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SLOWED PHOTORESPONSE RECOVERY AND AGE-RELATED DEGENERATION IN CONES LACKING G PROTEIN-COUPLED RECEPTOR KINASE 1

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1. INTRODUCTION

The vertebrate retina is a specialized neural network that contains very sensitive signal transducers—the rod and cone photoreceptors. Rods function in near darkness (scotopic) and are responsible for vision in dim light, while cones operate in bright light (photopic) and provide high acuity color vision. In the human retina, rods are the dominant photoreceptor cell type and comprise about 95% of all photoreceptor cells, while cones comprise only 5%. Yet in a bright light environment, normal cone function is essential for visual perception since rods become saturated and rendered nonfunctional.

Retinitis pigmentosa (RP) and Age-related macular degeneration (AMD) are two major causes of visual loss due to photoreceptor degeneration. In RP, rod degeneration results in night blindness and loss of peripheral vision. Inevitably, cones are also lost following the disappearance of rods through unknown mechanisms, resulting in blindness.¹ AMD is the leading cause of visual impairment and legal blindness in elderly people in the Western world. In AMD, central vision is compromised initially, and although rod cell death occurs prior to cone loss, it is the subsequent cone cell death that eventually leads to complete visual loss.^{3–5} During the last decade, significant advances have been accomplished in understanding the mechanisms leading to rod cell death; however, those underlying cone cell loss are still poorly delineated. This is due to the paucity of cones in the mammalian retina that makes the study of normal cone function and disease-related processes difficult.

The neural retinal leucine zipper (*Nrl*) knockout (KO, ^{-/-}) mouse has a pure-cone retina due to a cell fate switch from rod to S cone during retinal development.⁶ Electroretinography (ERG) analysis of *Nrl*^{-/-} mice reveals that the amplitude of light-adapted ERG responses elicited by maximum stimulus did not change significantly up to 31 weeks of age, suggesting the cones in these mouse retinas survive without rod function.⁶ In this study, we describe an age-dependent retinal degeneration in the pure-cone *Nrl*^{-/-} mice lacking G protein-coupled receptor kinase 1 (GRK1). The *Nrl*^{-/-} *Grk1*^{-/-} mice provide a useful model for studying the molecular, cellular and biochemical pathways of cone cell death in AMD and other retinal diseases.

2. METHODS

2.1. Animals

Nrl^{-/-}*Grk1*^{-/-} mice were generated by crossing the *Nrl*^{-/-} mice⁶ with the *Grk1*^{-/-} mice⁷ as described previously.⁸ The *Nrl*^{-/-} were generously provided by Anand Swaroop and Alan Mears (University of Michigan), and the *Grk1*^{-/-} mice were provided by Jason Chen (University of Utah). Both the *Nrl*^{-/-} and the *Nrl*^{-/-}*Grk1*^{-/-} mice were born and maintained in total darkness. All animals were treated according to the guidelines established by the Institute for Laboratory Animal Research.

2.2. Immunoblot Analysis

Three mice from each genotype and each age group were killed at mid-day under room light. From each animal, retina was dissected from one eye and the other eye was prepared for histological and immunohistochemistry analysis (data not shown). The retinas were flash frozen on dry ice and kept at -80°C until use. Frozen retinas were homogenized and an equal amount of proteins from each retina was resolved on replicate 11.5% SDS-PAGE and transferred to PVDF membranes (Millipore Corp., Bedford, MA). The blots were incubated with rabbit polyclonal antibodies against mouse cone arrestin (mCAR/LUMIj),⁹ S or M opsin⁸ followed by a horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody and visualized by an Enhanced Chemiluminescence (ECL) Kit (Amersham, Arlington Heights, IL).¹⁰

2.3. Electrophoretography (ERG)

ERGs were recorded as previously described.¹¹ Mice were dark-adapted overnight, and their eyes were dilated with topical administration of phenylephrine (2.5%) and tropicamide (0.5%). Mice were anesthetized via an intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine HCl (10 mg/kg), and the cornea anesthetized with 0.5% topical tetracaine. The mouse was placed in an aluminum foil-lined Faraday cage and a DLT fiber electrode placed on the right cornea. A platinum reference electrode was placed on the lower eyelid and another ground electrode on the ipsilateral ear. Photoc stimuli of 10-μs duration were delivered through one arm of a coaxial cable using a Grass PS22 xenon flash. The cable delivered the flash 5 mm from the surface of the cornea, and flashes were attenuated with neutral density filters held to a window of fixed f-stop. Dark-adapted maximum responses (mesopic ERG) were measured using the non-attenuated light stimulus (10⁰). Photopic ERGs were measured using the same stimulus with a 6-foot candle (fc) white background light delivered through the other arm of the coaxial cable. The half-amplitude bandwidth of the system was 0.01 – 100 Hz. Responses were recorded on a Coopervision/Nicolet Electrovisual Diagnostic System, and amplified potentials were displayed on a storage oscilloscope for viewing and photographic recording.

3. RESULTS

3.1. Delayed photoresponse recovery in *Nrl*^{-/-}*Grk1*^{-/-} mice

GRK1 is responsible for light-dependent phosphorylation of both S and M cone opsins in the mouse retina.⁸ To evaluate the physiological effect of the GRK1 deletion on phototransduction in cones, we recorded ERGs from *Nrl*^{-/-} and *Nrl*^{-/-}*Grk1*^{-/-} mice.

As shown in Figure 1A, both *Nrl*^{-/-} and *Nrl*^{-/-}*Grk1*^{-/-} mice have no ERG response to low intensity light stimulation, which caused a strong rod response in wildtype (WT) mouse, consistent with the pure-cone phenotype of the *Nrl*^{-/-} mouse.⁶ Strong ERG responses were elicited in both *Nrl*^{-/-} and *Nrl*^{-/-}*Grk1*^{-/-} mice with high intensity light (Figure 1B). Interestingly, the amplitude of the b-wave is reduced by approximately 50% with 6-fc white background light (6-fc bgd) in the *Nrl*^{-/-}*Grk1*^{-/-} mouse, in contrast to that of the *Nrl*^{-/-} mouse

whose b-wave amplitude is not significantly affected by the background light (Figure 1B & C).

We also investigated the time course of recovery of dark-adapted ERGs in *Nrl*^{-/-} and *Nrl*^{-/-}*Grk1*^{-/-} mice. Paired-flash ERG responses of dark-adapted mice were recorded using high intensity (10⁰) white flashes at inter-stimulus intervals (ISIs) specified in seconds (s) to the left of the traces (Figure 2). Complete recovery of the ERG responses in the *Nrl*^{-/-} mice was achieved in about 0.625 sec, but for the *Nrl*^{-/-}*Grk1*^{-/-} mice, the ERG responses were not totally recovered even after 5 sec, suggesting a delayed recovery of cone responses in *Nrl*^{-/-}*Grk1*^{-/-} mice.

3.2. Age-dependent cone degeneration in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina

Morphological studies show that the outer nuclear layer (photoreceptor layer) of the *Nrl*^{-/-}*Grk1*^{-/-} mice is significantly thinner than that of the age-matched *Nrl*^{-/-} mice, and is progressively thinner with advancing age, suggesting an age-dependent photoreceptor degeneration in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina (X. Zhu *et al.*, in preparation). ERG analyses of retinal function of mice reared in total darkness reveal that the b-wave amplitude of mesopic ERGs decreases rapidly with increasing age in the *Nrl*^{-/-}*Grk1*^{-/-} mice, while that of the *Nrl*^{-/-} mice remains unchanged until 9 months of age (Figure 3). Animals reared in 12:12 hr cyclic light and bright constant light have similar ERG b-wave amplitude to those of the same genotype raised in total darkness (X. Zhu *et al.*, in preparation), suggesting that the functional decrease and degeneration of the *Nrl*^{-/-}*Grk1*^{-/-} photoreceptors are dependent on age but independent of light under these conditions, in contrast to the light-induced rod degeneration in the *Grk1*^{-/-} mouse retina.⁷

3.3. Both S and M cones die with age in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina

To determine if both S and M cones degenerate in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina, we determined protein expression levels by immunoblot analyses for S and M opsins, as well as mCAR, which is expressed in all cones.^{8, 9} The expression levels of all three photoreceptor-specific proteins decrease dramatically with age in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina but do not change significantly in the *Nrl*^{-/-} mouse retina up to 11 months of age (Figure 4). These results suggest that both S and M cones degenerate with age in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina.

4. DISCUSSION

Morphological and biochemical analysis of the *Nrl*^{-/-} retina suggest a complete lack of rods and increased number of S cones.⁶ Single cell recordings reveal that the *Nrl*^{-/-} photoreceptors have similar responses to the cones of the rhodopsin^{-/-} mice,^{12, 13} which are used as a cone animal model for studying cone function.¹⁴ Therefore, the photoreceptors of the *Nrl*^{-/-} mouse retina are functionally and biochemically cones.

In biochemical experiments utilizing these pure-cone retinas, both S and M opsins are phosphorylated following light exposure and cone arrestin preferentially binds to light-activated, phosphorylated cone opsins, suggesting that cones in the *Nrl*^{-/-} mouse retina may have a similar signal shutoff pathway to that of the WT rods.⁸

GRK1 is expressed in all rods and cones in the rod-dominant human, monkey and mouse^{8, 15–17} and in cone-dominant chicken retina.¹⁸ GRK1 phosphorylation of light-activated rhodopsin is required for normal inactivation of rhodopsin *in vivo*.^{7, 19} Although a cone-specific GRK (GRK7) exists in other species,^{16, 20–22} GRK1 is the only opsin kinase found in the mouse retina^{16, 22, 23} and is responsible for light-dependent phosphorylation of both S and M cone opsins.⁸ ERG analysis of cone photoresponses of the *Grk1*^{-/-} mouse reveals that

GRK1 plays a critical role in the inactivation of murine cone phototransduction.¹⁷ In the studies presented here, we demonstrate that when the *Grk1* gene is simultaneously knocked out in the *Nrl*^{-/-} mice, the pure-cone retinas of the *Nrl*^{-/-}*Grk1*^{-/-} animals have delayed photoresponse recovery. Both their retinal function and photoreceptor-specific protein expression levels decrease dramatically with age, indicating that lack of GRK1 function in cones leads to cone cell degeneration, in addition to the delayed photoresponse recovery reported previously.

In another study using single cell recordings of the *Nrl*^{-/-}*Grk1*^{-/-} photoreceptors, Pugh and collaborators have confirmed the critical role of GRK1 in cone phototransduction shutoff and have shown that the shutoff of M opsin is more dramatically delayed than that of S opsin in the *Nrl*^{-/-}*Grk1*^{-/-} retina.²⁴ These results suggest a potential alternative shutoff pathway for S opsin in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina. Our biochemical experiments show that both S and M opsins decrease with age, but S opsin decrease appears to be slower than that of M opsin in the *Nrl*^{-/-}*Grk1*^{-/-} retina (Figure 4B). Further immunohistochemistry studies are needed to accurately quantitate if M cones die earlier or more rapidly than S cones in the *Nrl*^{-/-}*Grk1*^{-/-} retina. Because the cone cell degeneration in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina is light-independent, we postulate that GRK1 may play other roles that are related to regulation of cellular proliferation and/or apoptosis in cone photoreceptors. Experiments are underway to define the molecular mechanisms leading to cone cell death in the *Nrl*^{-/-}*Grk1*^{-/-} mouse retina. These mice provide an excellent model for studying the molecular pathways of cone photoreceptor degeneration and for testing preventive and therapeutic strategies for rescuing cone function, thus preserving vision, in various retinal degenerative diseases.

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Figure 1. Electrophysiology. A. Dark-adapted ERG responses with low stimulus intensity (10⁻³) from WT, *Nrl*^{-/-} and *Nrl*^{-/-} *Grk1*^{-/-} mice. B. Dark- and light-adapted ERGs with high stimulus intensity (10⁰) from *Nrl*^{-/-} and *Nrl*^{-/-} *Grk1*^{-/-} mice. C. Graph representation of the b-wave amplitude from ERG recordings of *Nrl*^{-/-} and *Nrl*^{-/-} *Grk1*^{-/-} mice. The data represent mean ± Standard Deviation (SD) of six mice of each genotype.

Figure 2.

Recovery of ERG responses after a conditioning flash in *Nrl*^{-/-} and *Nrl*^{-/-} *Grk1*^{-/-} mice. A high intensity white flash was delivered after a conditioning flash at the same intensity at inter-stimulus intervals (ISIs) specified in seconds (s) to the left of the traces. Responses obtained without the preceding conditioning flashes are marked as controls.

Figure 3. Maximum b-wave amplitude of dark-adapted ERG responses in *Nrl*^{-/-} and *Nrl*^{-/-}*Grk1*^{-/-} mice.

Figure 4.

Immunoblot analysis of cone arrestin, S and M Opsin. *Nrl*^{-/-} (A) and *Nrl*^{-/-}*Grkl*^{-/-} mice (B) reared in total darkness were killed, and one retina was dissected and frozen. Frozen retinas were homogenized and equal amount of proteins were applied to a SDS-PAGE. In each panel, a representative immunoblot and a histogram representing quantitative data (Mean $\pm$ SEM) from 3 immunoblots are shown per age group.