Defects in Vestibular Sensory Epithelia and Innervation in Mice with Loss of Chd7 Function: Implications for Human CHARGE Syndrome

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ABSTRACT

CHD7 is a chromodomain gene mutated in CHARGE syndrome, a multiple anomaly condition characterized by ocular coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia, and ear defects including deafness and semicircular canal dysgenesis. Mice with heterozygous Chd7 deficiency have circling behavior and semicircular canal defects and are an excellent animal model for exploring the pathogenesis of CHARGE features. Inner ear vestibular defects have been characterized in heterozygous Chd7-deficient embryos and early postnatal mice, but it is not known whether vestibular defects persist throughout adulthood in Chd7-deficient mice or whether the vestibular sensory epithelia and their associated innervation and function are intact. Here we describe a detailed analysis of inner ear vestibular structures in mature mice that are heterozygous for a Chd7-deficient, gene-trapped allele (Chd7Gt/H11001). Chd7Gt/H11001 mice display variable asymmetric lateral and posterior semicircular canal malformations, as well as defects in vestibular sensory epithelial innervation despite the presence of intact hair cells in the target organs. These observations have important functional implications for understanding the clinical manifestations of CHD7 mutations in humans and for designing therapies to treat inner ear vestibular dysfunction. J. Comp. Neurol. 504:519–532, 2007. © 2007 Wiley-Liss, Inc.

Indexing terms: mouse mutant; inner ear; innervation; sensory epithelia

CHD7, a recently identified chromodomain gene, is mutated in human CHARGE syndrome, a multiple congenital anomaly condition characterized by ocular coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia, and ear abnormalities including deafness and vestibular dysfunction, and brainstem/cranial nerve dysfunction (Lin et al., 1990; Byerly and Pauli, 1993; Vissers et al., 2004). CHARGE affects 1 in 8,500 to 1 in 12,000 births (Kallen et al., 1999; Issekutz et al., 2005). Partial or complete hypoplasia of the semicircular canals is present in almost all patients with CHARGE (Tellier et al., 1998), making the vestibular phenotype a reliable and consistent manifestation of CHARGE syndrome. Current diagnostic criteria for CHARGE include semicircular canal dysgenesis as a major criterion (Verloes, 2005). Highly variable penetrance of other CHARGE features has been noted, even in monozygotic twins, suggesting epigenetic influences on gene expression and/or function (Johnson et al., 2005).

Between 60 and 80% of CHARGE patients are heterozygous for CHD7 mutations, including nonsense, deletion, and missense mutations; however, no correlations have
been found between specific mutations and associated phenotypes (Vissers et al., 2004; Sanlaville et al., 2005; Aramaki et al., 2006; Jongmans et al., 2006; Lalani et al., 2006). Human CHD7, located on chromosome 8q12.1, is a member of the family of chromodomain helicase DNA-binding genes, which code for proteins that are presumed to affect chromatin structure and gene expression (Woodage et al., 1997). The CHD7 protein may regulate expression of genes that are essential for development of the inner ear and other CHARGE-affected tissues (Bosman et al., 2005; Lalani et al., 2006).

We have established a mouse model for CHARGE that enables us to study vestibular development, pathology, and potential for future interventions (Hurd et al., 2007). Heterozygous $\text{Chd7}^{\text{Gt}/+}$ mice were generated from gene-trapped embryonic stem cells containing a β-galactosidase reporter cassette (Hurd et al., 2007). Heterozygous $\text{Chd7}^{\text{Gt}/+}$ and other $\text{Chd7}$-deficient mice are a good model of CHARGE syndrome, because they exhibit semicircular canal defects, circling behaviors, variable hearing loss, and increased numbers of hair cells in the organ of Corti, whereas homozygous $\text{Chd7}^{\text{GtGt}}$ mice are embryonic lethal (Kiernana et al., 2002; Bosman et al., 2005; Hurd et al., 2007). Mature inner ear phenotypes in $\text{Chd7}$-deficient mice are less well understood, and there is no information about the structure and function of vestibular epithelia and innervation with $\text{Chd7}$ deficiency.

The inner ear is a complex organ responsible for hearing and balance. The sensory epithelium and the primary neurons for hearing and balance develop from an ectodermally derived placode that forms the otocyst (Fritzsch et al., 2005). A series of invaginations, fusion events, and differential growth activities lead to the formation of the hearing organ (cochlea) and balance organs (vestibular system) (Fekete, 1996). The vestibular system is comprised of five sensory patches: the maculae of the saccule and utricle and one crista ampullaris for each of the three semicircular canals (anterior, posterior, and lateral) (Barald and Kelley, 2004; Desai et al., 2005a,b). The sacculus and utricle perceive linear acceleration through a patch of sensory epithelium called the macula (Desai et al., 2005b). Each of the three semicircular canals perceives angular acceleration through a sensory patch called the crista ampullaris. Together, they function to maintain head position and balance.

At one end of each canal, a bulbous cavity called the ampulla becomes visible by embryonic day 15 in mice (Morsli et al., 1998). Each of the three ampullae contains a sensory epithelium organ called a crista ampullaris, which contains sensory hair cells that connect with vestibular nerve endings (Goldberg et al., 1985; Desai et al., 2005a). All five vestibular sensory epithelia consist of a mosaic of mechanosensitive hair cells and non-sensory supporting cells. Each crista is found in a bulbous expansion that becomes visible at one end of the canal by embryonic day 15 in the mouse.

Here we report the results of an extensive evaluation of mature $\text{Chd7}^{\text{Gt}/+}$ inner ears. We observed several abnormalities in the gross morphology of $\text{Chd7}^{\text{Gt}/+}$ semicircular canals and in the vestibular sensory epithelia and their innervation. These data indicate a requirement for $\text{Chd7}$ in vestibular development and innervation, with important consequences for designing rational therapies for CHARGE-related balance disorders.

### MATERIALS AND METHODS

#### Mice

$\text{Chd7}^{\text{Gt}/+}$ mice were generated and characterized as previously described (Hurd et al., 2007). $\text{Chd7}$ mutant (Gt) and wildtype alleles were also genotyped as previously described (Hurd et al., 2007). Mice were maintained by backcrossing with C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) or 129S1/SvImJ mice (Jackson Laboratory) to generation N2. Mice analyzed were male or female adults, ranging from 3 weeks to 11 months in age. All procedures were approved by The University of Michigan University Committee on Use and Care of Animals (UCUCA).

$\text{Chd7}^{\text{Gt}/+}$ (n = 25) and wildtype (n = 8) littermate mice were studied. Gross morphologic examination was performed on all ears. Among these mice, 19 $\text{Chd7}^{\text{Gt}/+}$ mice and four littermate wildtype mice were designated for epifluorescence analysis; 6 $\text{Chd7}^{\text{Gt}/+}$ mice and three littermate wildtype mice were designated for electron microscopy analysis. Mice from the entire range of ages (3 weeks to 11 months) were used for each of the analyses and were found to have similar morphologies, indicating that no obvious developmental or aging changes occurred within this age range.

#### Dye filling of inner ears

Mice were anesthetized with a mixture of xylazine (0.0024 mg/kg) and ketamine (0.012 mg/kg) and then decapitated. Temporal bones were removed and fixed locally by opening the oval and round windows, opening a perforation in the apical end of the cochlea, and gently perfusing 4% paraformaldehyde. Once fixed, ears designated for epifluorescence were instilled with fast green dye (100 mg in 10 ml phosphate-buffered saline [PBS]; Sigma, St. Louis, MO) through the oval window with a microcanula connected to a 1-ml syringe. Once filled with dye, ears were observed and photographed on a Leica MZ-12 stereomi-
microscope with a digital SPOT Insight QE camera. Once dye distribution in the inner ear was recorded, the bone was dissected away to expose the membranous labyrinth and surrounding soft tissues. The dye appeared to fill both the endolymphatic and perilymphatic fluid spaces of the inner ear, likely due to ruptured membranes that resulted in connections between the two spaces. Ears that were dye-filled were further used for epifluorescence as described below. No dye filling was performed on ears designated for electron microscopy.

**Epifluorescence analysis of vestibular sensory epithelia**

Tissues were permeabilized with 0.3% Triton X-100 in PBS (pH 7.3-7.4) for five minutes. After rinsing in PBS, they were treated with Image-iT® FX signal enhancer (Invitrogen, Carlsbad, CA) for 30 minutes. After additional rinsing, some specimens were blocked in normal goat serum (1%) and bovine serum albumin (0.1%) for 30 minutes. To label 200-kDa neurofilament, tissues were incubated for 1 hour in monoclonal mouse anti-neurofilament 200 antibody (Sigma, no. N0142) diluted 1:50. The carboxyterminal tail segment of enzymatically dephosphorylated pig neurofilament H-subunit was used as the immunogen. This antibody localizes in neurofilaments of molecular weight 200 kDa in rat spinal cord extract by immunoblotting (manufacturer’s technical information). The pattern of anti-neurofilament staining in our study is similar to that previously reported with other anti-neurofilament antibodies in inner ear neurons (Dau and Wenthold, 1989; Berglund and Ryugo, 1991).

After rinsing, the tissues were incubated for 30 minutes with rhodamine-conjugated goat anti-mouse secondary antibody (Jackson Immunochemicals, West Grove, PA, code no. 115-025-146, lot # 61962) diluted 1:200. To label F-actin, Alexa Fluor 488-conjugated phalloidin (Invitrogen, cat. no. A12379) diluted 1:300 was added to the secondary antibody. Phalloidin labels actin, which is abundant in stereocilia and cell junctions, and enhances identification of cell types in the sensory epithelium (Meites and Raphael, 1994; Raphael et al., 1994). Tissues were mounted on microscope slides with ProLong Gold Antifade (Invitrogen). Tissue analysis and epifluorescence photomicroscopy were performed on a Leica DMRB microscope with 40× and 100× objective lenses.

**Scanning electron microscopy (SEM)**

For SEM analysis, temporal bones were removed, the cochleae were exposed, and the samples were fixed in 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolein in 0.1 M cacodylate buffer (pH 7.2-7.4) (Osborne and Comis, 1991). Tissues were processed by using the OTOTO method (Osborne et al., 1984). Samples were dehydrated in increasing concentrations of ethanol and critical point dried with CO2 in a SamDri 790 (Tousimis, Rockville, MD). Samples were examined on an Amray 100B microscope and photographed digitally. Adobe (San Jose, CA) Photoshop 9.0 was used to colorize and merge immunofluorescence images and to adjust contrast, brightness, size, and sharpness of all images.

**RESULTS**

Chd7+/− mice exhibit circling behaviors, embryonic semicircular canal defects, and β-galactosidase reporter expression in CHARGE-relevant tissues; homozygous Chd7+/− embryos do not survive beyond embryonic day 10.5 (Hurd et al., 2007). A full description of the Chd7+/− mouse is provided elsewhere (Hurd et al., 2007). Briefly, the Chd7+/− allele contains retroviral vector sequences upstream of the ATG-containing exon 2 of Chd7. This abolishes proper splicing between exons 1 and 2 and is predicted to disrupt normal CHD7 protein because the mutant allele lacks upstream sequences necessary for ribosomal entry and translation. Phenotypes of Chd7+/− mice resemble other heterozygous Chd7-deficient mice (Boisman et al., 2005), demonstrating that they are a good model of CHARGE syndrome.

**Variable defects in lateral and posterior semicircular canals of Chd7+/− mice**

For a full description of normal inner ear morphology including the types of hair cells, innervation, and ampulla and canal morphology, we refer the reader to Desai et al. (2005a,b) and Goldberg et al. (1990). The overall morphology of the bony and membranous labyrinths from right and left ears of Chd7+/− mice (n = 50 ears) and wildtype littermates (n = 16 ears) was examined stereoscopically. All wildtype mice had normal inner ear morphology, as detailed in Figure 1A,B (see list of abbreviations). Inner ears were injected with fast green dye through the oval window. The fluid spaces in the normal inner ear are continuous through the cochlea and the three semicircular canals that open into the vestibule. One end of each canal widens to form the ampulla. The non-ampullated end of the lateral canal opens directly into the vestibule. The non-ampullated ends of the anterior and posterior canals join to form the common crus before entering the vestibule. The anterior semicircular canal and its corresponding ampulla were present in all Chd7+/− ears and appeared normal.

Abnormalities of lateral canal morphology were observed in all Chd7+/− inner ears examined (n = 50; Table 1). Defects ranged in severity from mild alteration of canal shape to complete truncation. In some mice, the lateral canals were truncated (Fig. 1C), and others appeared as complete, patent loops with a smaller diameter, leading to a more circular morphology compared with wildtype (“loop” canal, n = 18/50; Fig. 1D–F). Marked variability was observed in the diameter of the arc of the canal and of the canal lumen in these ears. Other lateral canals were found to have a point of luminal narrowing along their arcs, which, on dye filling, resembled a bird’s beak (“bird-beak” canal, n = 13/50; Fig. 1F–J). Most commonly, the beak was noted at the non-ampullated end of the canal, just proximal to its opening into the vestibule, although occasionally it was found near the midsection of the canal or, in two ears, near the ampullated end. The constriction was mild in some ears, but in others, the connection between the non-ampullated end and the vestibule was narrow and could be confirmed only by dye instillation (Fig. 1G) and/or epithelial dissection (Fig. 1H).

The most severe lateral canal defect observed in Chd7+/− mice was the complete truncation of the non-ampullated end (n = 19/50; Fig. 1C,1D). The length of the canal proximal to the truncation varied from greater than half of the usual canal circumference to no more than a tiny bud off the ampulla. On epithelial dissection, the lateral ampulla was present in most ears. In four of 50 Chd7+/− ears, both the ampulla and corresponding crista
Fig. 1. Lateral canal defects in Chd7<sup>−/−</sup> inner ears. Stereoscopic images of the vestibular portion of inner ears from wildtype (A,B,D) and mutant Chd7<sup>−/−</sup> (C,E–J) mice. A,B: Fast green dye-filled right ear from a 21-day-old wildtype mouse, with the ventral aspect pointing down and anterior to left (A) or back (B). The anterior canal (ac) is round and has one ampullated end (aa). The non-ampullated end of the anterior canal meets the oval-shaped posterior canal (pc) to jointly form the common crus (cc). Part of the lateral canal (lc) is visible. The lateral canal enters directly into the vestibule (v). C: The lateral canal in a left Chd7<sup>−/−</sup> ear, before dye-filling, is truncated (lt) at the midpoint of the canal arc, at the non-ampullated end. D: An elliptical-shaped wildtype right lateral canal within its bony capsule, before dye-filling. E: A loop-shaped lateral canal from a left Chd7<sup>−/−</sup> ear is smaller and more circular than in wildtype (D). F: The lateral canal from a right Chd7<sup>−/−</sup> ear demonstrates a bird-beak (bb) deformity, with a narrowing of the canal lumen near the non-ampullated end. G: A severe right bird-beak lateral Chd7<sup>−/−</sup> canal tapers toward the vestibule, such that dye barely penetrates the narrowing membranous vestibular tissues. H: After bone removal from the canal in G, a thin epithelial connection (ec) is seen between the end of the canal and the vestibule (v). The lateral (lcr), anterior cristae (acr), utricle (ut), and corresponding vestibular nerve bundles (vn) are seen. I: In the left Chd7<sup>−/−</sup> ear, the lateral ampulla is absent (arrowhead) and the lateral canal is truncated (lt). J: In another left Chd7<sup>−/−</sup> ear, the lateral ampulla is absent and is replaced by a narrow epithelial lumen (arrowhead). The membranous connection between the bird-beak deformity and the vestibule is present at the other end of the canal epithelium (white arrow). Scale bar = 1 mm in A (applies to A–C) and D–J.
were absent (Fig. 1I,J). Of the four Chd7Gt/H11001 ears missing the lateral ampulla, the lateral semicircular canal formed a complete but smaller loop in one ear, a bird-beak in another ear, and was truncated in the other two ears.

Variability in lateral semicircular canal defects was frequently observed not only between different Chd7Gt/H11001 mice but also between the right and left ears of the same mouse (Tables 1, 2). Of the 13 mice with at least one loop deformity of the lateral canal, 5 had bilateral loops, 6 had a bird-beak deformity of the contralateral canal, and 2 had a contralateral lateral canal truncation. Variability in loop size was often noted between the ears of mice with bilateral loops. Of the 12 mice with at least one lateral canal truncation, 7 had bilateral truncations, 3 had a contralateral bird-beak, and 2 had a contralateral loop. The extent of lateral canal truncation also varied between ears in some mice. The intra-animal variability was higher for the beak morphology than for the loop and truncated lateral canals. Only 2 of the 11 mice with at least one beak had bilateral beaks. Again, the site and degree of narrowing was not identical between the ears of those mice with bilateral beaks. No mice were missing the lateral ampulla in both ears. In total, 14/25 mice had similar, though not necessarily identical, malformations of the lateral canal between ears, and 11/25 had different malformations of the lateral canal between ears.

### TABLE 1. SUMMARY OF SEMICIRCULAR CANAL AND SENSORY EPITHELIAL DEFECTS IN CHD7Gt/H11001 MUTANT MICE

<table>
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<tr>
<th>Mouse no.</th>
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<th>Lateral</th>
<th>Post</th>
<th>Ant</th>
<th>Tech. used</th>
<th>Lateral morpho</th>
<th>Posterior innervation</th>
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Abbreviations: abs, absent; amp., ampulla; IF, immunofluorescence; —, not done; nl, normal; L, left ear; pres, present; rud., rudimentary; R, right ear; SEM, scanning electron microscopy; sm., small; trunc, truncation; red, reduced.

### TABLE 2. CORRELATIONS BETWEEN LATERAL SEMICIRCULAR DEFECTS AND CIRCLING BEHAVIORS IN CHD7Gt/H11001 MICE

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<th>No. of non-circlers</th>
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</table>

Total no. of mice: 25
We also noted dysplasia of the posterior canal in Chd7Gt/H11005 mice. A complete posterior canal was present in the majority of Chd7Gt/H11001 ears (n = 42/50); however, the morphology varied from that of wildtype littermates. Due to the small size and irregular surfaces of the specimens, it was difficult to orient the posterior canal properly to take accurate measurements of the canal size. Subjectively, the canal arc diameter appeared smaller, the canal lumen wider, and the common crus shorter, such that the canal assumed a more circular, lumen wider, and the common crus shorter, such that the canal arc diameter appeared smaller, the canal lumen wider, and the common crus shorter, such that the canal appeared as a cavity of almost normal width, associated with epithelial lumen (Fig. 2B–D). In other cases, it appeared as a patulous common crus formed a markedly widened bony cavity that, on micro-CT inspection, a dense white nerve bundle was observed to contain a patulous common epithelial lumen (Fig. 2B–D). In other cases, it appeared as a cavity of almost normal width, associated with a small, truncated out-pouching from the superior end, where the posterior canal would otherwise be (n = 2/8, not shown). On gross observation during dissection, the posterior crus was always present within the bony posterior ampulla, regardless of the presence of a distinct posterior canal (n = 50/50; Fig. 2D). The size and shape of the posterior canal often varied between ears of the same animal and was bilaterally absent in only one of the seven mice with absence of a distinct posterior canal (Table 1).

Sensory epithelium abnormalities in Chd7Gt/H11001 mice

Following stereoscopic examination, the three cristae and the utricular and saccular maculae from Chd7Gt/H11001 (n = 31) and wildtype littermate (n = 7) ears were stained for actin and neurofilament and viewed as whole mounts with epifluorescence. This preparation method allows for detailed analysis of the entire sensory epithelium. Stereocilia and cell adherens junctions contain actin filaments, so actin was used as a marker to delineate surface morphology, cell borders, and stereocilia presence and configuration. Similarly, neurofilament staining was used to define the nerve fibers and terminal calyces that surround the flask-shaped, type I vestibular sensory cells in each organ. Axons of the vestibular branch of the eighth cranial nerve form calyces around type I hair cells and button-type terminals at the basal end of type II hair cells (Goldberg et al., 1985; Desai et al., 2005a,b). Due to the challenging nature of the tissue preparation, not all five sensory organs were preserved in every specimen (Table 1).

Each of the five vestibular organs exhibited sensory cells with intact stereocilia organized in a step-like fashion, surrounded by supporting cells (Fig. 3). Like its corresponding anterior ampulla, the anterior crista was present in all Chd7Gt/H11001 (n = 27) and wildtype (n = 5) ears examined (Fig. 3). The anterior cristae of both Chd7Gt/H11001 and wildtype mice assumed a typical saddle shape and were comprised of sensory cells with a normal stereocilia bundle configuration (Fig. 3). In both Chd7Gt/H11001 and wildtype mice, the sensory epithelium was completely divided at its midpoint by a non-sensory area, the septum cruciatum, which is characteristic of murine anterior and posterior cristae (Fig. 3A,D). On stereoscopic brightfield inspection, a dense white nerve bundle was observed to enter the anterior crista on the undersurface of both Chd7Gt/H11001 and wildtype ears (Fig. 1H and data not shown). Neurofilament staining of Chd7Gt/H11001 and wildtype ears demonstrated a dense array of calyx-shaped nerve terminals that surrounded the cell bodies of stereocilia-bearing sensory cells (Fig. 3B,E). In deeper planes of the crista, the nerve terminals could be traced back toward branch points and common nerve fibers (data not shown). An area of dense neurofilament staining was noted in Chd7Gt/H11001 and wildtype ears at the mid-portion of the septum cruciatum (Fig. 3E,F); a similarly dense area was noted when the deepest, most central portions of the cristae beneath the stereocilia-bearing surface were examined (data not shown). The anterior cristae of Chd7Gt/H11001 mice resembled those of wildtype mice in surface morphology and innervation pattern. Subjectively,
they appeared to be of similar size, but this was not measured.

The lateral crista ampullaris was present in all wildtype ears and in all Chd7Gt/H11001 ears that had a lateral ampulla (n = 21 Chd7Gt/H11001; n = 8 wildtype ears; Fig. 4). No lateral sensory epithelium was present in the four Chd7Gt/H11001 ears lacking a lateral ampulla. The shape, surface morphology, and neurofilament staining pattern of lateral cristae from Chd7Gt/H11001 mice resembled those in wildtype ears (Fig. 4). Unlike posterior and anterior cristae, lateral cristae of the wildtype lack a septum cruciatum. All Chd7Gt/H11001 ears also lacked a septum cruciatum, but one ear did have a small, hair cell-free patch in the lateral crista ampullaris reminiscent of a septum cruciatum. This may be a rare variant of unclear significance. On high-magnification view, the Chd7Gt/H11001 lateral crista hair cell bundle configuration was normal, and nerve calyces were clearly associated with sensory cells (Fig. 4C,F). The width of the lateral crista in some Chd7Gt/H11001 ears appeared smaller than in wildtype ears on both stereoscopic examination of epithelium and on whole mount preparation, and the vestibular nerve bundle entering the crista was visibly smaller than wildtype on stereoscopy. These findings were present independent of the degree of severity of the lateral canal dysplasia.

By immunofluorescence, the posterior crista ampullaris was present in all Chd7Gt/H11001 (n = 26) and wildtype (n = 8) ears examined, and abnormalities of the morphology and/or innervation of the posterior crista ampullaris were noted in all 26 Chd7Gt/H11001 ears. The morphologic defects ranged from an apparent reduction in the surface area of the saddle-shaped sensory epithelium to a flattening of the saddle into a round, patch-like epithelium (Fig. 5). The posterior septum cruciatum appeared normal in three Chd7Gt/H11001 ears and was absent in two Chd7Gt/H11001 ears, both of which had a small, patch-like sensory epithelium (Fig. 5). The other 21 Chd7Gt/H11001 ears had a small area devoid of stereocilia at the edge of the sensory epithelium, which resembled a rudimentary septum cruciatum (Fig. 5K). This area was located near the center of the crista. In most instances, only one such area was present. When two hair cell-free patches were present, the areas were located on opposite (paramedial) positions in the crista. On high-magnification of these ears, the hair cell bundle configuration and orientation appeared normal (data not shown).

Posterior cristae from Chd7Gt/H11001 mice also displayed abnormalities in neurofilament staining. There was no evidence of any labeling of the terminal nerve calyces or axons in the majority of the Chd7Gt/H11001 posterior cristae (n = 18/26; Fig. 5). In this group of ears, when a complete or rudimentary septum cruciatum was present, the typically dense neurofilament staining was still noted in the plane beneath the epithelial surface in this region (Fig. 5). The remaining cristae demonstrated some terminal caly-
neurofilament staining pattern was noted in several ears cruciatum, and in most with rudimentary septa. A normal patch-like epithelia, in one with a complete septum cruciatum and epithelial morphology are not sufficient for posterior cristae innervation, and normal innervation does not guarantee normal epithelial morphology. Also of note, a normal posterior sensory epithelium in the absence of neurofilament staining was seen in mice as old as 11 months. Absence of neurofilament staining was noted bilaterally in five mice and only unilaterally in three.

The utricle and saccule were present in all Chd7^{Gt/+} and wildtype ears examined (utricle: n = 24 Chd7^{Gt/+}, n = 6 wildtype; saccule, n = 9 Chd7^{Gt/+}, n = 4 wildtype). The shape, hair cell morphology, and innervation pattern of the utricular and saccular maculae of Chd7^{Gt/+} mice was indistinguishable from wildtype animals (Fig. 6; Chd7^{Gt/+} utricle only). Otoconia appeared normal on all specimens as bright, refractile particles on stereoscopy (Fig. 1K–M). No apparent difference in utricular size was noted between wildtype and Chd7^{Gt/+} ears. The saccule in the mouse is difficult to obtain in its entirety; thus we were unable to make general conclusions regarding saccular size.

**Fine surface morphology demonstrated by SEM**

Vestibular epithelia from Chd7^{Gt/+} (n = 8) and wildtype (n = 3) ears were examined by SEM (Fig. 7). The pattern of stereocilia at the apical surface of the vestibular hair cells of all five sensory organs appeared normal in Chd7^{Gt/+} mice. Specifically, the stereocilia bundles appeared to be organized in the typical staircase pattern. The kinocilium was consistently present and was located on the side of the bundle displaying the longest stereocilia. Hair cell density appeared similar to that of wildtype. One Chd7^{Gt/+} lateral crista was found to have a paramedial circular patch of microvillus-bearing cells that resembled those seen in the septum cruciatum of the anterior and posterior cristae of wildtype mice (not shown).

**Correlation with behavioral phenotypes**

Nine of the 25 closely observed Chd7^{Gt/+} mice had circling behaviors. Circling mice appeared to circle rapidly and bidirectionally and exhibited this behavior for much of their waking hours, although they stopped to eat, rest, and procreate. Circling behavior in mice is often considered indicative of vestibular dysfunction; thus we attempted to correlate behavioral phenotypes to the observed inner ear defects of Chd7^{Gt/+} mice. Semicircular canal morphologies and sensory epithelial defects with associated circling behaviors are listed in Tables 1 and 2. Some lateral semicircular canal pathologies tended to be more strongly associated with circling than others. We attempted to correlate circling behaviors observed in some Chd7^{Gt/+} mice with underlying inner ear pathologies (Tables 1, 2). All 25 mice exhibited some degree of lateral canal malformation. Only nine of these exhibited circling, and all nine had severe bilateral defects of the lateral canal (truncation or bird-beak defect on each side). Only three mice with such severe bilateral lateral canal defects were non-circlers. All 13 mice with the less severe loop defect in at least one ear were non-circlers. No circling mouse was found to have a completely patent lateral ca-

![Image](https://example.com/image.png)
nal. Analysis of the corresponding $2 \times 2$ contingency table indicates that there is a strong association between circling and severe bilateral malformations of the lateral canal ($\chi^2 = 14.2$, df = 1, $P < 0.0001$). These data suggest that unimpeded fluid flow through a loop-type lateral canal may be associated with milder vestibular deficits.

We also examined whether posterior canal morphology and neurofilament staining defects were associated with circling behaviors. Of the 18 Chd7$^{Gt}$ mice with complete bilateral posterior canals, 6 circled and 12 did not. Of the six Chd7$^{Gt}$ mice in which one posterior canal was missing while the contralateral posterior canal was intact, two circled and four did not. One Chd7$^{Gt}$ mouse with bilateral absence of the posterior canal circled. Analysis of the $2 \times 2$ contingency table demonstrated that circling is not significantly associated with absence of the posterior canal ($\chi^2 = 0.198$, df = 1, $P = 0.656$); 6 of 9 circlers and 12 of 18 non-circlers had both posterior canals present. Thus, the strongest relationship observed was between circling behavior and lateral canal morphology.

Among five Chd7$^{Gt}$ mice with bilateral absence of neurofilament staining in the posterior crista, only one exhibited circling behavior. Lack of distal innervation of the posterior crista was not sufficient to induce circling. Unilateral absence of neurofilament staining in the posterior crista (with normal staining in the contralateral ear) was observed in three Chd7$^{Gt}$ mice and in no Chd7$^{Gt}$ mice that circled, but many contralateral ears could not be examined by immunofluorescence or were damaged during tissue processing. Finally, bilateral

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**Fig. 5.** Posterior crista ampullaris defects in the Chd7$^{Gt}$ mice. Epifluorescence images of the central portion of the posterior crista of wildtype (A–D) or Chd7$^{Gt}$ (E–M) ears stained for actin (A,E,H,K) and neurofilament (B,F,I,L), with merged images shown in C,D,G,J,M. **A:** The sensory epithelium is densely populated with hair cells, and the septum cruciatum (sc) is present at the center of the sensory epithelium. **B:** Terminal nerve calyces densely populate the crista (arrowhead). A dense area of neurofilament staining is seen beneath the epithelium of the septum cruciatum (sc). **C,D:** The nerve calyces surround hair cells in (arrowheads). **E,F:** Some mutant cristae (E) have hair cells with tall stereocilia, but the area of the sensory epithelium is small, the septum cruciatum (sc; arrow) is rudimentary and does not extend across the entire crista, and no nerve calyces are present (F). **G:** In the merged image, dense neurofilament staining is seen at the site of the rudimentary septum cruciatum (sc). **H:** The septum cruciatum resembles the wildtype crista in A but is wider and does not span the crista. **I,J:** No nerve calyces are present. **K–M:** A focal area of dense nerve calyx staining is present in the crista in the rudimentary septum cruciatum of the mutant crista. Scale bar = 20 μm in A (applies to A–C), E (applies to E–G), H (applies to H–J), and K (applies to K–M).
nerve calyces were present in one non-circling Chd7Gt/H11001 mouse and in one circling Chd7Gt/H11001 mouse. Of note, the pattern of staining was asymmetric in the circling Chd7Gt/H11001 mouse, with normal calyx density in one crista and sparse calyces in the other. Collectively, these data do not provide sufficient evidence for a link between the various combinations of posterior canal dysmorphologies or sensory epithelial defects and circling behavior.

Fig. 6. Normal utricles in Chd7Gt/H11001 mice. Epifluorescence images of a utricular macula of a Chd7Gt/H11001 ear stained for actin (A,B) and neurofilament (C), with merged image shown in D. The shape of the utricle is normal and the hair cells are distributed densely and evenly throughout the sensory epithelium. Nerve calyces densely populate the macula (C) and surround the sensory cells (D). Scale bar = 50 μm in A; 10 μm in B (applies to B–D).

Fig. 7. Normal ultrastructural anatomy of the vestibular epithelia in Chd7Gt/H11001 mice. Scanning electron micrographic images of wildtype (A,C,E) and Chd7Gt/H11001 (B,D,F) vestibular epithelia. The lateral cristae of both wildtype (A) and Chd7Gt/H11001 (B) ears are populated by hair cells in dense bundles, with a staircase orientation and long stereocilia on the kinocilium side. Dense bundles of stereocilia are also present in the posterior cristae of wildtype (C) and Chd7Gt/H11001 (D) ears. The hair cell bundles of wildtype (E) and Chd7Gt/H11001 (F) utricular maculae appear similar to each other. Scale bar = 10 μm in A,B: 1 μm in C,D; 5 μm in E,F.
DISCUSSION

The results of our experiments demonstrate severe and variable sensory epithelial defects in the vestibular system of mice with Chd7 deficiency. These defects include abnormalities in vestibular sensory epithelial innervation and are associated with structural defects in the lateral and posterior semicircular canals.

Hair cells and innervation

Balance disorders in humans and mice can occur due to abnormal development, loss, or dysfunction of vestibular hair cells, or they can be due to defects in vestibular neurons and accessory structures (Anagnostopoulos, 2002). We found that in some Chd7Gt/H11001 mice, vestibular hair cells were present despite absence of vestibular nerve innervation. Posterior cristae of some Chd7Gt/H11001 mice contained hair cells even though nerve calyces were missing. The presence of sensory cells in the absence of afferent innervation has implications for the mechanisms of hair cell differentiation and survival. Prior studies in various experimental model systems have demonstrated that hair cells can develop and differentiate normally in the absence of neurons. Normal vestibular hair cell differentiation occurs in denervated embryonic chicken ears (Corwin and Cotanche, 1989), and fully formed, mature cochlear outer hair cells are present in mouse strains with severe nerve fiber loss, such as the neurotrophin-3 (NT-3) null mutant (Fritsch et al., 2004).

Together, these findings suggest that hair cell development is not dependent on neuron-directed influence. Here we show that vestibular hair cells can survive into relatively advanced ages (11 months) without associated innervation. The persistence of mature hair cells in the absence of afferent innervation suggests that innervation is not necessary for long-term vestibular hair cell survival. This may be of importance for clinical interventions aimed at inducing nerve growth toward the denervated hair cells. It is not known whether reduced or absent innervation of the posterior crista in Chd7Gt/H11001 mice is due to absence of primary vestibular neurons or lack of appropriate signaling from the sensory epithelium.

Comparisons with other Chd7-deficient mice

Some of the gross morphologic and behavioral abnormalities of Chd7Gt/H11001 mice are similar to those found in other heterozygous Chd7-deficient lines derived from ENU mutagenesis (Bosman et al., 2005). Similarities include variability in semicircular canal defects between mice and between right and left ears of the same mouse, as well as defects of the non-ampullated end of the lateral canals (Bosman et al., 2005; Hurd et al., 2007). In Chd7Gt/H11001 mice, the lateral crista was usually present, although in a few ears, the lateral ampulla was missing whereas a portion of the anterior end of the lateral canal remained. Other Chd7-deficient mice also have variable posterior canal defects, ranging from the canal and ampullae being reduced in size to being completely absent (Bosman et al., 2005; Hurd et al., 2007). Defects in the posterior crista and its innervation in Chd7Gt/H11001 mice occur despite an intact posterior canal and intact hair cells. This is of interest both for understanding developmental mechanisms and for designing therapies.

Mutations leading to semicircular canal abnormalities have been identified in at least 24 mouse genes (Anagnostopoulos, 2002). Some of these genes (BMP4, Jagged1, Fgf10, EphB2) encode signaling molecules that may be direct targets of CHD7 transcriptional regulation (Cowan et al., 2000; Tsai et al., 2001; Pauley et al., 2003; Thompson et al., 2003). Genes that encode proteins whose primary function is in the cell nucleus (Otx1, Hmx3/Nkx5.1, Prx1/2, Hoxa2, Hoxa1b1, Nor-1, Dlx5, Gbx2, Pou3f4, Foxg1, Fkh10, kr/majB, Sox2) may also be targets of CHD7 transcription, or may act as protein-protein interacting partners in CHD7 complexes (Gavalas et al., 1998; Hulander et al., 1998; Morsli et al., 1998; ten Berge et al., 1998; Wang et al., 1998, 2004; Acampora et al., 1999; Depew et al., 1999; Phippard et al., 1999; Rossel and Capecci, 1999; Petri et al., 2002; Kiernan et al., 2005; Lin et al., 2005; Sartori et al., 2006; Choo et al., 2006). Aside from Chd7, only a few other genes (Hmx2/3, Dlx5, Fgf10) are known to be critical for development of both the semicircular canals and their cristae (Wang et al., 1998, 2004; Pauley et al., 2003; Chang et al., 2004).

The time course of vestibular organ development and gene expression likely influences the nature and severity of inner ear defects in both humans and mice. In mice, the semicircular canals are formed between embryonic days 11.5 and 13 (Martin and Swanson, 1993). The anterior and posterior semicircular canals develop from a common vertical outpouching in the dorsal otocyst, whereas the lateral canal develops from a horizontal outpouching in the middle part of the otocyst (Morsli et al., 1998). Chd7Gt/H11001 mice have consistent defects of the lateral semicircular canal, ranging from truncations to reduced loop or bird-beak-shaped canals. The lateral semicircular canal is the last canal to form in mice and humans and is the most common canal disrupted in humans (Jackler et al., 1987). The lateral canal is also the most commonly disrupted canal in Chd7-deficient mice, raising the possibility that Chd7 is required for cellular proliferation, survival, or differentiation during the critical period of lateral canal development. CHD7 may also have a similar role in human semicircular canal development. In mice, the posterior crista forms at embryonic day 11.5, followed by the anterior and lateral cristae and the macula utriculi at embryonic day 12 (Morsli et al., 1998). The anterior and lateral cristae are believed to share a common developmental origin (Morsli et al., 1998). In Chd7Gt/H11001 mice with a missing lateral ampulla, the associated crista ampullaris is typically also missing.

The complex nature of the developing inner ear may involve direct signaling between sensory epithelia and their associated semicircular canals. Fibroblast growth factors expressed by the sensory cristae influence the outgrowth of semicircular canals, and it is thought that canal development requires prior specification by sensory tissues (Chang et al., 2004). However, there is also evidence that canal development is required for proper formation of the sensory epithelia. Foxg1 null mice develop lateral semicircular canals despite the absence of a lateral crista (Pauley et al., 2006). Because the anterior and lateral cristae share a common developmental origin, the anterior crista may be required for the formation of both the lateral and anterior semicircular canals.

Chd7Gt/H11001 mutant mice have intact lateral cristae but severely malformed lateral semicircular canals. Given these observations, it is difficult to explain why the posterior cristae of Chd7Gt/H11001 mice have abnormal structure and innervation without more severe disruptions of the
posterior canal. To our knowledge, Chd7<sup>Gt<sup>−</sup></sup> mice are the first mutant mice in which formation of the posterior crista is abnormal despite an intact posterior semicircular canal. This observation suggests that a complicated signaling relationship between the sensory and non-sensory epithelium is critical for correct inner ear morphogenesis. Because many earlier studies used in situ hybridization or other histological approaches to analyze inner ear epithelia of mutant mice, it is also possible that subtler defects in the subcellular organization of the sensory epithelium organization might have been missed.

**Inter- and intra-animal variability**

We observed considerable variability in canal morphology, sensory epithelial morphology, and posterior crista innervation between Chd7<sup>Gt<sup>−</sup></sup> mice and between right and left ears of the same mice. This variable expressivity is characteristic of CHARGE syndrome in humans (Aramaki et al., 2006). The reasons for asymmetry of mammalian Chd7 inner ear phenotypes are not clear. We hypothesize that the array of lateral canal defects and posterior crista morphologies represents a continuum of phenotypic size that the array of lateral canal defects and posterior

ies using conditional expression of right/left body symmetry genes. Further study might have been missed.

**Circling behaviors**

In Chd7<sup>Gt<sup>−</sup></sup> mice, truncation or narrowing of the lateral canal is correlated with circling behavior, whereas the presence of a complete canal is not. The lack of circling behavior in one mouse with bilateral lateral canal truncations and others with unilateral bird-beak is notable. This mouse may have had subtler head bobbing behaviors not detected in our analysis or might harbor a suppressor mutation in a Chd7 modifier gene(s). Also, although one mouse with bilaterally absent posterior canals circled, asymmetric posterior canal defects and bilateral complete posterior canals were noted in circling and non-circling mice. Similarly, some mice lacking innervation of the posterior cristae circled, whereas others did not. Thus, the data are insufficient to attribute the circling to any single cause or site of pathology in the sensory epithelium.

Many strains of circling mice have inner ear defects, but some circling mutant mice lack structural abnormalities of the inner ear, suggesting that central or other peripheral lesions may cause or contribute to circling behavior (Nolan et al., 1995; Rabbath et al., 2001). Several parameters of the circling behavior may shed light on its origins. These include the uni- versus bi-directionality of the circling, the extent to which circling continues throughout life, correlations with the presence of hair cells, and the relationship to other positional pathologies such as head bobbing or shaking. Many circling mice have severe hearing loss, but at least one Chd7-deficient strain (Edy<sup>++</sup>) has only mildly raised compound action potentials, which indicates normal function of hair cells (Kiernan et al., 2002). Therefore, it is possible that Chd7-deficient vestibular hair cells are functional and could be stimulated to provide signals to the central nervous system. The presence of circling in other mutant mice with profound bilateral inner ear dysfunction (pirouette, spinner) also suggests that unilateral or asymmetric inner ear defects are not required for circling (Beyer et al., 2000; Mitchem et al., 2002). Further studies will determine the contribution of vestibular nerve function as well as visual, cerebellar, and basal ganglia involvement in the circling behaviors of Chd7<sup>Gt<sup>−</sup></sup> deficient mice. Correlations between circling mice and an inner ear phenotype have been examined in other mouse mutants. All circling Ecl mice display bilateral lateral semicircular canal malformations, which vary in severity among animals and between ears of the same animal (Cryns et al., 2004). This is consistent with what we have observed with Chd7<sup>Gt<sup>−</sup></sup> mice.

**Genetic background effects**

Chd7 is a member of the chromdomain family of proteins that are proposed to regulate chromatin via ATP-dependent helicase and DNA binding activities (Woodage et al., 1997). Chd7 also has important protein-protein interaction domains (SANT and BRK), which likely contribute to its functional roles (Woodage et al., 1997). The interacting proteins and downstream target genes of Chd7 are not known, but clues can be obtained from other organisms. In Drosophila melanogaster, the Chd7 orthologue kismet functions as a transcriptional activator, with important roles in transcriptional elongation by RNA polymerase II and in maintaining segmental identity during development (Daubresse et al., 1999). We hypothesize that Chd7 activates transcription of genes that are critical for inner ear development. Additional studies are needed to identify these downstream target genes, some of which may also be required for normal inner ear development.
SENSE SENSORY EPITHELIAL DEFECTS WITH LOSS OF CHD7

Clinical relevance

Inner ear defects manifest more consistently than other clinical features in CHARGE individuals and are therefore useful for diagnosis (Amiel et al., 2001). The vestibular problems appear to be a dominant co-morbidity in CHARGE patients, who experience postural and motor difficulties throughout their lives (Abadie et al., 2000). Thus the use of Chd7 mutant mouse models is important for understanding this vestibular pathology. We describe several findings that are common to Chd7Gt/H11001 mice and CHARGE patients. Similar to humans, all mice with Chd7 mutations have vestibular defects. Specific findings common to CHARGE patients and Chd7Gt/H11001 mice are partial or complete semicircular canal hypoplasia, reduced canal function, asymmetric defects, and substantial variability between ears and between subjects, even among siblings (Tellier et al., 1998; Jongmans et al., 2006; Morimoto et al., 2008). Our mouse studies also suggest that vestibular sensory epithelia may be disrupted in CHARGE individuals.

Some of our findings raise possibilities for future therapies. The presence of hair cells without innervation in the posterior cristae suggests that therapies aimed at attracting growth of neurons toward hair cells in the sensory epithelium may be beneficial. The presence of an intact lateral crista in the ampulla of a defective lateral canal implies that novel surgical treatments to shunt endolymphatic fluid via an artificial canal could promote fluid movement though the ampulla. Such a “canal-plasty” might result in stimulation of the intact lateral sensory epithelium and restore function. Future studies will be aimed at exploring these therapeutic options and uncovering the mechanisms whereby Chd7 regulates early developmental events in the semicircular canals and related vestibular tissues.

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