Ames Waltzer Deaf Mice Have Reduced Electroretinogram Amplitudes and Complex Alternative Splicing of Pcdh15 Transcripts

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PURPOSE. Mutations of PCDH15, the gene encoding protocadherin-15, cause either nonsyndromic deafness DFNB23 or Usher syndrome type 1F (USH1F) in humans and deafness with balance problems in Ames waltzer (av) mice. Persons with USH1 usually begin to exhibit signs of retinitis pigmentosa (RP) in early adolescence, but av mice are reported to have functional retinas. In this study, the auditory, visual and molecular function of the retina has been evaluated by ERG for potential therapies for USH1. (Invest Ophthalmol Vis Sci. 2006;47:3074–3084) DOI:10.1167/iovs.06-0108

METHODS. Hearing thresholds of mice between 6 and 10 weeks of age were measured by auditory brain stem response (ABR). Immunohistochemistry and histology were used to examine the effect of homozygosity of Pcdh15av-5J on stereocilia bundles of inner ear hair cells and the photoreceptor cells of the retina. Scotopic and photopic Ganzfeld ERGs were recorded from homozygous Pcdh15av-5J and Pcdh15av-Jfb mice at different ages. Heterozygous littermates served as control subjects. Measurements of the width of the outer nuclear layer (ONL) and the length of rod photoreceptor outer segment (ROS) were made.

RESULTS. Homozygous Pcdh15av-5J mice have profound hearing loss and disorganized stereocilia bundles of inner ear hair cells. Compared with heterozygous littermates, homozygous Pcdh15av-5J and Pcdh15av-Jfb mutant mice had scotopic ERG amplitudes consistently reduced by approximately 40% at all light intensities. The b-to-a-wave ratio confirmed that the a- and b-waves were reduced proportionally in homozygous mutant mice. Histologic measurements of retinal sections revealed no significant differences in either the ONL width or the ROS length as a function of genotype. The protocadherin-15 labeling pattern with antisera PB303 in the retina of both heterozygous and homozygous Pcdh15av-5J mice was indistinguishable from the wild type. Wild-type Pcdh15 av have many alternatively spliced isoforms. A novel isoform was found in the retina of homozygous Pcdh15av-5J mice, which appears to circumvent the effect of the mutant allele (IVS14-2A→G), which causes skipping of exon 14, a shift in the translation reading frame and a premature stop codon in exon 15.

CONCLUSIONS. Pcdh15av-5J and Pcdh15av-Jfb mice do not faithfully mimic the RP found in USH1 due to mutations of PCDH15, but have significantly attenuated ERG function in the absence of histologic change. The decline in ERG amplitude with a preserved b-to-a-wave ratio suggests a role for Pcdh15 in retinal function and/or generation of the ERG potentials. Understanding the molecular mechanism by which av mice circumvent degeneration of the retina might offer insights into potential therapies for USH1.
Pcdh15av-3J, Pcdh15av-5J, and Pcdh15av-Jfb mice at 2.5 to 8 months of age (Supplementary Table 1). For mice homozygous for these four mutant alleles of Pcdh15, the ERGs were reported to be indistinguishable from those of control mice. Moreover, the retinas of homozygous Pcdh15av-3J and Pcdh15av-5J mice were examined histologically, and no obvious histopathology was reported. Pcdh15av-3J, originally designated Pcdh15av-J19, was initially identified through complementation testing at The Jackson Laboratory (Bar Harbor, ME) and has been partially characterized at the molecular level. The Pcdh15av-3J mutation is an A-to-G transition mutation of a consensus splice site that causes skipping of exon 15. Pcdh15av-5J mice exhibit circling behavior, show severe disorganization of inner ear hair bundles, and lack an auditory-evoked brain stem response (ABR). Stereocilia are actin-filled projections on the apical surface of hair cells that are specialized for mechano-transduction of sound.

To determine whether either Pcdh15av-3J or Pcdh15av-5J mice are models of the RP feature of USH1, we evaluated the retinal phenotype of these two av alleles using electroretinograms (ERGs) and histology. We found that Pcdh15av-3J and Pcdh15av-5J had reduced a- and b-wave amplitudes but had an unaffected implicit time and b-to-a wave ratio at the ages measured. We do not understand the biological basis of these stably reduced ERG potentials in Pcdh15av-3J or Pcdh15av-5J mice. However, we provide molecular genetic data that support the hypothesis that there are alternative splice isoforms of the Pcdh15 precursor that are sufficiently functional in the retina, but not in the ear.

Materials and Methods

Mice

Animal procedures were conducted in accordance with the National Institutes of Health Animal Care and Use Committee Protocols 1126-03 and 1101-02 and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We obtained heterozygous C57BL/6J-Pcdh15av-1J/J mice from The Jackson Laboratory. The Pcdh15av-1J mutant allele arose spontaneously in offspring segregating a gastrin-releasing peptide receptor (Grpr) null allele (B6.129-Grprtm1Jfb/J) at the fifth backcross generation and maintained afterward on a C57BL/6J background in our NIDCD/NIH mouse colony.

Immunohistochemistry of Mouse Inner Ear

The inner ears of the P5 C57BL/6J and Pcdh15av-3J mice were dissected from temporal bones and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 hours at room temperature and visualized as wholemounts. Auditory and vestibular sensory epithelia were dissected, immunostained, and mounted (ProLong Antifade Kit; Invitrogen, Carlsbad, CA). Actin labeling, using rhodamine-phalloidin, was performed as described previously.

Immunohistochemistry of Mouse Retina

Wild-type (C57BL/6J), heterozygous, and homozygous Pcdh15av-3J mice were perfused transcardially with 4% paraformaldehyde in PBS. The fixed eye tissues were removed immediately and immersed in the same fixative at 4°C for 2 hours. Tissues were cryoprotected, embedded in OCT compound, and cut at 10 μm-thickness. Retinal sections were blocked in 10% normal goat serum for 1 hour and incubated with affinity-purified rabbit anti-proteocadherin 15 antibody (PB303) at 4°C overnight. After several washes in PBS, sections were incubated with AlexaFluor 568 conjugated goat anti-rabbit IgG (Invitrogen). 4′,6-Diamino-2-phenylindole (DAP) was used as a counterstain. PB303 antisem is directed toward the cytoplasmic domain of protocadherin 15 (GenBank accession number, AAG53891; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and was validated previously by Western blot analyses and colocalization with an Hc-Red tagged protocadherin 15 cytoplasmic domain expressed in human lymphoblast cells. No signal was detected in control lymphoblastoid cells stained with PB303.

Histologic Evaluation of the Mouse Retina

Eyes from heterozygous and homozygous mice were fixed for histology either by transcardial perfusion with 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer followed by overnight immersion in the same fixative or by immersion in the same fixative for at least 24 hours immediately after enucleation from freshly euthanized animals. Perfusion-fixed eye tissue was trimmed, postfixed in 1% osmium tetroxide/dH2O for 1 hour, and embedded in Araldite resin (Electron Microscopy Science, Hatfield, PA). Sections of 0.5-μm thickness were cut along the vertical meridian passing through the optic nerve and stained with 0.1% toluidine blue. Immersion-fixed tissue was similarly trimmed and marked for orientation and embedded in methacrylate for sectioning at 1 to 2 μm and stained with hematoxylin and eosin. To evaluate the histologic difference between heterozygous and homozygous mutant mice, measurements of the width (micrometers) of the outer nuclear layer (ONL), the number of photoreceptor cells, and the length of rod photoreceptor outer segments (ROS) were performed at 200-μm intervals and in retinal sections from three heterozygous and three homozygous Pcdh15av-3J mutant mice. Measurements were made on digital photomicrographs using a 60× objective.

Electroretinography

ERGs were recorded in female heterozygous and homozygous Pcdh15av-3J mice at 5 weeks and 3 months of age and in female heterozygous and homozygous Pcdh15av-5J mice at ages 5 to 8 weeks, 5 months, and 11 months. Heterozygous and homozygous mice of each age group were from the same litter. Each mouse was evaluated by ERG at only one age.

Mice were dark adapted for 12 hours before intraperitoneal administration of ketamine (80 mg/kg) and xylazine (4 mg/kg). The pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine HCl. Body temperature was maintained near 38°C with a heating pad. ERGs were recorded with gold wire loops placed on the cornea with a drop of methylcellulose after applying 1% proparacaine topical anesthetic. Gold wires were placed on the sclera at the limbus as the differential electrodes, and a ground wire was attached to the left paw.

Scotopic ERG responses were elicited in the dark-adapted state using single xenon photostrobe flashes (PS3) Photic Stimulator; Grass-Telefactor, West Warwick, RI) delivered in a Ganzfeld light-integrating sphere with interstimulus intervals of 3 to 60 seconds, depending on stimulus intensity. A stimulus intensity range of ~6.9 to +0.6 log cd · s/m2 was obtained using neutral-density filters (Wratten; Eastman Kodak, Rochester, NY). Responses were filtered using low-pass (1 kHz) and high-pass (0.1 Hz) filters and a 60-Hz line frequency filter and amplified 5000 times (C5511 AC amplifier; Grass-Telefactor). Photopic responses were elicited in the light-adapted state on a rod-suppressing white background of 34 cd/m2, with single flashes at 2-second interstimulus intervals. Twenty responses were averaged for each light intensity level. a-Waves were measured from the prestimulus baseline to the initial trough. b-Waves were measured from the baseline or from the a-wave trough when present. Implicit times were measured from flash onset to the a- and b-wave maximum. The amplitude of the positive scotopic threshold response (pSTR) was measured from baseline to the initial peak, and the amplitude of the negative (n)STR was measured from the initial trough to the peak.
measured from the baseline to the trough after the pSTR in the intensity range $-6.9$ to $-3.4 \log \text{cd} \cdot \text{s/m}^2$.

Intensity-response amplitude data were displayed conventionally on log-log coordinates or log-linear coordinates. In mice, the ERG responses from threshold to approximately $-1.5 \log \text{cd} \cdot \text{s/m}^2$ (scotopic range) reflect activity initiated exclusively by the rod photoreceptors. Above this range, b-wave amplitudes are influenced both by cone photoreceptor activity and the emergence of the a-wave. ERGs in the upper intensity range were graphed, to track the a-wave responses. Scotopic a-wave responses are almost exclusively the result of rod activity. In the mouse, cones contribute very little to the dark-adapted a-wave.

The Naka-Rushton function

$$\frac{V}{V_{\text{max}}} = \frac{I^n}{I^n + I^n_{\text{max}}}$$

was fitted to scotopic b-wave amplitudes, measured as a function of stimulus intensity ($I$), to evaluate changes in ERG amplitude and sensitivity. The Naka-Rushton function is useful in describing the scotopic b-wave intensity–response relationship and evaluating the effect of retinal disease in humans. The least-square fits were iteratively performed by computer (OriginPro 7.5 software; Originlab Corp. Northampton, MA) to determine the values of $n$, $k$, and $V_{\text{max}}$ for each mouse. $V_{\text{max}}$ is the maximum of the b-wave amplitude, $k$ is the intensity at which the b-wave amplitude reaches half saturation (inversive of sensitivity), and $n$ is a dimensionless constant related to the slope of the intensity–response function.

Auditory-Evoked Brain Stem Response

Hearing thresholds of mice between 6 and 10 weeks of age were measured by auditory-evoked brain stem response (ABR). Four wild-type C57BL/6J, five Pcdh15$^{+/-}$ heterozygotes, and six Pcdh15$^{+/-}$ homozygous mice were tested. The mice were anesthetized with intraperitoneal injections of 2.5% averitin (0.015 mL/g body weight). All recordings were conducted in a soundproof chamber on a heating pad with an auditory evoked potential diagnostic system (Intelligent Hearing Systems, Miami, FL) with high-frequency transducers, as previously described. Responses to 30-ms clicks and 8, 16- and 32-kHz tone bursts were recorded. Thresholds were detected by decreasing sound stimulus intensity until no reproducible ABR pattern could be observed. Wild-type mice were interspersed throughout the testing as normal control subjects.

Sequence Analysis

Genomic DNA was isolated (DNeasy Tissue Kit; Qiagen, Valencia, CA) from tail biopsy specimens from the homozygous Pcdh15$^{+/-}$ mice. We screened the reported 34 exons and intronic boundaries of Pcdh15 by PCR amplification from genomic DNA and direct sequencing of the PCR products. The Pcdh15$^{+/-}$ mutation was detected by amplification and direct sequencing of exon 15 using primers 15 forward (F), 5'-TGGGTGATTTAAACCCCTTG-3' and 15 reverse (R), 5'-TGGGTGATTAAACCCCTTG-3', which amplified a 636-bp product. Pcdh15$^{+/-}$ mice were genotyped by directly sequencing Pcdh15 exon 17 PCR products amplified using the primers 5'-GATCTTCG-CATCCAATGCACG-3' and 5'-AGCATTTGTCGTGTTGTAAT-3' and mouse genomic DNA as a template.

Reverse Transcription–PCR

Poly(A)$^+$ RNA was isolated from adult brain, ear, and eye of the homozygous Pcdh15$^{+/-}$ mice (Poly(A)Pure; Ambion, Austin, TX) and was reverse transcribed with an oligo dT primer and reverse transcriptase (PowerScript; BD-Clontech, Palo Alto, CA). cDNA was PCR amplified (LA-Tag; Takara Mirus, Madison, WI) and primers Pcdh15-F, 5'-TACTGCCTGGACTCGTGTCGACTAAAGATGAAACT-3' and Pcdh15-R, 5'-GTTACGACCAAGAAAAGAATCGCCCAGGTAAAAT-3' were subcloned and both strands fully sequenced.

Results

Sequence Analysis

We confirmed that the Pcdh15$^{+/-}$ mutation is an intronic A-to-G transition (IVS14-2A$\rightarrow$G; Fig. 1A) that is predicted to give rise to a smaller cDNA, suggesting that exon 15 was skipped, which was confirmed by sequencing. The IVS14-2A$\rightarrow$G mutation abolished a PvuII restriction endonuclease recognition site (CATCGT) found in the splice acceptor site of intron 14. PCR-RFLP digestion with PvuII produced an uncut 636-bp fragment in Pcdh15$^{+/-}$ homozygotes, 469- and 167-bp fragments in the wild-type animals and all three fragment sizes in the heterozygotes.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932939/)
cause skipping of exon 15 and a premature stop codon in exon 16 (Figs. 1B, 1C).20 The exon 15 splice site mutation also abolishes a PvuII restriction fragment length polymorphism (RFLP) was performed on genomic DNA from the Pcdh15av-5J homozygotes, heterozygotes, and wild types. Digestion with PvuII resulted in an uncut fragment of 636-bp for the mutant allele and fragments of 469- and 167-bp for a homozygous wild-type mice (Fig. 1D).

To rule out the possibility that the A-to-G substitution (IVS14-2A→G) is a common benign polymorphism, we sequenced exon 15 of Pcdh15 from 11 different wild-type mouse strains (Balb/CJ, C58/J, SEA/GnJ, P/J, RF/J, SWR/J, AKR/J, C57BL/6J, C57BL/6J, C57BL/6J, C57BL/6J, and DBA/2J). All 11 strains had an A at position IVS14-2 (GenBank accession number, AF281899). We also demonstrated experimentally that exon 15, found in the Pcdh15av-5J homozygotes, was not included, as predicted, in Pcdh15 mRNA (Fig. 1C).

**Auditory Evaluation**

At weaning (21 days), the homozygous Pcdh15av-5J mice exhibited hyperactivity, circling, and head tossing that continued throughout adulthood. Pcdh15av-5J homozygous mice also did not exhibit a startle reflex, indicative of a hearing impairment. We measured ABR thresholds in six affected mice at 7 weeks of age. All the homozygous mice had profound hearing loss with a threshold over 100 dB sound pressure level for click and 8-, 16- and 32-kHz pure tone stimuli. The homozygous wild-type littermate control mice had normal thresholds detected for the click and pure-tone stimuli tested (Fig. 2).

The Pcdh15av-5J homozygous mice had inner ear hair cell abnormalities. In the organ of Corti and the vestibular sensory epithelium, as early as E17.5, stereocilia bundles were disorganized, and the kinocilia were misplaced (data not shown). We also examined Pcdh15av-5J homozygotes and heterozygotes at P5. Similarly, the homozygous Pcdh15av-5J mice at P5 had disorganized stereocilia hair bundles in the apical, middle, and basal turns of the organ of Corti (Figs. 3D–F), whereas the heterozygotes had the expected wild-type V-shaped pattern of stereocilia hair cell bundles of the organ of Corti (Figs. 3A–C). Using antisera PB303 directed against an epitope of the cytoplasmic domain of protocadherin 15, we observed a wild-type staining pattern of stereocilia in degenerating hair cells of homozygous Pcdh15av-5J and Pcdh15av-Jfb mice (data not shown).

**Retinal Evaluation of av− Mice**

The ERGs of 5- to 8-week-old mice were recorded, to evaluate retinal function at a young age, but at a time when retinal responses and anatomy are mature. Shown in Figure 4A are typical scotopic ERG waveforms from a phenotypically wild-type heterozygous Pcdh15av-Jfb and a homozygous Pcdh15av-Jfb mouse. ERGs from the heterozygous mouse had amplitudes and waveforms similar to those in the wild-type C57BL/6J mice,29 including the ratio of b- to a-wave. Waveforms from the homozygous Pcdh15av-Jfb mouse resembled those of its heterozygous littermate in shape and timing but had reduced amplitudes. The photopic ERG of the heterozygous Pcdh15av-Jfb mouse (Fig. 4B) shows typical waveforms with threshold at −1.4 log cd·s/m² and a normal implicit time and amplitude. The homozygous wild-type littermates were similar to the heterozygotes in all respects except for a small reduction in amplitudes. Figure 4C shows averaged STRs from the 5-week-old littermates.
old heterozygous *Pcdh15^av-Jfb* and homozygous mutant *Pcdh15^av-Jfb* mice at an intensity of −5.4 log cd·s/m². The pSTR and nSTR are the most sensitive components of the ERG and are markers of inner retinal activity.30 The responses in the homozygous *Pcdh15^av-Jfb* mice were scaled down compared with those in heterozygous *Pcdh15^av-Jfb* without a change in timing or shape similar to the changes seen in the a- and b-waves.

Figure 5 shows the intensity–response data from the 5- to 8-week-old heterozygous and homozygous mutant *Pcdh15^av-Jfb* and *Pcdh15^av-Jfb* mice (*n* = 5). Compared with heterozygous littermates, the homozygous mutant mice had consistently reduced scotopic (Fig. 5A) and photopic (Fig. 5B) a- and b-wave amplitudes at all intensities. The log-log plots emphasize the fact that the ratio of the heterozygous-to-homozygous amplitudes was remarkably similar across intensities. The b-to-a-wave ratio shown in Figure 5C confirms that the a- and b-waves were reduced proportionally in the homozygous mice, since the b-to-a-wave ratio of the *Pcdh15^av-Jfb* and *Pcdh15^av-Jfb* homozygous mice did not differ from that in the heterozygous mice at any intensity. Statistical comparisons between the b-wave intensity response data of the homozygous and heterozygous *Pcdh15^av-Jfb* and *Pcdh15^av-Jfb* mice were performed with *V* max (the maximum of the b-wave amplitude) and *k* (semisaturation intensity) obtained from the Naka-Rushton function (Fig. 6, Table 1). The insets in Figure 5A show fits (red lines) of the equation to the log-linear data in the scotopic range. Scotopic a-wave and photopic b-wave responses at various ages were compared by measuring the amplitude at maximum stimulus intensity. Comparisons were made across age and genotype by two-way ANOVA with Bonferroni posttests.

Because RP in USH1 is a progressive disorder and the effects of the *av* mutations may change with age, retinal function was also measured at later ages in the *Pcdh15^av-Jfb* and *Pcdh15^av-Jfb* mutant mice. Age-related changes in scotopic b-wave parameters are illustrated in Figure 6 and Table 1. In the 5-week- and 3-month-old *Pcdh15^av-Jfb* homozygous mice, *V* max was significantly reduced (~40%) compared with that in the heterozy-

**Figure 4.** Electroretinogram (ERG) waveforms recorded from a representative heterozygote and homozygous *Pcdh15^av-Jfb* 5-week-old mouse. (A) Scotopic response; (B) photopic response; (C) STR from averaged traces (*n* = 3, 4) at −5.4 log cd·s/m².
gous littermates ($P < 0.01$, $n = 5$; $P < 0.05$, $n = 3$, respectively). The $V_{\text{max}}$ data from the 5-week ($P < 0.05$, $n = 3$) and 5-month ($P < 0.05$, $n = 4$) $Pcdb15^{av-5J}$ homozygous mice showed a similar significant reduction compared with their heterozygous littermates (Fig. 6B, Table 1). At 11 months, the difference were not significant, because of a greater relative decline in $V_{\text{max}}$ in the heterozygous mice with age. In the $Pcdb15^{av-5J}$ mice there was no statistically significant effect of genotype on sensitivity ($k$) but a small, but significant, effect was seen in the $Pcdb15^{av-Jfb}$ mice ($P < 0.05$; Figs. 6C, 6D; Table 1). As might be predicted from the a- and b-wave relationship at 5 weeks, a-wave reduction seen at later ages was proportional to the b-wave changes in mice homozygous for $Pcdb15^{av-5J}$ or $Pcdb15^{av-Jfb}$ and was significantly reduced across ages (Table 1). There was a significant reduction of b-wave $V_{\text{max}}$ with age in heterozygous and homozygous mice for both mutations but no age-dependence of sensitivity and no effect of age on the difference in ERG parameters between the heterozygous and homozygous mutants.

Histologic measurements of retinal sections along the vertical meridian from 3-month-old homozygous $Pcdb15^{av-5J}$ and 6-month-old homozygous mutant $Pcdb15^{av-Jfb}$ mice revealed no significant differences in either the width of the ONL in cell number, or in the length of the ROS as a function of genotype (Fig. 7). ONL width in micrometers also revealed no difference between the heterozygous and homozygous mice (data not shown). Thus, no structural abnormalities were seen that correlated with or could explain the ERG differences.

Immunohistochemistry of the mouse retina used a previously validated,14 affinity-purified antisera, PB303, that recognizes an epitope of the cytoplasmic region of protocadherin 15 encoded by exons 32 to 34, (Fig. 8, left). In the wild-type retina of the mouse with specimens labeled with antisera PB303, there was dense labeling of protocadherin 15 in the photoreceptor inner segments (IS), the outer plexiform layer (OPL), the inner nuclei layer (INL), and the ganglion cell layer (GCL) and a diffuse staining pattern in the inner plexiform layer (IPL). No staining was observed in the retinal pigment epithelium (RPE). We previously reported a similar pattern of labeling of the photoreceptor cells by antisera PB303 in normal monkey and human retinas.14 Unexpectedly, we also observed a wild-type pattern of protocadherin 15 labeling with PB303 in the retina of both heterozygous and homozygous $Pcdb15^{av-5J}$ mice (Fig. 8, middle, right). Despite the IVS14-2A→G mutation, which results in a premature translation stop codon in exon

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**Figure 5.** Dark-adapted ERG intensity-response curves from 5-week-old heterozygous ($n = 5$) and homozygous ($n = 5$) $Pcdb15^{av-5J}$ and heterozygous ($n = 3$) and homozygous ($n = 4$) $Pcdb15^{av-Jfb}$ mice. (A) b-Wave amplitude ±SE on a log-log scale. Inset: example of the Naka-Rushton function fitted to b-wave data on a log-linear plot. (B) a-Wave amplitude ±SE on log-log scale. (C) b-to-a-Wave ratio ±SE on log-linear scale.
16, our immunohistochemical findings indicate that homozygous Pcdh15<sup>av-5J</sup> mice must express an isoform of protocadherin 15 that has a cytoplasmic domain recognized by antisera to Pcdh15 that has a cytoplasmic domain recognized by antisera to Pcdh15.

**Splice Variants of Pcdh15**

The Pcdh15<sup>av-5J</sup> mutant exon 15 splice acceptor site causes skipping of exon 15 and would be expected to introduce a premature stop codon in exon 16, thereby truncating protocadherin 15 after the fourth extracellular (EC) domain. To understand how homozygous Pcdh15<sup>av-5J</sup> mice could produce a protocadherin 15 with a normal localization in the retina, we screened retinal and inner ear cDNA from wild-type C57BL/6J mice for mutant exon 15 splice acceptor site mutations. We found a novel protocadherin 15 isoform that excludes exons 3 through 15 (Fig. 9A), there may be a splice variant of Pcdh15 that circumvents the premature stop codon in exon 16 and gives rise to a functional but shorter splice variant of Pcdh15 after the fourth EC domain.20 To understand how homozygous Pcdh15<sup>av-5J</sup> mice could produce a protocadherin 15 isoform that excludes exons 3 through 15 (Fig. 9A), there may be a splice variant of Pcdh15 that circumvents the premature stop codon in exon 16 and gives rise to a functional but shorter splice variant of Pcdh15 after the fourth EC domain.20 To understand how homozygous Pcdh15<sup>av-5J</sup> mice could produce a protocadherin 15 isoform that excludes exons 3 through 15 (Fig. 9A), there may be a splice variant of Pcdh15 that circumvents the premature stop codon in exon 16 and gives rise to a functional but shorter splice variant of Pcdh15 after the fourth EC domain.20

To test for the presence of such a transcript, we amplified murine retinal cDNA using a forward primer located in exon 1 and a reverse primer located in exon 19 (Figs. 9A, 9B).14,18 Therefore, we considered the possibility that in homozygous Pcdh15<sup>av-5J</sup> mutant mice, because of the exon 15 acceptor site mutation (IVS14-2A→G), there may be a splice variant of Pcdh15 that circumvents the premature stop codon in exon 16 and gives rise to a functional but shorter version of protocadherin 15 that has an intact cytoplasmic domain encoded by exons 32 to 34. We further analyzed the possibility that other presumably null alleles of Pcdh15 such as Pcdh15<sup>av-2J</sup> and Pcdh15<sup>av-Jfb</sup> produce transcripts without exons that include the mutant alleles. RT-PCR analyses with primers designed from sequence within exons 2 and 6 revealed at least three different splice isomers of Pcdh15 produced in wild-type animals that either included or excluded exons 3 and 4 (Figs. 9A, 9B). Moreover, in the wild-type mice, three transcripts were found in an RT reaction with a forward primer located within exon 11 and a reverse primer located within exon 19 (Figs. 9A, 9B). Therefore, we considered the possibility that in homozygous Pcdh15<sup>av-5J</sup> mutant mice, because of the exon 15 acceptor site mutation (IVS14-2A→G), there may be a splice variant of Pcdh15 that circumvents the premature stop codon in exon 16 and gives rise to a functional but shorter version of protocadherin 15 that has an intact cytoplasmic domain encoded by exons 32 to 34.

**DISCUSSION**

Homozygous Pcdh15<sup>av-5J</sup> mice are deaf and hyperactive and show circling behavior. The organ of Corti and vestibular sensory epithelia of these mutant mice have disorganized hair cell stereocilia bundles and a misplaced kinocilium, consistent with the deafness and vestibular phenotype reported for six other av alleles.18–20 At 5 weeks and older Pcdh15<sup>av-5J</sup> and Pcdh15<sup>av-Jfb</sup> homozygous mice had significantly reduced a- and b-wave ERG amplitudes (−40%). With respect to the visual system, however, the Pcdh15<sup>av-5J</sup> and Pcdh15<sup>av-Jfb</sup> mice differed from other reported av mice,21 which do not show an abnormal retinal phenotype. Apart from the possibility that different mutations of Pcdh15 may differ in their phenotypic effects (Supplementary Table S1, http://www.iovs.org/cgi/content/full/47/7/3074/DC1), we have no other likely explanation for the difference in ERG findings. However, other studies in mice with similar auditory and vestibular phenotypes caused by mutations in nonsyndromic deafness or Usher-related genes (e.g., Cdb23, Myo7a, and Myo6a) also show ERG reduction without histologic changes at the light microscopy level.21–33 Cadherin 23 and myosin VIIa colocalize with protocadherin 15 in photoreceptor synapses. Protocadherin 15 is also found in the outer segments of mice. Thus, these proteins may affect photoreceptor function at more than one level.
there may be an augmented bipolar cell response.36 b-wave amplitudes can be reduced similarly and significantly without morphologic effects detectable by light microscopy.34 Aging studies in normal C57BL/6j mice have shown that mild or moderate alterations in photoreceptor morphology with age also produce ERG amplitude reductions with preserved b-to-a-wave ratio and waveform configuration.29 Even in the absence of detectable photoreceptor morphologic changes, ERG a- and b-wave amplitudes can be reduced similarly and significantly with age.35 These studies support the possibility that Pcdh15 mutations produce subtle alterations in photoreceptor morphology and function, resulting in the findings we observed.

A decline in retinal function with a preserved b-to-a-wave ratio, normal sensitivity and implicit time, and absence of histologic change are not typical findings in animal models of photoreceptor diseases or RP.36,37 For example the P23H rhodopsin transgenic rat, a model for RP, shows a preferential reduction of the a-wave35,38 with a relative preservation of the scotopic b-wave in early-stage degeneration. The a-wave originates directly from suppression of photoreceptor dark-current by a stimulus, whereas the b-wave reflects postsynaptic bipolar cell activity.39,40 The effects on the a-wave could be explained by a proportional reduction in the product of the ONL width and the ROS length.38 In addition, there may be an augmented bipolar cell response.35

Thus, neither the mutations in this study nor those previously investigated in mice constitute functional models of the RP associated with Usher syndrome.

We considered various other ways that a mutation of Pcdh15 could affect the ERG. One possibility is reduced stimulus intensity at the retina caused by preretinal media opacity or even a loss of quantum catch from reduced rhodopsin density in the rod outer segments. However, these disorders would cause a rightward shift in the intensity–response curve and a loss of sensitivity that was not seen in this study. As shown in Figure 5A, the amplitude curve of the Pcdh15<sup>5<sup>va</sup>j</sup> mice was combined (±SE, n = 3). Two-way ANOVA of cell counts and ROS measurements by retinal position indicate no significant effect of homozygosity for either recessive av mutant allele.

**TABLE 1.** ERG a-Wave and b-Wave Data from Pcdh15<sup>5va</sup>j and Pcdh15<sup>5va</sup>j<sup>b</sup> Homozygous and Heterozygous Mice

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<th>Pcdh15&lt;sup&gt;5va&lt;/sup&gt;j</th>
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<tr>
<td>Age</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; ± SE</td>
<td>k* ± SE</td>
<td>P</td>
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<tr>
<td>5 weeks</td>
<td>Heterozygous</td>
<td>851 ± 67</td>
<td>4.78E-4</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>Homozygous</td>
<td>516 ± 49</td>
<td>7.54E-4</td>
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<tr>
<td>5 months</td>
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<td>697 ± 73</td>
<td>2.80E-4</td>
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<td>(n = 3)</td>
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<td>395 ± 38</td>
<td>4.27E-4</td>
</tr>
<tr>
<td>Pcdh15&lt;sup&gt;5va&lt;/sup&gt;j&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2-Way ANOVA</td>
<td>a-Wave</td>
<td>2-Way ANOVA</td>
</tr>
<tr>
<td>Age</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; ± SE</td>
<td>k* ± SE</td>
<td>P</td>
</tr>
<tr>
<td>5 weeks</td>
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<td>1004 ± 148</td>
<td>4.13E-4</td>
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<tr>
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<td>4.05E-4</td>
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<tr>
<td>5 months</td>
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<td>815 ± 112</td>
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<td>5.12E-4</td>
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<tr>
<td>11 months</td>
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<td>559 ± 58</td>
<td>3.90E-4</td>
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<tr>
<td>(n = 3)</td>
<td>Homozygous</td>
<td>315 ± 32</td>
<td>3.90E-4</td>
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Two-way ANOVA analysis on the effect of genotype, age, and interaction.

* Data from scotopic b-wave fitted with the Naka-Rushton function.
† Amplitude at the maximum intensity (0.6 log cd·s/m²).

**Figure 7.** Quantitation of photoreceptor nuclei in the outer nuclear layer (ONL) and measurements of rod outer segment length (ROS) in homozygous and heterozygous mutant mice. Measurements and counts were performed every 200 μm from the optic nerve (ON) to an area near the peripheral edge of retinal sections taken along the vertical meridian of the eye. Results from Pcdh15<sup>5va</sup>j and Pcdh15<sup>5va</sup>j<sup>b</sup> mice were combined (±SE, n = 3). Two-way ANOVA of cell counts and ROS measurements by retinal position indicate no significant effect of homozygosity for either recessive av mutant allele.
and Pcdh15av-Jfb homozygous mice was shifted vertically downward, rather than displaced along the stimulus-intensity axis. Another possible explanation for this ERG amplitude reduction in the Pcdh15av-5J and Pcdh15av-Jfb homozygous mice involves a passive scaling of intraretinal resistance across which the current of cellular generators is converted to the ERG voltage. This was proposed as a possible explanation for reduced ERG amplitudes in aging mice without detectable retinal morphologic changes. In the normal retina the retinal pigment epithelium layer (RPE) offers the highest resistance to electrical current within the ocular tissues when measuring the ERG with extraocular electrodes. This trans-RPE resistance, termed the “R membrane,” is created by tightly coupled gap junctions between the individual RPE cells. A resistance change would affect the distribution of currents between the retinal pathway and the remote location of the ERG electrodes and result in a scaled amplitude reduction without a waveform change. The fact that there was no expression of Pcdh15 in the RPE detected by immunohistochemistry (Fig. 8) suggests that if the resistance change is a mechanism of ERG reduction, it is more likely that it occurs in another retinal layer, such as the inner–outer limiting membrane. Our immunohistochemistry showed staining of protocadherin 15 in several layers of the retina. A previous study localized this protein to the OLM in mice. The possibility that there is a structural alteration in av mice albeit beyond the level of detection by light microscopy, which could cause a resistance change in the retina, is consistent with observations in zebrafish with reduced levels of protocadherin 15b. These animals had reduced optokinetic and ERG responses associated with improperly arranged photoreceptor outer segments. We are planning a series of measurements to explore whether the transretinal resistance is altered in young av mice. We are also exploring measurements of the c-wave which originates across the RPE as a measure of RPE integrity in these animals. Several other factors, such as age, size, sex, and strain, as well as certain technical variables can affect ERG amplitude measurements. In addition, different mouse strains can exhibit ERG differences in amplitude, waveform, and b-to-a-wave ratio. However, in the present study, the heterozygous and homozygous littermates were matched for all these factors, and both av alleles examined were backcrossed with C57BL/6J for at least six generations.

Humans affected with Usher syndrome type 1 have profound congenital deafness, RP, and vestibular areflexia. We have shown that some missense mutations of PCDH15 cause only nonsyndromic deafness (DFNB23). Four profoundly deaf individuals (aged...
13–44 years) from two DFNB23 families were examined by ERG and funduscopy and were found to have normal retinal function. This is not an isolated observation. In addition to PCDH15, missense or less deleterious mutations in at least two other USH1 genes (USH1C and CDH23) cause nonsyndromic recessive deafness (DFNB18 and DFNB12, respectively).11,46

The av mouse has often been suggested as an animal model for Usher syndrome type 1F, which is associated with mutations in environmental cofactors between mice and humans,48

Pcdh15av-5J on 3, 4, and 17. In full-length in-frame isoforms of

b-wave amplitudes attenuated by 17%, whereas mouse model.51 Two waltzer alleles (Cdh23v-2J and Pcdh15av-5J) mutant retinna, using PB303 directed toward the cytoplasmic domain of Pcdh15, show staining patterns indistinguishable from that in the wild-type (Fig. 8). Thus, in homozygous Pcdh15av-5J mice there is an in-frame isoform of Pcdh15 without exon 17. This isoform may partially compensate in the eye for the loss of one of the protocadherin 15 isoform(s) containing exon 17.

In the retina of homozygous Pcdh15av-5J mice, we found a novel splice variant predicted to encode only 6 of the 11 EC domains, a transmembrane domain, and a cytoplasmic domain. Furthermore, our immunohistochemistry of wild-type and Pcdh15av-ls5J mutant retinas, using PB303 directed toward the cytoplasmic domain of Pcdh15, show staining patterns indistinguishable from that in the wild-type (Fig. 8). Thus, in homozygous Pcdh15av-ls5J mice there is an in-frame isoform of Pcdh15 without exon 17. This isoform may partially compensate in the eye for the loss of one of the protocadherin 15 isoform(s) containing exon 17.

Other suggested mouse models of USH1 also do not have RP. In humans, most reported mutations of MYO7A encoding unconventional myosin VIIa are associated with USH1. Six of the nine alleles of MYO7a mutant (shaker 1) that were examined by ERG show a reduction of 20% to 30% in the a- and b-wave amplitudes compared with control mice. Homozygous MYO7a P6268 mice also show reduced phagocytosis in the retinal pigmented epithelium (RPE) but have otherwise normal retinas.50

In humans, mutations in CDH23 are associated with USH1D and nonsyndromic deafness DFNB12 and waltzer is the mouse model. Two waltzer alleles (Cdh23v and Pcdh15av-5J) have mildly affected ERGs. Cdh23v mutant mice have ERG a- and b-wave amplitudes attenuated by 17%, whereas Cdh23v-2J have faster implicit time for both a- and b-waves, but no retinal histopathology changes have been observed in either mouse. The afer mouse, deficient in harmonin, is another animal model of USH1. This mouse was reported to have peripheral retinal degeneration, but normal ERGs when compared with heterozygous littermate controls.

In summary, our study further characterized the Pcdh15av-5J and Pcdh15av-ls5J allele and presented evidence of significantly attenuated but stable ERGs in both mutants but without a corresponding histopathology of the retina. We speculate that in the retina of the various Ames waltzer mice described to date, physiologically relevant isoforms of Pcdh15 are present and we predict that a mouse that has a nonfunctional Pcdh15 for all the retina-relevant isoforms of Pcdh15 will have RP. This hypothesis can be tested by constructing a mouse with an appropriate deletion of Pcdh15 and examining the retinal phenotype.

In conclusion, some strains of av mice are incomplete models of the pathophysiology of USH1, but will nevertheless be useful in understanding the functions of potentially complimentary isoforms of protocadherin 15 in the retina.45 Our observation also raises the possibility of stimulating the production of a compensatory isoform of human protocadherin 15 in the retina as a therapy for some of the mutant alleles associated with USH1F.

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References
