Otoconial agenesis in tilted mutant mice

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Abstract

The sense of balance is one of the phylogenetically oldest sensory systems. The vestibular organs, consisting of sensory hair cells and an overlying extracellular membrane, have been conserved throughout vertebrate evolution. To better understand mechanisms regulating vestibular development and mechanisms of vestibular pathophysiology, we have analyzed the mouse mutant, tilted (tlt), which has dysfunction of the gravity receptors. The tilted mouse arose spontaneously and has not been previously analyzed for a developmental or physiological deficit. Here we demonstrate that the tilted mouse, like the head tilt (het) mouse, specifically lacks otoconia and consequently does not sense spatial orientation relative to the force of gravity. Unlike other mouse mutations affecting the vestibular system (such as pallid, mocha and tilted head), the defect in the tilted mouse is highly penetrant, results in the nearly complete absence of otoconia, exhibits no degeneration of the sensory epithelium and has no apparent abnormal phenotype in other organ systems. We further demonstrate that protein expression in the macular sensory epithelium is qualitatively unaltered in tilted mutant mice.

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1. Introduction

Orientation in space and maintenance of equilibrium is a fundamental requirement for the survival of complex organisms. Consequently, the vestibular system constitutes one of the phylogenetically oldest and most highly conserved of the sensory systems (Romer, 1964). Disturbances of vestibular function constitute a leading health problem in the elderly and result in significant morbidity with substantial economic consequences (NIH, 1991). Despite the prevalence of problems with balance, little is known about the molecular mechanisms controlling the development and the pathophysiology of the vestibular organs. This lack of knowledge appears to be due in part to the small size and difficult accessibility of the vestibular sense organs, and the consequent limited availability of tissue for biochemical study.

The availability of mutations affecting vestibular function, especially in mice (Deol and Lane, 1966; Tan-Creti, 1969; Lim et al., 1978; Trune and Lim, 1983a; Kitamura et al., 1991) and more recently in zebrafish (Riley and Grunwald, 1996; Whitfield et al., 1996), will allow the identification of genes that are essential for vestibular development and vestibular physiology. The cloning of such genes will allow the characterization of proteins required for normal vestibular development and physiology. Unfortunately, many of the mouse mutants with a vestibular phenotype are not particularly promising for study because they have multi-organ involvement, penetrance is poor or the phenotype leads to degeneration of vestibular structures. In our search for a highly penetrant and specific mutation affecting the vestibular system, we have identified only three mouse mutants, tilted (tlt; Chr 5, 28.0; Lane, 1986; Lane, 1987), head tilt (het; Chr 17, 4.1; Sweet, 1980) and tilted...
The recessive mutation in these mouse mutants is phenotypically restricted to the gravity receptor system and exhibits a high degree of penetrance. Preliminary morphologic investigation of the \textit{tlt} mouse indicates that the only detectable deficiency is an absence of otoconia or, infrequently, the presence of a few giant otoconia.

In mammals, otoconia consist of dense particles (0.1–25 μm in length) containing a protein matrix and inorganic calcium carbonate crystals in the form of calcite (Carlstrom, 1963; Mann et al., 1983; Pote and Ross, 1986). Otoconia are embedded in a gelatinous membrane which together form the otoconial membrane (or otoconial complex) (Lim, 1984). The otoconial membrane overlies the ciliated sensory epithelium of the utricular and saccular maculae and by virtue of its high mass enhances the sensitivity to gravity and other forces due to linear acceleration.

The otoconial membrane and the internal organic matrix of otoconia consist of glycoproteins (otoconins) and sulfated glycosaminoglycans (GAGs), primarily complexed in the form of proteoglycans (Schrader et al., 1973; Ross et al., 1985; Munyer and Schulte, 1991; Pote and Ross, 1991; Suzuki et al., 1992; Tachibana and Morioka, 1992; Fermin et al., 1995; Suzuki et al., 1995a). In mammals, the major otoconial protein has a molecular weight in the range of 90–100 kDa (otoconin-90) and has calcium-binding properties (Pote and Ross, 1986, 1991). In contrast, the major otoconial protein of \textit{Xenopus laevis} has a molecular mass of only 22 kDa (otoconin-22). Otoconin-22 has been purified and sequenced and was found to be 37% identical to phospholipase A2 (Pote et al., 1993). The otoconin(s) of calcitic otoconia in higher animals have not been further characterized.

In mice, the first seeding of calcium crystals in otoconia is observed on embryonic day 14 (Veenhof, 1969). Calcification reaches its highest rate on embryonic days 15 and 16. Otoconial growth progresses from a trigonal/spindle-shape to a dumbbell/barrel shape and is essentially complete by postpartum day 7 (Veenhof, 1969; Salamat et al., 1980; Lim, 1984).

The \textit{tlt} mouse \((\text{tlt}; \text{Chr} 1, 59.9; \text{Lim et al., 1978})\) arose spontaneously in a \(p^{3tl}/p^{3tl}\) stock and has subsequently been bred onto a C57BL/6J background and cryopreserved at the Jackson Laboratories (Lane, 1986). The homozygous \textit{tlt} mouse \((\text{tlt/tlt})\) often holds its head in a slightly tilted position. Under normal laboratory conditions the animals show no other behavioral abnormalities (e.g., circling, head-tossing). However, when dropped into water, the mice cannot find the surface and would drown if not rescued. The \textit{tl} locus was previously mapped to mouse chromosome 5 between the \textit{hammer toe} (\(\text{Hm}\)) and \textit{Kit} (Kit\(^{W<cp}\)) locus (\(\text{Hm} 6.9 \text{ tlt} 16.4 \text{ Kit}^{W<cp}\)) (Lane, 1986, 1987). No additional behavioral, histopathological or genetic studies have been performed on this mouse. The \textit{thd} mouse also has a phenotype restricted to macular superstructures but exhibits a high frequency of giant otoconia (Lim et al., 1978). Analysis of \textit{het} mice suggests a phenotype very similar to \textit{tl}t with a complete absence of otoconia (J. Schimenti, personal communication, and our preliminary observation).

In this paper, we demonstrate that the \textit{tlt} phenotype is unique compared to other vestibular mouse mutants such as \textit{pallid} (Trune and Lim, 1983b), \textit{mocha} (Rolfsen and Erway, 1984) and \textit{thd head} (Lim et al., 1978), in that \textit{tlt} phenotypic defect appears to be limited to the macular gravity receptor organs and the penetrance of the defect is close to 100%. Furthermore, we demonstrate that \textit{tlt/tlt} mice lack otoconia but have normal-appearing gelatinous membranes and normal sensory epithelia in all vestibular organs as well as in the organ of Corti.

2. Methods

2.1. Mice

The \textit{tlt} mouse, obtained from frozen embryo stocks at the Jackson Laboratory, was maintained in a C57BL/6J genetic background under specific pathogen free conditions (SPF). To maintain the genetic background of these mice, alternate matings were carried out, first, between confirmed heterozygous mice \((+/\text{tlt})\) (generation 1) and, second, between a wild-type C57BL/6J mouse and a \textit{tlt/tlt} mouse (generation 2). \(+/\text{tlt}\) showed no abnormal phenotype and both male and female \textit{tlt/tlt} mice were fertile. Mice used in this study were derived from a cross between either heterozygous or homozygous parents. Some of the animals examined were also derived from an F2 intercross between \textit{tlt/tlt} mice in the C57BL/6J strain and \textit{Mus musculus castaneus}. No functional or anatomic differences were observed between F2 intercross mice and inbred C57BL/6J mice.

2.2. Functional testing of the vestibular system

The phenotype of \textit{tlt/tlt} mice can be determined by the observation of head tilting behavior which was seen in about 50–75% of the homozygous mice. Because detection of this behavior was subjective and it was not always present, the \textit{tlt} phenotype was assessed in all subjects by observing their behavior when dropped from a height of 20 cm into a deep tank of water. After being dropped into water, control mice promptly resurfaced and swam to the side of the tank with nose and tail maintained above the water line (this is normal swimming behavior for mice). In contrast, when \textit{tlt/tlt} mice were dropped into water, they spiraled underwater and required immediate rescuing to prevent drowning.
(this is non-swimming behavior). When dropped into water, a small percentage (<2%) of tltltlt mice, circled on the water surface with their nose but not their tail held out of the water. Alternatively, some mice performed somersaults, backflips or corkscrews in the water. These mice could find the water surface but occasionally needed rescuing and are referred to as intermediate swimmers. All intermediate swimmers were tested at least two times. The analysis of swimming behavior is similar to the technique used by Lim et al. (Lim et al., 1978) but simplifies the categories of swimming into three groups instead of five and is based on qualitative observation of swimming behavior.

2.3. Functional testing of the auditory system

Auditory function was tested by recording auditory brainstem response (ABR) thresholds to 0.1-ms clicks and 8-ms tone pips at frequencies of 3, 4, 6, 8, 10, 12, 16, 20, 25, 30, 40 and 50 kHz. Detailed methodology is described by Henry (1979) and Henry and Chole (1979).

2.4. Preparation of the membranous labyrinth for histopathological analysis

Mice (most 3–5 weeks of age) were anesthetized with ketamine (80 mg/kg body weight) and xylazine (16 mg/kg). Once anesthetized, they were perfused through the left heart with 3–5 ml of a physiological buffer such as lactated Ringer’s solution or phosphate buffered saline (PBS), followed by 5 ml of fixative (either 1% OsO₄ in Dalton’s buffer or 4% paraformaldehyde in PBS). After perfusion, the mice were decapitated, their bullae removed and opened to reveal the cochlea. The stapes was removed from the oval window and small holes were made with a sharpened steel pick at the cochlear apex and in the scala tympani just rostral to the inner ear. The specimens were immersed in 1% OsO₄ for 5–10 min, washed in buffer, dehydrated and infiltrated with plastic as described above. After infiltration, each inner ear was cut by hand into three thick sections (approx 0.5 mm thick). The cuts were oriented parallel to the modiolus of the cochlea. These sections were then embedded in 1.5-mm thick layers of plastic with one cut surface facing the bottom of the embedding mold (Peel-a-Way, R-40). After polymerization, the specimens were examined by light and phase-contrast microscopy.

Following examination as whole mounts by bright-field and phase-contrast microscopy, each vestibular organ was cut by hand with razor blades into several thick sections (0.25–0.50 mm thick). The cuts were oriented perpendicular to both the endolymphatic surface and the long axis of the sensory epithelium. These thick sections were placed in polypropylene embedding molds containing a 1.5-mm thick layer of plastic. One cut surface of the epithelium was placed in contact with the bottom of the mold. After polymerization, the thick sections were examined by phase-contrast microscopy. Selected thick sections were semi-thick and thin-sectioned on an ultramicrotome for examination by bright-field and transmission electron microscopy (TEM) (JEOL, 100-S), respectively.

Hair-cell density was quantified in thick sections through the middle of the utricular and saccular maculae in controls (wild type and heterozygotes) and tltltlt mice. The sections were imaged at 1250× with a phase-contrast microscope (Wild M-20 equipped with a Javelin camera and Data Translations frame grabber and Foster-Findley image processing software). A line was drawn at the endolymphatic surface of the sensory epithelium. The nuclei of the Type I and Type II vestibular hair cells beneath the line were marked, the marks counted, and sensory-cell density calculated relative to the measured length of the drawn line. Density was
expressed as hair cells per millimeter of the macular surface.

2.5. Tissue preparation for biochemical analysis

Mice were sacrificed by cervical dislocation, and then decapitated. The temporal bones were rapidly removed from the skull, cleared of soft tissues and immersed in Freon-12 chilled to its melting point with liquid nitrogen and freeze-dried at −40°C for 3 days. Using fine microblades and hairpoints, the maculae of the sacculus and utricule, and otoconial membranes were dissected in the freeze-dried state at room temperature with a relative humidity of 40% or less. Samples were weighed on quartz fiber balances. Complete procedural details are described in Thalmann (1971, 1976). Otoconia were separated from the surrounding gelatinous membrane and other contaminants by three cycles of suspension in 0.1% SDS/NaAc, pH 7.4, followed by sonication for 3 min and centrifugation at 10,000×g for 1 min. The otoconial proteins were then extracted with 100 mM EDTA, pH 7 for 24 h at 4°C. EDTA was removed by dialyzing against 0.1 M MgCl₂ at 4°C overnight. The samples were subsequently dried and analyzed as described below. Supernatants were also retained, dried and analyzed as described below.

2.6. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

For 2D-PAGE, freeze-dried samples were solubilized in a mixture containing 8 M urea, 4% CHAPS, 65 mM dithioerythritol, 40 mM Tris-HCl, pH 9.5, and a trace of bromphenol blue. 2D-PAGE was carried out on an immobilized pH gradient (IPG, Pharmacia 3-10) in the first dimension and using the DALT technique (Anderson, 1988) in the second dimension. The gel gradient was poured with the aid of a computerized gradient maker (Angelique, Large Scale Biology, Rockville, MD). Proteins were visualized with the silver staining procedure described by (Oakley et al., 1980). Gels were scanned on a Pharmacia LKB Image Master 1-dimensional flatbed scanning densitometer in transmitted light mode. Subtractive analysis of protein patterns was carried out using Image Master software (Pharmacia Biotech, Piscataway, NJ) on an NEC Image 486DX/66 desktop computer (Boxborough, MA). Most experiments were spiked with three protein standards, carbamyl anhydrase, ovalbumin, and β-galactosidase. However, to verify that no proteins were obscured, all experiments were also run without added standards.

3. Results

3.1. Tlt/tlt mice have a highly penetrant defect in vestibular function

Tltlt mice, maintained in the C57BL/6J genetic background, were identified by their inability to swim. Of 500 progeny examined from a homozygous mating, 490 (98%) were not able to swim and 10 (2%) showed an intermediate ability to swim (Table 1). The phenotype of 280 progeny from a heterozygous mating was also examined. Of these, 56 (20%) were not able to swim and 2 (0.7%) showed an intermediate ability to swim (Table 1). The percentage of mice derived from the heterozygous cross that had a swimming phenotype was slightly lower than the predicted Mendelian pattern of inheritance of a recessive gene (20.7 vs. 25%). How-

![Fig. 1. Auditory brainstem response thresholds in tilted mice. ABR thresholds were averaged for five F2 tiltlt mice derived from a backcross between C57BL/6J and Mus musculus castaneous (circles). Also shown are average thresholds determined behaviorally for 77 laboratory mice (strains: NMRI (Ehret, 1974); CBA/J (Berlin et al., 1968; Birch et al., 1968)) (triangles). Error bars indicate standard deviations.](image)

Table 1

<table>
<thead>
<tr>
<th>Matinga</th>
<th>Total number of progeny screened</th>
<th>Not able to swim</th>
<th>Intermediate ability to swim</th>
<th>Normal ability to swim</th>
</tr>
</thead>
<tbody>
<tr>
<td>tiltlt×tilt+</td>
<td>280</td>
<td>56 (20%)</td>
<td>2 (0.7%)</td>
<td>222 (79.3%)</td>
</tr>
<tr>
<td>Predicted</td>
<td></td>
<td>(25%)</td>
<td>(0%)</td>
<td>(75%)</td>
</tr>
<tr>
<td>tiltlt×tiltlt</td>
<td>500</td>
<td>490 (98%)</td>
<td>10 (2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Predicted</td>
<td></td>
<td>(100%)</td>
<td>(0%)</td>
<td>(0%)</td>
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aMating to determine the inheritance of the tilt phenotype in the C57BL/6J genetic background.
Fig. 2. Whole mounts and semi-thick sections of the mouse saccular macula. Phase contrast images viewed from the endolymphatic surface in (A) control mouse, (B) tilted mouse. At this power the darkly-stained myelinated nerve fibers (nf) can be seen approaching the sensory epithelium from the lateral side (lower edge of photomicrograph) and medial side of the epithelium. A: Nerve fibers are indistinct because of the thick layer of otoconia (o) over the epithelium. B: Nerve fibers are clearer because no otoconia are present. One-μm sections of the saccular macula stained with toluidine blue and azure II and viewed by phase contrast microscopy; C: control mouse; D: tilted mouse. The sensory epithelium is similar in both mice. Type I (I) hair cells are surrounded by nerve chalices. Type II (II) hair cells are shorter and less darkly stained. Stereociliary bundles (st) can be seen projecting from both cell types into the endolymphatic space. Nuclei of the supporting cells (sc) form a nearly continuous row adjacent to the basal lamina (bl). Cross-sectioned nerve fibers (nf) are visible in the connective tissue beneath the epithelium. The otolithic membrane, visible in both (C) and (D), consists of the subcupular meshwork (not labeled, just above epithelium), honeycomb layer (hl) and the gelatinous layer (gl). The only obvious difference between the maculae in (C) and (D) is the lack of otoconia (o) in the tilted mouse (D).

Fig. 3. Saccular macula from a tilted mouse with intermediate swimming ability viewed from the endolymphatic surface. Five ‘giant’ otoconia are present overlying the sensory epithelium. Nerve fibers (nf) are visible at lateral and medial edges (compare to Fig. 2B).
ever, this difference is not statistically significant at a level of 0.05 (uncorrected 2 sided Z-test, $P=0.097$). These data indicate that the penetrance may be slightly less than 100% and that the swimming test is a fairly reliable method to score the phenotype of these mice.

3.2. Tlt/tlt mice have normal hearing

ABR thresholds were determined in five 4-month-old tlt/tlt mice. Thresholds for clicks averaged $11 \pm 2$ (s.d.) dB sound pressure level (SPL). Thresholds for tone pips are shown in Fig. 1. The thresholds of the tlt/tlt mice were comparable to published data (Henry, 1979; Ehret, 1983; Fay, 1988), and those recorded from wild-type mice in our laboratory.

3.3. Histopathological analysis of the tlt/tlt mouse

Gross examination of the major organs from tilted mice showed normal organ systems, including the middle ear and the bony and membranous labyrinths. All subdivisions of the membranous labyrinth were identified in tlt/tlt mice and were found to be grossly similar in size and shape to those of controls.

High-power ($1250 \times$) phase-contrast and bright field microscopic examination of the saccular and utricular maculae from tlt/tlt mice (3–5 weeks of age) revealed normal-appearing sensory epithelia and ototoxic membranes and in nearly all animals, a complete absence of otoconia (Fig. 2B,D). Both ears had the same histopathological defect, regardless of the direction and degree of head-tilting behavior. The maculae from heterozygous and wild-type mice ($n = 7$) were histologically normal and contained a full complement of otoconia (Fig. 2A,C). The presence of otoconia over the sensory epithelium and myelinated nerve fibers beneath the epithelium (blackened by fixation with osmium tetroxide) prevented quantification of hair cell density in the whole mount preparations.

In 50 tlt/tlt mice examined, the inner ears were dissected and in all cases of mice that could not swim, no otoconia were found in either the saccule or utricle. In the rare mouse that showed an intermediate ability to swim, otoconia were either missing entirely or one to several ‘giant’ otoconia were identified in one or both saccules only (Fig. 3). Thus the phenotype of tlt/tlt mice correlated very well with the pathological changes seen in the inner ear.

To determine whether otoconia were ever formed in tlt/tlt mice during development then degenerated, late embryonic stage and newborn temporal bones were examined. Plastic-embedded thick sections of embryonic and newborn ears were covered with a droplet of immersion oil and viewed with a stereozoom microscope (Nikon, SMZ-U). The maculae were identified by their locations relative to the semicircular canals, cristae and oval window. A moderately thick layer of otoconia was visible over the utricular and saccular maculae in all E18.5-day temporal bones from control mice. In contrast, no otoconia were visible over either maculae in E17.5-day embryonic or newborn tlt/tlt temporal bones (data not shown). Phase-contrast microscopic examination ($625 \times$) of the macula in the E18.5-day controls and newborn tlt/tlt mice revealed well-differentiated vestibular hair cells with stereocilia. In the E17.5-day tlt/tlt maculae stereociliary bundles were infrequently seen (data not shown).

After examination as whole mounts (Figs. 2A,B, and 3), the vestibular organs were each cut into several thick sections and examined by phase-contrast, then semithick sectioned and examined by bright-field microscopy (Fig. 2C,D). In control mice, a layer of highly refractive otoconia were clearly visible over the macula of the saccule and utricle. In tlt/tlt mice, the gelatinous portion of the otolithic membrane was visible over the endolymphatic surface of the maculae but it contained no embedded otoconia. Type I hair cells were identified by their nerve chalice and stereociliary bundle. Type II
hair cells were identified by their stereocilia and the position of their nuclei near the surface of the epithelium. It is important to note that compared to the section shown in Fig. 2C, Fig. 2D was overstained to clearly show the normal-appearing subdivisions of the otolithic membrane in the tltlt mouse. In lighter stained sections of the tltlt maculae, the nuclei and nucleoli of the sensory and supporting cells were easily seen. There was no evidence of pyknotic or degenerating cells in any of the sectioned specimens. In three controls, the density of hair cells (Types I and II combined) averaged 159 ± 10 per mm in the saccular macula and 187 ± 21 per mm in the utricular macula. In five tltlt mice, hair-cell density averaged 174 ± 18 per mm in the saccular macula and 191 ± 25 per mm in the utricular macula. There was no significant difference between tilted mice and controls with respect to hair-cell density (P = 0.17–0.80). The total number of hair cells per macula was not determined.

In both tltlt and control mice, the type I vestibular hair cells had flask shapes, narrow necks and a slightly convex endolymphatic surfaces (Fig. 4 and data not shown). Their nuclei were located about 2/3 of the distance between the surface of the epithelium and the basal lamina. The basal pole and lateral membrane of the Type I hair cells were entirely surrounded by a nerve chalice. The Type II vestibular hair cells were shorter with a more uniform width from apex to base; their nuclei were located closer to the endolymphatic surface of the maculae. In both types of hair cells, stereocilia could be seen projecting from the endolymphatic surface of the cell into the overlying otolithic membrane (Figs. 1C,D, and 3). In some sections, the size of the surrounding nerve chalice appeared larger in tltlt mice compared to controls. Nuclei of the supporting cells formed a nearly continuous row adjacent to the basal lamina (Fig. 1C,D). By TEM, the apical 2/3 of the supporting cells were filled with dilated cisternae of rough endoplasmic reticulum containing a homogeneous material of moderate electron density (Fig. 4).

To determine whether or not the tilted mutation affects the sensory epithelium of the cochlea, hair cells were counted and densities determined (Bohne and...
In five control mice, the organ of Corti averaged $6.21 \pm 0.22$ mm in length and had a density of $115 \pm 3$ inner hair cells and $395 \pm 17$ outer hair cells per millimeter length. In two $\text{tilt/tilt}$ mice, the organ of Corti averaged $6.17 \pm 0.14$ mm in length and had densities of $114 \pm 3$ inner hair cells and $393 \pm 6$ outer hair cells per millimeter. No differences were found between the control and $\text{tilt/tilt}$ mice with respect to the light/phase contrast microscopic appearance of the cochlear duct, including the sensory and supporting cells, nerve fibers and tectorial membrane.

3.4. Analysis of protein expression in the macular sensory epithelium

To determine whether the $\text{tilted}$ mutation resulted in any changes in protein expression patterns within the macular epithelium, 2D-PAGE was performed on freeze dried preparations of the sensory epithelium and otoconial membrane. Approximately 200 proteins were easily discernible in two dimensional protein maps of pooled maculae (maculae sacculi and utriculi) (Fig. 5A,B). Most notably, in both control and $\text{tilt/tilt}$ mice, otoconin 90 protein was not seen in these epithelial preparations (arrow in panels A, B and C). Alignment with added reference proteins, and computer-assisted subtractive analysis was used to compare the relative abundance of other proteins expressed in the sensory epithelium of control and $\text{tilt/tilt}$ mice. This analysis revealed no major reproducible differences in three gels analyzed from both control mice and from $\text{tilt/tilt}$ mice. Several regions showed clusters of proteins that suggested subtle differences in concentration between $\text{tilt/tilt}$ and control mice. Whether these differences are significant will require further analysis. Additionally, to confirm that no differences in protein patterns were masked by the protein standards added in the gels shown in Fig. 5, several other sets of gels were also run without added standards (data not shown).

3.5. Analysis of protein expression in the otoconial membrane

2D-PAGE analysis of sonicated and EDTA extracted otoconia from control mice identified a prominent broad smear with a $pI$ centered at 2.9 and a molecular weight centered at 83 kDa. The molecular weight of this protein is in agreement with that of otoconin-90 identified by Pote and Ross (1986, 1991) in the rat. In addition, four minor proteins were detected showing $pI$s ranging from 5.0 to 5.3 and molecular masses ranging from 50 to 62 kDa. Analysis of the gelatinous membrane (dried supernatant after removing otoconia) from control mice did not reveal the presence of otoconin-90 (data not shown). However, all of the minor protein constituents of otoconia were detected.

4. Discussion

In this paper we characterized the phenotypic defect in the $\text{tilt/tilt}$ mutant mouse. Unlike other described mouse mutations that affect otoconial development (for example $\text{pallid}$ (Trune and Lim, 1983a), $\text{mocha}$ (Rolfsen and Erway, 1984) and $\text{tilted head}$ (Lim et al., 1978)), the $\text{tilted}$ mutation results in the complete agenesis of otoconia, apparently without other developmental or physiological sequelae. Otoconia are composed of a proteinaceous organic matrix and an inorganic phase composed of calcium carbonate crystals. These protein-calcium carbonate complexes are then attached to and partially embedded in a gelatinous matrix. The gelatinous membrane containing the morphologically distinct gelatinous and honeycomb layers and subcupular meshwork, together with the otoconia, compose the otocorial complex.

The nature of the mutation in the $\text{tilt/tilt}$ mouse is difficult to predict. The anatomic defect could result from several genetic lesions. For example, one of the core proteins of the organic component in the otoconia may be mutant or missing. As discussed below, several otoconial core proteins can be identified by gel electrophoresis, the major one being otoconin-90 which accounts for over 90% of the otoconial protein content. The function of otoconin-90 and the other unidentified core proteins is not known, but it is likely that these proteins interact and are essential for the controlled nucleation of the inorganic crystal component of otoconia. A point mutation or small deletion could affect the ability of the core proteins to associate with one another, to nucleate the calcium carbonate crystal or to affect the stability of otoconia. Alternatively, one of these proteins may be missing or reduced in quantity which would also disrupt otocorial structure or stability.

A second possible genetic defect in the $\text{tilt/tilt}$ mouse could affect proteins that regulate the concentration of calcium ions or other divalent cations in endolymph. Such proteins could include ion channels that may be specifically expressed in the inner ear. However, normal function of the semicircular canals and cochlea in the $\text{tilted}$ mouse tends to rule out the possibility of altered endolymphatic calcium homeostasis (Salt et al., 1989). Furthermore, the ampullae of the three semicircular canals are immediately adjacent to the utricle, and no significant calcium gradient could exist between these two fluid spaces. A third possible mutation would affect the interaction or binding of otoconia to the otolithic membrane. Failure to localize all of the components of otoconia to the correct anatomic location may preclude normal assembly.

The above mentioned genetic defects all affect specific components of otoconia. Alternatively, the mutation in the $\text{tilt/tilt}$ mouse may be more global in nature.
and genetically upstream from the above mentioned proteins. Such a mutation could involve a transcription factor that regulates the expression of one or several otoconial core proteins, matrix attachment proteins or proteins that regulate ion concentrations. Because the anomalous defect in the \textit{tlt/tlt} mouse appears to be restricted to the sacculus and utricle, one would expect such a transcription factor or specific combination of transcription factors to be expressed specifically in the sensory epithelium of these organs.

The agenesis of otoconia in \textit{tlt/tlt} mice suggests that a protein constituent of otoconia may be missing or altered. 2D-PAGE was therefore used to analyze proteins present in the otoconial complex and in the underlying sensory epithelium. An abundant protein that migrated in a molecular weight range similar to that described for rat otoconin-90 (Pote and Ross, 1986) was present in samples derived from the otoconial complex of control mice. We refer to this protein as mouse otoconin-90. Mouse otoconin-90 appeared as a broad smear and has an isoelectric point (\(pI\)) of 2.9. Both the smearing and the acidic \(pI\) support the notion that otoconin-90 is highly glycosylated as previously postulated on the basis of its staining properties (Ross et al., 1985) and the electrophoretic properties of avian otoconin-90 (Fermin et al., 1995).

The absence of otoconin in \textit{tlt/tlt} mice allowed a clear view of the underlying gelatinous membrane. Although the gelatinous membrane could not be separated from the underlying sensory epithelium in \textit{tlt/tlt} mice, 2D-PAGE analysis of sensory epithelium together with the overlying gelatinous membrane failed to identify a protein similar to otoconin-90. However, otoconin-90 was also not detected in samples of the sensory epithelium of control mice (Fig. 5A) or in the gelatinous membrane of control mice from which otoconia had been removed. The absence of otoconin-90 in the macular epithelium and gelatinous membrane was unexpected because of the generally held view that the macular sensory epithelium is the source of the organic components of the otoconia (Lim, 1980). Notably, mRNA for osteopontin, the only other identified mammalian otoconial protein, is restricted in its expression to the sensory portion of the macular epithelium (Takemura et al., 1994).

The origin of otoconin-90 is not known. Otoconin-90 may be synthesized as a precursor protein, similar to that of other matrix proteins such as collagen (Olsen, 1991). An otoconin precursor protein would be expected to have a higher molecular weight and most likely would also differ in its \(pI\). If such a precursor were present in the sensory epithelium it would therefore not be recognized on our gels. Alternatively, otoconin-90 or its precursor may be secreted from another site within the vestibular labyrinth. One such site is the perimacular non-sensory tissue which also contains the major site of active ion transport in the vestibular system (Wangemann, 1995).

A final possibility is that otoconin-90 is not being produced in the adult mouse at detectable levels. Examination of vestibular development demonstrates that the rate of production of otoconia is greatest between embryonic days 15–17, although otoconia continue to grow at a significant rate until about postnatal day 7 (Veenhof, 1969; Lim, 1973; Anniko, 1980; Salamat et al., 1980). Thereafter, the extent of otoconial production and growth is controversial (Preston et al., 1975; Lim, 1980; Ross et al., 1985; Takemura et al., 1994; Suzuki et al., 1995b).

Minor otoconial proteins were observed in otoconia isolated from control mice. The minor otoconial bands of the mouse range in molecular mass from 50 to 62 kDa, roughly comparable to those observed in the rat (Pote and Ross, 1986). All the minor bands exhibit an acidic \(pI\), consistent with their presumed glycoprotein/proteoglycan nature. Of the minor otoconial proteins, only osteopontin has been identified based on immunohistological evidence (Takemura et al., 1994). The lowest, very weak band, seen in our 2D-PAGE analysis corresponds roughly to published coordinates for osteopontin. However, this has not been verified by immunological analysis or by amino acid sequencing. In a recent publication, minor proteins for the entire otoconial complex of the chicken were reported to be actin, tubulin and myosin (Fermin et al., 1995). However, these bands were not detectable in mouse otoconia. The electrophoretic mobility of these chicken proteins seems to correspond to that of albumin and transferrin and therefore may have arisen from serum contamination of the otoconial preparations.

In control mice, the gelatinous membrane can be separated from the otoconia by centrifugation. 2D-PAGE analysis of the gelatinous membrane identified several proteins, some of which correspond in their positions to the minor bands of purified otoconia. Pote and Ross (1986) and Lim (1980) have reported that the fibrous material of the otoconial matrix is continuous with the gelatinous membrane. The analysis presented here is consistent with this hypothesis only for a subset of otoconial proteins. In preparations of the gelatinous membrane fraction contamination from the underlying epithelium, especially adhering stereocilia, must also be considered. However, the absence of actin, a major constituent of stereocilia, rules out any significant contamination in our preparation.

Overall, only minor quantitative differences between the protein profiles of the maculae from \textit{tlt/tlt} and control mice were observed. Because we have not identified the source of all otoconial proteins, it is not possible to rule out that a protein essential for the production of otoconia is missing, or produced in insufficient amounts to account for the phenotype in the \textit{tlt/tlt} mouse. Fur-
ther studies will address the spatial and temporal patterns of expression of otoconial proteins and eventually their corresponding genes. The identification of the gene responsible for the tilted phenotype will also be important to understand otoconial development.

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