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Late-onset hearing loss in a mouse model of DFN3 non-syndromic deafness: morphologic and immunohistochemical analyses

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Abstract

Recently, we reported that homozygous males and females of a mouse model of DFN3 non-syndromic deafness generated by the deletion of *Brn-4* transcription factor showed profound deafness due to severe alterations in the cochlear spiral ligament fibrocytes from the age of 11 weeks, whereas no hearing loss was recognized in young female heterozygotes. It is known that a part of obligate female carriers of DFN3 showed progressive hearing loss. In the present study, we examined the late-onset effect of *Brn-4* deficiency on the hearing organ of the mouse. About one third of heterozygous female mice revealed late-onset profound deafness at the age of 1 year. Furthermore, in these deafened heterozygotes, characteristic abnormalities in Reissner's membrane attachment and type II fibrocytes in the suprastrial zone became evident under light microscope, similar to homozygous female mice. A significant reduction in the immunoreactivity of connexin 26 (Cx26), connexin 31 (Cx31), Na,K-ATPase and Na-K-Cl cotransporter in the spiral ligament fibrocytes was observed in aged heterozygotes showing late-onset profound deafness. The late-onset phenotype observed in heterozygous mutant mice, being consistent with the progressive deafness observed in human female heterozygotes, may be explained by alterations of the ion transport systems in the spiral ligament fibrocytes.

Key words: *Brn-4*; Heterozygous female mouse; Cx26; Cx31; Na,K-ATPase; Na-K-Cl cotransporter

1. Introduction

Brn4/RHS2/Pou3f4, encoding one of the mammalian class III POU transcription factors, is the gene responsible for DFN3, which was first discovered as non-syndromic deafness in 1995 (de Kok et al., 1995). Among the human gene diseases, DFN3, an X chromosome-linked non-syndromic mixed deafness, is a clinical phenotype that includes conductive hearing loss, perilymphatic flow during stapes surgery, and progressive sensorineural hearing loss. Recently, by using a gene-targeting technique, we demonstrated that the sensorineural hearing loss in a mouse model of DFN3 was brought about by a disturbance in the spiral ligament fibrocytes that lead to a reduction of the endocochlear potential (EP), which is essential for the depolarization of mechano-sensory hair cells. Furthermore, another research group described that targeted mutagenesis of the *Brn4/Pou3f4* in mice brought about abnormalities in the oval window and the stapes, which may explain the conductive deafness (Phippard et al., 1999).

It was reported that a part of obligate female carriers with DFN3 showed progressive hearing loss from a young age (Cremers and Huygen, 1983; Arellano et al., 2000). The present study was designed to assess the similarity of phenotypes between human DFN3

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and the mouse model in the aspect of late-onset hearing loss in females. In addition, we tried to elucidate the underlying mechanism of the sensorineural hearing loss based on the reductions in the immunoreactivity of Cx26, Cx31, Na,K-ATPase, and Na-K-Cl cotransporter in the fibrocytes of the cochlear lateral wall of 1-year-old *Brn-4* deficient mice.

2. Materials and methods

2.1. Brn-4 deficient mice

To construct a targeting vector a 1.3-kb *Acc*I–*Bam*HI fragment and a 6-kb *Eco*RI fragment were isolated from a J1 genomic DNA library and used as short and long homologous sequences, respectively. The PGKneo cassette containing its own polyadenylation site flanked by a pair of loxP sequences, and a diphtheria toxin A chain gene cassette without the polyA signal were used for positive and negative selections, respectively. The vector was linearized by *Not*I before electroporation. To generate mutant mice, electroporation of J1 embryonic stem cells derived from 129/sv mice with a targeting vector was performed and cell clones were selected with G418. This clone was injected into blastocysts from C57BL/6 mice and the resulting chimeras were mated to C57BL/6 mice. The confirmation of germ line transmission was described as elsewhere (Minowa et al., 1999).

The care of the animals used in this study was approved by the Institute for Experimental Animals of Tohoku University School of Medicine.

2.2. Auditory brainstem responses

For the auditory brainstem response (ABR) measurements mice were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg) and then maintained in a head holder within an acoustically and electrically insulated and grounded test room. Stainless steel needle electrodes were placed on the tympanic bulla (positive lead) and scalp vertex (negative lead). The ABRs were obtained using an evoked potential recording system (NEC, Tokyo, Japan). Acoustic stimuli evoked by a click were delivered to the animals through a loudspeaker. The peak amplitude was measured as the peak-to-trough and the threshold was defined as 1 μV.

2.3. Histology

For light microscopy, animals were anesthetized by sodium pentobarbital and the temporal bullae were removed. Cochleas were fixed by perilymphatic perfusion via the round window and a small hole on the apex with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), overnight at 4°C. After rinsing with 0.01 M phosphate buffer saline (pH 7.4), the specimens were decalcified with 0.12 M EDTA (pH 7.2) at room temperature, dehydrated with ethanol, and embedded in paraffin. Serial sections of 6 μm thickness were prepared in the mid-modiolar plane and every 20th section was stained with hematoxylin-eosin.

2.4. Immunohistochemistry

For the immunostaining, the mounted sections were deparaffinized, rehydrated, and exposed to 5% normal goat serum in PBS for 1 h, then incubated at room temperature overnight in primary antibody. Commercially obtained antibodies, including polyclonal rabbit anti-connexin 26 (Zymed Laboratories, USA), rabbit anti-mouse Cx31 antiserum (ALP, USA), and monoclonal mouse anti-Na-K-Cl cotransporter (ARP, USA), were employed at dilutions of 1:200, 1:100, and 1:640 000, respectively with 1% bovine serum albumin (BSA) in PBS. After washing with PBS, the sections were flooded for 1 h with a 1:300 dilution of biotinylated goat anti-rabbit immunoglobulin (Dako, Denmark) for both Cx26 and Cx31 and a 1:500 dilution of biotinylated goat anti-mouse immunoglobulin (Dako, Denmark) for Na-K-Cl cotransporter in 1% BSA-PBS. After washing with PBS again, the sections were flooded for 1 h with Vectastain ABC reagent (Vector Laboratories, USA), and thoroughly rinsed in PBS. The sites of the bound primary antibodies were visualized by development for 10 min in 3,3′-diaminobenzidine (DAB)-H_2O_2 substrate medium prior to dehydration and cover slipping.

For the immunostaining of Na,K-ATPase, the deparaffinized sections were exposed to 5% normal rabbit serum in PBS for 1 h and incubated at room temperature overnight in commercially obtained chick antiserum against Na,K-ATPase (Cortex Biochem, USA) at a dilution of 1:2000 with 1% BSA in PBS. After washing with PBS, the sections were flooded for 1 h with a 1:500 dilution of biotinylated rabbit anti-chick IgG (Chemicon, USA) in 1% BSA-PBS. Subsequent steps were carried out as described above.

Control sections were processed in parallel in each protocol and included substitution for the primary antibody with a similar dilution of non-immune serum.

3. Results

ABR analysis was performed for all the female mice including 11–15-week-old heterozygotes, 1-year-old wild-type heterozygotes and homozygotes (Fig. 1). All

of the young heterozygotes showed a normal range of ABR thresholds of 24.1 ± 3.5 dB SPL (average ± standard deviation, $n = 10$), similar to those of wild mice (Minowa et al., 1999). The aged wild-type mice suffered a slight increase in the ABR thresholds with an average value of 53.9 ± 10.8 dB SPL ($n = 7$) which may have been due to the aging effect, based on the genetic background of the mouse strain used here (C57BL/6). Profound deafness with 100 ± 8.7 dB SPL ($n = 3$) was observed in the 1-year-old homozygotes. Interestingly, about one third of the heterozygote female mice showed profound deafness with an average ABR thresholds of 96.3 ± 6.5 dB SPL ($n = 4$) after 1 year, whereas the ABR thresholds of 50.3 ± 15.2 dB SPL ($n = 10$) of the others were distributed within the range of the wild-type 1-year-old mice.

Histological analysis of the inner ear structure was also performed on *Brn-4* wild and mutant female mice

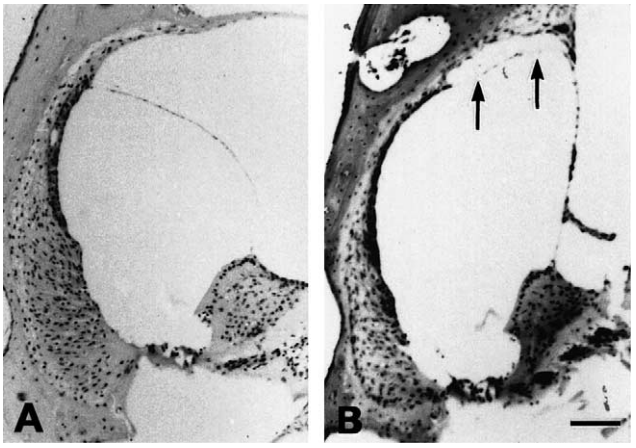

Fig. 2. A, B: Light microscopical photos of the basal turn of the cochlea in the 1-year-old mice. A: Wild-type mice. B: Mutant mice show abnormalities in the Reissner's membrane attachment and type II fibrocytes in the suprastrial zone (arrows). Bar = 100 μm.

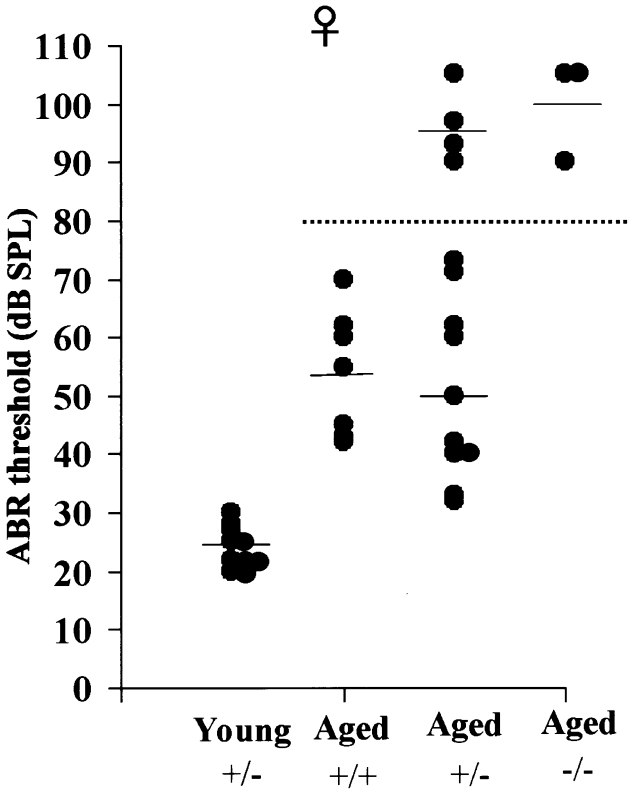

Fig. 1. Scatterplots of auditory brainstem response (ABR) thresholds for 11–15-week-old (young) and 1-year-old (aged) female mice. Young heterozygotes show a normal range of ABR thresholds with 24.1 ± 3.5 dB SPL (average ± standard deviation, young +/−). 1-year-old wild mice show higher ABR thresholds with 53.9 ± 10.8 dB SPL (aged +/+), which may be due to the aging effect on the hearing of the genetic background mouse strain (C57BL/6). Aged homozygotes (aged −/−) show profound deafness with 100 ± 8.7 dB SPL. About one third of the heterozygote female mice show profound deafness with 96.3 ± 6.5 dB SPL, whereas the ABR thresholds with 50.3 ± 15.2 dB SPL of the others are distributed within the range of the wild-type aged mice (aged +/−).

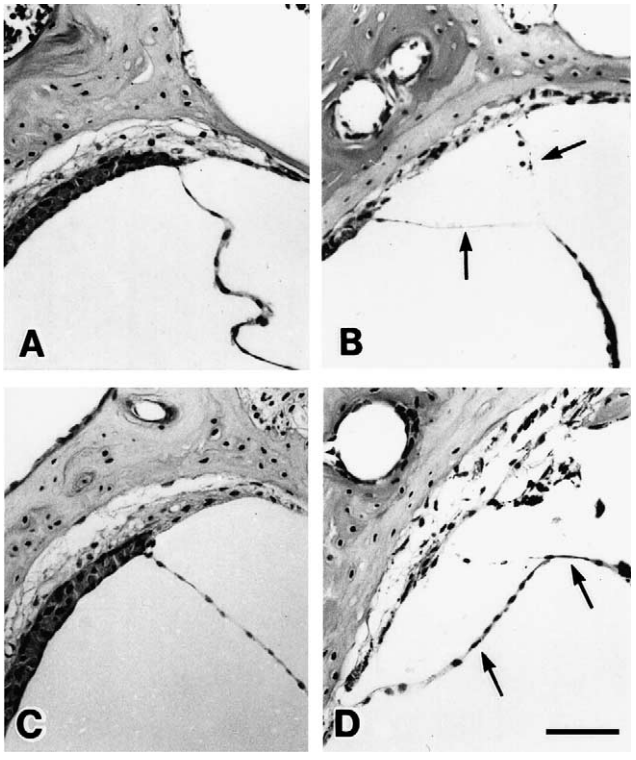

Fig. 3. A–D: High-magnification photos under light microscope of the suprastrial zone in the basal turn of the cochlea of 1-year-old female mice. A, C: The Reissner's membrane is firmly attached to the boundary between the suprastrial fibrocytes and the stria vascularis in the wild mice (A) and heterozygotes with good ABR thresholds (C). B, D: The detachment of the Reissner's membrane and the ballooning of the suprastrial zone are observed in the homozygotes (B) (arrows) and the heterozygotes with poor ABR thresholds (D) (arrows). Bar = 50 μm.

at the age of 1 year. No gross abnormality of the inner ear in 11-week-old *Brn-4* deficient mice was identified by extensive histological observations with a light microscope (Minowa et al., 1999). In the present study, degeneration of the organ of Corti and atrophy of stria vascularis were observed in the cochlea of all 1-year-old mice. However, abnormalities in the Reissner's membrane attachment and type II fibrocytes of the suprastrial zone were observed in the mutant mice (Fig. 2B), but not in the wild mice (Fig. 2A). The detachment of the Reissner's membrane and the ballooning of the suprastrial zone, presumably due to the degenerative changes in the fibrocytes of the 11-week-old mutants detected by ultrastructural observations, became evident by light microscopy in the 1-year-old mutant mice. High magnification of the 1-year-old female wild mice revealed that the Reissner's membrane was firmly attached to the boundaries between the suprastrial fibrocytes and the stria vascularis (Fig. 3A). The detachment of the Reissner's membrane and the ballooning of the suprastrial zone were observed in the 1-year-old homozygous mutant mice (Fig. 3B). Abnormalities both in the Reissner's membrane attachment and in the suprastrial zone were also detected in all of the 1-year-old heterozygotes suffering profound hearing loss (Fig. 3D), but not in the 1-year-old heterozygotes with a relatively elevated ABR thresholds due to the aging effect (Fig. 3C). These observations suggested that the late-onset deafness of the heterozygotes was similar to that of human DFN3 females.

We next elucidated the expression of proteins in the cochlear lateral wall, including Cx26, Cx31, Na,K-ATPase, and Na-K-Cl cotransporter. Dense immunoreactivity of Cx26 was expressed in the spiral ligament type I fibrocytes and the strial basal cells of the wild-type mice (Fig. 4A). Due to the aging effect based on the genetic background of the mouse strain, the immunoreactivity of Cx26 in the type II fibrocytes under the outer sulcus and spiral prominence epithelium was weak. This result coincided with that reported by Ichimiya (Ichimiya et al., 2000). Similarities also appeared in the heterozygous mice showing good ABR thresholds (Fig. 4C). The immunoreactivity of Cx26 was reduced definitely over the spiral ligament type I fibrocytes and the type II fibrocytes in the suprastrial zone in both the homozygotes (Fig. 4B) and the heterozygotes with profound deafness (Fig. 4D) as compared with that of the wild mice. On the other hand, the strial basal cells showed no reduction of Cx26 immunoreactivity.

The immunostaining of Cx31 was distributed clearly

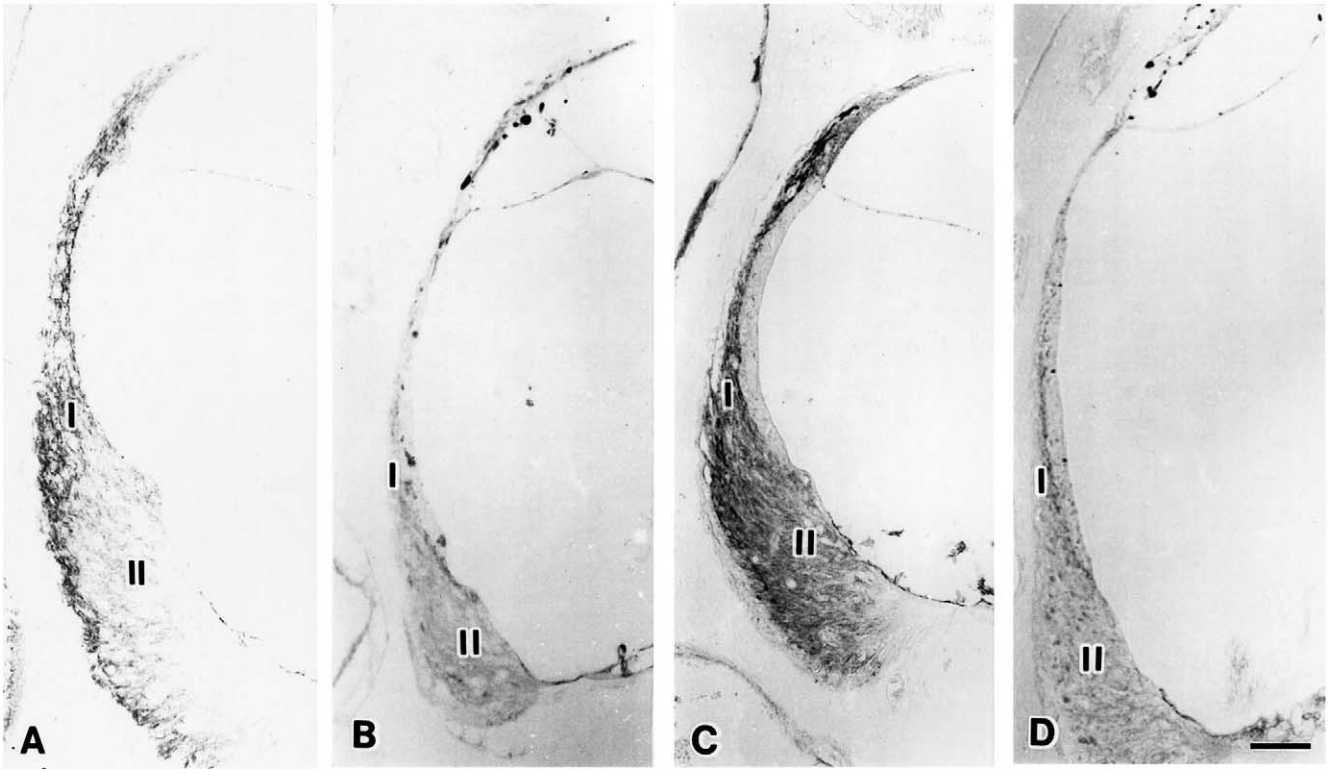

Fig. 4. A–D: Representative photomicrographs of immunohistochemical staining using antibodies against connexin 26 in the basal turn of the cochlea of 1-year-old mice. A, C: Dense immunostaining of Cx26 is observed in the type I fibrocytes (I) of the cochlear lateral wall and the strial basal cells in both the wild-type mice (A) and the heterozygous mice with good ABR thresholds (C). Weak immunostaining is shown in the type II fibrocytes (II). B, D: Immunoreactivity of Cx26 is clearly decreased in the fibrocytes of the spiral ligament and suprastrial zone, but not strial basal cells, in both homozygotes (B) and heterozygotes (D) with poor ABR thresholds. Bar = 50 μm.

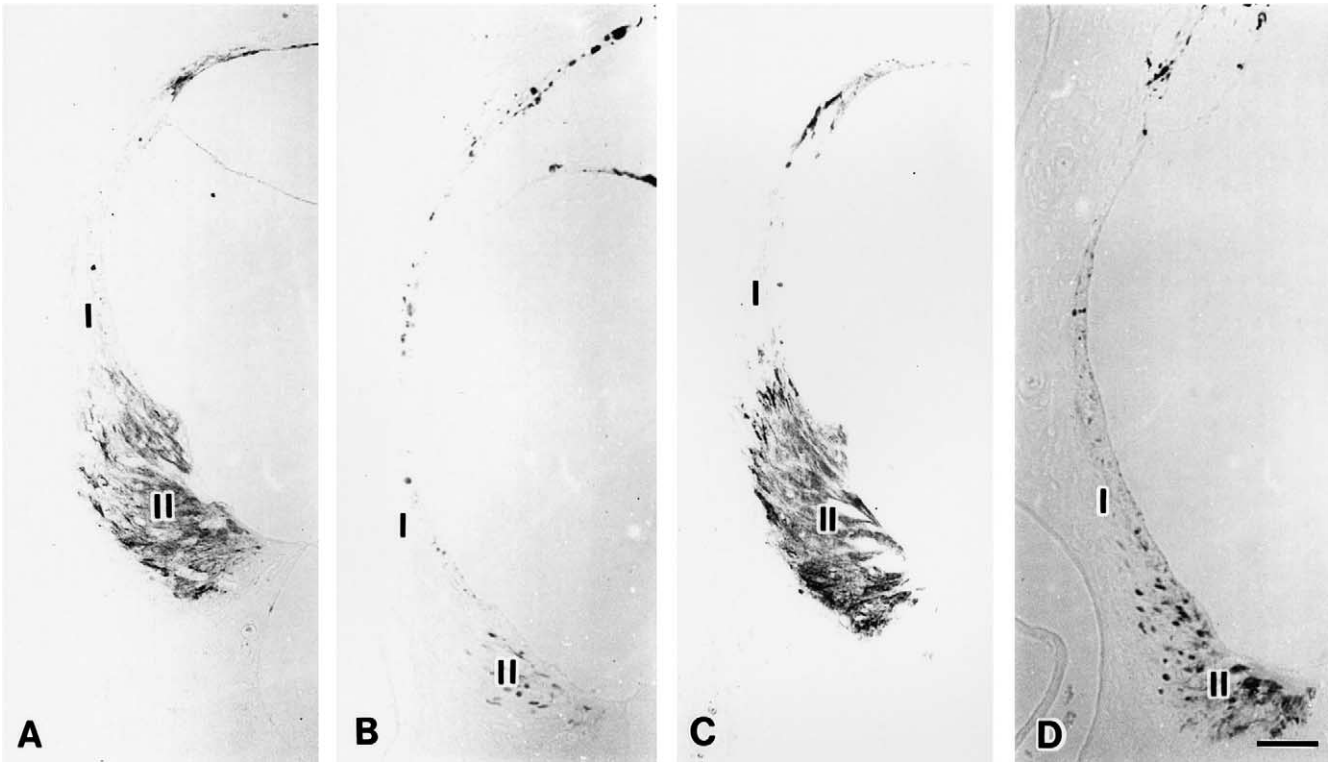

Fig. 5. A–D: Representative photomicrographs of immunohistochemical staining using antibodies against connexin 31 in the basal turn of the cochlea of 1-year-old mice. A, C: The immunostaining of Cx31 is apparently distributed in type II fibrocytes (II) of the spiral ligament and the suprastrial zone from both the wild-type mice (A) and the heterozygotes with good ABR thresholds (C). B, D: The immunoreactivity of Cx31 in the type II fibrocytes is definitely reduced in both the homozygotes (B) and the heterozygotes with poor ABR thresholds (D). I, type I fibrocyte area; bar = 50 μm.

in type II fibrocytes of the spiral ligament and the suprastrial zone in both the wild-type mice (Fig. 5A) and the heterozygotes with good ABR thresholds (Fig. 5C). However, the immunoreactivity of Cx31 in the type II fibrocytes was reduced definitely in both the homozygotes (Fig. 5B) and the heterozygotes with poor ABR thresholds (Fig. 5D).

Strong immunoreactivity of Na,K-ATPase was observed in both the strial marginal cells and the type II fibrocytes of the cochlear lateral wall in the wild-type mice (Fig. 6A). In the heterozygotes, dense immunoreactivity of Na,K-ATPase was observed in the mice with good ABR thresholds (Fig. 6C). In the homozygous mice (Fig. 6B) and the heterozygous mice with poor ABR thresholds (Fig. 6D), the immunoreactivity of Na,K-ATPase was evidently decreased in the spiral ligament type II fibrocytes and was slightly decreased in the strial marginal cells.

As with Na,K-ATPase, strong immunoreactivity of Na-K-Cl cotransporter was also observed in the strial marginal cells and the spiral ligament type II fibrocytes of both the wild-type mice (Fig. 7A) and the heterozygotes with good ABR thresholds (Fig. 7C). For homozygous mice (Fig. 7B) and heterozygous mice with poor ABR (Fig. 7D), the immunoreactivity of Na-K-Cl cotransporter was apparently decreased in the type II fibrocytes of the spiral ligament and less decreased in the marginal cells.

4. Discussion

Our previous study indicated that all of the 11-week-old heterozygous female mice showed a completely normal phenotype of the cochlear function and morphology (Minowa et al., 1999). In the present experiment, however, ABR measurements demonstrated that about one third of 1-year-old heterozygous female mice showed clearly profound deafness, while the rest of female mice showed moderate hearing loss due to the aging effect based on the genetic background of the C57BL/6 mice, as reported by other papers (Hunter and Willott, 1987; Hequembourg and Liberman, 2001). As well as the inbred C57BL/6 mouse strain, the inbred 129/sv mouse strain, which was the other genetic background of our study, also exhibited slightly elevated ABR thresholds even at 9 weeks of age (Zheng et al., 1999). However, the hybrids between C57BL/6 mice and 129/sv mice showed normal ABR thresholds at 15 weeks of age in this study. The ABR results sug-

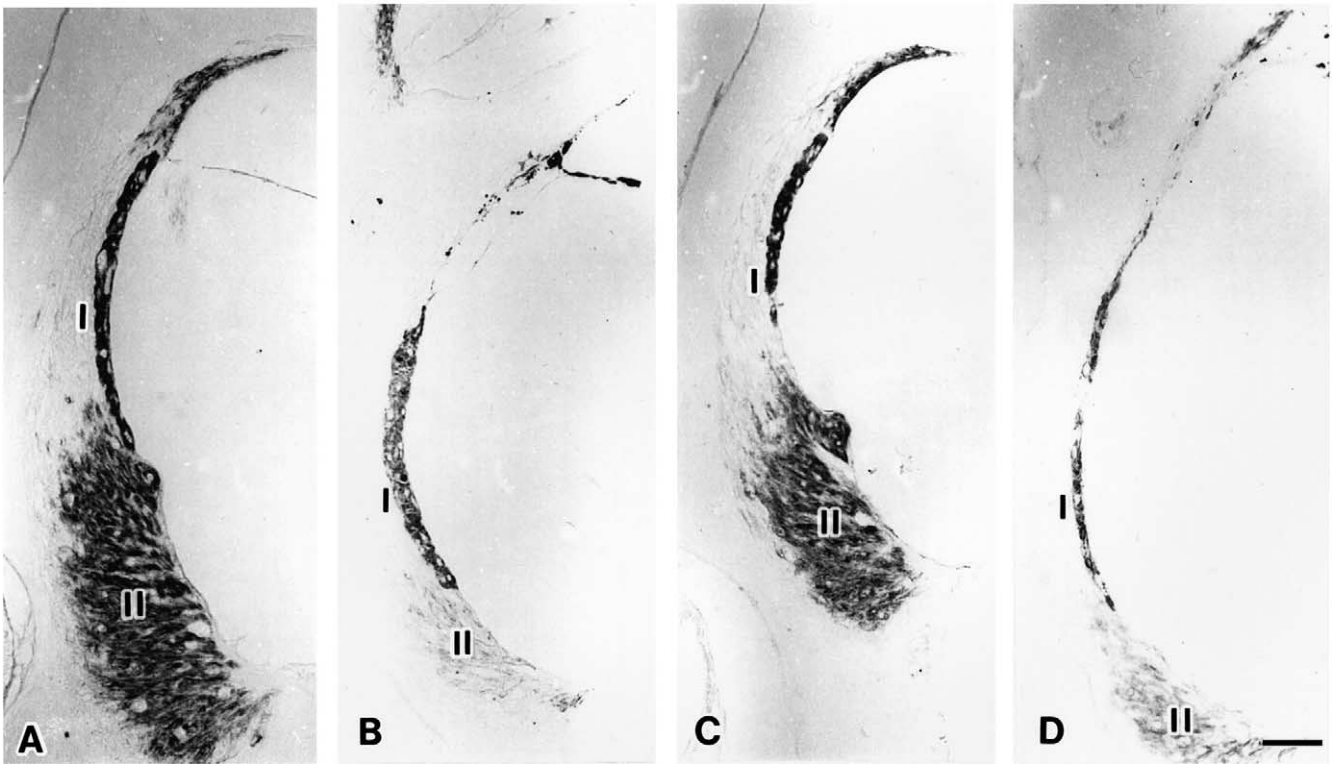

Fig. 6. A–D: Representative photomicrographs of immunohistochemical staining using antibodies against Na,K-ATPase in the basal turn of the cochlea of 1-year-old mice. A, C: Strong immunoreactivity of Na,K-ATPase is observed in both the strial marginal cells and the type II fibrocytes (II) of cochlear lateral wall in wild-type mice (A) and in heterozygous mice with good ABR thresholds (C). B, D: The immunoreactivity of Na,K-ATPase is evidently decreased in type II fibrocytes of the spiral ligament and the suprastrial zone, but is slightly decreased in the strial marginal cells in both the homozygous mice (B) and heterozygous mice with poor ABR thresholds (D). I, type I fibrocyte area; bar = 50 μm.

gest that the two different hearing loss genes of the two mouse strains may complement each other, hence the hearing of the hybrids may be normal. Even in 1-year-old mice, these hybrids only showed moderate hearing loss that was within the range of hearing loss of the genetic background. As also described by Mikaelian (Mikaelian et al., 1974), the degeneration of the organ of Corti and the atrophy of the stria vascularis in the cochlea were also observed in all 1-year-old mice of our study. Recently, Hequembourg and Liberman qualitatively evaluated the loss of hair cells, ganglion cells, and fibrocytes and suggested that loss of the type IV cell class of fibrocytes may be a primary cause of age-related hearing loss in the C57BL/6 mouse strain (Hequembourg and Liberman, 2001). In our study, no obvious degeneration of the spiral ligament fibrocytes was observed in the 1-year-old wild-type mice probably due to the thin sections used here (6 μm).

We found that Reissner's membrane attachment was abnormal and that the type II fibrocytes in the suprastrial zone were disordered in the homozygous mice and heterozygous female mice with poor ABR thresholds, but not in the heterozygous female mice with good ABR thresholds. These findings imply that one third of heterozygous mutant female mice of the *Brn-4* null allele had progressive or late-onset deafness. Clinical data have shown that a part of obligate female carriers with DFN3 had progressive hearing loss (Cremers and Huygen, 1983; Arellano et al., 2000). As described above, there exist some common characteristics between human female heterozygotes and the mice model heterozygotes. One is late-onset and/or progressive phenotype expression and the other is relatively low penetrance of the phenotype occurrence. Accordingly, *Brn-4* mutant mice could be a unique model of human DFN3 non-syndromic hereditary deafness that can provide useful tools for analyzing the mechanisms underlining gradually developing functional defects of the inner ear that lead to the severe hearing impairment. Although the mechanisms remain largely unknown, they could have some relevance to the mode of cellular X chromosome inactivation by which X-linked *Brn-4* alleles are randomly selected for expression. As a result of X inactivation in a very early stage of embryonic development (Gardner and Lyon, 1971), the cochlear fibrocytes derived from otic mesenchyme should consist of a mixture of *Brn-4* expressing cells and non-expressing cells to form the mosaic pattern of the adult cochle-

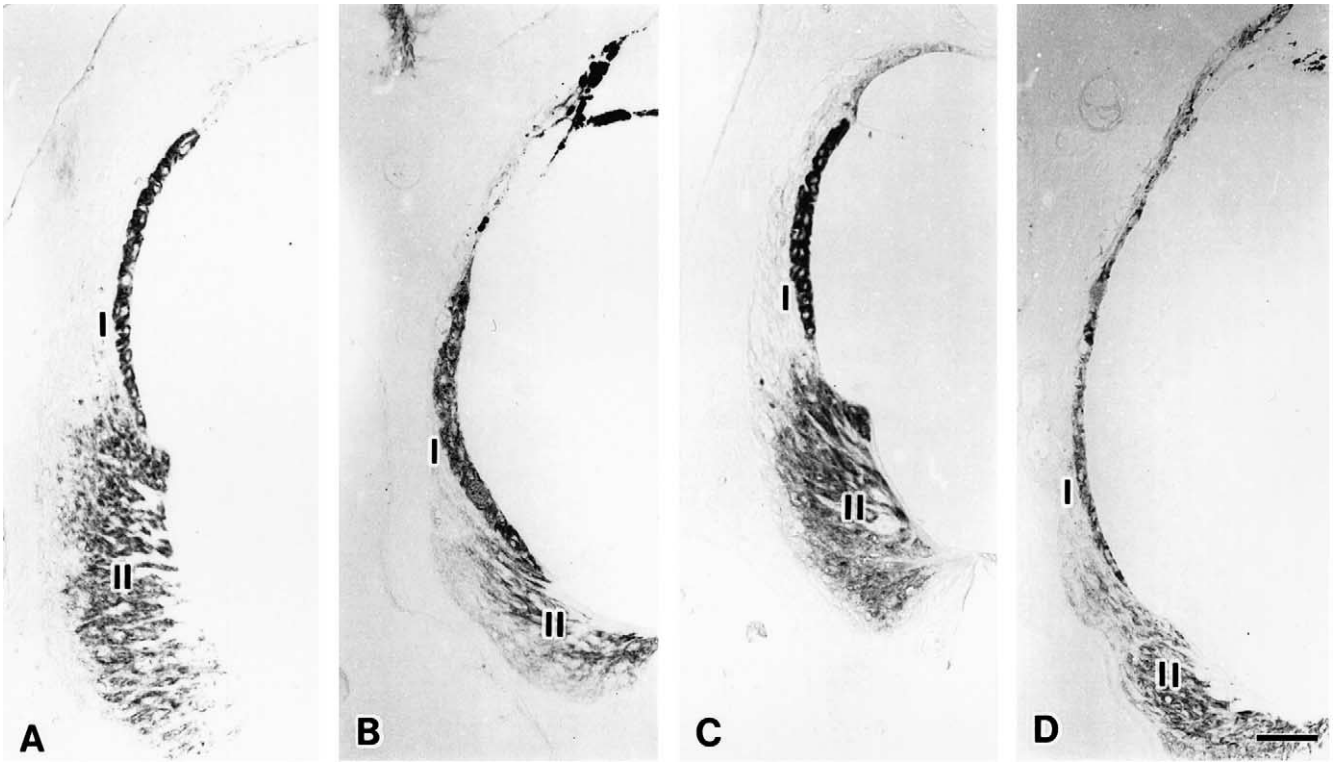

Fig. 7. A–D: Representative photomicrographs of immunohistochemical staining using antibodies against Na-K-Cl cotransporter in the basal turn of the cochlea of 1-year-old mice. Strong immunoreactivity of Na-K-Cl cotransporter is observed in the strial marginal cells and the type II fibrocytes (II) of the cochlear lateral wall from both the wild-type mice (A) and the heterozygotes with good ABR thresholds (C). For homozygous mice (B) and poor ABR heterozygous mice (D), immunoreactivity of Na-K-Cl cotransporter is clearly decreased in the type II fibrocytes of the spiral ligament, and less decreased in the marginal cells compared with the wild-type mice. I, type I fibrocyte area; bar = 50 μm.

ar spiral ligament. The content of the non-expressing cell population and the sizes of the patches of the mosaicism in these fibrocytes could vary from mouse to mouse (Wareham et al., 1983; Wareham and Williams, 1986) resulting in the variable extent of functional defects of the cochlear spiral ligament, which could be one reason for the relatively low penetrance of the heterozygous female profound deafness. The heterogeneity of the genetic background among the heterozygous females should also be taken in account. However, the late-onset and/or progressive phenotype expression still remains unexplained.

The high K^+ ion concentration and approximately 100 mV EP of the cochlear endolymph are essential for the auditory function in the mammalian cochlea. Kikuchi et al. (1995) suggested that the fibrocytes within the cochlear lateral wall were coupled by gap junctions and could be classified into a connective tissue cell gap junction system. Mutations in the *GJB2* gene encoding Cx26, an essential gap junction protein, can cause both recessive and dominant forms of non-syndromic deafness (Kelsell et al., 1997; Estivill et al., 1998). We previously reported that the generation and maturation of the EP may depend upon the postnatal development of gap junctional communication among the fibrocytes in the cochlear lateral wall and the Na,K-ATPase activity of the type II and suprastrial fibrocytes (Xia et al., 1999). Mutations in the *GJB3* gene encoding Cx31, another gap junction protein, can also lead to both recessive and dominant forms of non-syndromic

Table 1
Immunostaining of Cx26, Cx31, Na,K-ATPase and Na-K-Cl cotransporter in the spiral ligament type II fibrocytes of the 1-year-old mice

Antibody type	Gene type			
	Wild	Homozygote	Heterozygote, good ABR thresholds	Heterozygote, poor ABR thresholds
Connexin 26	1+	1+	1+ to 2+	1+
Connexin 31	3+	0 to 1+	3+	0 to 1+
Na,K-ATPase	3+	0 to 1+	3+	0 to 1+
Na-K-Cl cotransporter	3+	1+ to 2+	3+	1+ to 2+

Staining is based on a subjectively estimated scale from 0 = non-reactive to 3+ = strongly reactive.

deafness (Xia et al., 1998; Liu et al., 2000). According to our recent data, Cx31, which was only expressed in the spiral ligament fibrocytes in the developing cochlea, would contribute to a specific auditory function at high-frequency regions in a different way as Cx26 (Xia et al., 2000).

Na,K-ATPase and Na-K-Cl co-transporter in the basolateral plasma membrane of strial marginal cells and in type II fibrocytes of the cochlear lateral wall play an important role in the recycling of K^+ and the generation and maintenance of the EP (Schulte and Adams, 1989; Schulte and Schmiedt, 1992; Crouch et al., 1997; Sakaguchi et al., 1998).

The theory of recycling K^+ from the perilymph to endolymph is widely accepted (Steel, 1999). Mesenchymal fibrocytes of the cochlear lateral wall play an important role in K^+ recycling processes. The immunostaining of Cx26, Cx31, Na,K-ATPase and Na-K-Cl cotransporter in the spiral ligament type II fibrocytes of the 1-year-old mice was summarized in Table 1. From this table, it was indicated that as well as the immunoreactivity of Na,K-ATPase and Na-K-Cl cotransporter, the immunoreactivity of Cx26 and Cx31 in the fibrocytes of the cochlear lateral wall was also clearly decreased for the 1-year-old homozygotes and the heterozygotes with profound deafness. The reduction of immunoreactivity of Cx26, Cx31, Na,K-ATPase and Na-K-Cl cotransporter in the disordered fibrocytes of the cochlear lateral wall could injure the pathway of the K^+ recycling in the adjacent connective tissue system of fibrocytes and cause deafness.

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References

Arellano, B., Camacho, R.R., Berrocal, J.R.G., Villamar, M., Castillo, I.D., Moreno, F., 2000. Sensorineural hearing loss and mondini dysplasia caused by a deletion at locus *DFN3*. Arch. Otolaryngol. Head Neck Surg. 126, 1065–1069.

Cremers, C.W.R.J., Huygen, P.L.M., 1983. Clinical features of female heterozygotes in the X-linked mixed deafness syndrome (with perilymphatic gusher during stapes surgery). Int. J. Pediatr. Otorhinolaryngol. 6, 179–185.

Crouch, J.J., Sakaguchi, N., Lytle, C., Schulte, B.A., 1997. Immunohistochemical localization of the Na-K-Cl co-transporter (NKCC1) in the gerbil inner ear. J. Histochem. Cytochem. 45, 773–778.

de Kok, Y.J.M., van der Maarel, S.M., Bitner-Glindzicz, M., Huber, I., Monaco, A.P., Malcolm, S., Pembrey, M.E., Ropers, H.H., Cremers, F.P.M., 1995. Association between X-linked mixed deafness and mutations in the POU domain gene *POU3F4*. Science 267, 685–688.

Estivill, X., Fortina, P., Surrey, S., Rabionet, R., Melchionda, S., D'Agruma, L., Mansfield, E., Rappaport, E., Govea, N., Mila, M., Zelante, L., Gasparini, P., 1998. Connexin 26 mutations in sporadic and inherited sensorineural deafness. Lancet 351, 394–398.

Gardner, R.L., Lyon, M.F., 1971. X chromosome inactivation studied by injection of a single cell into the mouse blastocyst. Nature 231, 385–386.

Hequembourg, S., Liberman, M.C., 2001. Spiral ligament pathology: a major aspect of age-related cochlear degeneration in C57BL/6 mice. J. ARO 2, 118–129.

Hunter, K.P., Willott, J.F., 1987. Aging and the auditory brainstem response in mice with severe or minimal presbycusis. Hear. Res. 30, 207–218.

Ichimiya, I., Suzuki, M., Mogi, G., 2000. Aged-related changes in the murine cochlear later wall. Hear. Res. 139, 116–122.

Kelsell, D.P., Dunlop, J., Stevens, H.P., Lench, N.J., Liang, N.J., Parry, G., Mueller, R.F., Leigh, I.M., 1997. Connexin26 mutations in hereditary non-syndromic sensorineural deafness. Nature 387, 80–83.

Kikuchi, T., Kimura, R.S., Paul, D.L., Adams, J.C., 1995. Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. Anat. Embryol. 191, 101–118.

Liu, X.-Z., Xia, X.J., Xu, L.R., Pandya, A., Liang, C.Y., Blanton, S.H., Brown, S.D.M., Steel, K.P., Nance, W.E., 2000. Mutations in connexin31 underlie recessive as well as dominant non-syndromic hearing loss. Hum. Mol. Genet. 9, 63–67.

Mikaelian, D.O., Warfield, D., Norris, O., 1974. Genetic progressive hearing loss in the C57/bl6 mouse. Relation of behaviorial responses to cochlear anatomy. Acta Otolaryngol. (Stockh.) 77, 327–334.

Minowa, O., Ikeda, K., Sugitani, Y., Oshima, T., Nakai, S., Katori, Y., Suzuki, M., Furukawa, M., Kawase, T., Zheng, Y., Ogura, M., Asada, Y., Watanabe, K., Yamanaka, H., Gotoh, S., Nishi-Takeshima, M., Sugimoto, T., Kikuchi, T., Takasaka, T., Noda, T., 1999. Altered cochlear fibrocytes in a mouse model of DFN3 nonsyndromic deafness. Science 285, 1408–1411.

Phippard, D., Lu, L., Lee, D., Saunders, J.C., Crenshaw, E.B., III, 1999. Targeted mutagenesis of the POU-domain gene, *Brn/Pou3f4*, causes developmental defects in the inner ear. J. Neurosci. 19, 5980–5989.

Sakaguchi, N., Crouch, J.J., Lytle, C., Schulte, B.A., 1998. Na-K-Cl cotransporter expression in the developing and senescent gerbil cochlea. Hear. Res. 118, 114–122.

Schulte, B.A., Adams, J.C., 1989. Distribution of immunoreactive Na^+,K^+-ATPase in gerbil cochlea. J. Histochem. Cytochem. 37, 127–134.

Schulte, B.A., Schmiedt, R.A., 1992. Lateral wall Na,K-ATPase and endocochlear potentials decline with age in quiet-reared gerbils. Hear. Res. 61, 35–46.

Steel, K.P., 1999. The benefits of recycling. Science 285, 1363–1364.

Wareham, K.A., Williams, E.D., 1986. Estimation of the primordial pool size of the mouse liver using a histochemically demonstrable X-linked enzyme in the adult female mouse. J. Embryol. Exp. Morphol. 95, 239–246.

Wareham, K.A., Howell, S., Williams, D., Williams, E.D., 1983. Studies of X-chromosome inactivation with an improved histochemical

technique for ornithine carbamoyltransferase. Histochem. J. 15, 363–371.

Xia, A-P., Kikuchi, T., Hozawa, K., Katori, Y., Takasaka, T., 1999. Expression of connexin 26 and Na,K-ATPase in the developing mouse cochlear lateral wall: functional implications. Brain Res. 846, 106–111.

Xia, A-P., Ikeda, K., Katori, Y., Oshima, T., Kikuchi, T., Takasaka, T., 2000. Expression of connexin31 in the developing mouse cochlea. NeuroReport 11, 2449–2453.

Xia, J-h., Liu, C-y., Tang, B-s., Pan, Q., Huang, L., Dai, H-p., Zhang, B-r., Xie, W., Hu, D-x., Zheng, D., Shi, X-l., Wang, D-a., Xia, K., Yu, K-p., Liao, X-d., Feng, Y., Yang, Y-f., Xiao, J-y., Xie, D-h., Huang, J-z., 1998. Mutations in the gene encoding gap junction protein β-3 associated with autosomal dominant hearing impairment. Nat. Genet. 20, 370–373.

Zheng, Q.Y., Johnson, K.R., Erway, L.C., 1999. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear. Res. 130, 94–107.