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Vestibular dysfunction in vitamin D receptor mutant mice

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ABSTRACT

The vitamin D endocrine system is essential for calcium and bone homeostasis. Vitamin D deficits are associated with muscle weakness and osteoporosis, whereas vitamin D supplementation may improve muscle function, body sway and frequency of falls, growth and mineral homeostasis of bones. The loss of muscle strength and mass, as well as deficits in bone formation, lead to poor balance. Poor balance is one of the main causes of falls, and may lead to dangerous injuries. Here we examine balance functions in vitamin D receptor deficient (VDR−/−) mice, an animal model of vitamin D-dependent rickets type II, and in 1α-hydroxylase deficient (1α-OHase−/−) mice, an animal model of pseudovitamin D-deficiency rickets. Recently developed methods (tilting box, rotating tube test), swim test, and modified accelerating rotarod protocol were used to examine whether the absence of functional VDR, or the lack of a key vitamin D-activating enzyme, could lead to mouse vestibular dysfunctions. Overall, VDR−/− mice, but not 1α-OHase−/− mice, showed shorter latency to fall from the rotarod, smaller fall angle in the tilting box test, and aberrant poor swimming. These data suggest that VDR deficiency in mice is associated with decreased balance function, and may be relevant to poorer balance/posture control in humans with low levels of vitamin D.

1. Introduction

The hormonally active form of vitamin D, dihydroxyvitamin D_3 [1,25$(OH)_2$D] is a seco-steroid which binds to nuclear vitamin D receptors (VDR) [1]. 1,25$(OH)_2$D is converted from 25-hydroxyvitamin D [25OHD] by the mitochondrial enzyme 1α-hydroxylase (1α-OHase) [2–5]. 1,25$(OH)_2$D is a key regulator of calcium and phosphorus, and controls bone growth, cell proliferation, differentiation, and immune mechanisms [6–9]. Mutant mice deficient in 1α-OHase (1α-OHase−/−) are similar to VDR knockout (VDR−/−) mice, showing growth retardation, severe rickets and osteomalacia [7,10–12], and phenotypically resemble symptoms of human rickets [10,11,13].

Both VDR and 1α-OHase are expressed in the human and rodent CNS [14–21]. VDR−/− mice have numerous phenotypic abnormalities, including anxiety [22], food neophobia [23], aberrant nest-building [24,25], brain calcification [26] and impaired swimming [27,28]. In addition, VDR mutation accelerates neural degeneration in the cochlea [29], suggesting that otovestibular physiology may be affected in these mice. This possibility becomes particularly interesting, given a strong correlation between inability to swim and abnormal vestibulation [30].

In line with this, association between vitamin D and muscle strength [31], muscular/motor impairments have been suggested in VDR−/− mice [28,32]. Normal motor coordination requires balance and equilibrium [33], and poor balance is one of the main causes of falling, especially in elderly people. Potential effect of the vitamin D/VDR system on balance abilities and falls is not well established [34]. Here we examined whether vitamin D/VDR dysfunctions may cause vestibular deficits in mice. For this, we applied accelerating rotarod, tilting platform, rotating tube and swim tests to VDR−/− and 1α-OHase−/− mice. In addition, analyses of VDR expression in the vestibular organ were performed in the present study.

2. Materials and methods

2.1. Animals

Subjects were male and female VDR−/− and wild type (WT) mice on 129S1 and NMRI genetic backgrounds, and 1α-OHase−/− and WT mice on C57BL/6 background. VDR−/− mice were bred from the line originally generated by Prof. Kato (Japan) [10], on 129S1 background strain. The mutation was also transferred in our labora-

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tory (by approximately 10 backcrosses) to NMRI background strain. 1α-OHase−/− mice were provided by Prof. Dardenne (Canada) [35], and maintained by heterozygous crosses on C57BL/6 background strain. To normalize blood mineral ion levels, VDR−/− and 1α-OHase−/− animals received a special "rescue" diet, containing 2% Ca, 1.25% P, and 20% lactose (Lactamin AB, Sweden). The animals were experimentally naïve and housed individually or 2–4 per cage (20.7 cm × 36.5 cm), with food and water freely available.

In rotarod and tilting platform tests, 19 adult mice on 129S1 background (5–12 months), and 39 mice on NMRI background (young and adult mice aged 1–4 and 5–12 months, respectively) were used. In rotating tube and swim tests, 21 NMRI mice (6–12 months) were used, in addition to 11 young (1–5 months) 1α-OHase−/− and WT mice on C57BL/6 background. All animal experiments reported here were approved by the Animal Experiment Committee, State Provincial Office of Finland and performed according to EU legislation. Blood was taken from 25 WT and VDR−/− male and female mice (on 129S1 background) by heart puncture, to analyze plasma Ca^{++} and P^{++} levels.

2.1.1. Genotyping

Genotyping of the mice was confirmed by PCR with DNA prepared from tail tissue. For the VDR mutant mice four primers were used to amplify a 166 bp VDR (forward, 5′-CTG CTC TTC TTA CAG GGA TGG-3′ and reverse, 5′-GAC TCA CCT GAA GAA ACC CTT G-3′) and 400 bp Neo (forward, 5′-ATC TTC TGT CAT CTC ACC TTG C-3′ and reverse, 5′-CAA GCT CTT CAG CAA TAT CAC G-3′) band from the targeted allele. 1α-OHase−/− genotype was confirmed by using 2 primers (forward 5′-GTC CCA GAC AGA GAC ATC CGT-3′ and reverse 5′-GCA CCT GGC TCA GGT AGC TCT TC-3′).

2.2. Apparatus and procedure

In the balance tests the motion was created with a DC-motor. The motor was controlled with a programmable DC-power source (Hewlett-Packard E3633). A control program for the DC-power was developed using Visual Basic.net (Microsoft).

Rotarod was a plastic rod 3.2 cm in diameter, 7 cm long, and elevated 22.5 cm from the floor. The surface of the rod was lightly roughened to minimize missteps (hind leg slips). Mice were placed on the rod at slow (4 rpm) rotational speed. Speed gradually increased over a 120-s test session up to 28 rpm. The rotarod apparatus was placed inside a sealed box with camera and infrared light inside. The camera was attached to computer to allow digital video recording. The test was stopped if the mouse fell, allowing measurement of the latency to fall. The experiment consisted of three trials per day (2 learning trials and 1 test trial).

Tilting box was mounted directly on the axis of the DC-motor consisted of 6-mm thick square Plexiglas box, with glass floor to prevent scratching the surface. The outside of the box was 20 cm long, 10.8 high and 10 cm wide. The speed of tilting was 13.9°/s. Mice were placed in one end side of the box, with its snout facing the opposite end. The small transparent plastic "wall" was put inside the box to avoid the movement of the animal to the opposite side before the tilting of box started. The test was performed in the dark. The angle of falling down (angle at which the mouse began to slide down to the opposite end) and the latency to fall were measured from the video recording. Also, the ability to keep posture while falling down was recorded based on whether the mouse had all four paws on the ground when the tilting started.

Tilting tube was a 44.5-cm Plexiglas tube. Because animals were placed inside of the tube, three different diameters (4.1, 3.4 or 2.4 cm) were used, adjusted according to the size of the mice. Ends of the tube were closed to prevent the animal's escape. The tube was rotated with the speed of 35 rpm. The following measurements were observed: angle of rotation (45° or 90°) when the mouse fell, and the capability of the mouse to correct its posture when rotated. Between subjects, each apparatus was thoroughly cleaned using wet and dry cloths.

Swim test. All mice tested for balance performance were also tested in the swim test, as described earlier [28]. A glass cylinder (20 cm in diameter) was filled with warm water (22–25 °C) to a depth of 20 cm. The mice were individually lowered into the water, and their ability to swim was assessed for 3 min. Normal swimming behavior was recorded when animal kept horizontal body position, with its nose above the surface. Abnormal swimming was recorded when animal's position was vertical, with nose pointing upward. Sinking episode was scored when the position of the mouse was vertical and its nose went below the surface of the water. The water was changed between tested animals.

2.3. Histological analyses

WT and VDR−/− mice on 129S1 background were sacrificed with CO_2. Bulla was fixed with 4% formaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 2 h, and then washed thrice with PBS. Saccule, utricle and ampullae were isolated from the vestibulum under stereomicroscope. After opening the chambers, the vestibular organs were incubated in FITC-labeled phalloidin (50 μg/ml; Sigma–Aldrich, USA) for 40 min, and then in 4′,6-Diamidino-2-phenylindole (DAPI; Sigma–Aldrich, USA) for 10 min. After washing with PBS for 3 × 5 min, the Saccular macula and utricular macula were carefully dissected out and mounted with Gel Mount™ Aqueous Mounting Medium (Sigma–Aldrich, USA).

2.4. Immunofluorescent staining of VDR

Under general anesthesia with methyl parahydroxybenzoate (0.2 mg/kg) and benzethon chlorid (25 mg/kg), the animals were perfused with 4% formaldehyde in 0.1 M PBS following cardiac perfusion removal of the blood with PBS. The bulla was removed and further fixed with the same fixative solution overnight. After washing with PBS, decalcification with 10% EDTA was performed at room

Table 1
Body weight of mutant mice vs. to WT controls in both genders.

	WT mice		*U*-test	Mutants		*U*-test	*U*-test	
	Males	Females	Between genders	Males	Females	Between genders	Between genotypes	
							Males	Females
129S1	32 ± 5.7	23 ± 1.1	***p* < 0.0003****	22.5 ± 0.5	22.4 ± 2.1	NS	***p* < 0.02***	NS
NMRI	52.9 ± 9.2	36.1 ± 8.4	***p* < 0.0008****	34.2 ± 5.03	28.4 ± 6.07	***p* < 0.004***	***p* < 0.0002****	***p* < 0.008***
C57BL/6	26.6 ± 3.2	22.8 ± 1.5	***p* < 0.05***	19.6 ± 4.7	18.7 ± 3.7	NS	***p* < 0.003***	NS

Mutants: vitamin D receptor mutant (VDR−/−) on 129S1 and NMRI background strains, and 1 alpha-hydroxylase knockout (1α-OHase KO) on C57BL/6 strain. Data were analyzed using *U*-test between genders (WT male vs. female mice, mutant male vs. female mice) and genotypes (WT males + females vs. mutant males + females).
Data presented as mean ± S.D. Significant difference is $^{*}p < 0.05$, $^{**}p < 0.005$. Statistically significant data are in highlighted font. Statistically not significant—NS. WT males of all strains are bigger compared to both genders of mutants. Females of both genotypes are lighter than males of both genotypes, while male and female mutants had almost the same weight.

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, de-paraffinized with xylene, and passed through gradient ethanol until a final PBS wash. Sections were digested with 0.1% trypsin at 37 °C for 30 min, washed with PBS and 0.1% Tween-20 for 3× 2 min, and incubated with 1:20 pre-immune goat serum at room temperature for 30 min. Sections were incubated with rabbit polyclonal antibody to VDR, 1:10 (Abcam, UK) overnight at 4 °C. For the negative control, the primary antibody was omitted. After washing with PBS-T, sections were incubated with Alexa Fluor® 568 labeled goat antiserum against rabbit IgG (1:400, Invitrogen, USA) at room temperature for 60 min, followed by incubation with 10 ng/ml DAPI (Sigma–Aldrich, USA) for 10 min. Sections were mounted with Gel Mount™ Aqueous Mounting Medium (Sigma–Aldrich, USA) after washing with PBS/Tween-20 solution.

2.5. Confocal microscopy

The sections were studied under an Olympus microscope IX70 with ANDOR IQ (FITC fluorescence at 488 nm with a laser beam; Alexa Fluor® 568 fluorescence at 568 nm with a laser beam; DAPI with a 340–380 nm filter).

2.6. Statistical analyses

All data were analyzed by SPSS 11.5, and are expressed as mean ± S.D. Balance data were analyzed using the Mann–Whitney *U*-test. A probability of less than 0.05 was considered statistically significant. Since there were no obvious differences between sexes in any measure, data from both sexes were combined.

3. Results

VDR mutant and 1α-OHase−/− mice were significantly lighter compared to WT mice of both genders (Table 1). Ca^{++} plasma levels were slightly non-significantly lower in VDR−/− mice (1.69 ± 0.32 vs. 2.51 ± 0.15). Plasma P^{++} levels were slightly higher in mutants compared to the WT controls (4.89 ± 0.88 vs. 4.27 ± 0.56; $p < 0.05$).

Mouse balance abilities, assessed in the rotarod, tilting box and swim tests, are summarized in Table 2. In the rotarod test, we found significant difference between WT and VDR−/− mice on 129S1 background. While both genotypes improved their performance in this test after training, 12-month-old mutants showed significantly shorter time to fall than did 12-month-old WT controls. The mean latencies of the three trials were not significantly different between 6-month-old VDR−/− and WT mice. In the same test, WT and VDR−/− mice on NMRI background performed on the rod equally well.

In the tilting box test, 12-month-old VDR−/− mice on 129S1 background had smaller angle of falling, whereas WT mice demonstrated better balance and fell at a higher angle. Also, older mutants were not able to keep posture when falling, compared to the WT mice (Table 2). NMRI WT and VDR−/− mice fell down at the same angle, and showed unaltered postural control.

In the rotating tube test, the WT and VDR−/− mice on NMRI background also showed no balance or postural deficits (Table 2), and both genotypes were able to correct posture when rotated 90° without falling, or stood up after falling down. Although mutants performed more rotations with the tube, they showed less balance than the WT mice; however, the difference was not statistically significant.

In the swim test, VDR−/− mice showed abnormal swimming behavior, such as vertical swimming, and sinking. In contrast, WT 129S1 controls showed normal swimming behavior. We also did not observe circling or turning behavior (Table 2). VDR−/− mice on

Table 2
Balance and motor behavior of vitamin D receptor mutant (VDR−/−) and 1 alpha-hydroxylase knockout (1α-OHase KO) mice comparing to WT mice on several genetic backgrounds.

Tests	6–12 m.o 129S1 WT	6–12 m.o 129S1 VDR (−/−)	<12 m.o 129S1 WT	<12 m.o 129 VDR (−/−)	1–4 m.o NMRI WT	1–4 m.o NMRI VDR (−/−)	1–5 m.o C57BL/6 WT	1–5 m.o C57BL/6 1α-OH-ase KO (−/−)
Rotarod								
Mean of latency to fall down (s)	75.4 ± 22.0	66.4 ± 31.3	91.6 ± 21.3	**58.2 ± 26.6***	24.4 ± 9.3	30.8 ± 16.0	46.0 ± 11.7	42.7 ± 19.0
Tilting box								
Lat to slide down (s)	3.9 ± 0.6	3.9 ± 0.7	3.8 ± 0.4	3.5 ± 0.5	4.3 ± 0.5	3.9 ± 0.5	3.9 ± 0.8	4.0 ± 0.3
Angle° of fall	51.2 ±7.7	46.2 ± 8.8	48.4 ± 4.9	**42.5 ± 6.8***	44.1 ± 9.0	42.7 ± 7.9	46.7 ± 14.1	44.1 ± 5.3
Ability to keep posture position %	80.0 ± 40.0	37.0 ± 51.0	100.0 ± 0.0	**10.0 ± 30.0***	80.0 ± 40.0	80.0 ± 40.0	90.0 ± 0.0	70.0 ± 50.0
Tilting tube								
Rotated at 45° %	Not tested	Not tested	Not tested	Not tested	20.0 ± 40.0	9.0 ± 30.0	20.0 ± 40.0	100.0 ± 0.0
Rotated at 90°, but not fell down %	Not tested	Not tested	Not tested	Not tested	20.0 ± 40.0	18.0 ± 40.0	20.0 ± 40.0	0.0 ± 0.0
Fell down, but corrected one's posture %	Not tested	Not tested	Not tested	Not tested	20.0 ± 40.0	20.0 ± 40.0	20.0 ± 40.0	0.0 ± 0.0
Rotated with the tube all the time %	Not tested	Not tested	Not tested	Not tested	30.0 ± 50.0	50.0 ± 50.0	20.0 ± 40.0	0.0 ± 0.0
Good balance performance %	Not tested	Not tested	Not tested	Not tested	10.0 ± 30.0	9.0 ± 30.0	20.0 ± 40.0	0.0 ± 0.0
					<6 m.o	6–8 m.o		
Swim test								
Normal horizontal swimming %	100.0 ± 0.0	**25.0 ± 46.0****	100.0 ± 0.0	**0.0 ± 0.0*****	100.0 ± 0.0	**40.0 ± 50*****	10.0 ± 0.0	10.0 ± 0.0
Abnormal vertical swimming %	0.0 ± 0.0	**87.0 ± 35.0*****	0.0 ± 0.0	**100.0 ± 0.0*****	0.0 ± 0.0	**70.0 ± 50*****	0.0 ± 0.0	0.0 ± 0.0
Sinking %	0.0 ± 0.0	**75.0 ± 46.0*****	0.0 ± 0.0	**100.0 ± 0.0*****	0.0 ± 0.0	**60.0 ± 50*****	0.0 ± 0.0	0.0 ± 0.0
Turning %	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**0.0 ± 0.0**	Not observed	Not observed	Not observed	Not observed

Data are presented as the percentage of the number of animals (of total per group) showing the respective phenotypes.
Data are presented as mean ± S.D. in all tests. Age presented as months (m.o).
*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (*U*-test) between the genotypes. Statistically significant data are in highlighted font.
VDR mutants showed impaired motor and balance functions.

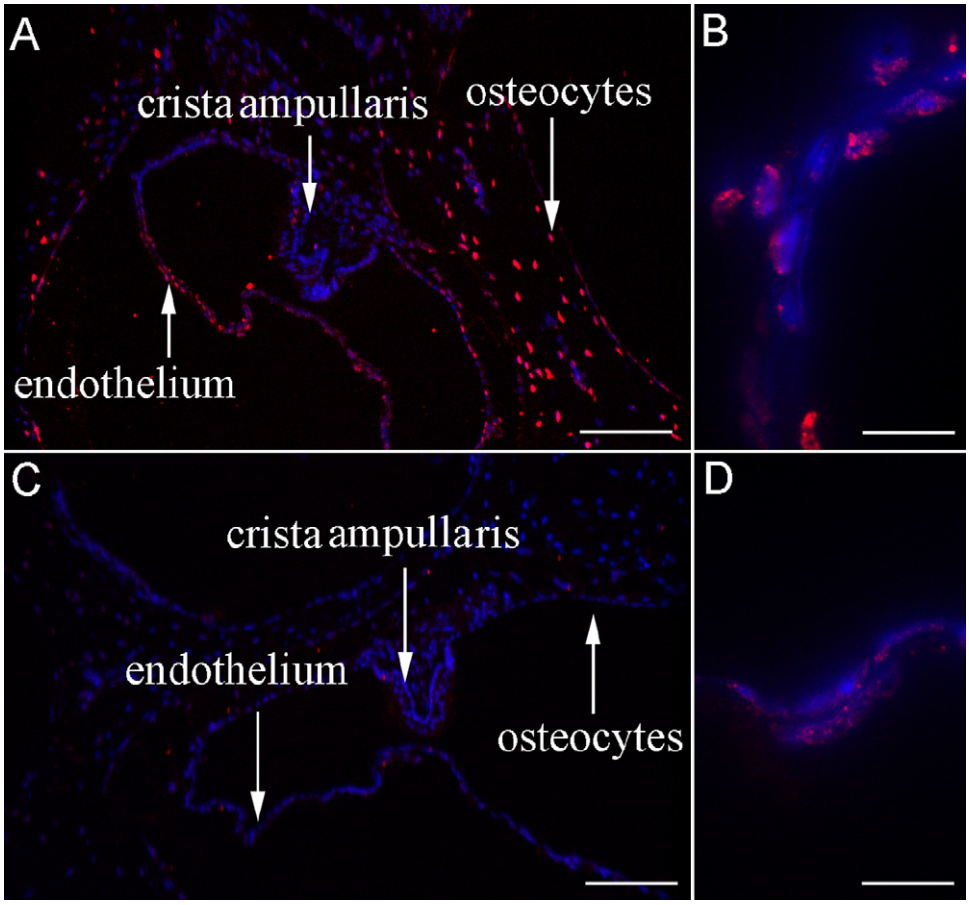

Fig. 1. VDR is expressed in the vestibular organ of WT mice using immunofluorescent confocal microscopy. VDR protein (red) appeared in the nuclei of the epithelium of crista ampullaris, ampullar endothelium, and surrounding osteocytes (A) as well as in the nucleus of the membranous semicircular canal endothelium (B) of WT mice. VDR is undetectable in the crista ampullaris, ampullar endothelium, surrounding osteocytes (C), and the membranous semicircular canal endothelium (D) in VDR−/− mice. Nuclei are stained with DAPI (blue). Scale bar = 9.8 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

NMRI genetic background showed similar deficits in their swimming. Normal horizontal swimming appeared in all WT mice, even in aged WT mice, compared to much younger VDR mutants (which showed aberrant vertical swimming and frequent sinking). However, the swimming abilities in young VDR mutants (<5 months) were better than in adult (5–13 months) mutants.

Adult 1α-OHase−/− mice showed unaltered swimming ability in contrast to VDR mutants of both strains. Balance perfor-

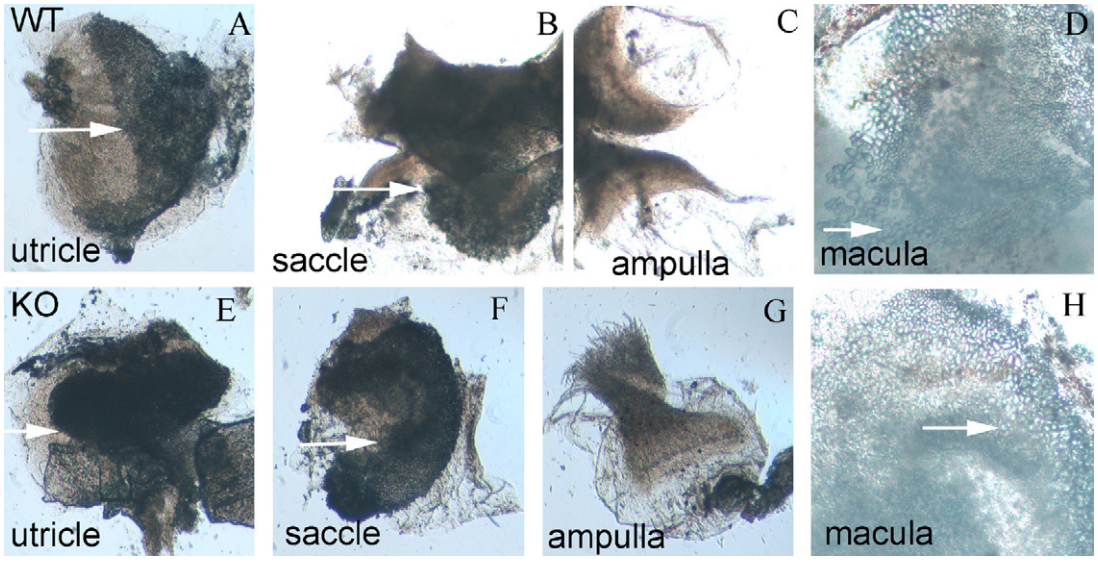

Fig. 2. Morphology of vestibular organ in WT and VDR−/− mice is demonstrated with phase contrast microscopy. The utricle, saccule, and ampulla end organ are normally developed in both WT (A–C) and VDR−/− (E–G) mice. The crystal statolith of macula (indicated with arrows) is formed in both WT (D) and VDR−/− (H) mice.

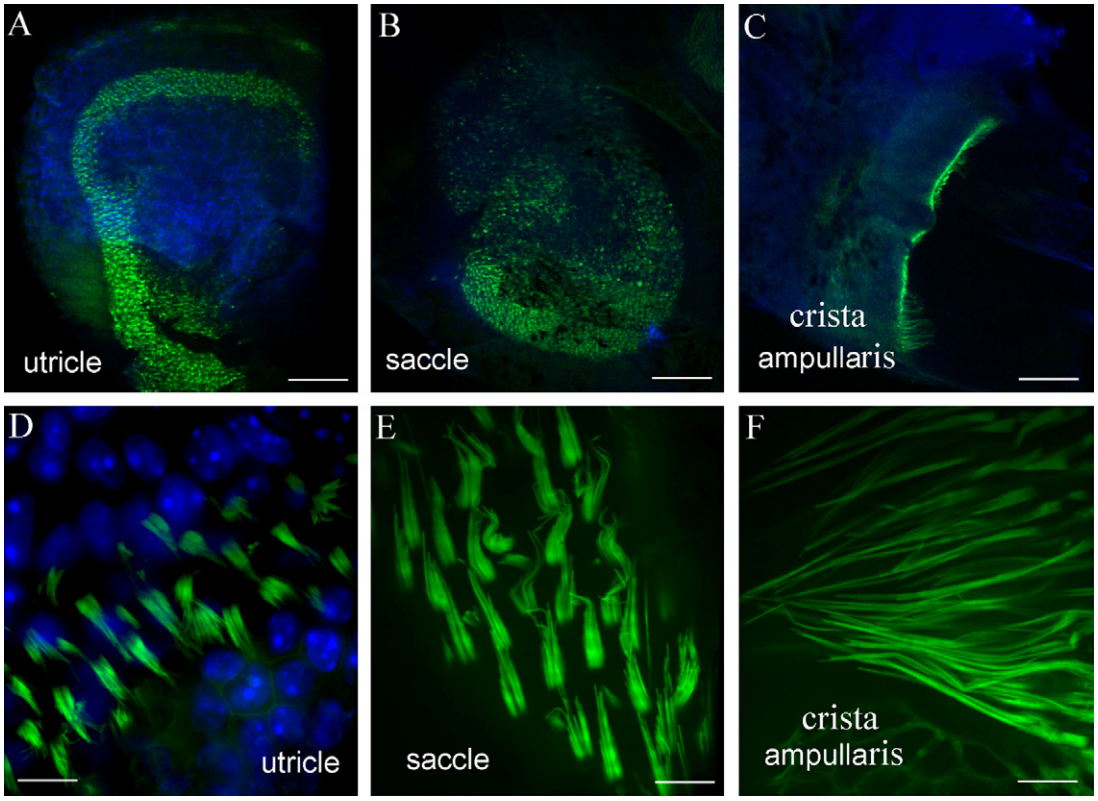

Fig. 3. The vestibular hair cells with normal stereocilia are demonstrated by probing the F-actin staining in WT mice. Hair cells with much longer stereocilia are in the crista ampullaris (A and D) than that in the utricle (B and E) and saccle (C and F). Nuclei are stained with DAPI (blue). Scale bar = 9.8 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

mance tested in all other tests was also normal in these mice (Table 2).

Finally, our immunohistological analyses showed that VDR were detected in the nuclei of epithelium of the endothelial lining of the crista ampullaris, of the membranous semicircular canal, and the surrounding osteocytes in WT mice (Fig. 1A and B). In VDR−/− mice, the VDR expression was not such strong as in WT mice (Fig. 1, panels C and D). Saccular macula, utricular macula, and crista ampullaris were morphologically normal in VDR−/− mice (Fig. 2), also showing unaltered fine structure of stereocilia (Fig. 3).

4. Discussion

In general, our study led to several important observations. First, we detected robust VDR expression in the vestibular organ implying the important role of the vitamin D/VDR system in vestibular functions. Second, we observed aberrant swimming (vertical position, sinking) as a consistent phenotype in VDR−/− mice on both (129S1 and NMRI) genetic backgrounds. Third, we found marked abnormalities of postural control in rotarod and tilting box tests in VDR mutants on 129S1 background. Finally, we showed that 1α-OHase−/− mice on C57BL/6 background did not exhibit any balance defects, and are similar to their WT littermates. It is likely that the vestibular defect is a result of a lack of appropriate gene transcription because of the VDR knockout. Since our VDR−/− mice express truncated VDR because of the second start signal [36], it is possible that the truncated receptor may mediate some membrane effects [37,38].

Since the swim test [30,39–41] is a well-known method to assess motor/balance functions in rodents, poor swimming of VDR−/− mice is in line with the possibility of their abnormal vestibular functions. Likewise, the rotarod and accelerating rotarod tests are well-known tests for balance, motor coordination and motor learning assessment [42–44]. While VDR−/− mice on 129S1 (but not NMRI) background demonstrated impaired rotarod performance, both genotypes had normal motor memory, since by the third trial all animals (independent of age, sex or genotype) learned equally well.

In the tilting box test, aged VDR−/− mice on 129S1 background again showed poorer balance, as assessed by smaller angle of fall, and inability to keep posture after falling. The angle in NMRI VDR−/− mice was also smaller (vs. WT mice), but this difference was not statistically significant. Also, in the rotating tube test, NMRI VDR mutants and C57BL/6 1α-OHase−/− mice had more rotations with the tube than their WT littermates, but the difference was not significant.

Various disorders with impaired vitamin D actions (including vitamin D deficiency or genetic defects in 1α-OHase or VDR genes) lead to rickets or osteomalacia. Our VDR and 1α-OHase mutant mice are an animal model of type 1 and 2 vitamin-dependent rickets [7,10,11,35,45].

1α-OHase−/− mice have elevated serum calcidiol (25OHD) [35]. 25OHD is the major circulating form of vitamin D in mammals [46], and binds to VDR with 100 times lesser affinity than $1,25(OH)_2D$ [47]. *In vitro* studies reported calcidiol as an active hormone [48]. Thus, the normal balance function in our 1α-OHase−/− mice might be through an activation of apoVDR or VDR activation by calcidiol or other ligand. Our observations are consistent with data showing significant association between calcidiol level and muscle strength in humans [49]. Also, another important aspect is the background strain differences. For example, mice of the C57BL/6 strain are considered to be good swimmers [50], prompting further experimental investigation of 1α-OHase−/− mice on other background strains, such as 129S1, in order to understand how background genes from parental strains may interact with the mutated gene.

Importantly, VDR mRNA is expressed in semicircular canal duct [51], the main component of the vestibular system, which is in agreement with the importance of vitamin D for balance functions. VDR protein expression was almost undetectable in the VDR KO mouse vestibular organ, due to expression of the truncated form of VDR (as reported by Bula et al. [36]). The other two VDR−/− mouse strains [9,13] do not express VDR at all, therefore it might be interesting to compare vestibular function in those animals.

Recently, we showed that 129S1 VDR mutants have progressive sensorineuronal hearing loss [29]. Balance is controlled through the vestibular system, which is contained in the inner ear, and anatomically linked to the hearing system. The functioning of the vestibular system depends on information from many systems, including hearing (as well as vision and muscle feedback), and impaired vestibular physiology in VDR−/− mice may therefore represent a common disordered "otovestibular" pathway triggered by vitamin D/VDR deficiency.

Finally, a previous study attributed abnormal swimming in VDR−/− mice to motor impairments, but did not assess the mouse vestibular functions [28]. Our findings confirmed poor swimming in VDR−/− mouse strains, but showed impaired balance performance of VDR−/− mice on 129S1 background in rotarod and tilting box tests. Collectively, these data suggest that vitamin D is important for balance control, and its dysfunction may cause to reduced balance functions.

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