

Progressive Degeneration of Stereocilia in Cochlear Hair Cells in Hearing-impaired Kuru² Mice

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Abstract. *Background:* We previously isolated a mouse strain, kuru², which exhibits abnormal behavior and hearing impairment. To investigate the etiology of this impairment, the ultrastructure of the inner ear was examined. *Materials and Methods:* The morphologies of the cochlea and the vestibule of control (Jcl:ICR) and mutant mice were analyzed by electron microscopy. In some experiments, the mice were cross-mated and their offspring examined. *Results:* The mutant mice displayed progressive degeneration of the stereocilia in the cochlea. The stereocilia started to degenerate on post-natal day 10 and, subsequently, the hair bundles continued to degenerate. On day 18, degeneration of the stereocilia was complete. In contrast, the vestibule was intact. *Discussion:* Many mutant mice display hearing impairment. These mice demonstrate a characteristic morphology of the inner ear and, since correlations may be made with corresponding human diseases, the current results could contribute to the further understanding of hearing impairment mechanisms.

Hearing impairment is one of the most common sensory disorders in humans. One in about 1,000 children has a prelingual disorder and, since various factors influence the hearing function, their identification is essential. However, clinical studies are hampered by the inaccessibility of the inner ear. Moreover, most diseases are not lethal, hence autopsy will not reveal early stage disease. Many suitable mice models have been established for human hearing impairments. Previously, we reported a mutant mouse, kuru², which exhibits hearing impairment with abnormal behavior such as circling, ataxic gait, head tossing, hyperirritability and an epileptic tendency. Examination of the inner ear showed that neuronal degeneration occurred in the spiral ganglion

cells of the cochlea (1). However, while degeneration of the spiral ganglion occurred in the mouse, it was observed at a late stage of life. There was a time difference between the start of degeneration and the onset of hearing loss.

In this study, we examined the ultrastructure of the inner ear in both young and aged mice and attempted to demonstrate early morphological changes in the cochlea associated with hearing impairment.

Materials and Methods

Animals. Closed colony Jcl:ICR (wild-type control) and kuru² mice were maintained on commercial breeding food (CA-1, Clea Japan, Tokyo, Japan) and tap water. These mice were mated according to the experimental plan and used for the examination. All the animal experiments were performed according to our animal care facility guidelines.

Evaluation of hearing acuity. Hearing acuity was evaluated by auditory brain stem response (ABR) with several power levels of a repetitive clicking sound (9.5/second). The mice were anesthetized with 1.5% isoflurane and ABR was measured by Synax 1200 (NEC Medical Systems, Tokyo, Japan). The electrodes were placed at the base of the ears and the cathode on the vertex of the cranium.

Histological examination. The inner ears of both mice were used in the histological analysis. The cochlea and vestibule were excised and fixed with 1.2% glutaraldehyde in phosphate-buffered saline (PBS) fixative for scanning electron microscopy (SEM) examination. In some experiments, the cochlea was further fixed for 2 days by pouring a small amount of fixative into the small holes made at the apex. After fixation, the samples were dehydrated by the critical point drying method, coated with Au-Pd sputter, and scanned by JSM-8500LV scanning electron microscopy (JEOL, Tokyo, Japan) at 15 kV.

For transmission electron microscopy (TEM), the temporal bones of mice were fixed for 10 days in 4% paraformaldehyde in PBS at 4°C. After fixation, the specimens were decalcified in 2% EDTA for 2 weeks. The cochlea and vestibule were dissected from the temporal bone and secondarily fixed with 1% osmic acid in PBS for one hour on ice. Tissues were dehydrated and embedded in epoxy resin. After sequential sectioning by an ultramicrotome, the sections were stained with uranyl acetate and lead citrate and examined by H-7500 electron microscopy (Hitachi, Tokyo, Japan).

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Key Words: Mutant mouse, hearing impairment, cochlea, stereocilia, degeneration.

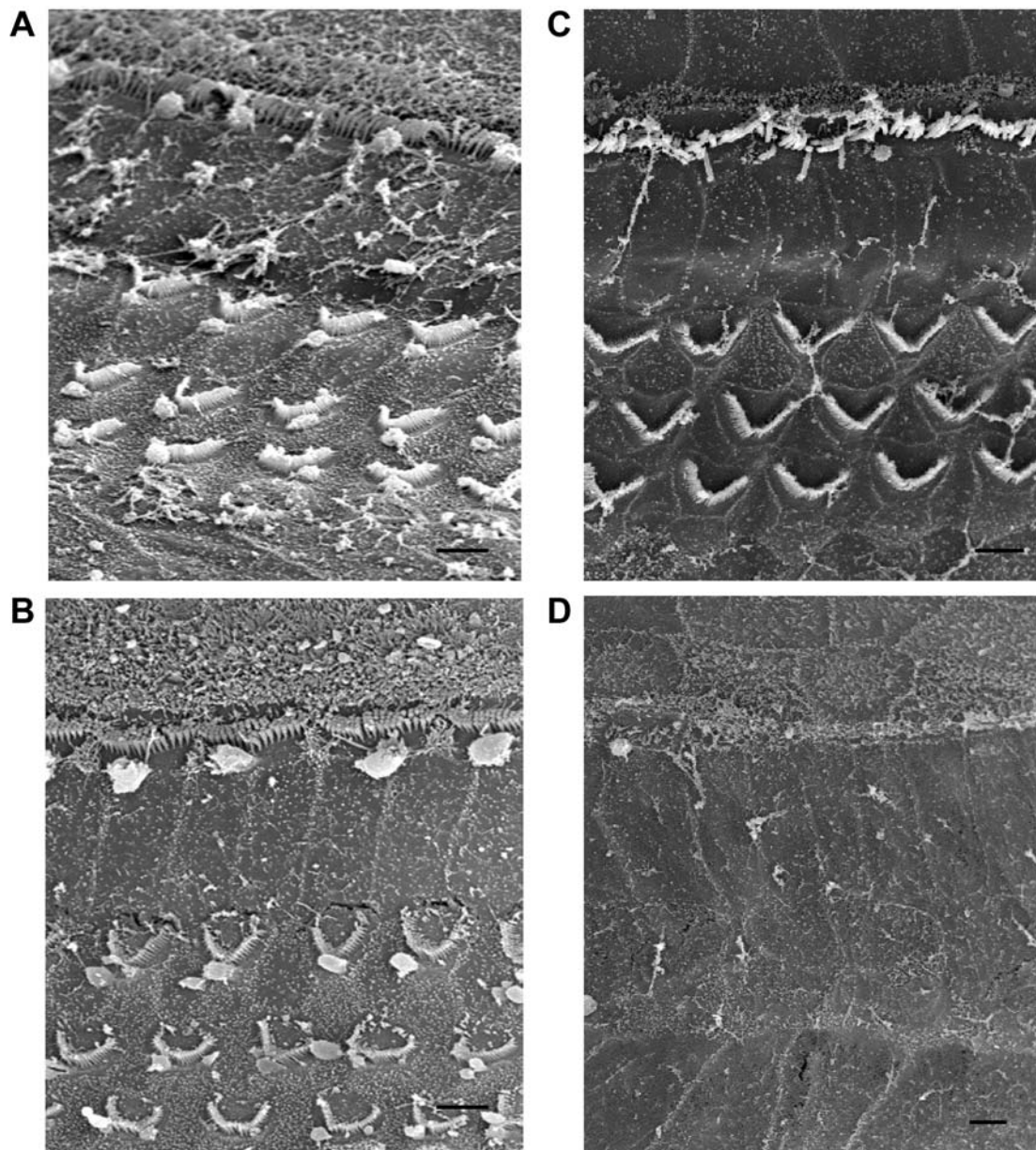


Figure 1. SEM analysis of cochlear hair cells in wild-type ICR and mutant mice. On day 10 after birth, the inner and outer hair cells grew and displayed ordinary development in both the wild-type (A) and *kuru*² (B) mice. On the contrary, in 7-week-old mice, significant differences were observed in the wild-type (C) and *kuru*² (D) mice. Both hair cells were mature in the control mice. However, hair bundles in the mutant mice were severely degenerated and mostly undetectable at this stage. Bars indicate 5 µm.

Results

Previously, we had not found gross abnormalities in the central nervous system or in formation of the cochlea and vestibule in the mutant mice by Hematoxylin-Eosin staining under light microscopy (1). These findings were reconfirmed in this study (data not shown). However, unlike the normal morphology of the inner ear in wild-type animals, defluxions

of stereocilia in cochlea hair cells were observed in elderly mutant mice. Therefore, the morphologies of their hair cells were further investigated using electron microscopy.

Both wild-type ICR and *kuru*² mice demonstrated ordinary development of the inner and outer layers of the hair cells in the organ of Corti at 10 days after birth (Figure 1). No differences, including numbers or shapes of the inner and outer hair cells, were detected in the two groups until a

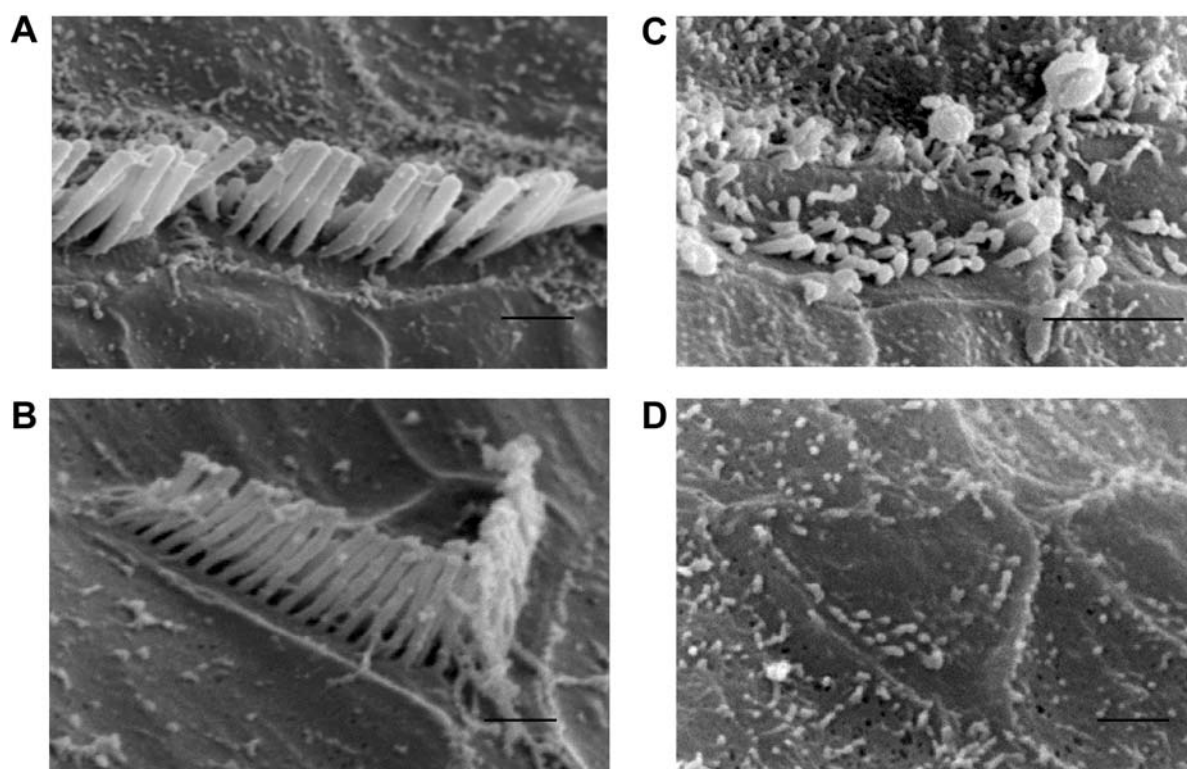


Figure 2. High magnification images of stereocilia in cochlear hair cells. Images of the inner (upper) and outer (lower) hair cells of the wild-type (left; A, B) and *kuru*² (right; C, D) mice at the age of 4 weeks. While the inner and outer hair cells of the wild-type mouse were well developed and intact, the hair cells of *kuru*² were degraded. The stereocilia of the inner hair cell were extremely short, while those of the outer hair cells were almost untraceable. Bars indicate 1 µm.

later stage of development. At 7 weeks after birth, both the inner and outer hair cells were well matured in wild-type mice. As in normal development, the stereocilia had a V-shaped appearance in the outer cell layers. The outer hairs were longer than the inner hairs. Moreover, the inner hair cells were set inside the outer hair cells. In contrast, the mutant mice had severely degenerated inner hairs and barely detectable outer hairs.

Higher magnification SEM images exhibited more precise changes in the *kuru*² mice (Figure 2). The inner hair cells had extremely short stereocilia, which were mostly degenerated without loss of the cell body. Alignment of the stereocilia was disrupted. More remarkable changes were observed in the outer hair cells, which were severely degenerated, with obliterated and untraceable stereocilia. The findings suggested diachronic degeneration of the hair cells after formation of the organ of Corti. To confirm this progressive deterioration of the hair cells, the outer hair cells of *kuru*² mice were sequentially examined (Figure 3). On day 7, the shape of the stereocilia was normal, as demonstrated previously. The stereocilia started to degenerate on post-natal day 12 and deterioration of the outer hair bundles progressed until day 18. Afterwards,

degeneration occurred through disappearance of the V-shaped conformation. These observations suggested that degeneration was predestined to occur by an unidentified underlying mechanism such as insufficient intrinsic factors or lack of homeostasis, rather than by external factors such as intoxication or traumas.

In the previous study, the genetic pattern of *kuru*² mice was assumed to be autosomal recessive, based mainly on the pedigree. Nevertheless, the final determination of the genetics underlying the phenotype is important for further analysis. Since degeneration of the hair cells was prominent and easy to identify, we attempted to confirm whether the stereocilia, spiral ganglion and hearing acuity were affected in the offspring of cross-breeding. Wild-type Jcl:ICR mice were mated with *kuru*² mice and their cross-bred offspring were further analyzed. We evaluated degeneration of the spiral ganglion of the control, *kuru*², and their offspring. Unlike *kuru*² mice, electron microscopic examination failed to demonstrate a decrease in spiral ganglion cell numbers in the cross-bred offspring (Figure 4) (1). The cell density of the spiral ganglion neuron was close to that of the control wild-type mice and there was a significant difference between the cross-bred offspring and *kuru*² mice

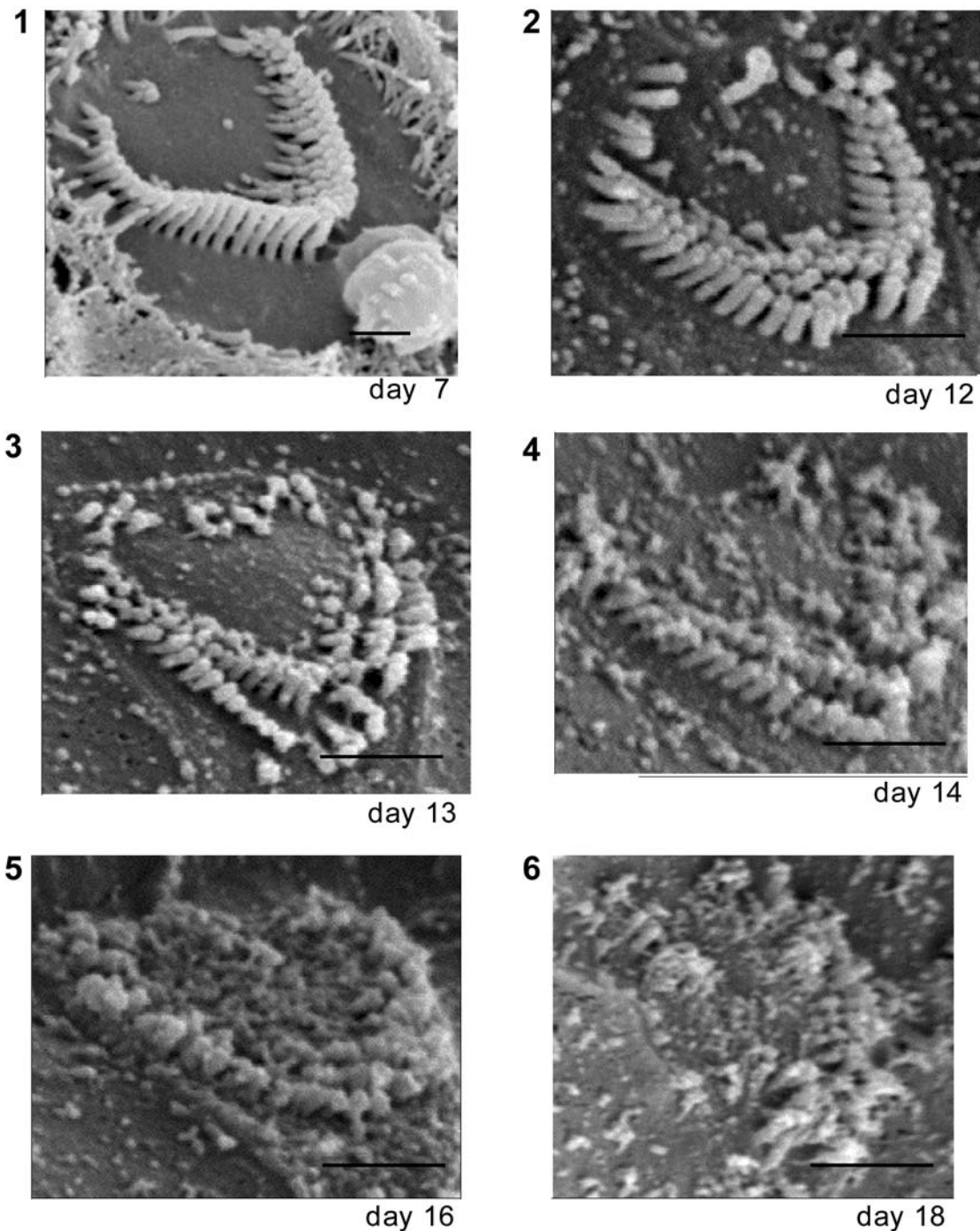
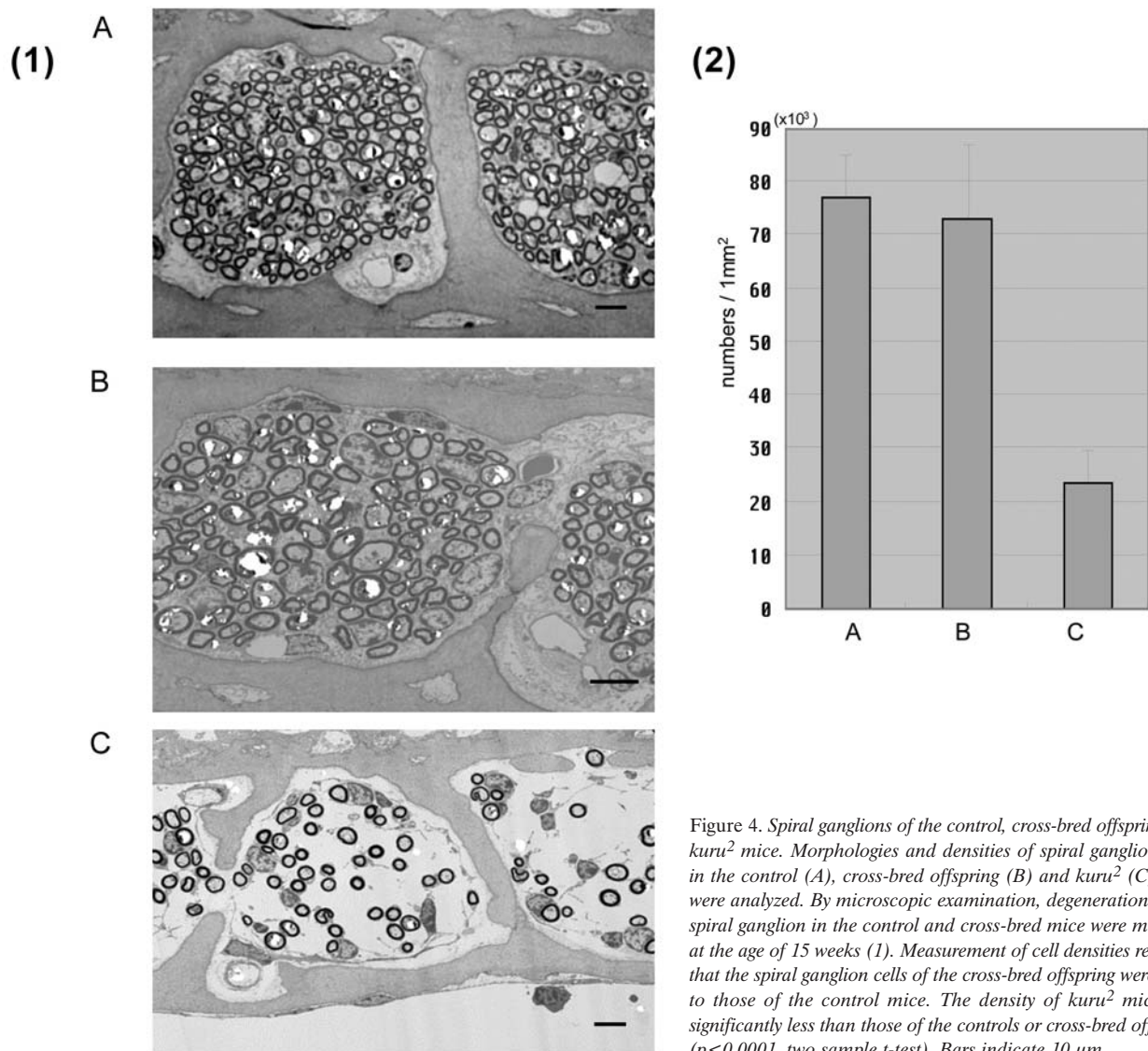


Figure 3. Changes in the outer hair cells in the *kuru*² mouse. On day 7, the shape of the stereocilia in the outer hair cell was normal, but began to degenerate on post-natal day 12 and deterioration of the outer hair bundle progressed until day 18. Degeneration was accomplished by disappearance of the V-shaped conformation. Bars indicate 1 µm.

($p < 0.0001$). Histology of the hair cells revealed that the stereocilia of the cross-bred offspring were intact even in elderly mice (Figure 5A). The auditory brain stem response was also evoked in the cross-bred offspring. I-V as well as

VI and VII peaks were clearly recognized in the mice. Thus, the phenotype of the cross-bred offspring was different from that of the *kuru*² mice (Figure 5B). These findings confirmed the autosomal recessive pattern of inheritance.



Next, we attempted to address the morphology of the vestibular sensory hair cells in *kuru*² mice. The mutant mouse demonstrated circling behavior and ataxic gait. Since *kuru*² mice had hearing impairment and degeneration of the sensory hair cells in the cochlea, degeneration in the vestibular hair cells was possible, but was not supported by the results, even in elderly mice. However, the hair cells in the cochlea were collapsed in *kuru*² mice (Figure 6A). Nonetheless, both the stereocilia and kinocillium of the vestibular hair cells were intact and clearly identified in the same individuals (Figure 6B).

Discussion

The mutant mice, *kuru*², displayed progressive deterioration of the stereocilia in the cochlea. The stereocilia began to

degenerate from 10 to 18 post-natal days. This contrasted to the vestibule, which was found to be almost intact in the histological analysis.

Characteristic changes in the inner ear have been investigated in each mouse with a hearing disorder. For example, the *Brn-4* gene encodes a *Pou* transcription factor and it is expressed throughout the inner ear in mesenchymal cells of both the cochlea and vestibule during development, but not in tissues derived from neuroepithelial or neuronal cells. The *Brn-4* knock-out mouse exhibited profound hearing impairment. It had no pathological features of other structures of the inner ear, including inner and outer hair cells, the stria vascularis, the spiral ganglia and the auditory nerve. The organ of Corti had a well-differentiated structure consisting of both hair

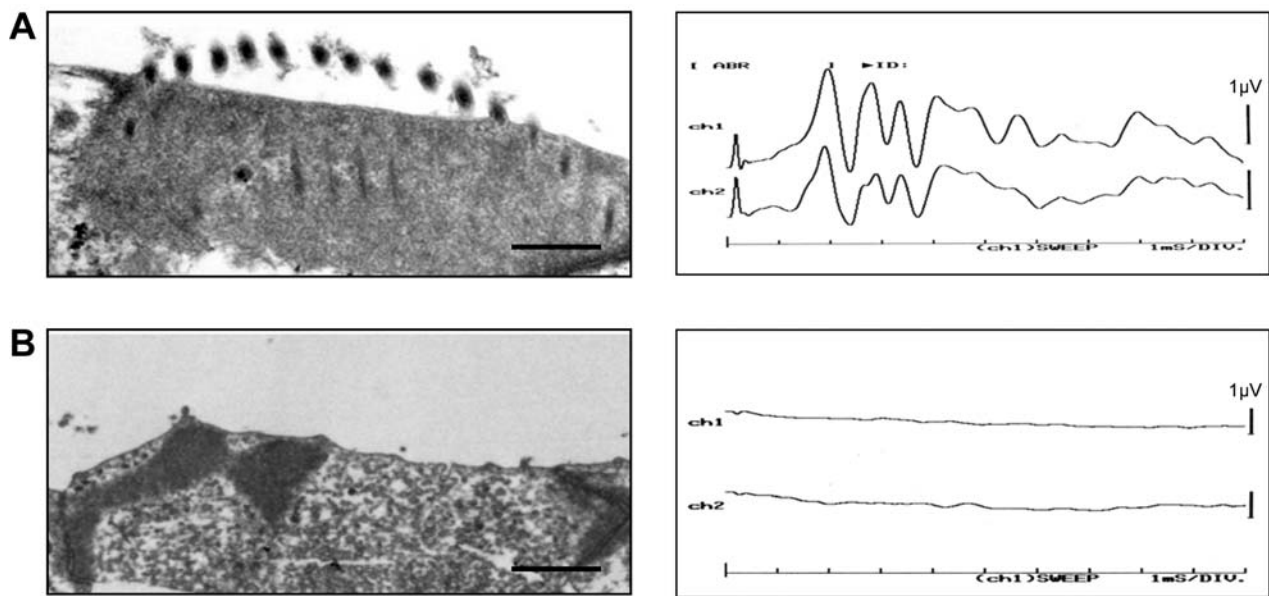


Figure 5. Histological and electrophysiological differences between the cross-bred offspring and the *kuru*² mice. TEM analysis of the outer hair cell (left) demonstrated the existence of stereocilia in the cross-bred offspring (A), but not in *kuru*² (B) mice at the age of 15 weeks. Bars indicate 1 μ m. The auditory brain stem response (right) demonstrated preservation of hearing acuity in the cross-bred offspring (A). Responses were not evoked in the mutant *kuru*² mice (B).

cells and several types of supporting cells, such as pillar and Deiters' cells resting on the basilar membrane. No gross morphological changes were observed in the conductive ossicles or cochlea. However, electron microscopy revealed severe ultrastructural alterations in cochlear spiral ligament fibroblasts (2).

In another case, mutation in *Col 11a2* was predicted to affect the triple domain of the collagen protein. Targeted disruption of the gene in the mouse also caused hearing loss. In the mouse, there were no detectable differences in the inner and outer hair cells, non-sensory epithelial cells, organ of Corti, neural structures or stria vascularis. However, morphological abnormality was seen in the tectorial membrane. Electron microscopy revealed loss of organization of the collagen fibrils (3).

Furthermore, otogelin, an N-glycosylated protein that is encoded by the gene, *Otog*, is present in acellular membranes, such as the tectorial membrane in the organ of Corti and otoconial membranes in the vestibule. Targeted disruption of *Otog* resulted in both hearing loss and severe imbalance. Ultrastructural analysis revealed large regions that contained either a few scattered wavy, fibrillar structures or extremely dense, rod-like structures, roughly parallel to the longitudinal axis of the tectorial membrane in the cochlea. In the vestibule, displacement and disorganized otoconial membranes were reported (4).

The morphology of the inner ear in these and many other hearing-impaired mice has been precisely reported. These

findings could provide valuable information on the pathogenesis of the disease in each mouse. Furthermore, diversity in the histological findings suggests distinctive functions of the affected genes.

The unconventional myosin genes *Myo 6*, *Myo 7a* and *Myo 15* are essential for hearing and mutations of these genes produce morphological changes of the affected mice. Despite the expression of each gene in multiple organs, mutations result in identifiable phenotypes in the vestibulocochlear organ. However, in the cochlea, their mutants show characteristic stereocilia defects. The *Myo 7a* mutant demonstrates disorganized stereocilia bundles similar to the mutation of *Cdh 23* (5), *Pcdh 15* (6, 7), or *Sans* (8). The *Myo 15* mutant shows short stereocilia similar to the pirouette mutant (*pi*) (9), while the *Myo 6* (Snell's waltzer) mutant shows early fusion of the stereocilia (10). By comparing the stereocilia in each mouse and viable double mutants, functions of *Myo 15* distinct from those of *Myo 6* and *Myo 7a* were demonstrated in the development or maintenance of the stereocilia (11).

In the current study, we demonstrated the sequential changes in the stereocilia in the *kuru*² mouse. We also demonstrated the dissimilarity of hair cell changes in the cochlea and vestibule. Since no morphological changes have been investigated in the mouse, the presentation of these abnormalities may contribute to the further understanding of hereditary hearing impairments mechanisms.

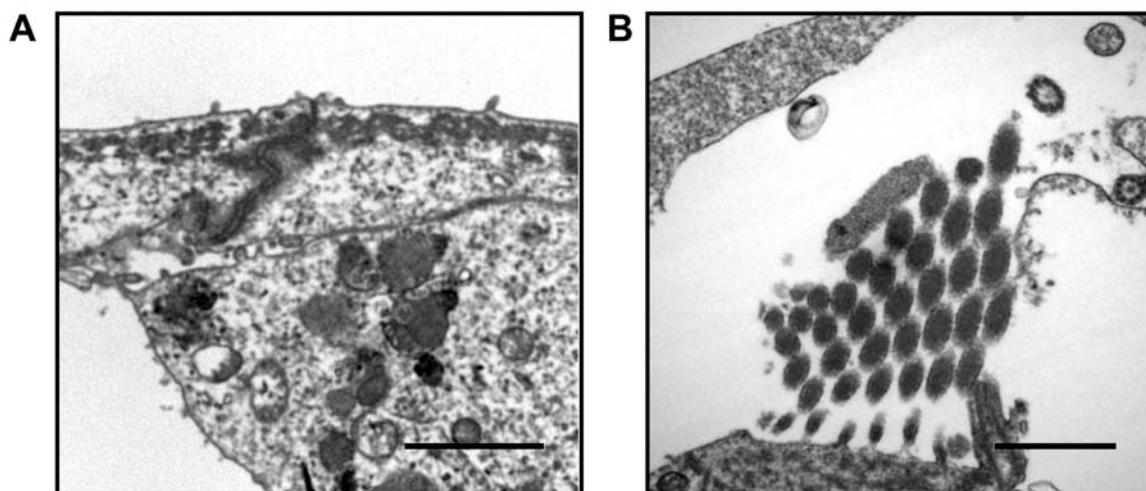


Figure 6. Comparison of cochlear and vestibular hair cells in the *kuru*² mice. TEM analysis of outer hair cells in the cochlea demonstrated degenerated stereocilia in the *kuru*² mouse at the age of 15 weeks (A). This finding was consistent with previous results. However, when the vestibules were examined, both stereocilia and kinocilium of the hair cells were preserved in the same individual (B). The finding is characteristic of *kuru*² mice. Bars indicate 1 µm.

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