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Progressive Hearing Loss in Mice with a Mutated Vitamin D Receptor Gene

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Key Words
Calcium · Ganglion cell · Hearing loss · Vitamin D · Caspase

Abstract
Background: Both hypo- and hypervitaminosis D can cause sensorineural hearing loss, and aural symptoms due to vitamin D insufficiency are especially common during gravidity. Hormonal forms of vitamin D regulate transcription by binding with the high-affinity vitamin D receptor (VDR). ***Objective:*** To assess the effects of impaired vitamin D action in VDR knockout (KO) mice on hearing, cochlear morphology, and cochlear gene expression. ***Materials and Methods:*** Eighteen young male and female mice (10 VDR KO and 8 wild type, WT, ≤6 months old), 33 adult male and female mice (16 VDR KO and 17 WT, between 7 and 14 months old), and 11 aged male and female mice (5 VDR KO and 6 WT, ≥15 months old) on 129S1 genetic background were studied. Auditory thresholds were evaluated by auditory brain stem response. Morphological changes were analyzed using plastic embedding and light microscopy. The expression of key genes (known to play a role in the regulation of cochlear function), and caspase 3 activity, were assessed using immunofluorescent confocal microscopy. ***Results:*** There was a statistically significant difference between the young and the adult groups, and between the adult and aged groups of WT mice. There was also a statistically significant difference between the adult and aged groups in VDR KO mice, and between the young WT group and the young VDR KO group. Spiral ganglion cell loss was observed in the basal turn of adult VDR KO mice, a phenomenon infrequently found in WT mice. Expression of connexin 26, KCNJ10, and transient receptor potential channel vanilloid subfamily 4/6 was not affected by VDR KO-mediated hearing loss. Caspase 3 activation was detected in the spiral ganglion cell and its satellite cells, stria vascularis, spiral ligament fibrocytes, and the organ of Corti in both genotypes. However, the percentage of positive cells and the staining intensity were lower in the VDR KO (compared to the WT) mice. ***Conclusion:*** These data suggest that sensorineural hearing loss progressively developed at an earlier age in VDR KO mice. While the fundamental gene expressions in the cochlea were not influenced by VDR mutation, it resulted in decrease of caspase 3 activation, which may be one of the factors underlying accelerating age-related hearing loss observed in VDR KO mice.

Introduction

The active forms of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25(OH)2D_3] and 25-hydroxyvitamin D_3, have several important biological roles including the regulation of calcium homeostasis, cellular differentiation, in-

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hibition of tumor cell proliferation, immune function, and brain development [McGrath et al., 2004; Veenstra et al., 1998]. All these actions are at least partially mediated by activation of the vitamin D receptor (VDR), a member of the steroid/nuclear receptor superfamily of ligand-activated transcription factors [Dusso et al., 2005; Holick, 2004, 2006; Lou et al., 2004; Ylikomi et al., 2002]. Vitamin D deficiency is a common factor in the reduction of bone-mineral density, and is associated with an increased risk of several chronic age-related diseases [Javaid et al., 2006; Zittermann, 2003]. Vitamin D deficiency, VDR malfunction, hypoparathyroidism, and hypervitaminosis have long been suggested to be potential causes of sensorineural hearing loss [Brookes and Morrison, 1981; Brookes, 1983, 1985; Cohen et al., 1979; Ikeda et al., 1989, 1987; Ishida et al., 2001; Ziporyn, 1983].

Mutant mouse lacking functional VDR – VDR knockout (KO) mouse – is a powerful tool for exploring the vital functions of vitamin D. Nevertheless, VDR KO mice require a special diet for survival, a problem that has only recently been solved [Li et al., 1998]. The behavioral phenotype of VDR KO mice include muscular and motor impairments, swimming disturbances and increased anxiety, reported by our group [Kalueff et al., 2004, 2006b] and further studied by others [Burne et al., 2006, 2005].

Our previous study also showed that a severe calcification developed in VDR KO mice in the thalamus [Kalueff et al., 2006a] – the brain structure that projects to the inferior colliculus of the auditory pathway and connects with the auditory cortex, as has recently been extensively investigated in both animals and humans [de la Mothe et al., 2006; Devlin et al., 2006; Pearson et al., 2007; Sigalovsky and Melcher, 2006; Takayanagi and Ojima, 2006]. Upon sustaining damage, neuronal retrograde degeneration was observed in the auditory system, and auditory brain stem response (ABR) amplitude decreased after inferior colliculus ablation [Kaga et al., 1999; Yamada et al., 2000]. Vitamin D also plays a trophic role in differentiation and maturation of neurons by promoting neurite outgrowth [Brown et al., 2003; Taniura et al., 2006]. Collectively, this suggests that hearing physiology may be affected in individuals with vitamin D/VDR dysfunctions.

In the present study, we analyzed hearing threshold levels and cochlear morphology to evaluate possible changes in the inner ear in VDR KO mice. In addition, gene expression and caspase 3 activation were also studied here.

Materials and Methods

Animals

Eighteen young male and female mice (10 VDR KO and 8 wild type, WT, $\leq$6 months old), 33 adult male and female mice (16 VDR KO and 17 WT, between 7 and 14 months old), and 11 aged male and female mice (5 VDR KO and 6 WT, $\geq$15 months old) were studied. VDR KO mice were initially generated at the University of Tokyo (Japan) [Yoshizawa et al., 1997]. All mice used were littermates on 129S1 genetic background produced by heterozygous crosses.

The VDR KO and WT mice used in the present study were maintained in a virus/parasite-free facility (temperature 24 $\pm$ 1°C, humidity 50 $\pm$ 5%), and exposed to a 12-hour light, 12-hour dark cycle. Lights were turned off at 19:00 and on at 7:00. The animals were experimentally naive and housed individually in transparent plastic cages (13 $\times$ 12 $\times$ 14 cm), with food and water freely available. To eliminate hypocalcaemia and rickets in the VDR KO mice, all mutant animals were fed a special rescue diet containing 2% calcium, 1.25% phosphorus, and 20% lactose (Lactamin AB, Sweden). The plasma Ca^{2+} level was only slightly lower in the VDR KO group (2.11 + 0.26 mM) than in the WT group (2.49 + 0.07 mM), and was also slightly below the normal levels reported previously for adult 129S1 mice (2.31–2.36 mM). All animal experiments were approved by the Ethical Committee of the University of Tampere. Animal care and experimental procedures were conducted in accordance with the European legislation.

Genotyping

On D21 postpartum, pups were weaned and tail clips were taken for genotyping performed using the polymerase chain reaction method on DNA prepared from tail tissue. Four primers were used to amplify a 166-bp VDR band (forward, 5′-CTG CTC TTC TTA CAG GGA TGG-3′, and reverse, 5′-GAC TCA CCT GAA GAA ACC CTT G-3′) and a 400-bp Neo band (forward, 5′-ATC TTC TGT CAT CTC ACC TTG C-3′, and reverse, 5′-CAA GCT CTT CAG CAA TAT CAC G-3′) from the targeted allele. After being genotyped, mice were assigned to different cages based on their genotype.

ABR Measurement

To assess auditory thresholds, BioSig32 (Tucker Davis Technologies, USA) was used for ABR threshold recording in both VDR KO and WT mice under general anesthesia with Domitor (0.8 mg/kg medetomidine hydrochloride) and Ketalar (80 mg/kg ketemine hydrochloride). A click duration of 50 μs and a repetition rate of 21.1/s were used for stimulation. Responses from 512 sweeps were averaged with a gain of 20 at each intensity level using a filter of 0.1–3 kHz. Thresholds were judged by visible repeatable responses.

Plasma Ca^{2+} Level Measurement

Before cardioperfusion, blood was taken from the heart to measure plasma Ca^{2+} level with atomic absorption spectroscopy (Yhtyneet Laboratoriot, Helsinki, Finland).

Plastic Embedding

The animals were perfused with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS (pH 7.4) following cardiac perfusion removal of the blood with 0.01 M PBS (pH 7.4) under general an-

esthesia with pentobarbital sodium (100 mg/kg). The bulla was removed and further fixed with the same fixative solution overnight. After washing with 0.1 M PBS, decalcification with 10% EDTA was performed at room temperature for 3 weeks. Dehydration was carried out by using 35, 70, 90, and 95% ethanol for 10 min each, and absolute ethanol twice for 15 min. After infiltrating with JB-4, samples were embedded at 4°C for 2 days. 2-μm sections were made with a microtone section machine (LEICA RM2265, Germany). The slides were stained with toluidine blue for light microscopy.

Immunofluorescent Confocal Microscopy

The animals were perfused with 4% formaldehyde in 0.1 M PBS (pH 7.4) following cardiac perfusion removal of the blood with 0.01 M PBS (pH 7.4). The bulla was removed and further fixed with the same fixative solution overnight. After washing with 0.1 M PBS, decalcification with 10% EDTA was performed at room temperature for 3 weeks. A standard paraffin embedding procedure was used and the samples were sectioned at 4-μm thickness. For immunofluorescent staining, the sections were heated at 60°C for 2 h, deparaffinized with xylene, and passed through gradient ethanol until a final PBS wash. The sections were digested with 0.1% trypsin at 37°C for 30 min, washed with PBS-T (0.1% Tween 20 was included) for 3 × 2 min, and incubated with 1:20 preimmunized goat serum at room temperature for 30 min. Different primary antibodies were used with incubation overnight at 4°C: rabbit polyclonal antibody to VDR, 1:10 (Abcam, UK, ab12129); mouse monoclonal antibody to synaptophysin, 1:200 (Sigma-Aldrich, USA); rabbit polyclonal antibody to connexin 26, 1:15 (Zymed, USA); rabbit polyclonal antibody to Kir4.1 (KCNJ10), 1:100 (Alomone Labs, Israel); rabbit polyclonal antibody to transient receptor potential channel vanilloid subfamily (TRPV) 4/6, 1:100 (Alomone Labs), and rabbit polyclonal antibody to caspase 3 active, 1:800 (R&D system, Inc., USA). For negative control, the primary antibodies were omitted in VDR, synaptophysin, connexin 26, KCNJ10, TRPV6, and caspase 3 active. The primary antibody was saturated with 0.4 mg/ml TRPV4 peptide containing 1% preinoculated goat serum overnight in the negative control for TRPV4 staining. After washing with PBS-T, the slides were incubated with FITC-conjugated goat antiserum against rabbit IgG (1:80, Sigma-Aldrich), Alexa Fluor® 568-labeled goat antiserum against rabbit IgG (1:400, Invitrogen, USA) or TRITC-conjugated goat antiserum against mouse IgG (1:200, Sigma-Aldrich, USA), which depended on the host primary antibody, at room temperature for 60 min, followed by incubation with 4′,6-diamidino-2-phenylindole (DAPI; 10 ng/ml, Sigma-Aldrich, USA) for 10 min. The slides were mounted with Gel Mount™ Aqueous Mounting Medium (Sigma-Aldrich, USA) after washing with PBS-T.

Confocal Microscopy

The immunofluorescently stained slides were observed under an Olympus microscope IX70 installed with ANDOR IQ (FITC fluorescence at 488 nm with a laser beam; Alexa Fluor® 568 and TRITC fluorescence at 568 nm with a laser beam; DAPI with a 340- to 380-nm filter). For caspase 3 activation quantification, the original confocal microscopy tiff images were evaluated with ImageJ 1.36b software. The positive percentage was calculated on the basis of counting the caspase 3 activation and total nuclear DAPI staining.

Statistics

Binomial test was used to compare the caspase 3 activation percentage between different groups. Student's t test was applied to compare the average ABR thresholds, spiral ganglion cell counting, and caspase 3 activation signal intensity between different groups. All data were presented as means ± SE.

Results

Hearing Loss in VDR KO Mice

An average threshold of 22 ± 1, 29 ± 2 and 73 ± 3 dB SPL was found in the young, adult and aged WT groups, respectively. There was a statistically significant difference between the young and adult WT groups ($p < 0.05$), and between the adult and aged WT groups ($p < 0.01$; fig. 1). In contrast, an average threshold of 32 ± 3, 35 ± 3, and 56 ± 8 dB SPL was found in the young, adult and aged VDR KO groups, respectively. Although there was no statistically significant difference between the young and the adult VDR KO mouse groups, we found a statistically significant difference between the adult group and aged group in VDR KO mice ($p < 0.05$; fig. 1). In addition, there was also a statistically significant difference between the young WT and VDR KO groups ($p < 0.01$), but not between the adult WT versus VDR KO, and aged WT versus VDR KO groups (fig. 1). ABR waveforms in VDR KO mice with hearing loss were disfigured when compared with WT mice (fig. 2a, b).

Cochlear Morphology

The cochlear structures including the organ of Corti, lateral wall, and spiral ganglion cells were fully developed in both WT (fig. 3a) and VDR KO mice (fig. 3b–d). The efferent nerve endings were detected using an antibody against synaptophysin underneath the inner hair cells and the outer hair cells (fig. 3c). An excessive layer of cells was found in the row of the outer hair cells in both VDR KO (fig. 3e) and WT (fig. 3f) mice. This does not seem to be an artifact because the 2-μm thickness of the sections cannot cover the overlap of hair cells. Condensed nuclear staining with toluidine blue of spiral ganglion cells in both VDR KO (fig. 3g) and WT mice (fig. 3h) is a sign of degeneration [Kawamura et al., 1997]. The volume of these cells was also extremely reduced. Numerous spiral ganglion cell losses were seen in the basal turn of adult VDR KO mice, which was infrequently found in WT mice (fig. 3i). The spiral ganglion cells were counted and the VDR-KO mice had, on average, 9.8 cells/mm^2 (SD 3.6) and the WT mice had 15.7 cells/mm^2 (SD 2.6). No statistically significant differences were found because of small sample size.

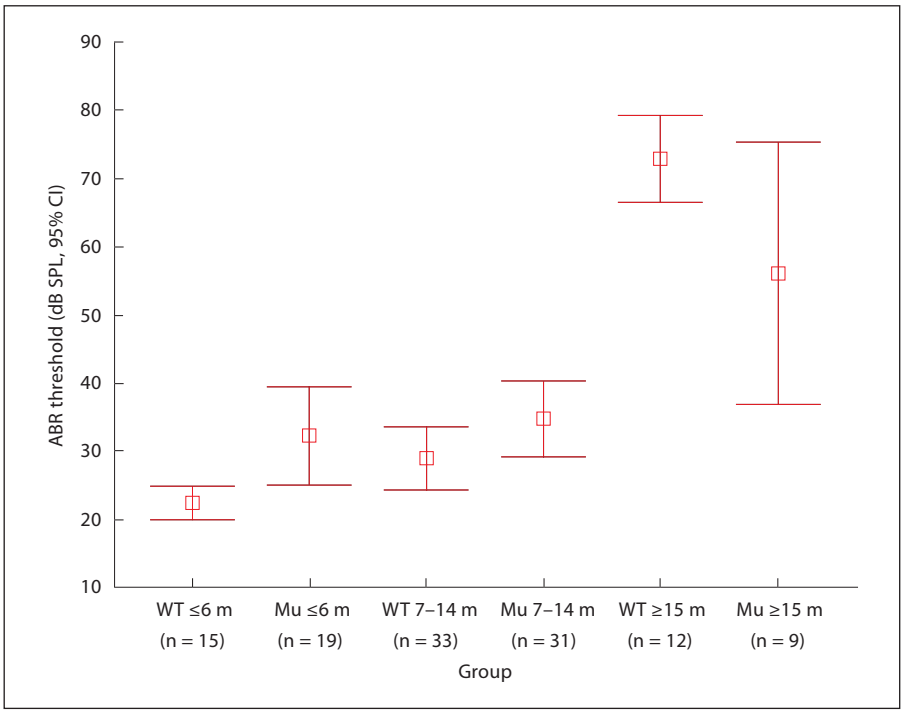

Fig. 1. VDR KO accelerated the development of hearing loss shown by ABR. Age-related ABR threshold elevation was found in both the WT and the VDR KO groups. Hearing loss developed significantly faster in the VDR KO group than in the WT group ≤6 months. Mu = Mutant, VDR KO. The number of ears that were measured is shown in parentheses.

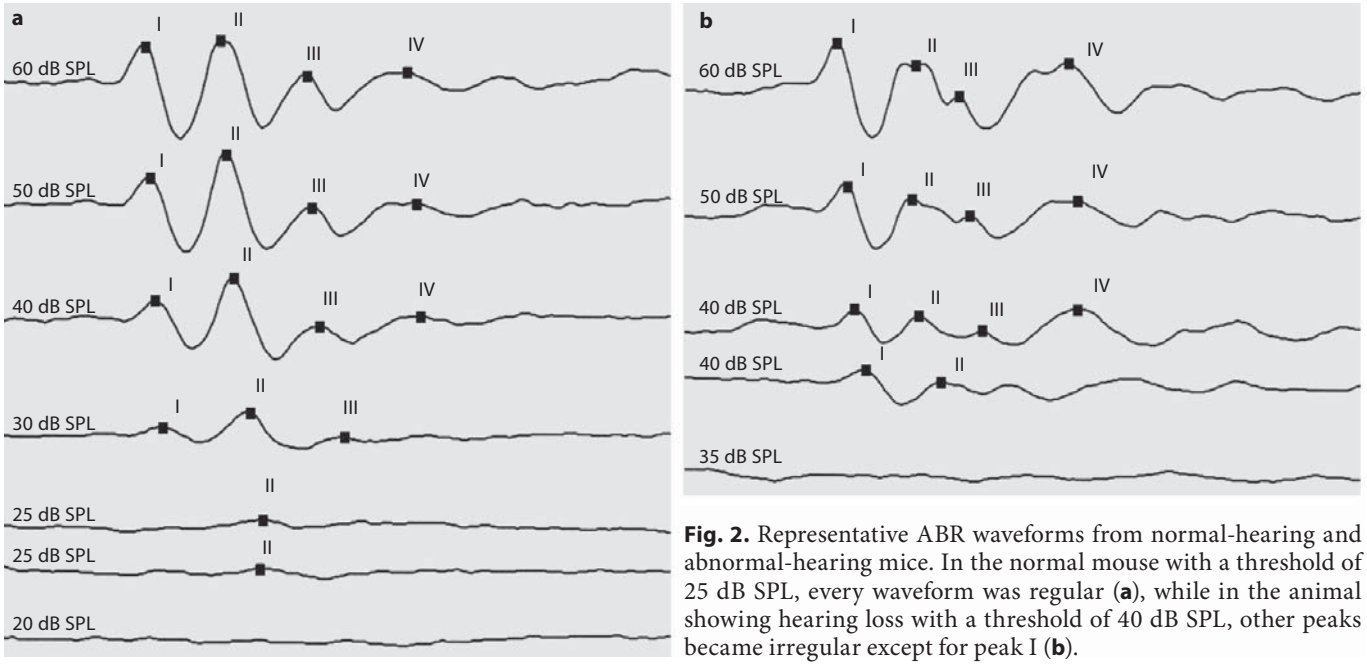

Fig. 2. Representative ABR waveforms from normal-hearing and abnormal-hearing mice. In the normal mouse with a threshold of 25 dB SPL, every waveform was regular (**a**), while in the animal showing hearing loss with a threshold of 40 dB SPL, other peaks became irregular except for peak I (**b**).

VDR Expression in the Cochlea

VDR was expressed in the nuclei of osteocytes of bulla, spiral ganglion cells, and Reissner's membrane cells, in the cytoplasm of spiral ligament fibrocytes and stria vascularis cells, and in both nuclei of the hair cells and inner sulcus cells in WT mice (fig. 4). VDR was not detectable in VDR KO mouse cochlea. There was no signal for VDR in the negative control slice when the primary antibody was omitted.

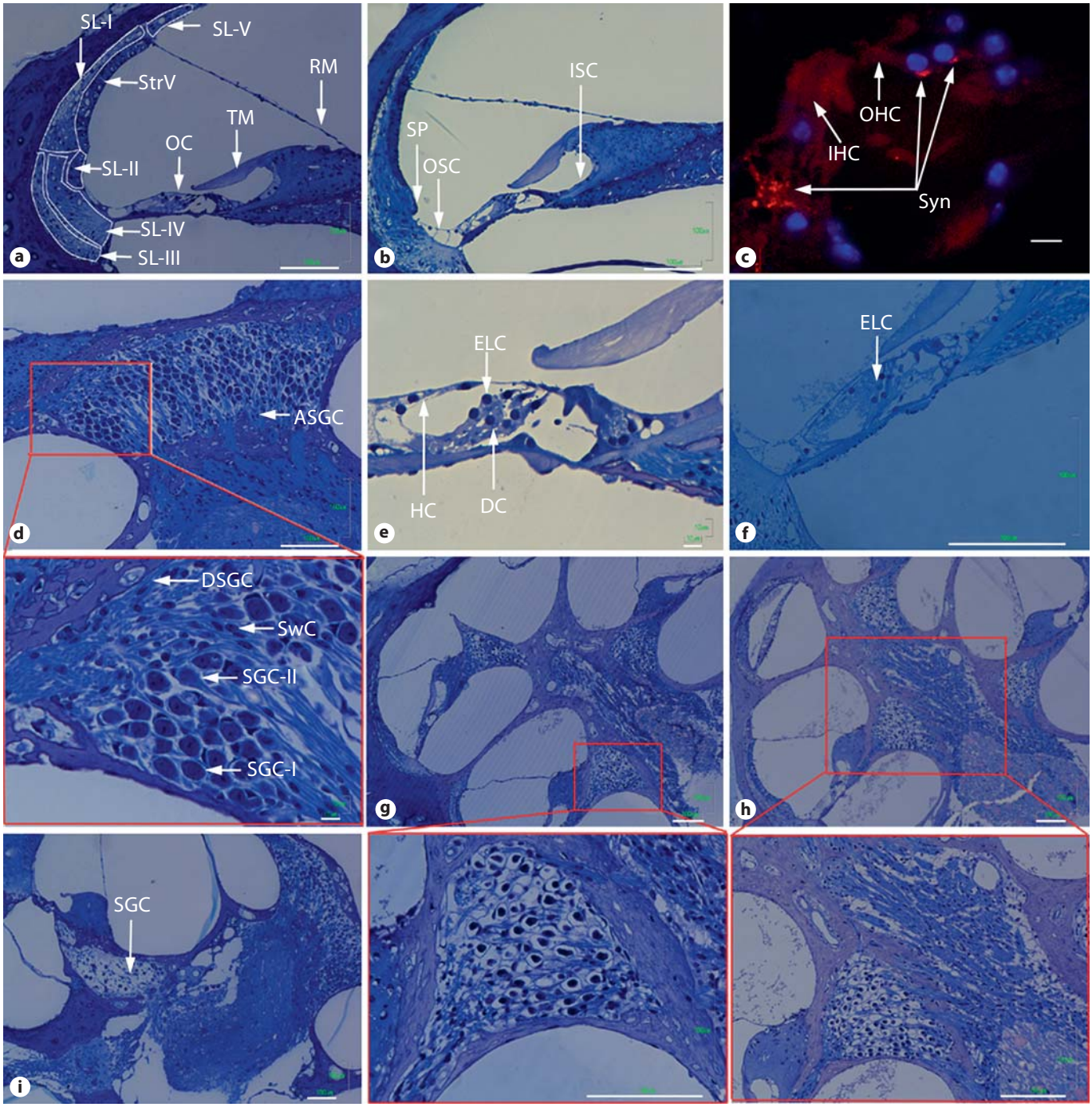

Fig. 3. Comparison of cochlear morphology between VDR KO and WT 129S1 mice. The cochlear structures including the organ of Corti, lateral wall, and spiral ganglion cells are fully developed in both WT mice (**a**) and VDR KO mice (**b–d**). **c** The efferent nerve endings are proven by detecting synaptophysin underneath the inner hair cells and outer hair cells. An excessive layer of cells appears in the row of the outer hair cells in both VDR KO mice (**e**) and WT mice (**f**). Degenerating spiral ganglion cells, which show condensed nuclear staining with toluidine blue and extremely reduced cellular volume, occurred in both VDR KO mice (**g**) and WT mice (**h**). Spiral ganglion cell loss was found in the basal turn of VDR KO mice (**i**). ASGC = Axonal process of the spiral ganglion cells; DC = Dieters cells; DSGC = dendritic process of spiral ganglion cells; ELC = excessive layer of cells; HC = Hensen's cells; IHC = inner hair cells; ISC = inner sulcus cells; OC = organ of Corti; OHC = outer hair cells; OSC = outer sulcus cells; RM = Reissner's membrane; SGC-I = spiral ganglion cell type I; SGC-II = spiral ganglion cell type II; SL-I, SL-II, etc. = spiral ligament fibrocyte type I, type II, etc.; SP = spiral prominence; StrV = stria vascular; SwC = Schwann cells; Syn = synaptophysin. Scale bar = 100 μm (**a**, **b**, **d**, **f–i**), 6.9 μm (**c**), 10 μm (**e**).

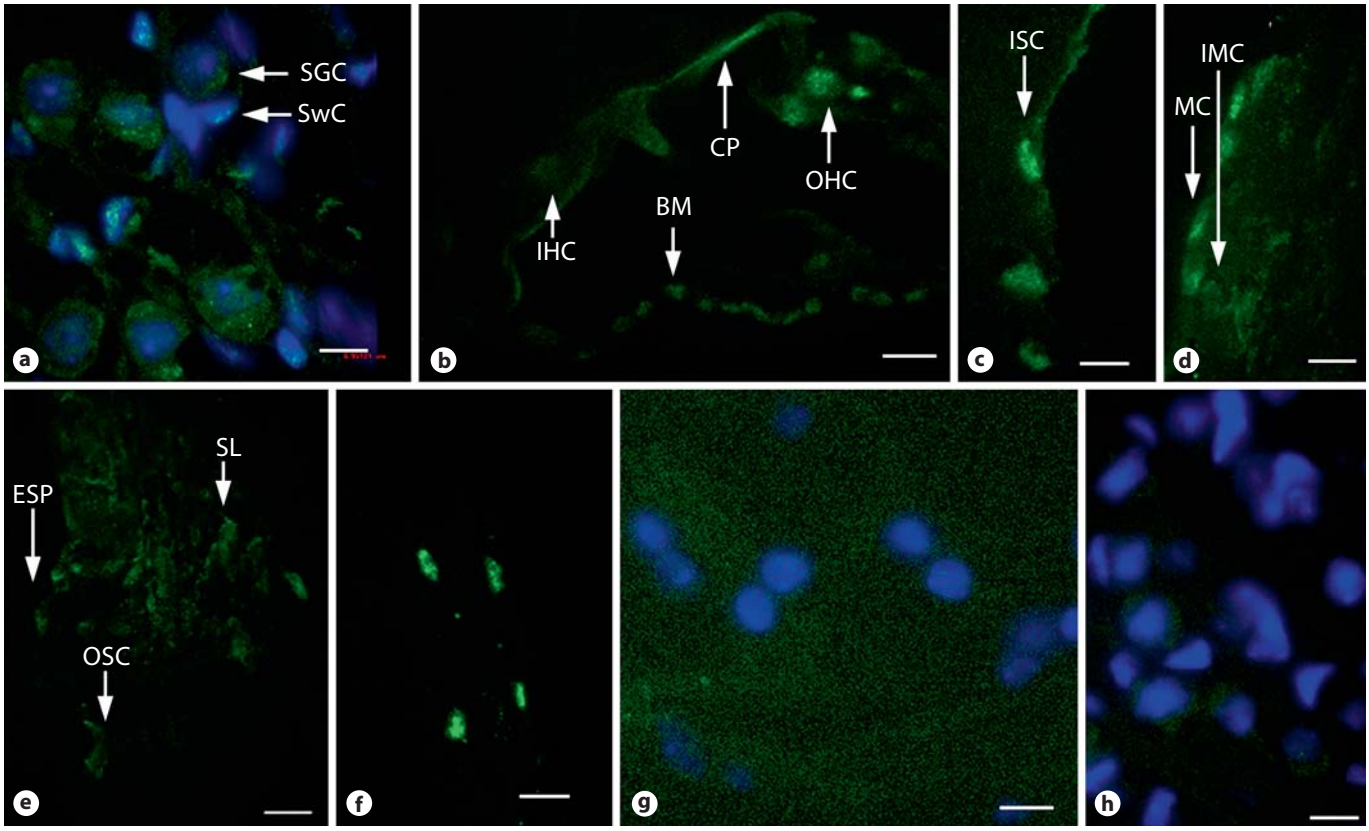

Fig. 4. VDR was expressed in the WT mouse cochlea. **a** VDR protein staining (green) was found in the nuclei of both spiral ganglion cells and Schwann cells. **b** In the organ of Corti, VDR was expressed in the nuclei of hair cells, the basilar membrane, and in the cuticular plate. VDR was also found in the nuclei of inner sulcus cells (**c**), marginal cells and intermediate cells of the stria vascularis (**d**), epithelium of spiral prominence, spiral ligament, and outer sulcus cells (**e**). **f** VDR was also expressed in the nuclei of osteocytes of bulla. **g** VDR was undetectable in VDR KO mouse cochlea. **h** There was no staining when primary antibody was omitted. **a**, **g**, **h** The nuclei were counterstained with DAPI. BM = Basilar membrane; CP = Cuticular plate; ESP = epithelium of spiral prominence; IMC = intermediate cells of the stria vascularis; MC = marginal cells of the stria vascularis; SGC = spiral ganglion cells; SL = spiral ligament. Scale bar = 6.9 μm.

Normal Expression of Connexin 26 and KCNJ10 in the Cochlea

Connexin 26 was weakly expressed in the Deiters' cells and Hensen's cells. Intense staining was found in the Claudius cells, inner sulcus cells, outer sulcus cells, type I spiral ligament fibrocytes, and the capillary of the stria vascularis, in both VDR KO and WT mice similarly (fig. 5a, b). There was no signal for connexin 26 in the negative control slice without the primary antibody. In conclusion, GJB2 gene expression was normal in VDR KO mice.

KCNJ10 expression was detected in the hair cells, supporting cells, inner sulcus cells, intermediate cells of the stria vascularis, and spiral ganglion cells in both WT and VDR KO mice with immunofluorescent confocal microscopy (fig. 5c, d). No KCNJ10 signal was detected in the negative control slice with primary antibody omitted.

Nonaffected TRPV4/6 Expression in the Cochlea

In the WT mouse cochlea, TRPV4 protein appeared in the cuticular plate of both inner and outer hair cells receiving the mechanic stimulation that comes from the hair bundle. Strong expression was also detected in the apical cytoplasm of both inner and outer pillar cells and their junction, inner sulcus cells, interdental cells, and in the matrix of the limbus, Reissner's membrane, spiral prominence, strial vascularis, spiral ligament fibrocytes, and spiral ganglion cells. TRPV4 expression was not changed in the cochlea of VDR KO mice (fig. 5e, f). No signal was found on the slice when the primary antibody

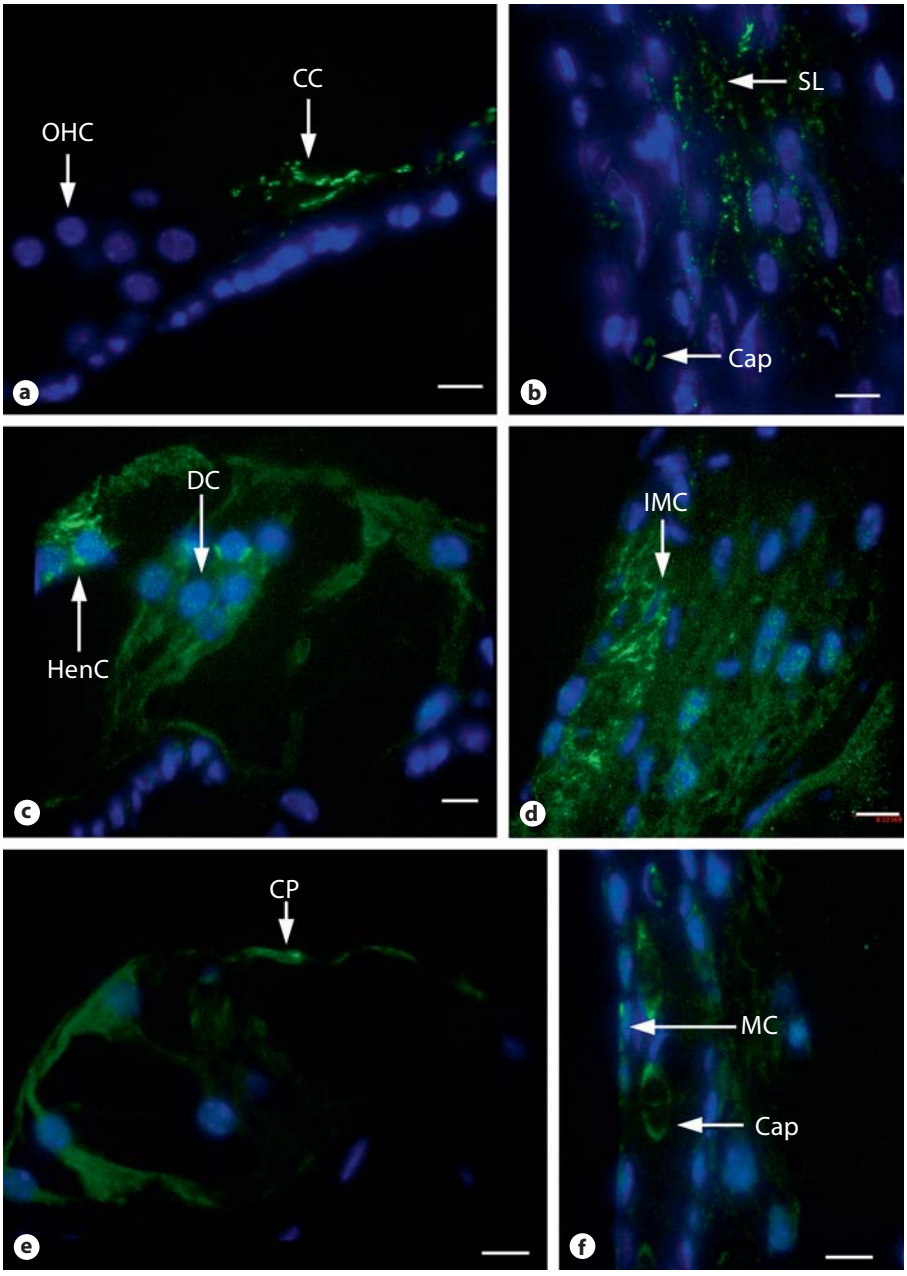

Fig. 5. The cochlear expression of connexin 26, KCNJ10, and TRPV4 was not affected by VDR KO. Connexin 26 protein was detected in Claudius cells in the organ of Corti (**a**), spiral ligament and capillary of the stria vascularis (**b**). KCNJ10 protein was expressed in Hensen's cells and Dieters cells in the organ of Corti (**c**), and marginal cells of the stria vascularis (**d**). TRPV4 was found in the cuticular plate in the organ of Corti (**e**), marginal cells and capillary in the stria vascularis (**f**). Cap = Capillary; CC = Claudius cells; HenC = Hensen's cells. Scale bar = 6.9 μm.

was saturated with TRPV4 peptide. There was very faint and variable expression of TRPV6 in the mouse cochlea.

Caspase 3 Is Activated in the Cochlea of Both WT and VDR KO Mice

Caspase 3 activation was performed in the cochlea of 6.5- and 10-month-old mice in both the WT and VDR KO groups. The activation was detected in cochlea cells, including the spiral ganglion cell and its satellite cells, stria vascularis, spiral ligament fibrocytes, and the organ of Corti (fig. 6). However, as can be seen in table 1, the percentage of positive cells and the staining intensity were lower in VDR KO (compared to the WT) mice. The activated caspase 3 showed a cytosolic and nuclear localization (fig. 6).

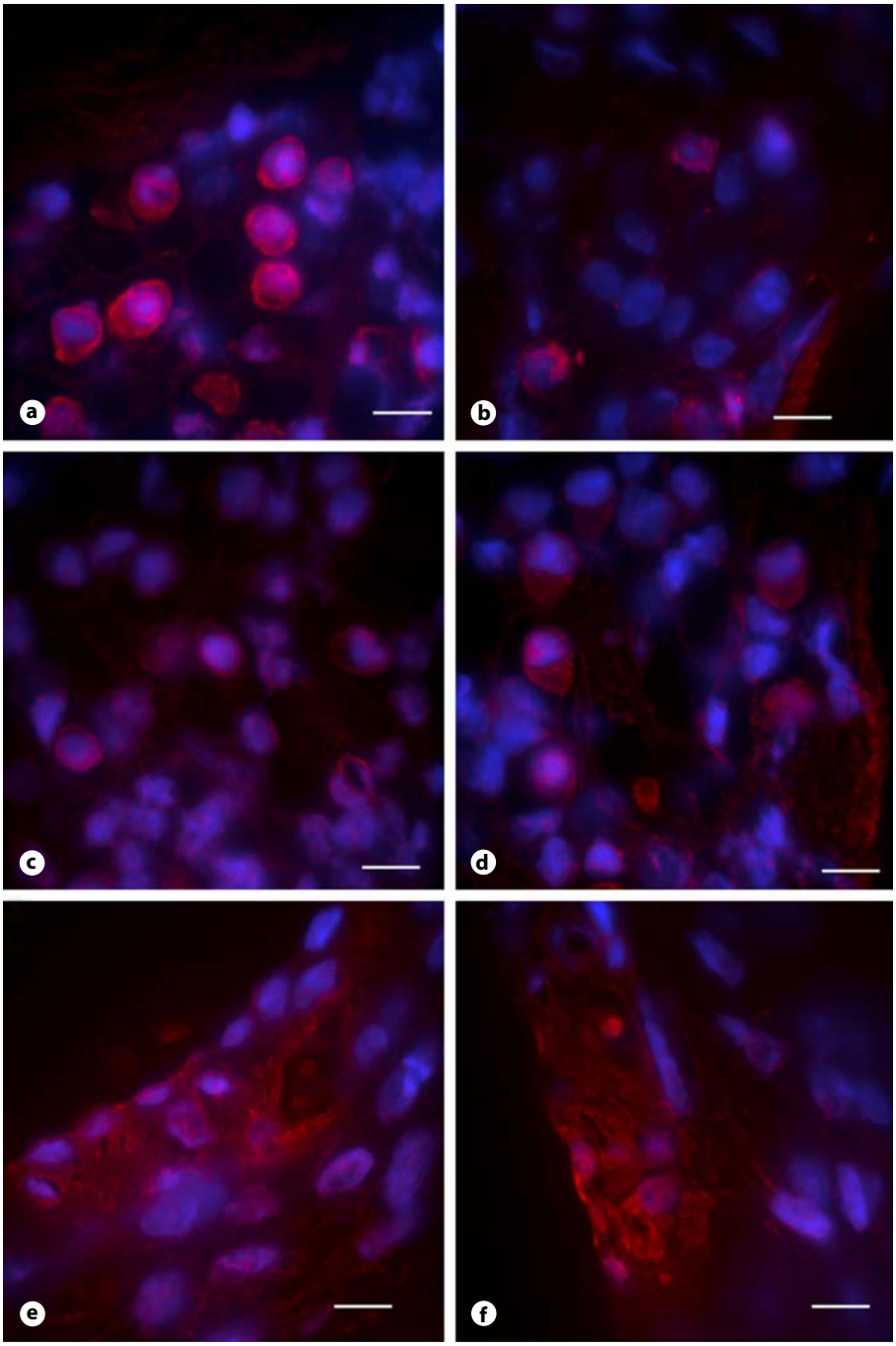

Fig. 6. Caspase 3 activation was decreased in the cochlea of VDR KO, compared to the WT mice. Caspase 3 activation (red) detected in both the cytosol and nucleus (blue) of neuron and satellite cells in the spiral ganglion of representative mice: a WT mouse at the age of 6.5 months (**a**), a VDR KO mouse at the age of 6.5 months (**b**), a WT mouse at the age of 10 months (**c**), and a VDR KO mouse at the age of 10 months (**d**). Caspase 3 activation was also detected in the stria vascularis of WT mice at the age of 6.5 months (**e**) and VDR KO mice at the age of 10 months (**f**). Scale bar = 9.6 μm.

Discussion

Most of the biological activities of 1α,25-dihydroxyvitamin D_3 require VDR, which translocates from the cytoplasm to the nucleus after ligand binding and then heterodimerizes with the retinoid X receptor [Mangelsdorf and Evans, 1995]. Our finding of VDR expression in the mouse cochlea implies that vitamin D is potentially important in maintaining hearing, which is in agreement with the previous reports [Brookes and Morrison, 1981; Brookes, 1983, 1985; Ziporyn, 1983]. Although VDR protein was not detected in the VDR KO mouse cochlea, which is at odds with reports on a truncated form of VDR in the same strain of VDR KO mice [Bula et al., 2005], this

Table 1. Caspase 3 activation in the spiral ganglion of WT mice and VDR KO mice

Group	Observed cells	Positive cells	Caspase 3 activation, %	Intensity of caspase 3 activation, AU
WT 6.5 months	157	65	41.40	1,538.45 ± 37.48
VDR KO 6.5 months	111	23	20.72	1,313.55 ± 12.44**
WT 10 months	189	80	42.33	1,212.83 ± 29.85
VDR KO 10 months	186	61	32.80	1,215.08 ± 12.80

** $p < 0.001$, statistically significant difference in caspase 3 activation intensity between VDR+/+ and VDR–/– mice at the age of 6.5 months. AU = Arbitrary unit.

can be explained by the difference in the epitope which was recognized by different antibodies. The antibody used in our study (Abcam, ab12129) was produced by inoculation with synthetic peptide corresponding to amino acids 5–19 of human VDR, whereas the antibody applied by Bula et al. [2005] was raised against a synthetic peptide mapping at the C-terminus of VDR of rat origin (Santa Cruz Biotechnology, sc-1008).

Our ABR results suggest that disruption of vitamin D-related bioactivity by partial deletion of the VDR gene accelerates age-related hearing loss. This auditory loss has been well documented in substrains of 129 mice [Ouagazzal et al., 2006; Zheng et al., 1999], and the finding that both WT and VDR KO mice show age-related hearing loss is also in agreement with the literature. However, there was a significant difference in threshold between young WT mice and young VDR KO mice, while there was no statistically significant difference between adult WT and VDR KO mice or between aged WT and VDR KO mice, indicating that a more rapid progressive hearing loss in mice was exaggerated by VDR mutation. The fact that there was no statistically significant difference between young VDR KO mice and adult VDR KO mice supports our hypothesis that age-related hearing loss developed earlier in VDR KO mice than in WT mice (before 6 months).

VDR exerts the biological role through regulating calcium and phosphate metabolism, and gene expression. The calcium and phosphate metabolism disorder can be compensated by feeding the animal a rescue diet, because the expression of Calbindin D 9K and plasma membrane calcium ATPase-1b (PMCA-1b) is VDR independent, whereas TRPV6 expression is VDR dependent [Bouillon et al., 2006; Song et al., 2003]. We were unable to restore the plasma calcium levels completely with the special diet, but the difference was rather small, which could also account for the hearing loss. Notably, TRPV6 expression in the brain is much higher than in the intestine, which may indicate that TRPV6 is a main player in brain calcium metabolism. Indeed, while TRPV6 decreased expression contributes to thalamic calcification [Nijenhuis et al., 2003] – a phenotype already reported in VDR KO mice [Kalueff et al., 2006a] – TRPV6 was not detected in the cochlea of either WT or VDR KO mice.

Analyzing our ABR data in VDR KO mice, we note that the elevated threshold and irregular ABR waveforms (except for wave I) can be explained as a result of decreased or abnormal brain stem neurons, since the cochlea, including the spiral ganglion cells, is normally developed. Deafness represents the late stages of neuronal degeneration, including large scale spiral ganglion cell loss (fig. 1i). Although 129S mice bear the potential of presbycusis, VDR KO can accelerate the process of aging as a consequence of downregulation of glial-cell-line-derived neurotrophic factor and nerve growth factor [Brown et al., 2003; Ohlemiller and Gagnon, 2004a, b; Ouagazzal et al., 2006; Taniura et al., 2006; Zhang et al., 2006; Zheng et al., 1999]. Thalamic calcification and degeneration may also induce brain stem lesions through the mechanism of retrograde degeneration [Kaga et al., 1999; Yamada et al., 2000].

Caspase 3 activation is also involved in age-related hearing loss and the mechanism of neuron degeneration may include both apoptosis and necrosis [Carloni et al., 2007; Riva et al., 2007]. Our finding of extensive activation of caspase 3 in the cochlea of WT 129S1 mice is in line with these reports. Both cytosolic and nuclear localization of cleaved caspase 3 protein in the cochlear cells indicates the role of caspase 3 activation in nuclear impairment [Kamada et al., 2005]. Except for the destroying effect, caspase 3 is also essential for the normal development of the auditory system [Morishita et al., 2001; Taka-

hashi et al., 2001], and its deficiency induces spiral ganglion degeneration and hearing loss in mice [Morishita et al., 2001]. While the mechanism sedimented from this phenomenon might be linked to caspase 3 regulating transcription through cleaving Ring1B in the nucleus [Wong et al., 2007], we also cannot rule out the possibility that decreased caspase 3 activation in VDR KO mice may be caused by the loss of some unknown gene expressions, which are controlled by VDR.

Several additional potential pathogenetic mechanisms have been tested in our study. TRPV4, a member of the TRP family, is a nonselective cationic channel that functions as a component of an osmotic/mechanical sensor [Liedtke et al., 2000, 2003]. Similar to TRPV4, the Drosophila protein Nanchung is essential for hearing in Drosophila [Kim et al., 2003]. TRPV4 mRNA and protein were also detected in murine cochlear hair cells, stria vascularis, and spiral ganglion cells, and hearing loss developed in TRPV4 KO mice [Liedtke et al., 2000; Shen et al., 2006; Tabuchi et al., 2005]. We studied the distribution of TRPV4 in the mouse cochlea using confocal microscopy. The expression pattern surrounding the endolymph edge also supports the hypothesis that TRPV4 is important in maintaining the fluid homeostasis of the endolymph, but this is not affected by VDR KO.

Potassium plays a significant role in maintaining the cochlear hair cell function. Connexin 26 is a fundamental structural protein in intercellular communication, especially K^+ circulation after the hair cell depolarization. Mutations in the GJB2 gene, which encodes the gap junction protein connexin 26, represent a major cause of prelingual, nonsyndromic, recessive deafness. These mutations are responsible for as much as 50% of such cases in many populations [Petersen and Willems, 2006]. We studied the connexin 26 protein in the mouse cochlea with immunofluorescent confocal microscopy and found that this fundamental structural protein was not affected by VDR KO.

Finally, inwardly rectifying K^+ channels regulate the resting membrane potential in many cells by contributing much of the resting K^+ conductance and maintaining low extracellular K^+ via spatial buffering mechanisms [Lagrutta et al., 1996; Reimann and Ashcroft, 1999]. Kir 4.1 (KCNJ 10) is expressed in the intermediate cells of the stria vascularis of the inner ear and helps to generate the endocochlear potential, which is the driving force of mechanoelectrical transduction by spatially buffering K^+ at a low level in a distinct intrastrial compartment [Hibino et al., 1997; Takeuchi et al., 2000]. Our results suggest that the system of generating the endocochlear potential was not disturbed by the VDR KO.

Conclusion

Our study shows that sensorineural hearing loss is progressively developed at an earlier age in VDR KO mice. Although the fundamental gene expressions in the cochlea were not influenced by VDR KO, caspase 3 activation disturbed by VDR genetic disruption may contribute to aberrant hearing phenotype in mice reported here.

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