Impairment of Vision in a Mouse Model of Usher Syndrome Type III

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PURPOSE. The purpose of this study was to obtain an Usher syndrome type III mouse model with retinal phenotype.

METHODS. Speed congenic method was used to obtain Clrn1 exon 1 knockout (Clrn1-/-) and Clrn1 N48K knockin (Clrn1 N48K/N48K) mice under A/J background. To study the retinal functions of these mice, we measured scotopic and photopic ERG responses. To observe if there are any structural abnormalities, we conducted light and transmission electron microscopy of fixed retinal specimens.

RESULTS. In 3-month-old Clrn1-/- mice, scotopic b-wave amplitude was reduced by more than 25% at the light intensities from -2.2 to 0.38 log cd/s/m², but scotopic a-wave amplitudes were comparable to those of age-matched wild type mice at all the light intensities tested. In 9-month-old Clrn1-/- mice, scotopic b-wave amplitudes were further reduced by more than 35%, and scotopic a-wave amplitude also showed a small decline as compared with wild type mice. Photopic ERG responses were comparable between Clrn1-/- and wild type mice. Those electrophysiological defects were not associated with a loss of rods. In Clrn1 N48K/N48K mice, both a- and b-wave amplitudes were not discernable from those of wild type mice aged up to 10 months.

CONCLUSIONS. Mutations that are Clrn1-/- biallelic cause visual defects when placed under A/J background. The absence of apparent rod degeneration suggests that the observed phenotype is due to functional defects, and not due to loss of rods. Biallelic Clrn1 N48K/N48K mutations did not cause discernible visual defects, suggesting that Clrn1-/- allele is more severely dysfunctional than Clrn1 N48K allele.

Usher syndrome type III (USHIII) is characterized by progressive loss of hearing and vision as well as by variable degrees of balance disorder.1,2 Onsets of both hearing and vision loss are highly variable. For example, vision in the peripheral field is lost in the first 2 to 3 decades of life, and thereafter central vision can last for 1 or 2 more decades.2-4 Clarin-1 (Clrn1) is currently the only gene associated with USHIII.5,6 The gene is predicted to encode a four transmembrane domains protein (CLRN1) with unknown function. Proteomics analysis of CLRN1-interacting proteins suggests that CLRN1 is associated with the regulation of actin homeostasis.7 Consistently, studies of CLRN1-deficient mouse7-9 and zebrafish10 indicate structural and functional defects in the stereocilia: actin-filled membrane structures responsible for the mechanosensation of auditory hair cells. Furthermore, recent study using CLRN1-deficient zebrafish suggests that CLRN1 is also potentially involved in synaptic transmission by hair cells.10 Currently, the role of CLRN1 in the retina is unknown, largely because there are no model animals that recapitulate the visual defects of USHIII.

Severities of Mendelian diseases, including Usher syndrome (USH), are affected by other genes collectively called modifiers.11-14 Accordingly, interpretations of phenotypic outcomes tend to be complicated by the genetic interactions between USH causative genes and their modifiers. Such genetic interaction is potentially one of the causes for variable onsets of hearing or vision loss observed in human USH patients.1,2,15,16 In mouse, studies of different genetic backgrounds were instructive to understand the effect of modifiers on the age-dependent deterioration of vision. For example, effects of different genetic backgrounds were clearly illustrated by the study of retinitis pigmentosa 1 homolog (RP1h) mutant allele, RP1h N48K Eap.17 When RP1h N48K allele was placed in C57BL/6j or DBA1/J backgrounds, it did not induce apparent retinal degeneration phenotype. When placed in the A/J background, however, the RP1h N48K allele induced severe age-dependent retinal degeneration. Unlike other genetic backgrounds, the A/J background predisposes mice to age-related retinal degeneration. Unlike other genetic backgrounds, the A/J background predisposes mice to age-related retinal degeneration.18,19 Which is a hallmark of many inherited blinding disorders.20 In human, age-related deterioration of vision is a natural process,21 and may affect the visual performance of USH patients. Thus, the A/J mouse is potentially a useful tool to exacerbate age-related retinal phenotypes that are observed in human USH patients but difficult to detect in most murine USH models.16

In the past, we and others generated and characterized murine models of USHIII8,9,22 There are two major mutations,15,6,23 causative to USHIII: Y176X mutation, which is commonly observed in Finnish USHIII patients and causes a premature termination of the translation, and N48K mutation, which is commonly observed in USHIII patients of Ashkenazi Jewish population and causes defects in the N-linked glycosylation of CLRN1. Mutation Y176X is considered functionally null, and thus the Clrn1 knockout would recapitulate this
genetic condition. Mutation N48K may be a hypomorphic allele as the progression of hearing impairment is slower in Clrn1N48K knockin mouse than in Clrn1 knockout mouse. While these models demonstrate hearing impairments, they did not exhibit visual phenotype. Thus, it has been difficult to quantify and compare the effects of different Clrn1 mutations to the retina. In this study, we backcrossed Clrn1 exon 1 knockout (Clrn1+/0) and N48K knockin (Clrn1N48K/C0) alleles to A/J strain, and obtained Clrn1-deficient and mutant mice with more than 96% of the genes derived from A/J background. These newly generated USHIII models allowed us to investigate the role of CLRN1 in the mouse retina and to compare these newly generated USHIII models to obtain more than 96% of the genes derived from A/J background.

Animals

All animal experiments employed procedures approved by Case Western Reserve University Animal Care Committee and conformed to both the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Associate of Research for Vision and Ophthalmology. Mice were maintained in a 12-hour light/12-hour dark (6 AM/6 PM) cycle and fed standard mouse chow. All electroretinogram experiments were performed under dim red light produced using a medium red filter (Rosco Laboratories, Inc., Stamford, CT, USA).

The Generation of Clrn1 Mutant Mice Under A/J Background

The Clrn1−/− and Clrn1N48K/C0 mice under C57BL/6J background8,9,22 were backcrossed to A/J background using Jackson Laboratory’s Speed Congenic Service. This method identifies the progenies which carried the highest percentage of targeted background by testing the cross-strain differences in the single nucleotide polymorphisms (SNPs; also called microsatellite polymorphisms). At Jackson, SNPs were scanned throughout the genome24,25 to confirm that Clrn1−/− and Clrn1N48K/C0 mice carried more than 96% of the genome from the A/J strain. Those mice were again backcrossed with A/J mice to obtain Clrn1−/− and Clrn1+/N48K offspring, which were then expanded into colonies at Case Western Reserve University. The presence of Clrn1−/− and Clrn1+/N48K alleles were confirmed by PCR amplification of genomic DNA (Supplementary Figs. S1A-C). The mice used for this study were negative to rd8 allele as confirmed using the PCR-based method,26 and thus our analyses were not affected by this mutation.26

The number of offspring we could obtain was lower than that for C57BL/6J mice under C57BL/6J background. Thus, our analyses were not affected by this mutation.26 We found that the A/J strain was difficult to breed. Accordingly, the number of offspring we could obtain was lower than that for the A/J strain, and obtained Clrn1-deficient and mutant mice with more than 96% of the genes derived from A/J background. These newly generated USHIII models allowed us to investigate the role of CLRN1 in the mouse retina and to compare the properties of Clrn1+/− and Clrn1N48K/C0 alleles. Thus, in conjunction with the previous characterizations of hearing phenotypes,8,9 this characterization of retinal phenotype is relevant to understanding the etiologies of human USHIII caused by Y176X and N48K mutations.

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Genotyping PCR

The genotypes of the A/J Clrn1+/− mice were confirmed by performing PCR using two sets of primer pairs: P3/P4 and C1/P4. P3 (5’-GGAGTAAGAAGTAGTTACACGG-3’, forward primer) is located upstream of exon 1 of Clrn1 and P4 (5’-GCATTCTCAAGCAATCAG-3’, reverse primer) is located downstream of exon 1. Together, P3/P4 amplifies a 2066 bp product for wild-type (WT) allele and a 782 bp product for Clrn1−/− allele. C1 (5’-TTTACGAGGCTTTTCTTCG-3’, forward primer) is located within exon 1, and when paired with P4, a 1035 bp product is produced for WT allele, while no product results from Clrn1−/− allele. Template DNA was mixed with dH2O, 0.5 U HotStarTaq (Qiagen, Hilden, Germany), the manufacturer-provided reaction buffer, 200 μM deoxyribonucleotide triphosphates (dNTPs), and 0.5 μM of each primer. Polymerase chain reaction conditions consisted of an initial denaturation/alkaline activation step of 30 minutes at 95°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C for P3/P4 or 55°C for C1/P4, and 165 seconds at 72°C, followed by a final elongation step of 7 minutes at 72°C. Allele Clrn1N48K was identified using PCR primers 2L (5’-CGACAGATGACTCATACCATTAAGCTATGTG-3’) and 2R (5’-CCTCTGTTTTTGTAAAAATTCAACACACAC-3’) which flank the loxP site present in the Clrn1N48K knock-in allele but not in the WT allele. This set of primers produced a 275 bp PCR product for the knock-in allele and a 230 bp product for WT allele. The reaction composition is identical to that given above for Clrn1+/− genotyping. Products were amplified using the following PCR conditions: 15 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 150 seconds at 72°C, concluded by a final elongation step of 10 minutes at 72°C. Brightness and contrast of the gel images were adjusted using photo editing software (Adobe Photoshop Cs6; Adobe Systems, Inc., San Jose, CA, USA). Only linear adjustments were made.

ERGs

We analyzed Clrn1−/− and WT mice by ERG at the ages of 3, 9, and 12 months. Clrn1N48K/C0 and WT mice were analyzed by ERG at the ages of 4 and 10 months. Mice were dark-adapted at least overnight before analysis. Under dim red light, mice were anesthetized with intraperitoneal injection using 10 μL/g body weight of 12.5 mg/mL ketamine and 0.5 mg/mL xylazine diluted with 1× PBS (Hyclone cat# SH30256.01). The pupils were dilated with 0.01% tropicamide. A contact lens electrode was placed on the eye, and a reference electrode and ground electrode were placed under the skin of the tail and between the ears. We recorded ERGs with a universal electrophysiological system (UTAS E-3000 MF; LKC Technologies, Inc., Gaithersburg, MD, USA). The light intensity was calibrated by the manufacturer and was computer-controlled. The mice were placed in a Ganzfeld dome, and scotopic and photopic responses to flash stimuli were each obtained from both eyes simultaneously. We noted variability in A/J mouse’s response to the anesthetics, which might have affected the ERG responses. Thus, at the end of the procedure, each eye was inspected for the presence of cataracts. If the mouse formed severe cataracts, indicated any signs of morbidity, or did not survive the procedure, data from these mice were excluded from the analysis.
**Immunofluorescence Staining**

Eye cups were fixed in 4% paraformaldehyde for 6 hours at room temperature, followed by incubation in a succession of phosphate buffer (0.02 M NaH2PO4, 0.08 M Na2HPO4) with increasing concentrations of sucrose (5%–20% wt/vol in phosphate buffer) at room temperature, and overnight incubation in a mixture of one part OCT (Sakura) to two parts SPB (20% sucrose wt/vol in phosphate buffer), at 4°C. Eye cups were then frozen and sectioned on a cryostat (CM1850; Leica Microsystems, Wetzlar, Germany) at −20°C. Fixed eye sections were blocked in 1.5% normal goat serum diluted in PBS with vol/vol 0.1% Triton X-100 (PBST) for 1 hour, incubated with primary antibody overnight at room temperature, washed, and then incubated in secondary antibody for 1 hour at room temperature, followed by a final wash and mounting. The following primary antibodies were used: mouse mAb anti-b-peripherin/rds (a gift from Brian Kevany, Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA); mouse mAb anti-rod opsin 1D4 (an antibody originally generated by Robert S. Molday et al., and a gift from Vera Moiseienko-Koval, Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA); rabbit pAb anti-anti-gal fibrillar acidic protein (catalog no. Z034; Dako, Santa Clara, CA, USA); rabbit pAb anti-PKCα (catalog no. SC-208; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); mouse mAb anti-PSD-95 (catalog no. 05-494; Upstate Biotechnology, Cambridge, MA, USA); mouse IgG anti-RIBEYE (catalog no. 612044; BD Transduction Laboratories, San Jose, CA, USA); mouse mAb anti-Go-alpha (catalog no. MAB073; Chemicon, Billerica, MA, USA); mouse mAb anti-Bassoon (Stressgen, Farmingdale, NY, USA); mouse mAb anti-Calbindin-D-28K (clone CB-955, catalog no. C9848; Sigma-Aldrich Corp., St. Louis, MO, USA); mouse mAb anti-Calretinin (catalog no. MAB1568; Chemicon); and mouse mAb anti-myosin VIIA (catalog no. MYO7A 138-1; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Goat anti-mouse Cy3, donkey anti-rabbit Alexa Fluor 488, or donkey anti-mouse Alexa Fluor 488 (Jackson ImmunoResearch) were used as secondary antibodies. Samples were visualized by confocal microscopy.

**Histology and Light Microscopy**

For light microscopy, mouse eyecups were fixed and processed as described above. The sections of fixed eye cups were stained with Hoechst 33342 nuclear staining and mouse mAb anti-rod opsin 1D4 as described above. After washing with PBST and mounting, the sections were imaged using a Leica DM6000B microscope. Brightness and contrast of differential interference contrast (DIC) images were adjusted using photo editing software (Adobe Photoshop CS6; Adobe Systems, Inc.); only linear adjustments were made. Composite images were made using image analysis software (Image-Pro Plus, Media Cybernetics, Rockville, MD, USA).

**Immunoblotting**

Whole retinal lysate was prepared by sonicating excised retina in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% IGEPA, CA-630, and 0.5% sodium deoxycholate) containing complete protease inhibitor (Roche Pharmaceuticals, Basel, Switzerland), mixing for 1 hour at 4°C, and then clearing by centrifugation. Proteins were resolved using SDS-PAGE, transferred to polyvinylidene fluoride membrane, blocked in PBS containing 0.1% Tween-20 and 5% dry milk, and probed with primary antibodies against rhodopsin, peripherin, or glial fibrillary acidic protein (GFAP; see section Immunofluorescence Staining for detailed antibody information). Membranes were washed in PBS containing 0.1% Tween-20 and incubated in secondary antibody conjugated with horseradish peroxidase. Tubulin probed by mAb anti-beta-tubulin (catalog no. E7, Developmental Studies Hybridoma Bank) was used for a loading control for each lane. Immuno-positive bands were quantified using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Electron Microscopy**

Eye cups were prepared using 4.5-month-old AJ Clrn1 mice along with age-matched WT AJ mice obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). Tissue sections were prepared as described previously20,29 and examined by a FEI Tecnai Spirit (T12) transmission electron microscope with a charge-coupled device camera (US-4000 4k X 4k; Gatan, Inc., Pleasanton, CA, USA).

**RESULTS**

**Characterization of Clrn1 Mice Under A/J Background**

In the past, we have analyzed the phenotypes of Clrn1 knockout (Clrn1<−/−> and Clrn1N48K knockin (Clrn1N48K/N48K) mice under C57BL/6J background. These Clrn1<−/−> and Clrn1N48K/N48K mice did not demonstrate any detectable defects in the retina.8,9,22 Compared with C57BL/6J mice, A/J mice demonstrate a pronounced age-related deterioration of vision, which is also a characteristic of the human visual system.17,18 To study the function of CLRN1 in context to age-related deterioration of vision, we generated Clrn1<−/−> and Clrn1N48K/N48K under A/J background. To investigate whether the loss of Clrn1 gene causes retinal abnormalities, visual functions of Clrn1<−/−> and WT control mice were compared by ERG analyses at different ages under scotopic conditions (Figs. 1, 2). At age 3 months, the Clrn1<−/−> mice displayed b-wave amplitudes significantly lower than WT counterparts (Figs. 1A, 2A). With the light stimuli at the intensities of −2.2 to 0.38 log cd·s/m2, the responses of Clrn1<−/−> mice were 25% to 31% lower than those of WT mice (P < 0.013 by t-test). At the same age, there was no significant difference in a-wave amplitude between Clrn1<−/−> and WT mice (Figs. 1A, right panel, 2B). B-wave originates from the secondary neurons,30 and thus the reduction in b-wave amplitudes observed in Clrn1<−/−> is likely caused by deficit in the synaptic communication between photoreceptors and secondary neurons, or deficits in the inner retina.

At age 9 months, b-wave amplitudes became further separated (Fig. 2A, 9 months), and a-wave amplitudes became significantly different between Clrn1<−/−> and WT mice (Fig. 2B). B-wave amplitudes of Clrn1<−/−> mice was 43% to 51% lower than those of WT mice (P < 0.001 by t-test) at the light intensities of −2.2 to 0.38 log cd·s/m2 (Fig. 2B, left and middle panel). A-wave amplitudes of Clrn1<−/−> mice were also significantly lower than those of WT mice by 30% to 40% for the three highest light intensities (−0.6, 0.38, and 1.6 log cd/s/m2). As a-wave originates from photoreceptor cells,31 this age-dependent decline suggests that photoreceptor function is compromised in Clrn1<−/−> mice. The above ERG responses were measured under scotopic conditions, and were primarily contributed by rod photoreceptors. To test the contribution of cone photoreceptors, we measured the ERG responses under photopic conditions. Under photopic conditions, Clrn1<−/−> and WT mice gave similar b-wave amplitudes under different intensities of light, suggesting that it was not the cone photoreceptor function, but the rod photoreceptor function which was primarily affected in Clrn1<−/−> mice (Fig. 3A).
In analyzing the phenotype caused by Clrn1<sup>−/−</sup>, it is crucial to note that WT A/J mice demonstrated age-dependent decline in both a- and b-wave amplitudes independent of the Clrn1 defect. For example, WT A/J mice demonstrated 52% and 59% decline in a- and b-wave amplitudes from age 3 to 12 months at the light intensity of 0.38 log cd·s/m<sup>2</sup> (WT, Figs. 1A, 1C). Thus, this age-dependent decline of ERG amplitudes in A/J background eventually masked the decline caused by Clrn1 deficiency (Fig. 2). At age 12 months, neither the a- nor b-wave amplitude was significantly different between Clrn1<sup>−/−</sup> and WT mice at all light intensities tested (P > 0.11 by t-test). ***P < 0.001. **P < 0.01. *P < 0.05 by t-test.

**Figure 1.** Electroretinogram responses of Clrn1<sup>−/−</sup> and WT mice in A/J background at age 3 to 12 months. Electroretinogram responses (Left) were measured under scotopic conditions. Amplitudes of b- (Middle) and a-wave (Right) were plotted as a function of light intensity. (A) At age 3 months, b-wave amplitudes were significantly lower in Clrn1<sup>−/−</sup> than in WT mice at the light intensities of −2.2, −0.6, and 0.38 log cd·s/m<sup>2</sup>. (B) At age 9 months, b-wave amplitudes of Clrn1<sup>−/−</sup> were significantly lower than those of WT mice at all the intensities tested. A-wave amplitudes of Clrn1<sup>−/−</sup> mice were also significantly lower than those of WT mice at the light intensities of −0.6, 0.38, and 1.6 log cd·s/m<sup>2</sup>. (C) At age 12 months, there was no significant difference in a- and b-wave amplitudes between Clrn1<sup>−/−</sup> and WT mice at all light intensities tested (P > 0.11 by t-test). **P < 0.001.

**Figure 2.** Age-dependent decline in ERG a- and b-wave amplitudes. Amplitudes of a- and b-waves (light stimuli: log cd·s/m<sup>2</sup>) were measured and compared between WT and Clrn1<sup>−/−</sup> mice at ages 3, 9, and 12 months. Both WT and Clrn1<sup>−/−</sup> demonstrated age-dependent decline of a- and b-wave amplitudes from 3 to 9 months. At age 9 months, a- and b-wave amplitudes of Clrn1<sup>−/−</sup> were lower than those of WT. From 9 to 12 months, additional age-dependent decline was observed in WT mice, and as a result a- and b-wave amplitudes became similar between Clrn1<sup>−/−</sup> and WT mice. The data are represented by mean ± SD. For these analyses, the numbers and ages of animals used were as follows: eight WT and seven Clrn1<sup>−/−</sup> mice at age 3 months, seven WT and seven Clrn1<sup>−/−</sup> at age 9 months, and eight WT and eight Clrn1<sup>−/−</sup> at age 12 months. ***P < 0.001. **P < 0.01. *P < 0.05. NS, not significantly different (P > 0.05) by t-test.
and WT mice (Figs. 1C, 2). In general, a- and b-wave amplitudes observed for 12-month-old Clrn1−/− and WT mice were similar to those observed for 9-month-old Clrn1−/− mice. Thus, from 9 to 12 months, the decline of ERG amplitudes caused inherently by A/J genetic background masked the decline caused by Clrn1 defect.

Dark adaptation is mediated by biochemical processes such as 11-cis-retinal regeneration and translocation of proteins associated with rhodopsin-mediated GPCR cascade. To understand the contribution of these processes, the rate of dark adaptation was compared between under Clrn1−/− and WT mice. Initially, the photoreceptor cells were photobleached with single-flash ERG (~0.2 log cd·s/m²) every 5 minutes for 3 minutes, and then dark-adapted again. The recovery of a-wave amplitudes was monitored with single-flash ERG (~0.2 log cd·s/m²) every 5 minutes for 60 minutes. There was no significant difference in recovery rate between Clrn1−/− and WT mice (P > 0.29 by t-test). Nine-month-old Clrn1−/− and WT mice were used for these two tests.

**Absence of Global Morphologic Abnormalities in the Ciliary and Synaptic Compartments of Clrn1−/− Retina**

Given assumptions that Usher syndrome is a cilia- or synapse-related disorder, and considering a possible role of CLRN1 in the organization of actin that often mediates protein trafficking or synaptic transmission, we tested the localization of two major ciliary trafficked proteins, rhodopsin, and peripherin/rds, in Clrn1−/− mice. Despite these assumptions, both rhodopsin and peripherin/rds localized properly to the rod outer segments of Clrn1−/− mice (Fig. 4), and protein expression levels were not significantly different between WT and Clrn1−/− mice as assessed by immunoblotting (Supplementary Fig. S2, P > 0.42). Thus, Clrn1−/− is not involved in the trafficking of those primary proteins in the outer segment.

The b-wave defect found in Clrn1−/− is possibly a sign that the photoreceptor synapse is affected. Ectopic synapse formation has been reported to be one of age-dependent phenotypes in A/J strain of mice. To examine if ectopic synapse formation is exacerbated in Clrn1−/− mice, we labelled the eye sections of 9-month-old Clrn1−/− and WT mice using an antibody against PSD95 to visualize the photoreceptor synaptic termini and another antibody against PKCζ to visualize the dendritic regions from rod on-bipolar cells. As anticipated, ectopic synapses formed in WT A/J mice as evidenced by aberrant innervation of PKCζ positive dendrites into the outer nuclear layer (Fig. 5A, WT, arrow), however there was no apparent difference in the extent of aberrant innervation between WT and Clrn1−/− mice (Fig. 5A, Clrn1−/−, arrow). The majority of synapses were formed appropriately in the outer plexiform layer as demonstrated by localization of PSD95-positive presynapse structures and PKCζ positive dendrites in the outer plexiform layer (Fig. 5A, OPL). A similar result was obtained for another on-bipolar cell marker Go α (Supplementary Fig. S3A). Likewise, dendrites of horizontal cells, labelled by anti-calbindin antibody, demonstrated similar innervation patterns in both WT and Clrn1−/−, mostly connecting appropriately at the outer plexiform layer (Supplementary Fig. S3B). Anti-calretinin, which is known to label amacrine and ganglion cells, did not show discernible differences in WT and Clrn1−/− mice.
and Clrn1<sup>−/−</sup> mice (Supplementary Fig. S3C). Calretinin-positive neurons stratified in three major layers of inner plexiform layers in WT and Clrn1<sup>−/−</sup> mice (Supplementary Fig. S3C). Photoreceptors are characterized by ribbon synapse. Ribbon synapses contain the ribbon structures, which play critical roles in regulating the synaptic vesicle cycle. Anti-Bassoon and RIBEYE labeled individual puncta, characteristic of photoreceptor ribbons. The distribution, size and number of ribbons were not significantly different between WT and Clrn1<sup>−/−</sup> mice (Supplementary Figs. S3D, S3E).

To further investigate the ultrastructural organization of photoreceptor synapse, we conducted transmission electron microscopy. In a photoreceptor ribbon synapse, a pair of horizontal processes associate with a rod synaptic terminus in a structure termed the triad. Photoreceptor ribbon synapse is further characterized by innervation of dendrite processes from bipolar cells. Those triads and bipolar processes were observed in rod spherules of WT and Clrn1<sup>−/−</sup> mice (Fig. 5B), further supporting the lack of structural abnormality in rod synapses.

To more globally test if Clrn1 defect is causing structural abnormality of retina, we conducted morphometric analysis of 9-month-old mice, when the most significant decline in the ERG responses was documented. Thicknesses of different retinal layers were measured using retinal sections transecting optic nerve heads, and compared between WT and Clrn1<sup>−/−</sup> retinas (Fig. 6A). We found no significant difference in either the outer segment length or outer nuclear layer thicknesses, suggesting that Clrn1 deficiency does not cause rod photoreceptor degeneration (Figs. 6B, 6C). Likewise, thicknesses of the outer plexiform layer were not significantly different between WT and Clrn1<sup>−/−</sup> mice (Fig. 6D). This is in contrast to CaBP4<sup>−/−</sup> mice, which demonstrated dramatic attenuation of b-wave and thinning of outer plexiform layer. We also did not observe significant changes in the thicknesses of inner nuclear and plexiform layers that consist of secondary neurons (Figs. 6E, 6F). Consistent with the results above, we found no significant difference in GFAP localization in the process of Müller cells between Clrn1<sup>−/−</sup> and WT mice (Fig. 4). Using immunoblotting, protein expression levels of GFAP were also assessed.

**Figure 5.** Deficiency of CLRN1 did not promote the formation of ectopic synapse. (A) Sections of fixed eye cups of 9-month-old WT (top row) and Clrn1<sup>−/−</sup> (bottom row) mice were probed with antibody against PSD95 (red), and PKCa (green). In WT and Clrn1<sup>−/−</sup>, the majority of synapses are formed appropriately in the outer plexiform layer (OPL). Images are single confocal optical sections. Scale bar: 5 μm. (B) Ultrastructure of rod spherule in WT (left) and Clrn1<sup>−/−</sup> (right) mice at age 4.5 months. Triad structures, characterized by association of a synaptic ribbon and two horizontal cell processes (H), are intact in the retinas of both WT and Clrn1<sup>−/−</sup>. Innervations of bipolar dendrites (Bi) were observed both in WT and Clrn1<sup>−/−</sup>. Scale bar: 100 nm.
Clrn1 measured between WT and hypothesis that advantage of the visual testing of A/J mice, we tested the mice were probed with nuclear staining dye (blue) and antibody against rhodopsin (red), and then imaged by light microscopy. Differential interference contrast images (gray scale) were merged with fluorescence images (blue and red). INL: inner nuclear layer; IPL: inner plexiform layer; IS: inner segment; ONL: outer nuclear layer; OS: outer segment. From optic nerve (ON), the thicknesses of OS (WT (n = 4) Clrn1N48K (n = 4)) were measured every 250 µm on superior and inferior sides. There was no significant difference in the thicknesses of any of the layers measured between WT and Clrn1+/− mice (n = 4 retinas from 4 animals for each group, P > 0.095 by t-test). Scale bar: 20 µm.

**Figure 6.** Deficiency of CLRN1 did not affect the morphology of retina. (A) Sections of fixed eye cups of 9-month-old WT (left) and Clrn1+/− (right) mice were probed with nuclear staining dye (blue) and antibody against rhodopsin (red), and then imaged by light microscopy. Differential interference contrast images (gray scale) were merged with fluorescence images (blue and red). INL: inner nuclear layer; IPL: inner plexiform layer; IS: inner segment; ONL: outer nuclear layer; OS: outer segment. From optic nerve (ON), the thicknesses of OS (B), ONL (C), OPL (D), INL (E), and IPL (F) were measured every 250 µm on superior and inferior sides. There was no significant difference in the thicknesses of any of the layers measured between WT and Clrn1+/− mice (n = 4 retinas from 4 animals for each group, P > 0.095 by t-test). Scale bar: 20 µm.

quantiatively on retinas collected at age 4.5 months, when progressive functional changes are anticipated to be dramatic in Clrn1+/− mice based on the time course of decay in ERG amplitudes (Fig. 2). This analysis also failed to detect significant difference between Clrn1+/− and WT (Supplementary Fig. S2, GFAP), suggesting that Clrn1 deficiency does not accelerate retinal degeneration. Taken together, these results suggest that the reduction in the a-wave amplitude is not a result of rod photoreceptor degeneration or compromised rod photoreceptor-bipolar connectivity. Instead, the observed deficiency is likely associated with the function of CLRN1.

**Characterization of Clrn1N48K Mice in A/J Background**

Previous comparison of Clrn1N48K/N48K and Clrn1+/− mice indicate that the onset of hearing loss is delayed for Clrn1N48K/N48K, although both Clrn1+/− and Clrn1N48K/N48K mice lose hearing eventually. Thus, this past study suggests that, unlike Clrn1−/− allele which is null, the function of Clrn1N48K product is dramatically attenuated but still present. Such attenuated function of Clrn1N48K is not sufficient to sustain hair cells, thus leading eventually to deafness. Taking advantage of the visual testing of A/J mice, we tested the hypothesis that Clrn1N48K is more functional than Clrn1+/− and thus constitutes a hypomorphic allele. To test this hypothesis, we investigated if Clrn1N48K/N48K exhibit declined a- and b-wave amplitudes as observed for Clrn1+/− mice. The visual performances of Clrn1N48K/N48K mice were compared with WT mice at age 4 and 10 months. Those two time points (4 and 10 months) were offset by 1 month compared with those selected for Clrn1+/− mice, because of an anticipation that onset of degenerative events are delayed in Clrn1N48K/N48K mice over Clrn1+/− mice based on hearing phenotype. At both age 4 and 10 months, the a-wave amplitudes were similar between WT and Clrn1N48K/N48K mice at all light intensities tested (Figs. 7A, 7B, right panel), suggesting that Clrn1N48K/N48K mutation did not cause an appreciable decline in photoreceptor function. Likewise, there were no detectable defects of the inner retinal response, as b-wave amplitudes were also similar for both WT and Clrn1N48K/N48K at both ages under all tested light intensities (Figs. 7A, 7B, left panel). The a- and b-wave amplitudes of these 10-month-old mice were similar to those of the 9-month-old WT mice (Figs. 1B, 7B, WT). Thus, visual performance of the A/J mouse strain does not decline significantly from age 9 to 10 months. These comparisons indicate that Clrn1N48K/N48K alleles did not exacerbate age-related decline in the a- and b-wave amplitudes, as Clrn1+/− alleles did.

**Discussion**

To the best of our knowledge, the A/J Clrn1+/− mouse is the first USHIII model that exhibits retinal phenotype. Contrary to Clrn1+/−, the lack of observable visual phenotype in A/J Clrn1N48K/N48K mouse suggests that Clrn1N48K is a hypomorphic allele compared with Clrn1+/− which is functionally null. This assumption is consistent with the delayed onset of hearing loss in Clrn1N48K/N48K mouse as compared to Clrn1+/− mouse in the previous study. In the past, it was challenging to obtain a visual phenotype in USHIII model animals. This study demonstrates that the A/J strain of mouse is a useful tool to obtain statistically and biologically significant attenuation of visual performance caused by deficiency in the Clrn1 gene which is causative to USHIII. A similar approach has been successful for obtaining a visual phenotype for the Rp1b mutant allele, suggesting the A/J strain is useful to analyze the
functions of human blindness causative genes whose phenotypic outcomes have been observed in other strains of mice.

The observed defects in scotopic ERG responses are suggestive of rod photoreceptor dysfunction in Clrn1<sup>+/−</sup> mice. Absence of differences in photopic ERG responses between Clrn1<sup>+/−</sup> and WT further suggests that defects are originating primarily from rods, and not from cones. This is consistent with clinical studies of USHIII in which rod photoreceptor cells are initially affected. In A/J Clrn1<sup>+/−</sup>, decline of ERG amplitude was progressive, characterized initially by b-wave defect followed by combined a- and b-wave defects. This b-wave defect is a characteristic of attenuated synaptic transmission from rod photoreceptors to secondary neurons. A similar progression was observed for CaBP4<sup>+/−</sup> mice which are defective in a calcium binding protein localized to photoreceptor synapses. CaBP4 deficiency dramatically reduced b-wave responses due to compromised calcium signaling in the photoreceptor synaptic terminal. This decline in the b-wave is then followed by eventual a-wave defects. Thus the defects originating at photoreceptor synapses can lead to depressed a-wave function as observed in this study of A/J Clrn1<sup>+/−</sup> mice. While the molecular mechanism causing compromised a- and b-wave amplitudes is unclear, we believe that the reduced ERG amplitudes is not due to a loss of photoreceptor cells, as no significant differences in a- and b-wave amplitudes between Clrn1<sup>+/−</sup> and WT mice at all the light intensities tested (P > 0.41 by t-test).

FIGURE 7. Electroretinogram responses of Clrn1<sup>N48K/N48K</sup> and WT mice in A/J background. Amplitudes of a- (right) and b- (left) waves were measured under scotopic conditions at age 4 (A) and 10 (B) months, and plotted as a function of light intensity. At both ages, there were no significant differences in a- and b-wave amplitudes between Clrn1<sup>N48K/N48K</sup> and WT mice at all the light intensities tested (P > 0.41 by t-test).

-Ush syndrome is a devastating disorder that drastically compromises the quality of life of those afflicted. Recent studies suggest that CLRN1 is possibly involved in the function of synapses in auditory hair cells of mouse and zebrafish. Other studies, however, failed to detect such synapse defects, and instead demonstrated roles of CLRN1 in stereocilia of hair cells that are not present in photoreceptor neurons. For the studies of Usher syndromes, the major challenges are that sensory deficits are observed in retina and cochlea which have no apparent functional similarities, and that animal models which demonstrate retinal deficits are often missing. In this regard, retinal ERG phenotype observed in A/J Clrn1<sup>+/−</sup> is a viable starting point to understand the difference and similarity of CLRN1 roles in the retina and cochlea.

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References


