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Photoreceptors in *whirler* mice show defective transducin translocation and are susceptible to short-term light/dark changes-induced degeneration

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Abstract

Usher syndrome combines congenital hearing loss and *retinitis pigmentosa* (RP). Mutations in the whirlin gene (*DFNB31/WHRN*) cause a subtype of Usher syndrome (USH2D). *Whirler* mice have a defective whirlin gene. They have inner ear defects but usually do not develop retinal degeneration. Here we report that, in *whirler* mouse photoreceptors, the light-activated rod transducin translocation is delayed and its activation threshold is shifted to a higher level. Rhodopsin mis-localization is observed in rod inner segments. Continuous moderate light exposure can induce significant rod photoreceptor degeneration. *Whirler* mice reared under a 1500 lux light/dark cycle also develop severe photoreceptor degeneration. Previously, we have reported that *shaker1* mice, a USH1B model, show moderate light-induced photoreceptor degeneration with delayed transducin translocation. Here, we further show that, in both *whirler* and *shaker1* mice, short-term moderate light/dark changes can induce rod degeneration as severe as that induced by continuous light exposure. The results from *shaker1* and *whirler* mice suggest that defective transducin translocation may be functionally related to light-induced degeneration, and these two symptoms may be caused by defects in Usher protein function in rods. Furthermore, these results indicate that both Usher syndrome mouse models possess a light-induced retinal phenotype and may share a closely related pathobiological mechanism.

1. Introduction

Usher syndrome is a clinically and genetically heterogeneous disease. It is the most common cause of combined sensorineural hearing impairment and *retinitis pigmentosa* (RP) (Smith et al., 1994). In some cases, vestibular dysfunction (Hallgren, 1959) and mental disturbances

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(Boughman et al., 1983) are also symptoms of the syndrome. Three major clinical types of Usher syndrome (type I, II, and III) can be distinguished based on the severity and progression of hearing loss and the age of onset of RP. Currently, ten different genes are known to be associated with the various subtypes of Usher syndrome (William, 2008; Kremer et al., 2006; Reiniers et al., 2006; Saihan et al., 2009; Riazuddin et al., 2012). However, even though there are reports about the ability of several of these Usher proteins to form complex through molecular interaction in photoreceptors (Maerker et al., 2008; Van Wijk et al., 2006; Yang et al., 2010), the disease mechanism of RP in Usher syndrome remains unknown.

Mutations in the *DFNB31/WHRN* gene, which encodes a protein called whirlin (Mburu et al., 2003), cause a subtype of Usher syndrome, type IID (USH2D) (Ebermann et al., 2007). In vertebrate retina, whirlin protein is expressed in the photoreceptor cells. In the photoreceptors, whirlin protein accumulates at cilium region and synaptic terminals (Kersten et al., 2010; Maerker et al., 2008; Van Wijk et al., 2006; Yang et al., 2010). *Whirler* mice have mutations in *DFNB31/WHRN* gene and are an accepted animal model for USH2D. *Whirler* mice have auditory dysfunction, and their cochlear hair cells have abnormally formed stereocilia (Holme et al., 2002). However, like several other naturally occurring Usher mouse models, *whirler* mice do not develop retinal degeneration (Mburu et al., 2003).

Previously, we have reported that the rod photoreceptors in *shaker1* mice, a well-accepted mouse model for USH1B, showed delayed rod transducin translocation with a shift of its light activation threshold to a significantly higher level (Peng et al., 2011). In rod photoreceptors, it has been suggested that the transducin translocation activated by a specific light threshold, may serve as a neuroprotective function for rods under conditions of high intensity light by reducing metabolic stress (Artemyev, 2008; Calvert et al., 2006; Kalra et al., 2007; Lobanova et al., 2007; Sokolov et al., 2002; Slepak and Hurley, 2008). Indeed, we have found that continuous exposure of *shaker1* mouse under even moderate intensity light could induce significant rod photoreceptor degeneration. Furthermore, when *shaker1* were reared under a moderate light (1500 lux/dark cycle), they develop severe retinal degeneration in less than 6 months (Peng et al., 2011). We have further observed that subretinal injection of wild type myosin VIIa could rescue both light-induced degeneration and delayed transducin translocation, indicating these symptoms are caused by defects in myosin VIIa (Zallocchi et al., 2011).

Here, we report that, similar to *shaker1* mice, the rod photoreceptors in *whirler* mice also show delayed transducin translocation with a shift of its light activation threshold to a significantly higher level and sensitivity to moderate light-induced photoreceptor degeneration. In addition, similar to previous reports for *shaker1* mice (Liu et al., 1999), *whirler* mice show immunostaining for rhodopsin in the inner segments, suggesting a possible rhodopsin mis-localization. Interestingly, we have found that, alternative short-term 1 hour moderate light exposure with 7 hours dark adaptation induces photoreceptor degeneration in both *shaker1* mice and *whirler* mice as severe as that induced by continuous light exposure. These light conditions do not affect strain/age matched wild type retinas. Our findings from these two mouse models indicate a clear connection between defective transducin translocation and light-induced degeneration. These results also show that,

similar to *shaker1* mice, *whirler* mice do indeed possess a robust retinal phenotype, which has likely been missed due to dim light conditions in most animal vivariums. More importantly, these results show that these two Usher syndrome mouse models, alluding to a closely related pathobiological mechanism.

2. Methods

2.1. Ethics Statement

All animal handling and procedures were performed in accordance with protocols for these studies that have been approved by the Boys Town National Research Hospital Institutional Animal Care and Use Committee (IACUC) and in accordance with NIH and USDA guidelines. Every effort was made to minimize discomfort and distress.

2.2. Animals

Pigmented *whirler* mice (*B6.Cg-Whrn^{wi}Tyrl^{b/+}/+*) were purchased from Jackson Laboratories (Bar Harbor, ME; Stock Number: 000571). These mice harbor a 526 base pair deletion abolishing the short C-terminal isoform and creating a frame shift that results in premature termination of the long isoform before the third PDZ domain (Mburu et al., 2003). Pigmented 129 Sv/J mice were used as wild type control mice. *Whirler* mice were back-crossed onto the pigmented 129 Sv/J background seven times for purposes of comparison with the wild type mice. In both *whirler* mice and control wild type mice the RPE65 transcript was amplified and sequenced and found to be of the L450 genotype (Danciger et al., 2000; Wenzel et al., 2001), therefore, both *whirler* and the wild type mice used in these studies harbor the L450 quantitative trait locus for RPE65, and their photoreceptors are thus inherently more sensitive to light induced damage than the mice with L450M in their RPE65, which are much less sensitive to light induced photoreceptor damage (Danciger et al., 2000; Redmond et al., 2007; Wenzel et al., 2001). Thus, under the same light conditions, the results may not be reproduced used in strains that are L450M for RPE65, which would have much higher resistance to light damage. The animals were kept in transparent cages under 12 hr. light/dark cycle at the Boys Town National Research Hospital (BTNRH) *vivarium*. Procedures for light/dark adaptation did not cause pain, discomfort, distress or morbidity. The animals were anesthetized with a mixture of ketamine 300 mg/kg and xylazine 30 mg/kg body weight, administered IP, prior to euthanizing by cervical dislocation to eliminate the potential for pain. Tissues were obtained after the animals were euthanized.

2.3. Antibodies

Antibodies against the following proteins were used: rhodopsin (Sigma, MO), centrin1 (Santa Cruz, CA), cytochrome C (Millipore, MA) and α subunit of rod transducin (CytoSignal, CA). Anti-R9AP antibody was a gift from Dr. V. Arshavsky, Duke University.

2.4. Light/Dark Adaptation

Light and dark adaptation, including continuous 6 day moderate light exposures and long term moderate light/dark cycle were performed exactly as described in an earlier paper where *shaker1* mice were analyzed (Peng et al., 2011). For alternative short-term light/dark

adaptation changes, the animals were first kept in transparent cages without any restraint in a lightproof darkroom for 8 hour dark adaptation, and then the animals were first exposed under 2000 lux light for 1 hour. Diffuse white fluorescent light were placed 4–6 inches above the cages and beside the cages on all four sides. Light intensity was measured inside the cage. After this one hour light exposure animals were kept in a darkroom for dark adaptation for 7 hours, and then the mice were exposed again to 2000 lux light for 1 hour. After this, the mice were dark adapted for 7 hours again. Such 2000 lux 1 hour-light exposure/7 hour-dark adaptation alternative changes were repeated for 2 weeks.

2.5. Immunocytochemistry

Methods used for immunohistochemical analysis have been described in detail in previous publications (Peng et al., 1997; Peng et al., 2000; Peng et al., 2003; Peng et al., 2011). Briefly, mouse eyes were removed from euthanized animals, and were fixed in 4% paraformaldehyde in 100 mM sodium phosphate buffer (PB, pH 7.3) at 4°C. The tissue was then transferred into 5% sucrose in PB at 4°C overnight and then 30% sucrose in PB at 4°C overnight. Retinal sections, cut with a Microm cryostat and mounted on gelatin-coated slides, were incubated first with 5% normal goat serum (Vector Laboratories) in PBS for 1 hour at room temperature, and then incubated with primary antibodies overnight at 4°C [anti-transducin $\alpha 1$ antibody 1:1000; anti-rhodopsin antibody 1:1,000; anti-R9AP 1:1000], followed by three washes in PBS. All incubation and wash buffers contained Triton X-100 (0.3%). The sections were then incubated with Alexa 594-conjugated anti-mouse immunoglobulin antibody or Alexa 488-conjugated anti-rabbit immunoglobulin antibody (Invitrogen, Eugene, OR) 1:250 for 2 hours. For double-immunostaining, retinal sections were incubated with mixed primary and secondary antibodies. The slides were then washed with PBS and coverslipped with 50% glycerol in PBS for viewing under a Zeiss AX10 microscope.

2.6. Serial tangential sectioning and western blotting

The method is modified based on that described by Sokolov et al. (Sokolov et al., 2002), and Martemyanov et al. (Martemyanov et al., 2003). After dark adaptation, mouse retina was isolated under various light conditions and then transferred into ice-cold Ringer's solution and immediately flat mounted on PVDF membranes, placed between two glass slides separated by 0.5 mm spacers and immediately frozen. For sectioning, the glass slides were separated, and the PVDF membrane with attached retina was mounted on the cryomicrotome specimen holder. Sequential 5 μ m tangential retinal sections were collected in 100 μ l of SDS-PAGE sample buffer. For dim light experiments, all the procedures before transferring the sections into sample buffer were performed under dim light. Aliquots for each sample were subjected to 12% of SDS-PAGE followed by Western Blotting. To standardize serial sectioning data from different experiments we ensured that all retina sample (round patch after trimming the curved edges) had a similar size around 2 mm; the orientation of the retina was guaranteed by the location of the standard protein marker: rhodopsin (rhodopsin must always only appear in the first 1–6 sections, if the retina was not properly oriented, rhodopsin would appear in other sections in the regions of inner segments, INL or OPL); rhodopsin must be at least in the first 4–5 sections to ensure the thickness of photoreceptor outer segment layer; each section had the same thickness and was collected in

the same amount of sample buffer; the protein concentration of each retina sample in different experiments was determined to ensure they are similar; for each lighting condition, at least 4 times of experiments that could fulfill the above requirements were repeated.

2.7

Fluorescence measurements, densitometry and co-localization indexes: Fluorescence intensity was estimated using imageJ and the following procedure: 1) Selection of region of interest (ROI); 2) calculation of Area (A) and Integrated Density (ID); 3) Selection of a background region and calculation of the Mean Grey Value (MGV); 4) Calculation of the Corrected Total Fluorescence (CTF).

$$CTF = ID - (A \times MGV)$$

Quantitative density analysis for western blotting was carried out using the Gel Analysis tool from Image J software.

Pearson's and Li's co-localization indexes were calculated using ImageJ as described in Zallocchi et al. 2012.

2.8. Counting total photoreceptor numbers

Quantification of photoreceptors was performed as described previously with no modifications (Peng et al., 2011).

2.9. Statistical analysis

The data presented in this work represent at least 4 replicates for all experiments described. Statistically analyzed data was subjected to the Student's t-test with Bonferroni correction.

3. Results

3.1. Photoreceptors in *whirler* mice show delayed rod transducin translocation, which may be due to a shift of its activation threshold

Light-induced transducin translocation in rod photoreceptors can be observed only when the exposure light intensity exceeds a threshold level (Sokolov et al., 2002). In wild type mice, after 10 min of 500 lux light exposure, a significant amount of transducin was translocated into the inner segments, cell bodies and the synaptic layer of rod photoreceptors (Figure 1A). In rod photoreceptors of *whirler* mice, light-induced transducin translocation was defective (Figure 1B–C). As shown in Figure 1B, in *whirler* mice, after 500 lux light exposure for 10 min, most of α -transducin remained in rod outer segments. There was little staining in rod inner segments (small arrows) and there was almost no staining of α -transducin in the synaptic terminals (OPL). However, when the light intensity was increased to 700 lux for 10 minutes, transducin in *whirler* photoreceptors was translocated to the inner segments (small arrows in Figure 1C) and synaptic terminals (large arrows), indicating the activation threshold in *whirler* mice was elevated to 700 lux compared to 200 lux for wild type retinas (Peng et al., 2011). When the light intensity was increased to 1500 lux for 1

hour, a significant amount of transducin in *whirler* photoreceptors was translocated to the synaptic layer (OPL, Figure 1D, large arrows). These results showed that transducin in *whirler* rods did move to the inner segments and synaptic terminals, suggesting that the translocation was not completely blocked though the activation threshold was elevated.

To further investigate the translocation of transducin in *whirler* mice, we have compared the fluorescence arbitrary units of transducin immune-staining in different layers of photoreceptors in wild type and *whirler* mice exposed under various light intensities and durations (Figure 1E–I). The results clearly showed that, in wild type mice, after dark adaptation, when the mice were exposed to 10 min of 200 lux light, transducin began to move into the inner segments, cell bodies and synaptic terminals (Figure 1F). In *whirler* mice, significant translocation of transducin into the inner segments, cell bodies and synaptic terminals was obvious only when the mice were exposed to 10 min of 700 lux light (Figure 1H). These results support the conclusions that transducin translocation in *whirler* mice is delayed and its activation threshold is shifted to 700 lux. This defective transducin translocation could be detected in *whirler* mice from 3-week-old to 15-month-old, indicating it is an age-independent inherent property of *whirler* photoreceptors.

3.2. Tangential serial section immunoblotting studies confirm defective translocation of α -transducin in *whirler* mice

To confirm the defective translocation of transducin in *whirler* mice, we used the most reliable quantitative method for detecting protein translocation in photoreceptors: tangential serial section immunoblotting (Sokolov et al., 2002). As shown in Figure 2, in wild type mice, after 10 min 500 lux light exposure, using rhodopsin and R9AP (Cao et al., 2010; He et al., 1998; Martemyanov et al., 2003) as the markers for outer segment (in the first 5–6 sections), centrin1 (Trojan et al., 2008) as a marker for connecting cilium and cytochrome C (Sokolov et al., 2002) as a marker for inner segments; transducin was clearly translocated into the inner segment region. However, in *whirler* mouse photoreceptors, under the same condition of 10 min 500 lux light exposure, the majority (>90%) of transducin remained located in the first 5–6 sections, mostly in the region of rod outer segment, indicating transducin translocation in *whirler* mice was delayed (using serial tangential section immunoblotting method, very similar pattern of transducin distribution during light/dark adaptation was found in four other *whirler* retinas). Interestingly, tangential section studies showed that, in *whirler* mice, rhodopsin's location, when compared with locations of R9AP, centrin1 and cytochrome C, was extended to section number 6–8, in the region of inner segment (compared to section 5 in wild type mice), suggesting there might be mislocalization of rhodopsin in *whirler* photoreceptors. To confirm this, we examined the localization of rhodopsin in *whirler* photoreceptors with immunocytochemistry. As shown in Figure 3, staining of rhodopsin in wild type mice was concentrated in the rod outer segments (Figure 3A). However, in *whirler* mice, rhodopsin staining was also found in the inner segments (Figure 3B, arrows), suggesting mislocalization of rhodopsin in mutant rod photoreceptors. This pattern of rhodopsin mislocalization is quite similar to that in *shaker1* mice (Figure 3C), which has been reported previously (Liu et al., 1999), suggesting a possible rhodopsin dislocation shared in the two Usher mouse models.

To confirm that the staining of rhodopsin in the inner segment in *whirler* mice is not due to an oblique sectioning, we performed double immunostaining using anti-R9AP and anti-rhodopsin antibodies on *whirler* mouse retinas. As shown in Figure 3D–F, in rod inner segments (**arrows**), there was rhodopsin staining that clearly did not co-localize with R9AP, verifying mislocalization of rhodopsin. Pearson's and Li's co-localization indexes for rhodopsin and R9AP were calculated on those areas of the inner segments (arrows). While Pearson's values were inconclusive (0.24 ± 0.05), Li's correlation index demonstrates segregation (-0.12 ± 0.02) between rhodopsin and R9AP distribution, which suggests mislocalization of rhodopsin in the inner segments.

3.3. *Whirler* mouse photoreceptors are susceptible to continuous moderate light exposure induced degeneration

In pigmented wild type mouse retina, moderate light exposure (< 2500 lux) will not cause significant rod degeneration (Hao et al., 2002; Wenzel et al., 2005). Previously, we have reported that in pigmented *shaker1* mice, continuous 6 day 2500 lux light exposure could induce significant rod degeneration (Peng et al., 2011). Here, we found that pigmented *whirler* mice were also much more susceptible than strain-matched pigmented wild type mice to continuous moderate light exposure-induced damage. As shown in Figure 4, under the same conditions, when compared with strain-matched pigment wild type mice (Figure 4A), after a continuous (6 days) moderate 2500 lux light exposure, the average number of photoreceptors in *whirler* mice (Figure 4B) was reduced by more than 25% (average of 8 *whirler* mice).

3.4. *Whirler* mice develop photoreceptor degeneration when they are reared under moderate-light/dark cycle

Similar to *shaker1* and other naturally occurring Usher mouse models, *whirler* mice did not develop retinal degeneration even at the age of 15-months when they were reared in regular animal facilities with day light intensity at the cage level that was dimmer than 200 lux for light/dark cycle (Figure 4C). However, when 1-month-old *whirler* mice were reared under condition of 2500 lux light (at the cage level) for 12 hours each day for 12/12 light/dark cycle, they developed significant photoreceptor degeneration in less than 6 months (the average rod number had reduced about 25% in 4 *whirler* mice). Under the same conditions, the strain/age-matched pigmented wild type mice did not develop statistically significant degeneration (Figure 4D).

3.5. Short-term light/dark changes-induced photoreceptor degeneration in both *shaker1* and *whirler* mice are as severe as that induced by continuous light exposure

Both *shaker1* and *whirler* mice show delayed rod transducin translocation and moderate light-induced photoreceptor degeneration. It is therefore possible that the light-induced photoreceptor degeneration is functionally related to delayed transducin translocation. Our results indicated that the most severe delay of transducin translocation in these two mouse models occurred during the first hour of light exposure after dark adaptation. When *shaker1* and *whirler* mice were exposed to light intensity higher than their elevated threshold for more than one hour, a significant amount of transducin was translocated out of the outer

segments. In these two mouse models, if the delay of transducin in the rod outer segments stresses their photoreceptors when they are exposed to moderate light, the most significant damage to their photoreceptors should occur during the first hour when they are exposed to light following dark adaptation.

Indeed, we found that when both *shaker1* and *whirler* mice were exposed to one hour 2000 lux light and then dark adapted for another seven hours in repeated cycles for 2 weeks, their photoreceptors suffer significant degeneration. Importantly, the degeneration was as severe as that induced by continuous 6 days 2500 lux light exposure. As shown in Figure 5A, repeated cycles of alternative short-term 1 hour moderate 2000 lux light exposure and 7 hour dark adaptation for 14 days could not induce detectable rod degeneration in pigmented age/strain-matched wild type mice. However, under the same conditions, the number of photoreceptors in both *shaker1* (Figure 5B) and *whirler* (Figure 5C) mice were reduced by more than 25% (average of 4 *shaker1* or *whirler* mice) indicating that repeated cycles of short-term 1 hour moderate light followed by 7 hours of dark adaptation (42 hours total of light exposure) could induce a photoreceptor degeneration in both *shaker1* and *whirler* mice comparable to that induced by 6 days (144 hours) continuous light exposure demonstrating a much more severe degenerative effect for the period of light exposure immediately following dark adaptation. These results are consistent with the notion that light-induced photoreceptor degeneration in these two Usher syndrome mouse models may be closely related to the retention of transducin in rod outer segments.

4. Discussion

Usher syndrome Type 2D (Maerker et al., 2008), which is caused by mutations in the gene *DFNB31/WHRN*, is characterized by congenital deafness and late onset progressive RP. *Whirler* mice, as a potential mouse model for USH2D, have mutations in the *DFNB31/WHRN* gene that result in a 526 base pair deletion which abolishes expression of the short carboxy-terminal isoform and truncates the long isoform before the third PDZ domain, resulting in congenital deafness (Ebermann et al., 2007; Mburu et al., 2003). However, *whirler* mice usually do not develop RP (Mburu et al., 2003). Recently, it has been reported that a mouse with a targeted disruption near the N-terminus of whirlin showed very late onset photoreceptor degeneration (Yang et al., 2010), confirming that mutations in the *DFNB31/WHRN* gene do cause some degree of retinal degeneration in mouse. Since mutations in *whirler* mice were found in the middle of the *DFNB31/WHRN* gene, it has been suggested that it may be possible that only mutations at the N-terminus cause retinal degeneration (Yang et al., 2010). Here, we show *whirler* mice actually are susceptible to moderate light-induced photoreceptor degeneration, indicating that *whirler* mice possess a robust retinal degeneration phenotype, but this phenotype is light exposure dependent. The fact that *whirler* mice express a truncated protein that lacks the third PDZ (PDZ3) domain suggests that this domain is important for light sensitivity to photoreceptor degeneration. PDZ domains are known for their role in protein-protein interactions (Kersten et al., 2010; Van Wijk et al., 2006) and in the case of whirlin's PDZ3 it may be key for the formation of a complex that regulates light-mediated processes such as α -transducin translocation as demonstrated in this work. because the fact that the light threshold of α -transducin translocation is increased but not obliterated in the *whirler* mice suggests that whirlin may

act as a facilitator or a regulator of the process. The presence of rhodopsin at the connecting cilia in whirler and shaker mice might reflect a defect in the regulation of protein trafficking to the apical microdomain of rod photoreceptors, a defect mechanistically dissimilar to rhodopsin mutants, such as Q344ter, which results in mislocalization throughout the entire cell (Concepcion and Chen, 2010).

Previously, we have reported that *shaker1* mice, another naturally occurring Usher mouse model, showed moderate light-induced photoreceptor degeneration (Peng et al., 2011). *Shaker1* mice have mutations in *Myo7a* gene, which encodes the actin motor protein, myosin VIIa. Mutations in the *Myo7a* gene cause another subtype of Usher syndrome, type 1B (USH1B) (Weil et al., 1995), which is characterized by severe congenital deafness and early onset/progressive RP. *Shaker1* mice are congenitally deaf and have vestibular areflexia. They are widely accepted as a mouse model for USH1B (Gibson et al., 1995; Self et al., 1998; Libby and Steel, 2001). Interestingly, similar to *whirler* mice, they do not develop RP. However, we have reported that, when *shaker1* mice were exposed to continuous moderate light (2500 lux) or reared under moderate light (1500 lux)/dark cycle for 6 months, they developed significant photoreceptor degeneration (Peng et al., 2011). Our results present here indicate that *whirler* mice, similar to *shaker1* mice, also possess a light exposure dependent retinal degeneration phenotype.

Why both *shaker1* and *whirler* mouse photoreceptors are more susceptible to light induced degeneration is unclear. In *shaker1* mice, sub-retinal injection of lentiviral vectors expressing wild type myosin VIIa could restore the transducin translocation phenotype in the *shaker1* mice and protected the photoreceptors from light-induced damage (Zallocchi et al., 2011), strongly suggesting that delayed transducin translocation and light-induced degeneration are directly related. Here we show that *whirler* mice have a similar delay in rod transducin translocation and light-induced degeneration, providing further support for the notion that the threshold shift in α -transducin translocation and photoreceptor degeneration are functionally closely related.

Light-activated translocation of transducin from the outer segments to the inner segments may reflect a critical neuroprotective function of rods that prevents damage from high intensity light and reduces the metabolic stress in rods under conditions of bright illumination (Calvert et al., 2006; Kalra et al., 2007; Lobanova et al., 2007; Slepak and Hurley, 2008). There have been reported that suggested defects in protein translocation could increase the susceptibility of light-induced photoreceptor degeneration (Chen et al., 1999; Kong et al., 2006; Peng et al., 2008) and prolonged activation of the phototransduction cascade has been associated with photoreceptor degeneration (Brill et al., 2007; Chen et al., 1999; Xu et al., 1997; Lem and Fain et al., 2004). Shifting the threshold of transducin translocation to a higher level, thus, may increase the metabolic stress in rods under conditions of bright illumination; and make these rods vulnerable to light-induced degeneration even under moderate light intensities. The activation threshold of both *shaker1* and *whirler* mice has been shifted to 700 lux. Transducin in their rods will be translocated only when the light intensity is higher than 700 lux. When these mice were exposed to light between 200 lux-700 lux, transducin was not translocated out of the outer segments. This

would increase the accumulation of oxygen free radicals in their photoreceptors with elevated metabolic stress.

In *shaker1* and *whirler* mice, after dark adaptation, due to the delayed transducin translocation, the first hour of light exposure over 200 lux is the period of most significant retention of α -transducin in their rod outer segments, which would result in hyperactivation of the phototransduction pathway causing oxidative damage. To further investigate whether light-induced degeneration in *shaker1* and *whirler* mice is closely related to the retention of transducin in the rod outer segments, we performed a short-term light/dark adaptation changes on these two mouse models. The duration of light exposure in this short-term light/dark adaptation experiments (42 hours) was much less than that in 6 days continuous light exposure (144 hours). However, our results indicated that 14 days of repeated cycle of one hour moderate light exposure after sufficient dark adaptation (7 hours) could induce photoreceptor degeneration as severe as that induced by 6 days continuous light exposure. The cyclic exposure represents 42 hours of light exposure, while the non-cyclic exposure represents 144 hours of light exposure. Thus by dark adapting between 1 hour light exposures, the retinal degeneration per unit light exposure was 3–4 times greater than that for continuous light. This comparative observation is consistent with the notion that retention of transducin in the outer segments plays an important role in light-induced degeneration.

Our results from *shaker1* (Peng et al., 2011) and *whirler* mice show that these two Usher syndrome mouse models have a very similar retinal phenotype. These two mouse models have mutations in two completely different genes: *Myo7a* and *DFNB31/WHRN*. These two genes encode two different proteins: myosin VIIa and whirlin. However, the loss of function of either of these two different proteins results in very similar pathobiological phenotypes in mice. Investigating the relation between these two proteins, therefore, will provide important clues for understanding the disease mechanism of delayed transducin translocation and light-induced degeneration, which is likely related to the progressive RP phenotype of Usher syndrome. Whirlin has been reported to interact with myosin VIIa *in vitro* (William, 2008; Delprat et al., 2005). They are both localized to the photoreceptor periciliary region. Whirlin, usherin, and G protein-coupled receptor 98 (GRP98) (all three USH2 proteins) have been known to bind to each other *in vitro* (Adato et al., 2005; van Wijk et al., 2006; Yang et al., 2010). They have been co-localized at the periciliary region in photoreceptors and may form a complex there (van Wijk et al., 2006; Wang et al., 2012; Yang et al., 2010). Whirlin is also capable of interacting with SANS, the protein encoded by the USH1G gene (Maeker et al., 2008). Therefore, it will be of interest to determine whether all these Usher syndrome subtypes share a common functional pathway in photoreceptor biology. Their interactions predict that the similar symptoms of delayed transducin translocation and light-induced degeneration could also be observed in mouse models for these Usher syndrome subtypes.

Preliminary results of gene therapy in *shaker1* mice indicate that both delayed transducin translocation and light-induced degeneration are caused by lack of myosin VIIa function (Zallocchi et al., 2011). In vertebrate retina, myosin VIIa is found in both RPE and photoreceptors. It is, therefore, difficult to determine which cell type is the major site

underlying these phenotypes in *shaker1* mice. Our results in *whirler* mice provide evidence that these two phenotypes likely result from defects in photoreceptors and may not be related to RPE function, because whirlin, the protein encoded by *DFNB31/WHRN*, has not been reported to be expressed in RPE. Based on this, we believe these two phenotypes are more likely a result of malfunction of myosin VIIa or whirlin in photoreceptors. Furthermore, both *shaker1* mice and *whirler* mice show mis-localization of rhodopsin. This observation combined with the defect in α -transducin translocation suggests that these two Usher syndrome-related proteins may in fact function in regulated protein transport between the inner and outer segments of photoreceptors. However, mislocalization of rhodopsin was observed in various degenerating photoreceptors. The mislocalization of rhodopsin observed in *shaker1* and *whirler* mice may be caused by degenerating photoreceptors. This is yet to be determined. Since mislocalization of rhodopsin was observed in one-month-old *shaker1* and *whirler* mice reared under dim light in *vivarium*, conditions that do not result in any measurable photoreceptor cell degeneration in these two models, we feel that mislocalization of rhodopsin in these two models is less likely a result of degenerating photoreceptors.

Finally, both mouse models for Usher syndrome display a common phenotype of moderate light-induced photoreceptor degeneration, suggesting exposure to higher intensity of light may play an important role in the development of RP in Usher syndrome mouse models.

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Whirler mouse photoreceptors show delayed rod transducin translocation.

Its activation threshold is shifted to a higher level.

Rhodopsin mis-localization is observed in rod inner segments.

Moderate light exposure can induce significant rod degeneration.

Short-term light/dark changes can induce rod degeneration.

Figure 1. Activation threshold for rod α -transducin translocation in *whirler* photoreceptors has been shift to 700 lux

Immunostaining of α -transducin on wild type (A) and *whirler* (WHR) (B, C and D) retinas after light adaptation for 10 min of 500 lux (A and B), 10 min of 700 lux (C), and 1 hour for 1500 lux (D). Small arrows indicate labeling of transducin in rod inner segments. Large arrows indicate labeling of transducin in the rod synaptic terminals. Arrow heads indicate labeled blood vessels. Scale bar=25 μ m. Fluorescence intensity of α transducin immunostaining in wild type (WT) and *whirler* (WHR) photoreceptors after dark adaptation (E) or exposure to different light intensities and durations (F-I). n=8 independent animals RPE=Retinal Pigment Epithelium; PRL=Photoreceptor Layer, OS=Outer Segments; IS=Inner Segments; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; DA=Dark Adaptation, AU=Arbitrary Units.

Figure 2. Tangential Serial Section Immunoblotting quantitative analysis of α -transducin and rhodopsin in wild type and *whirler* mouse photoreceptors

Upper panel: Western blots of tangential serial sections of a wild type mouse retina (left panel) and a *whirler* mouse retina (right panel) after being dark adapted for 8 hours and then light (500 lux) adapted for 10 min. The sections were cut starting from the top of the outer segments, and were immunolabeled by specific antibodies against rhodopsin (Rho) and transducin (Transd). R9AP was used as a marker for the location of rod outer segments. Centrin1 (Cen) was used as a marker for the connecting cilium. Cytochrome C (Cyt) was used as a marker for the locations of the rod inner segments. **Lower panel:** Densitometry profiles of the Western blots from the above lanes in which the densities of individual bands were expressed as a percentage of the total density of all bands representing each individual protein on the blot.

Figure 3. Mislocalization of rhodopsin in *whirler* photoreceptors

A–C: Immunostaining of rhodopsin (red) on wild type (A), *whirler* (WHR) (B) and *shaker1* (C) mouse retinas. **D–F:** Double labeling of anti-R9AP (green) and anti-rhodopsin (Rho) (red) on a *whirler* mouse retina (focusing on the photoreceptor layer). Arrows indicate staining of rhodopsin in the inner segments. RPE=Retinal Pigment Epithelium; PRL=Photoreceptor Layer; OS=Outer Segments; IS=Inner Segments; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer. Scale bar=25 μm.

Figure 4. A–C: Continuous light exposure induces more rod degeneration in *whirler* mice
A and B: Light micrographs of central retinal sections of a 3-month-old control wild type mouse (**A**) and a 3-month-old *whirler* mouse (**B**) after 6 days continuous 2,500 lux light exposure. RPE=Retinal Pigment Epithelium; PRL=Photoreceptor Layer; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; OS=Outer Segments; IS=Inner Segments. Scale bar=25 μ m. **C.** Average densities (n=8) of photoreceptors in the ONL of central retinal cross sections of 3-month-old wild type (WT) and 3-month-old *whirler* mice after 6 days continuous 200 and 2500 lux light exposure (LA). *Statistically significant differences between wild typeL and *whirler*L mice ($P < 0.05$). **D:** Kinetics of rod loss as a function of age in wild type and *whirler* mouse retinas reared under regular *vivarium* room light (< 200 lux at the cage level, WT, *whirler*) compared with that reared under 1500 lux light/dark cycle (WTL, *whirler*L). Data points represent quantitative measures of rod numbers (average of 4 mice) in the central parts of the retinas. *Statistically significant differences between wild typeL and *whirler*L mice ($P < 0.05$).

Figure 5. Short-term light/dark changes (1hour 2000lux light exposure and then 7 hours dark adaptation) induces more rod degeneration in *shaker1* and *whirler* mice. A, B and C: Light micrographs of central retinal sections of a 3-month-old control wild type mouse (A), a 3-month-old *shaker1* mouse (B) and a 3-month-old *whirler* mouse (C) after 14 days alternative 1 hour light (2000 lux) exposure and then 7 hours dark adaptation. RPE=Retinal Pigment Epithelium; ONL=Outer Nuclear Layer; OPL=Outer Plexiform Layer; OS=Outer Segments; IS=Inner Segments Scale bar=25 μ m. **D.** Average densities (n=8) of photoreceptors in the ONL of central retinal cross sections of 3-month-old wild type (WT), 3-month-old *shaker1* and 3-month-old *whirler* mice after 14 days alternative light/dark adaptation. *Statistically significant differences between wild type (WT) and *shaker1* and *whirler* mice ($P < 0.05$).