Assessment of Retinal Structure and Function in Ames Waltzer Mice

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PURPOSE. In humans, mutations in protocadherin 15 are known to result in Usher Syndrome type 1F (USH1F). Patients with USH1F are born with profound hearing loss and have visual problems that develop in late childhood. Based on the phenotypic hearing loss and an associated mutation in protocadherin 15 (Pcdh15), the Ames waltzer mice have been presented as potential models for USH1F. To determine whether the Ames waltzer is a model for retinopathy in USH1F, retinal structure and function were assessed in all four available alleles of the mouse.

METHODS. Activity of both the rod and cone pathways was evaluated by measuring electroretinograms (ERGs) in response to strobe flashes under dark- and light-adapted conditions, respectively. Retinas were processed with standard histochemical procedures, and retinal morphology was examined. The neural retina was dissected from normal pigmented mice at postnatal day (P)0, P5, P7, P20, P40, and P70, and the presence of Pcdh15 was determined by RT-PCR.

RESULTS. The amplitude and implicit time of both the rod- and cone-mediated ERG a- and b-waves were comparable between Ames waltzer mutants and heterozygous littersmates as old as 13 months. No evidence of retinal degeneration or disorganization was detected in mutant mice. Measures of retinal layer thicknesses were similar in mutant and wild-type control animals. Retinal expression of Pcdh15 was observed at all ages examined between P0 and P70.

CONCLUSIONS. Although Pcdh15 is present in neural retina, its role remains unclear. Mutations in the Pcdh15 did not result in retinal abnormalities in the four alleles of Ames waltzer tested in this study. The explanation for the absence of retinal phenotype in the Ames mouse should be helpful in understanding USH1F and developing treatments for this disorder. (Invest Ophthalmol Vis Sci. 2003;44:3986–3992) DOI:10.1167/iovs.02-1009

With the completion of the mouse and human genomes, mutations leading to inherited retinal diseases in humans are being identified at an accelerated rate (see Ret Net, www.sph.uth.tmc.edu/retnet/) provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). The identification of genetic defects associated with human diseases1 has led to the investigation of mice with orthologous genetic mutations.2 Before these mutant mice can be used in investigations into underlying disease mechanisms and to test potential treatments, it is imperative to evaluate the extent to which the animal model develops the correlated human disease. Thus, for any potential mouse model, it is critical first to characterize fully the mouse phenotype in comparison with the corresponding human disorder.

Usher syndrome (USH) is associated with a congenital loss of hearing, vestibular dysfunction, and photoreceptor degeneration and loss of visual function.3 USH has been segregated into general types that differ in severity and in the underlying genetic mutation. The rare type 3 for which the gene4,5 was recently identified is characterized by postlingual progressive hearing loss, progressive visual loss due to retinitis pigmentosa, and variable presence of vestibular dysfunction. For the less-severe type 2, the ERG is reduced, but a small waveform is recordable, and at least one genetic defect has been identified.6 For the most severe form, type 1, visual loss begins in childhood or early teens as night blindness and reduction of the electroretinogram (ERG).7 USH1 has been divided into several subtypes for which seven genes have been identified: USH1A,8,9 USH1B,10 USH1C,11,12 USH1D,13,14 USH1E,15 USH1F,16,17 and USH1G.18

The gene that harbors the Ames waltzer mutation encodes a novel protocadherin, Pcdh15.19 This result is of interest, because a recent study showed that patients with USH1F carry a recessive mutation in the human homologue of Pcdh15.20 Mutations in PCDH15 have been identified in two families, and in both families the mutations segregated with the disease phenotype and generated premature stop codons.21 Consistent with the phenotypes observed in the USH1F-affected families, expression of PCDH15 has been demonstrated in the human retina and cochlea by RT-PCR and immunohistochemistry.16 In the mouse, Pcdh15 is expressed throughout the neural and RPE layers of the retina between embryonic day (E)12 and E16.20 Furthermore, similar to the USH1F-affected families described earlier, mutations in Pcdh15 have been observed in mice with hearing loss associated with severe degeneration of cochlear and saccular sensory structures that is evident in adult animals.22 Herein, we report the retinal phenotype of the Ames waltzer mouse, a potential model for Usher syndrome type 1F (USH1F).
MATERIALS AND METHODS

Animals

The strains of mutant mice studied are listed in Table 1, and the corresponding mutation is illustrated in Figure 1. Heterozygous littermate controls were available for each line. All procedures using animals were approved by the local Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Transgenic Ames waltzer mutant mice, Pcdh15av-J, were obtained from a local breeding colony. The insertional mutant and methods for genotyping this line of mice have been described previously.24 We obtained Pcdh15av-TgN2742Rpw mutants from The Jackson Laboratory (Bar Harbor, ME). All four alleles were maintained in the C57BL/6J genetic background. All mice were genotyped by hybridizing Southern blot analysis containing genomic DNA or by PCR followed by restriction digest. For ERG analysis, mice were examined at either early (2–3.5 months) or late (≥8 months) adulthood. All comparisons between heterozygotes and homozygotes were made between age-matched litters. For morphologic analysis, four homozygous and four heterozygous littermates (three Pcdh15av-3J at 2.5, 7, and 9.5 months of age and one Pcdh15av-TgN2742Rpw at 13 months of age) were examined. Each mouse was anesthetized before eyes were removed and immersion fixed overnight in 10% formaldehyde for paraffin embedding or 2.5% glutaraldehyde and 2% paraformaldehyde for plastic embedding.

Reverse Transcription–Polymerase Chain Reaction

RT-PCR was used to detect expression of Pcdh15 mRNA in the neural retina. The technique consisted of two parts: synthesis of cDNA from RNA by reverse transcription (RT) and amplification of a specific cDNA by polymerase chain reaction (PCR). We chose primers to span a sufficiently large genomic fragment, so that amplification from contaminating DNA would not be possible under standard PCR conditions. Primers 903 and 765 (Fig. 1A) are more than 30 kb apart on the genomic DNA. In addition, to detect PCR amplification due to DNA contamination, reaction without reverse transcriptase (RT minus) was run in parallel with reaction containing reverse transcriptase (RT plus). The RT-minus and RT-plus reactions were used in separate PCRs and are referred to as − and + in Figure 2.

We performed RT reactions with commercial reverse transcriptase (Superscript; Gibco BRL) according to the manufacturer’s protocol. Total RNA was isolated from mouse retina at postnatal day (P)0, P2, P5, P60, and P70. We used C57BL/6J inbred mice, because Ames waltzer mice are maintained on this background. In general, PCR conditions were as follows. 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute. The annealing temperature was adjusted based on the melting temperature (Tm) of the primers. The primers used for RT-PCR were (forward) 5’-CCACTTCTGTCCCTTTGCTAGTCT-3’ and (reverse) 5’-CACAAAGGCATTGGCATTGCT-3’.

Table 1. List of Mutant Mice for ERG Analysis

<table>
<thead>
<tr>
<th>Allele</th>
<th>Homozygous Mice (n)</th>
<th>Heterozygous Mice (n)</th>
<th>Type of Mutation</th>
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<td>In-frame deletion</td>
</tr>
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<td>1</td>
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<tr>
<td>Pcdh15w-3J</td>
<td>3</td>
<td>2</td>
<td>Functional null</td>
</tr>
<tr>
<td>Pcdh15w-TgN2742Rpw</td>
<td>1</td>
<td>1</td>
<td>Functional null</td>
</tr>
</tbody>
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FIGURE 1. Schematic of the Pcdh15 cDNA and the predicted amino acid sequence showing the mutation in the different alleles of Ames waltzer. (A) Shows wild-type (+/+) Pcdh15 cDNA and changes in the cDNA associated with the Ames waltzer mutants (av alleles). Location of forward (903) and reverse (765) primers used for RT-PCR is shown on the wild-type cDNA. All the mutations in the different alleles of Ames waltzer are shown in the open rectangle labeled av alleles: Dash lines: in-frame deletion; arrowhead: transgene insertion site in the Tg allele; (*) the addition of a single base, which results in a frameshift mutation in the av allele. (B) Predicted amino acid sequence of the wild-type Pcdh15 protein and changes in the amino acid sequence as a result of mutation in the different alleles of Ames waltzer.
Figure 2. RT-PCR demonstrating expression of Pcdh15 in total RNA isolated from wild-type and mutant mouse retina. (A) RNA isolated from wild-type retina at P0, P2, P60, and P70. (B) RNA isolated from mutant retina at P70. The alleles Pcdh15-m2J (2J/2J) and Pcdh15-m3J (3J/3J) are shown here. Lane M: DNA size marker, PhiX174 phage DNA digested with HaeIII. (A, B, black arrows) 1.9-kb amplified fragment of Pcdh15. (B, open arrow) The housekeeping gene GAPDH. Reverse transcribed reaction with (+) and without (−) reverse transcriptase used as a template for PCR.

Electroretinography

Electroretinography is routinely used to monitor the progression of many retinal disorders, including RP and USH as the ERG waveform components decrease in amplitude over the course of retinal degeneration. Animals were dark adapted overnight, anesthetized intraperitoneally with a single dose of a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg) diluted in saline, and placed on a heating pad during the recording session. Occasionally, a 20% supplemental dose was administered to stimuli after a 10-minute light adaptation period during the recording session. Pupils were dilated with 1% tropicamide, 1% cyclopentolate hydrochloride, and 2.5% phenylephrine hydrochloride. Recordings were made with a stainless steel wire loop that contacted the corneal surface through a cheek served as ground and reference electrodes, respectively. Responses were amplified (1-1000 Hz), averaged, and stored on a signal-averaging system (UTAS; LKC Technologies, Gaithersburg, MD).

After the initial setup procedures, a dark-adapted intensity-response series was recorded by using a series of Ganzfeld flashes with intensities ranging from −4.2 to 0.5 log cd-sec/m². Cone ERGs were obtained to stimuli after a 10-minute light adaptation period during which the animals were exposed to a steady rod-desensitizing background light of 0.8 log cd-sec/m² presented in the Ganzfeld bowl. Cone responses to a series of flash intensities (−1.22 to 0.52 log cd-sec/m²) were elicited. The amplitude and implicit time of the ERG a- and b-waves were measured conventionally. For the a-wave, amplitude was measured from the prestimulus baseline to the peak. For the b-wave, amplitude was measured from the negative trough of the a- to b-wave peak. Implicit time, or time to peak, was measured from stimulus onset to the a-wave trough and b-wave peak.

Morphology

For histology, after anesthetic overdose with ketamine (160 mg/kg) and xylazine (32 mg/kg) followed by cervical dislocation, eyes were removed and immersion fixed overnight in 10% formaldehyde for paraffin embedding or 2% paraformaldehyde/2.5% glutaraldehyde for plastic embedding. Eyes were processed using standard procedures. For paraffin embedding, retinas were sectioned at 10 μm and stained with hematoxylin and eosin. Retinas embedded in plastic (EM Sciences, Fort Washington, PA), were sectioned at 1 μm and stained with toluidine blue. Digital images were taken of each retina (RT Spot camera; Diagnostic Instruments; Sterling Heights, MI). Measurements were made of each retinal layer, including the outer segments (OS), inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL), in a region approximately 100 μm from the optic nerve. Six sections from each animal were measured and averaged.

Figure 3. Retinal morphology in the Ames waltzer mutant Pcdh15m2J. Vertical retinal cross sections from a heterozygous (A) and a homozygous (B) Pcdh15m2J mutant mouse at 7 months of age taken approximately 100 μm from the optic nerve head. Each layer is labeled as outer segments (OS), inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and the ganglion cell layer (GCL). Scale bar, 25 μm.

Results

RT-PCR Analysis

Previous work in the mouse has shown that Pcdh15 is expressed throughout the neural and RPE layers of the retina between E12 and E16. To confirm expression of Pcdh15 in the mouse retina during postembryonic stages, we isolated RNA soon after birth (P0, P2, P5, and P10) and at adulthood (P40–P70) and used RT-PCR to detect Pcdh15 mRNA. Pcdh15 was expressed in the mouse retina at all ages tested. Four of the time points are shown in Figure 2A. We also tested RNA isolated from Pcdh15m2J (functional null allele) and Pcdh15m2J (in-frame deletion) and compared it to RNA isolated from wild-type retina and the housekeeping gene GAPDH. Results show that Pcdh15 is expressed in these mutants (Fig. 2B). It should be noted that the absence of a product in the RT-minus lane shows that the band seen in each RT-plus lane in Figure 2 was due to amplification of Pcdh15 cDNA by PCR.

Retinal Histology and Anatomy

Figure 3 includes a retinal cross section embedded in plastic from a representative Pcdh15m2J homozygous mutant at 7 months of age and a heterozygous littermate control. This line of Ames waltzer mouse is a functional null, because the mutation introduces a stop codon, thus disabling the functional region. No anatomic defects were noted in any retinas of homozygous or heterozygous mice embedded in either paraffin or plastic. Morphologic analysis was originally conducted with sections from paraffin-embedded retina. Retinal layer thicknesses were confirmed in plastic-embedded sections in which morphology is more reliably preserved (Table 2). Retinas from all animals included all normal retinal layers, and no abnormalities were noted in cellular structure as examined on...
the light microscope level. Photoreceptors were positioned in a normal polar fashion with IS and OS projecting continuously toward the RPE. To quantify this observation, the thickness of each layer (IS, OS, ONL, OPL, INL, IPL, and GCL) was measured in the functional null alleles of Pcdh15av-TgN2742Rpw and Pcdh15av-TgN2742Rpw-mutant and heterozygous littermate retinas at 2.5, 7, 9.5, and 13 months of age. All measurements in mutant and unaffected mice are listed in Table 2. These were comparable in all mice examined and are comparable to previously reported measurements on normal mice.27,28 The slight difference in OS length found in retinas embedded in paraffin compared with those embedded in plastic is most likely an artifact related to the embedding method.

In human cases of USH, photoreceptor degeneration can be detected with a standard ophthalmoscopic examination. Although symptoms of USH are manifested by the time of puberty in humans, aged animals were also examined to rule out late onset of retinal defects in mice. No evidence of cellular degeneration was found in any Ames waltzer mouse examined, and no differences were noted in aged compared with control animals.

### ERGs of Ames Waltzer Mutants

There are several lines of mutant mice in which abnormal function is detectable by ERG, even though no anatomic defects are noted.27,29,30 To determine whether the mutation in Pcdh15 in mice results in functional defects, electroretinography was used to evaluate rod- and cone-mediated function.28 ERGs were recorded to a series of increasingly intense flashes. Figure 4A presents a representative ERG recorded in response to the highest-intensity flash under dark-adapted conditions for each line examined and a corresponding heterozygous littermate. The peak amplitude of the ERG a- and b-waves in each mouse line is plotted in Figures 4B-E. Responses recorded under these conditions reflect primarily activity of the rod pathway.31 At low stimulus intensities, the responses were dominated by a large corneal positive waveform or b-wave. At higher stimulus intensities, a corneal negative a-wave became apparent that preceded the b-wave. As shown in Figures 4B-E, both the a- and b-waves increased in amplitude as the stimulus intensity increased. In each mutant mouse, the ERG waveforms overlapped those of heterozygous animals. Implicit times for the a- and b-wave were found to decrease with increasing flash intensity and were comparable in homozygous and heterozygous mice (data not shown).

To evaluate the cone pathway, responses to flashes of light were recorded under light-adapted conditions created by the presence of a background light of 0.8 log cd-sec/m² on which stimulus flashes were presented. This background light saturated all rod responses leaving only activity generated by cones and retinal cells in the cone pathway. Responses to a series of flashes of increasing intensity were recorded. Representative responses to the highest flash intensity are shown in Figure 5A. These responses were composed primarily of a positive b-wave of small amplitude at all flash intensities and almost no a-wave, compared with dark-adapted responses. In Figures 5B-E, amplitudes are plotted as a function of stimulus intensity for each mutant mouse and the corresponding heterozygous control, further showing no differences between homozygous and heterozygous animals. As in normal animals, response amplitude increases and implicit time decreased (data not shown) as a function of light intensity.

### DISCUSSION

Usher syndrome is the most common cause of sensory impairment in which deafness and blindness occur together. Affected persons are born with hearing loss and have progressive pigmentary retinopathy leading to blindness that develops in the second through fourth decades of life.3 This progressive retinal degeneration is apparent by fundus examination and dramatic reductions in ERG a- and b-waves over the course of the disorder.25,32 Clinically subdivided into types 1 to 5 based on the degree of deafness and the presence of vestibular dysfunction, USH1 is the most severe. Affected persons are born with profound deafness and have visual problems that develop in late childhood. Approximately 70% of USH1 is caused by mutations in myosin 7A (USH1B), with the second largest contribution to the USH1 genetic load at the USH1D-USH1F region on chromosome 10.33 A recent study showed that patients with USH1F carry a mutation in the human homologue of Pcdh15,16,17 a novel protocadherin gene associated with the Ames waltzer mutation in mice.19 Furthermore, expression of PCDH15 and Pcdh15 in the human and mouse retina, respectively, suggests a potential role for PCDH15/Pcdh15 in the eye. Because Ames waltzer mice have an orthologous mutation and marked evidence of auditory dysfunction,19,24,34 these mice were evaluated as a potential animal model for USH1F.

We have shown by electroretinography and light microscopy that retinal abnormalities associated with USH1F are not present in Ames waltzer mutant mice with orthologous mutations. Although hearing loss and vestibular dysfunction due to hair cell degeneration have been well established in these mice,24,34 no retinal abnormalities were detected. The possibility that retinal involvement might occur at a late onset was evaluated in 8- to 14-month-old mice. However, retinal morphology and ERG waveforms were comparable between homozygous and heterozygous littermates for all four alleles of Ames waltzer at all ages tested. In the Pcdh15av-TgN2742Rpw line of mice, the peak amplitude of light-adapted ERG waveform at the highest-intensity flash for the mutant was actually larger than the heterozygous control (Figs. 5A, 5E). However, this difference was not consistent throughout all flash intensities and was only present at the highest intensity where, in general, the greatest level of intersubject variability exists.26 Furthermore, the smaller response in both of these mice compared with the other three lines is most likely attributable to the advanced age of 14 months at the time of ERG.35 Differences in the pattern of expression and function of Pcdh15 in mice and humans may explain the absence of defect...
in the mutant mice. Although detailed characterization of the temporal and spatial expression pattern of human and mouse protocadherin 15 is needed to evaluate this possibility, results of previous work on another mouse are similar. Shaker-1 mice possess a mutation in the orthologous mouse myosin VIIa gene, responsible for USH1B in humans, but do not have the normally associated retinal defect. El-Amraoui et al. explained this by showing that myosin VIIa was not expressed in adult mouse photoreceptors but was expressed in human photoreceptors.

Protocadherins, a subclass of the cadherin superfamily, are expressed in neuronal tissues where they are thought to be involved in cell adhesion. However, details regarding their seemingly diverse functions remain unclear in both human and mouse. In the central nervous system, they are probably involved in synapse formation and maintenance, as they are spatiotemporally regulated during morphogenesis and have been shown to be localized at synaptic sites (reviewed in Refs. 39, 40). In mice with mutations in Pcdh15, hair cell stereocilia develop with orientation abnormalities, suggesting a role for Pcdh15 in structural polarity. Because the retina is also a highly polarized structure similar defects might be expected in the retina of the Ames waltzer mutant mice. Detailed ultrastructural analysis (electron microscopy) may be required to identify these defects.

It is possible that differences may exist in terms of how the gene is mutated. A protocadherin includes up to seven extracellular calcium-binding domains, one transmembrane domain, and a unique intracellular domain. The intracellular domain contains two well-conserved proline rich regions and thus probably represents the functional element. Two of the four mutants examined, Pcdh15<sup>avr</sup>-TgN<sup>2742Rpw</sup> and Pcdh15<sup>avr</sup>-3J, introduce stop codons near the extracellular portion of the transmembrane domain of Pcdh15. This results in a truncation at the transmembrane domain, making these mice functional nulls. However, the retinal phenotype of these animals did not differ from the Pcdh15<sup>avr</sup>-J and Pcdh15<sup>avr</sup>-2J, both of which are in-frame deletions. There is no report of a dominant-negative

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932921/)

**Figure 4.** Dark-adapted ERG waveforms (A) and a- and b-wave amplitudes (B-E). Representative waveforms from homozygous (thick trace) and heterozygous (thin trace) littermates for each mouse line are shown slightly offset from each other for comparison (A). Peak a- and b-wave amplitudes are plotted as a function of strobe flash intensity (B-E). Each mouse type is plotted separately comparing homozygous (closed symbols) and heterozygous littermates (open symbols) Pcdh15<sup>avr</sup>-J (B), Pcdh15<sup>avr</sup>-2J (C), Pcdh15<sup>avr</sup>-3J (D), and Pcdh15<sup>avr</sup>-TgN<sup>2742Rpw</sup> (E). For lines in which less than three homozygous or heterozygous mice were tested individual data are plotted. Otherwise, averaged data are plotted with error bars showing SEM.
mutation in the mouse Pcdh15 gene in the literature. It remains to be seen whether a dominant-negative mutation in Pcdh15 affects retinal function in mice.

Although the human USH1F mutation is 94% and 53% identical with the mouse sequence Pcdh15 DNA sequence in the extracellular and intracellular domains, respectively, differences in retinal phenotype may reflect underlying differences in the role of this protein in humans and mice. Mutations in the gene encoding myosin VIIa cause deafness and retinitis pigmentosa in humans (USH1B) and deafness and vestibular dysfunction in mice (shaker-1). In the mouse retina, myosin VIIa protein has been detected in retinal pigment epithelial cells but not in photoreceptors. In the adult human retina, myosin VIIa protein was present in both RPE and photoreceptors. Although several mutations linked to the shaker-1 locus have been identified to date, there is no report of retinal degeneration in any of the shaker-1 lines. A report was made showing minor deficits in retinal function in five of the nine alleles of shaker-1, appearing as a reduction in ERG amplitudes, not threshold, under mesopic conditions. However, these reductions do not resemble results obtained in mouse models of RP.

Based on the protocol described by Libby and Steel, the defect described in these mice may reflect a slowing of recovery after the presentation of stimuli at high light levels. Thus, we tested the ability of the Ames waltzer mice to recover after a high-intensity flash by using ERG but found no differences between homozygous and heterozygous littermates for all four lines of mice (data not shown). It is possible that the retinal disorder does not progress to detectable levels due to the short life span of the mouse compared with humans.

Finally, the phenotypic outcome of a particular “disease gene” in mice has been shown to vary according to the genetic background on which it resides due to the presence of modifier genes. All the Ames waltzer alleles described here were maintained in the C57BL/6J background. Whether a retinal phenotype may be observed when the Ames waltzer mutation is expressed in a different genetic background remains to be seen. If indeed a modifier gene is involved in the retinal phenotype rescue, these mice could be valuable in our attempt to understand the pathologic course of the disease in Usher syndrome 1F.
Acknowledgments

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