bars show ± 1 standard deviation. Group sizes were 5–9 animals.

The recovery of retinal responsiveness in reduced ambient light may be a feature therefore of normal retina; in which case it may be available in a wide range of retinal degenerations. Nevertheless, the value of light restriction for the degenerating retina will need to be tested cause by cause.

Some years ago (Stone et al., 1999) we proposed an 'oxygen toxicity hypothesis' to understand, and to provide a basis for exploring the late stage photoreceptor degenerations. We hypothesized that the depletion of photoreceptors by whatever cause would lead to a chronic rise of oxygen tension in the outer retina, and that this rise would be toxic to photoreceptors, closing a destructive cycle of photoreceptor death and rising oxygen levels in the outer retina (Fig. 8). The predicted rise in tissue oxygen levels in the outer retina has since been confirmed in rodent models of degeneration (Yu et al., 2004, 2000) and evidence that tissue oxygen levels are high in degenerating human retina can be seen in the thinning of retinal vessels which is characteristic of human retinal degenerations (Heckenlively, 1988), and in the protection which RP provides against hypoxic retinal disease (such as diabetic retinopathy) (Arden, 2001; Sternberg et al., 1984). Evidence for the second component of the hypothesis, that hyperoxia is toxic to photoreceptors, has been demonstrated directly in the rabbit (Noell, 1955), mouse and rat (Geller et al., 2005; Wellard et al., 2005; Yamada et al., 2001). Evidence of the mechanism of hyperoxia-induced photoreceptor death comes from studies of cell lines (Campian et al., 2004; Li et al., 2004; Scatena et al., 2004), the mitochondria of *Drosophila* (Walker and Benzer, 2004), and the retina (Shen et al., 2005), which show that hyperoxia damages cells, particularly their mitochondria (Pagano and Barazzzone-Argiroffo, 2003), by increasing the production of free radicals.

The present results suggest that an additional factor plays a role in this destructive cycle (Fig. 8). The stress which induces the death of some photoreceptors also downregulates function in the survivors. OSs are shortened and the a-wave reduced, suggesting a reduction in dark current. This reduction would reduce the activity of ATP-fuelled ion pumps in the inner segment, and therefore the photoreceptor's requirement for ATP. This in turn would reduce the production of ATP and the consumption of oxygen by mitochondria. This fall in consumption would tend to increase oxygen levels in the outer retina, and oxidative stress on surviving photoreceptors, again

Fig. 8. Scheme of the oxygen toxicity hypothesis, developed from Stone et al. (1999). Explanation in the text.

completing a destructive cycle (Fig. 8). Light restriction, by allowing the regrowth of outer segments, may reverse this second effect.

One prediction of this analysis is that the dual benefits of light restriction (slowing photoreceptor death, reversing damage) will be independent of the initial cause of photoreceptor depletion, and will not therefore be restricted to the human form of RP (Heckenlively et al., 1991) on which the P23H transgene was modelled. This would be an example of ‘mutation-independent’ therapy, as envisaged by Vaughan et al. (2003). This prediction remains to be tested; the outcome will be of clinical relevance.

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