Vitamin D-deficient diet rescues hearing loss in Klotho mice

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Abstract

Klotho-deficient mice exhibit a premature aging syndrome, a feature of which is mild hearing loss. In the present study, the hearing phenotype of Klotho mice was characterized to better determine how well this phenotype resembles presbycusis in humans. It was demonstrated that Klotho animals have auditory-evoked brainstem response (ABR) threshold shifts of 14-18 dB in response to pure tone stimuli of 4, 8, 16 and 32 kHz, and similarly, in response to clicks; however, cochlear histology and spiral ganglion neuron density appeared normal in these mice. It was further demonstrated that a vitamin D-deficient diet normalizes serum calcitriol ($1,25(OH)_2D_3$) levels and prevents hearing loss in Klotho mice. It is concluded that hearing loss in Klotho mice is caused by elevated renal 1α-hydroxylase expression and consequent excessive production of calcitriol. These findings implicate the vitamin D metabolic pathway in hearing loss and pose questions as to the mechanism by which elevated calcitriol levels mediate such hearing loss.

Keywords

Klotho, vitamin D, hearing, FGF23, presbycusis, calcitriol

Introduction

The Klotho mouse has been proposed as a model of human presbycusis and other age-related diseases. Mice with reduced *klotho* expression (*kl/kl*) display a mild hearing loss (Kamemori et al., 2002), short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis and emphysema (Kuro-o et al., 1997). In contrast, transgenic over-expression of *klotho* leads to a 19-31% increase in murine life span (Kurosu et al., 2005). Hence, it is posited that *klotho* is a regulator of life span and age-related degenerative disease and, in particular, hearing loss. The mechanism by which this occurs remains unclear.

Klotho is the co-receptor for fibroblast growth factor 23 (FGF23), and its expression in kidney is required for FGF23 to bind to, and signal through, the canonical FGF receptor, FGFR1(IIIc) (Urakawa et al., 2006). FGF23 is a hormone that regulates vitamin D metabolism by down-regulating the vitamin D-synthesizing enzyme 1-α-hydroxylase and up-regulating the vitamin D-catabolizing enzyme 24-hydroxylase in kidney (Shimada et al., 2004). FGF23 signaling is reduced in Klotho mice leading to high kidney 1-α-hydroxylase expression and high serum 1,25-dihydroxy vitamin D ($1,25(OH)_2D_3$) levels (Yoshida et al., 2002). Most aspects of the Klotho premature aging phenotype can be rescued with a vitamin D-deficient diet, including ectopic mineralization, infertility, growth retardation and skin atrophy (Tsujikawa et al., 2003). FGF23-klotho signaling promotes cell proliferation and prevents $1,25(OH)_2D_3$-induced apoptosis (Medici et al., 2008), suggesting that

premature aging in Klotho mice may be due to insufficient proliferation and/or excessive apoptosis.

The Klotho hearing loss phenotype is of particular interest given that it may represent an accelerated form of presbycusis, and if so, would facilitate examination of this phenomenon without excessive wait times for onset of disease. Kamemori *et al*. (2002) made a preliminary characterization of the Klotho hearing phenotype, by measuring an ABR threshold shift of 18 dB to pure tones of 8 kHz in Klotho animals, and demonstrating *klotho* expression in the stria vascularis of the cochlea. Based on these observations, Kamemori et al. (2002) hypothesized that *klotho* may regulate the ionic composition of the endolymph. They failed however, to investigate the hallmarks of presbycusis, such as onset and severity of hearing loss in the high frequency range.

Given the potential of the Klotho animal model to aid understanding of presbycusis, a more detailed analysis of the Klotho hearing loss phenotype was undertaken to determine how well this mimics presbycusis in humans. Furthermore, Tsujikawa et al's (2003) finding that high calcitriol levels mediate many aspects of Klotho disease, prompted an investigation of the effect of lowering calcitriol levels on the hearing of these mice. Results from this investigation are detailed herein.

Materials and Methods

Animals

Klotho mice with a 129Sv genetic background were generously provided by Makoto Kuro-o and were genotyped for the hypomorphic *kl* allele as described previously (Urakawa et al., 2006). The Walter and Eliza Hall Institute Animal Ethics Committee approved all experiments involving animals. Cohorts of mice were included in the diet study as follows +/+ ND 12 m, 9 f; *+/kl* ND 7 m, 10 f; *kl/kl* ND 7 m, 17 f; *kl/kl* DDD 17 m, 15 f. Cochear histology was examined for the following cohorts +/+ 3 m, 1 f; *kl/kl* 4 m.

Auditory-evoked brainstem response testing

Mice were anaesthetized by intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine and body temperature maintained at 37°C with a heat pad in a sound-attenuated, electrically shielded room. A loud speaker was placed 10 cm from the pinna of the test ear and computer-generated clicks and pure tone stimuli of 4, 8, 16 and 32 kHz (tone-pips, 1-ms rise/fall, 3-ms plateau) were presented with maximum intensities of 100-108 dB p.e. SPL. Auditory-evoked brainstem responses (ABRs) were recorded differentially using percutaneous stainless-steel needle electrodes positioned at the vertex of the skull (+ve) and on the snout (-ve) with a ground on the thorax. Signals were amplified by 10^5 and band pass filtered (150 Hz-3 kHz). The output of the filter was fed to a 16-bit analogue-to-digital converter (series 2 model, Tucker Davis Technologies, Alachua, FL, USA) and sampled at 20 kHz for a period of 12.5 ms following the stimulus onset. ABRs were averaged over 500 repetitions of the clicks or tone-pips presented at 33/s. Stimulus intensity was incremented in 5 dB steps from sub-threshold levels.

Average ABR traces were subsequently analyzed to determine ABR threshold using commercial software (Igor Pro v6.04, WaveMetrics, Portland, OR, USA). The threshold was defined as the lowest intensity stimulus that reproducibly elicited a Wave III ABR (2.5 to 3 ms latency) using a visual detection criterion (Coco et al., 2007).

Histology

Cochlear histology was collected from +/+ and *kl/kl* mice fed commercial mouse cubes (Barastoc WEHI, Ridley AgriProducts, Melbourne, VIC, Australia) containing 2000 IU/kg vitamin D. Mice were euthanized with an overdose of anesthetic (200 mg/kg ketamine and 40 mg/kg xylazine). A cannula containing 10% neutral buffered formalin (NBF) was inserted into the left ventricle to perfuse the animal. Cochleae were dissected from the temporal bones and post-fixed overnight in NBF at 4°C. Cochleae were then decalcified in 10% EDTA and embedded in Spurr's resin. Sections (2 μm) were collected in the mid-modiolar plane and stained with hematoxylin and eosin. Sections were imaged on a light microscope (Axioplan II, Carl Zeiss, North Ryde, Australia). Spiral ganglion neuron density was determined by counting the number of neurons with prominent nucleoli in the middle turn of Rosenthal's canal using commercial imaging (ImageJ64) software (Abramoff et al., 2004; Rasband, 1997-2009).

Vitamin D-deficient diet

Semi-pure diets were obtained from a commercial supplier (Specialty Feeds, Glen Forrest, WA, Australia). The normal diet (ND), AIN93G, contained 1000 IU/kg vitamin D. The vitamin D-deficient diet (DDD), SF03-009, was identical except that it contained no vitamin D. Both dams and their offspring were fed these diets to ensure that the dietary vitamin D status of experimental mice was set from the time of conception. Animals were fed *ad libitum*. We did not test the hearing of wild-type mice on DDD as they would have experienced significant morbidity associated with vitamin D deficiency and we considered it unethical to pursue these experiments given that this was not the focus of our research.

Serum calcitriol assays

Serum calcitriol levels were measured using a 1,25-dihydroxycholecalciferol^{125}I radioimmunoassay (RIA) kit (DiaSorin, Stillwater, USA) according to manufacturer's instructions.

Results

Hearing profile of Klotho mice

The +/+ and *kl/kl* mice were bred on the 129/SvJ background. This is a hearing-impaired strain that exhibits significantly elevated ABR thresholds before the age of 3 mo (Zheng et al., 1999). ABR testing of 3 wk old Klotho mice revealed that *kl/kl* mice had higher ABR thresholds than their +/+ littermates in response to clicks, as shown in Fig 1A, and in response to pure tone-pip stimuli of 4, 8, 16 and 32 kHz, as shown in Fig 1B. The observed mean threshold shifts of 14-18 dB for the *kl/kl*

mice were statistically significant at all frequencies tested (Student's t-test, $p<0.05$, Table 1).

Cochlear histology for 3 wk-old +/+ and *kl/kl* mice revealed no obvious morphological abnormalities in the organ of Corti (left), stria vascularis (middle) or Rosenthal's canal (right) (Fig 1C). Furthermore, spiral ganglion neuron densities of *kl/kl* mice were normal (Someya et al., 2009) (+/+ 1500±200, *kl/kl* 1900±300 cells/mm^2; mean±sd; n=4 per group; p=0.07 student's t-test).

Effects of eliminating vitamin D from the diet of Klotho mice

Three wk old *kl/kl* mice, raised from conception on a normal diet (ND) containing 1000 IU/kg vitamin D, had serum $1,25(OH)_2D_3$ levels 2-3 times higher than +/+ littermates. However, 3 wk old *kl/kl* mice, raised from conception on a vitamin D free diet (DDD), had normal serum $1,25(OH)_2D_3$ levels as shown in Fig 2A.

As indicated in Fig 2B, *kl/kl* mice fed ND died by 6 wks of age, whereas *kl/kl* mice fed DDD were viable to at least 100 d of age. Furthermore, as the weight by age plots of Fig 2C and D illustrate for male and female mice respectively, *kl/kl* mice on ND were runted whereas *kl/kl* mice on DDD had indistinguishable growth curves from +/+ littermates on ND. *kl/kl* mice on ND died before breeding age; however, *kl/kl* mice on DDD were fertile, and productive *kl/kl* x *kl/kl* matings were observed (data not shown).

To examine the effect on hearing of correcting the serum calcitriol levels of *kl/kl* mice, ABR thresholds were determined for 3 wk old +/+ and *kl/kl* mice fed DDD. As indicated in Fig 1A and B the ABR thresholds of *kl/kl* mice fed DDD were not significantly higher than those of +/+ littermates on ND.

Discussion

The Klotho mouse has been proposed as a model of age-related disease. It is in this context that we sought to investigate the hearing of these animals. Typically, presbycusis onset commences, and is more severe, in the high frequency range. This is certainly the case in wild type C57BL/6 animals that exhibit a rapid and progressive high frequency hearing loss with a 55 dB threshold shift in response to tones of 30 kHz by 200 d of age (Frisina et al., 2001; Henry et al., 1980) and a loss of distortion product otoacoustic emissions to 32 kHz by 5 mo of age (Jimenez et al., 1999). In the detailed ABR analysis of Klotho mice with a 129Sv genetic background we demonstrated a very subtle hearing loss at all frequencies tested that was present as early as 3 wks of age. Soon after this time, mice succumb to a plethora of age-related diseases that make up the Klotho syndrome. The finding that the Klotho hearing threshold shift was minor and occurred equally at all frequencies, casts doubt on the usefulness of the Klotho mouse as a model of human presbycusis per se; however, one cannot discount the potential role of dysregulated vitamin D metabolism in hearing loss more generally, and particularly the detrimental effect of high levels of systemic calcitriol.

Kamemori *et al*. (2002) proposed that hearing loss in Klotho mice may be due to disruption of endolymph ion homeostasis caused by loss of *klotho* expression in stria vascularis. We have demonstrated that normalizing $1,25(OH)_2D_3$ levels rescues hearing loss in Klotho mice. This indicates that hearing loss may be mediated indirectly by high systemic $1,25(OH)_2D_3$ rather than by direct lack of *klotho* expression in the stria vascularis. There are several mechanisms by which this may occur, the most likely of which is via induction of apoptosis within the auditory system. Evidence against this hypothesis was the observation that cochlear histology and spiral ganglion neuron density of Klotho mice were normal. Another likely effect of high calcitriol that could disrupt hearing is decreased mineralization of the middle ear ossicles. This process is regulated by vitamin D and Klotho mice display reduced bone mineral density and osteopenia (Kuro-o et al., 1997). This hypothesis remains to be tested.

There are few published cases linking hypervitaminosis D and hearing loss in humans (Cohen et al., 1979). High $1,25(OH)_2D_3$ levels in humans are rare because 24-hydroxylase generally converts much of this to inactive $1,24,25(OH)_3D$. In fact, hypervitaminosis D is difficult to achieve in humans given the wide range of tolerable dietary vitamin D doses. Dietary doses of up to 100 μg (4000 IU) per day have been reported without negative side affects (Vieth et al., 2001). Thus, the Klotho mouse does not model common human presbycusis, or in fact dietary-induced hypervitaminosis D; however, Klotho is a model of

dysregulated vitamin D metabolism, and it may transpire that calcitriol mediates some forms of human hereditary hearing loss.

High levels of systemic calcitriol mediate subtle hearing loss at all frequencies in Klotho mice. This may be due to calcitriol-induced apoptosis within the auditory system, although there was no loss of spiral ganglion neurons or disruption of the sensory epithelium noted in the present study. Another causative mechanism may be that high calcitriol stimulates demineralization of the ossicles, with resultant conductive hearing loss. Alternatively, high calcitriol may disrupt endolymph ion homeostasis, potentially via ion transporters in the stria vascularis. It remains to distinguish between these possibilities.

Figure Legends

Figure 1: ABR thresholds of 3 wk old mice were determined in response to **A)** clicks and **B)** pure tone-pips. Group means were compared by Student's t-test and significant p-values are noted. *kl/kl* mice fed ND displayed mild hearing loss that was rescued by removing calcitriol from the diet (DDD). Student's t-test was used to compare group mean ABR thresholds at each frequency and significant p-values (*) are indicated. **C)** Hematoxylin and eosin stained 2μm sections of cochleae revealed no obvious abnormalities in *kl/kl* mice; left 100x mag, bar = 200 μm; middle and right 400x mag, bar = 20 μm.

Figure 2: Results from experiments reducing dietary vitamin D ingestion by Klotho mice. **A)** Serum $1,25(OH)_2D_3$ levels of 3 wk old mice fed either ND or DDD. **B)** Survival; *kl/kl* mice fed ND died by 6 wks of age while *kl/kl* fed DDD were viable. **C)** Growth of male mice; *kl/kl* mice fed DDD displayed normal growth. **D)** Growth of female mice; *kl/kl* mice fed ND were runted, but *kl/kl* mice fed DDD displayed normal growth.

Acknowledgements

The authors acknowledge the financial support of the HEARing CRC, established and supported under the Australian Government's Cooperative Research Centres Program. AKW is supported by a grant from the National Institute of Health and National Institute for Deafness and Communication Disorders (HHS-N-263-2007-

247 00053-C). Dr James Fallon wrote the Igor procedure file used for ABR analysis.
248 Prof Howard Morris provided useful discussion.
249

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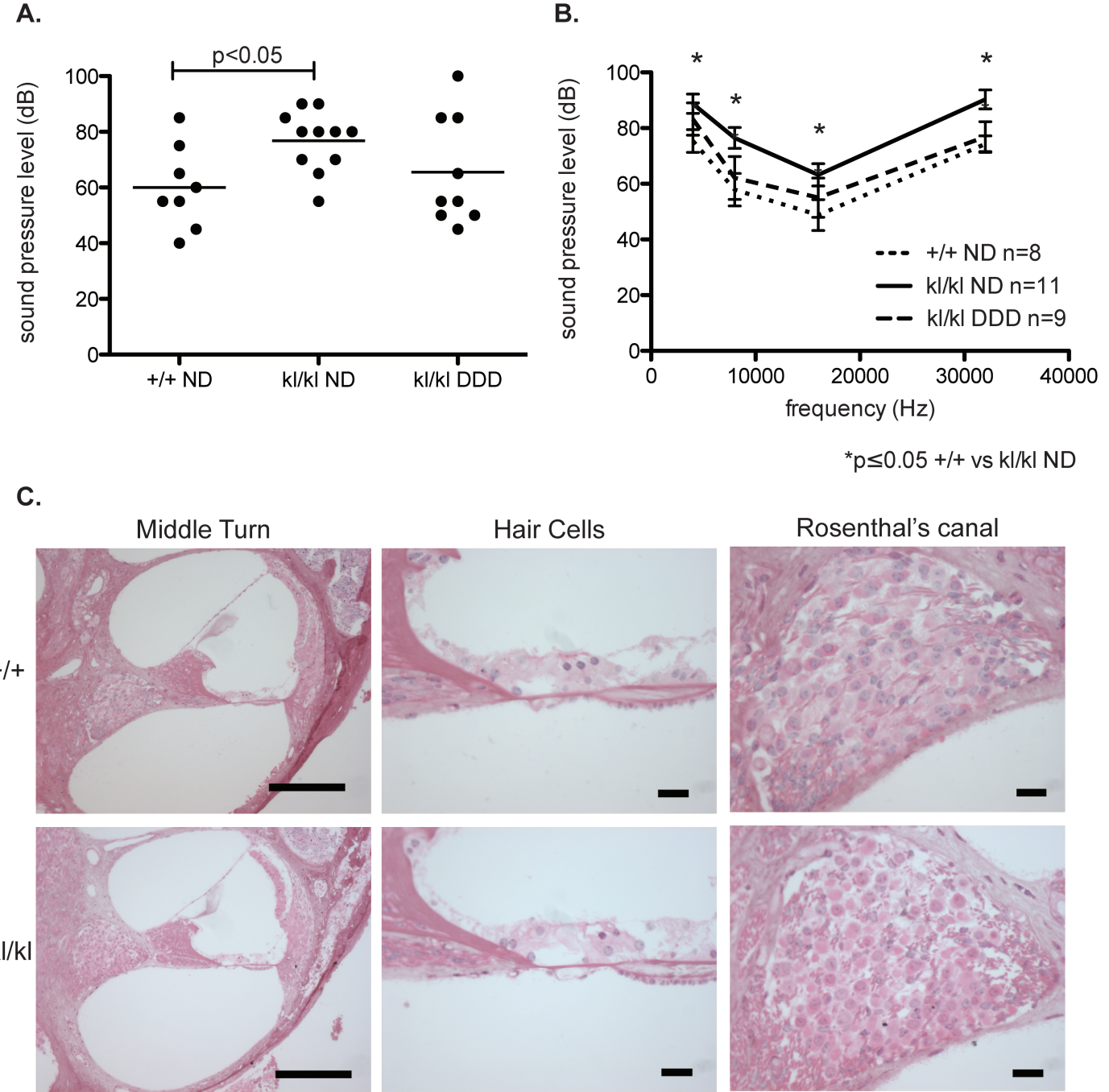
Table 1

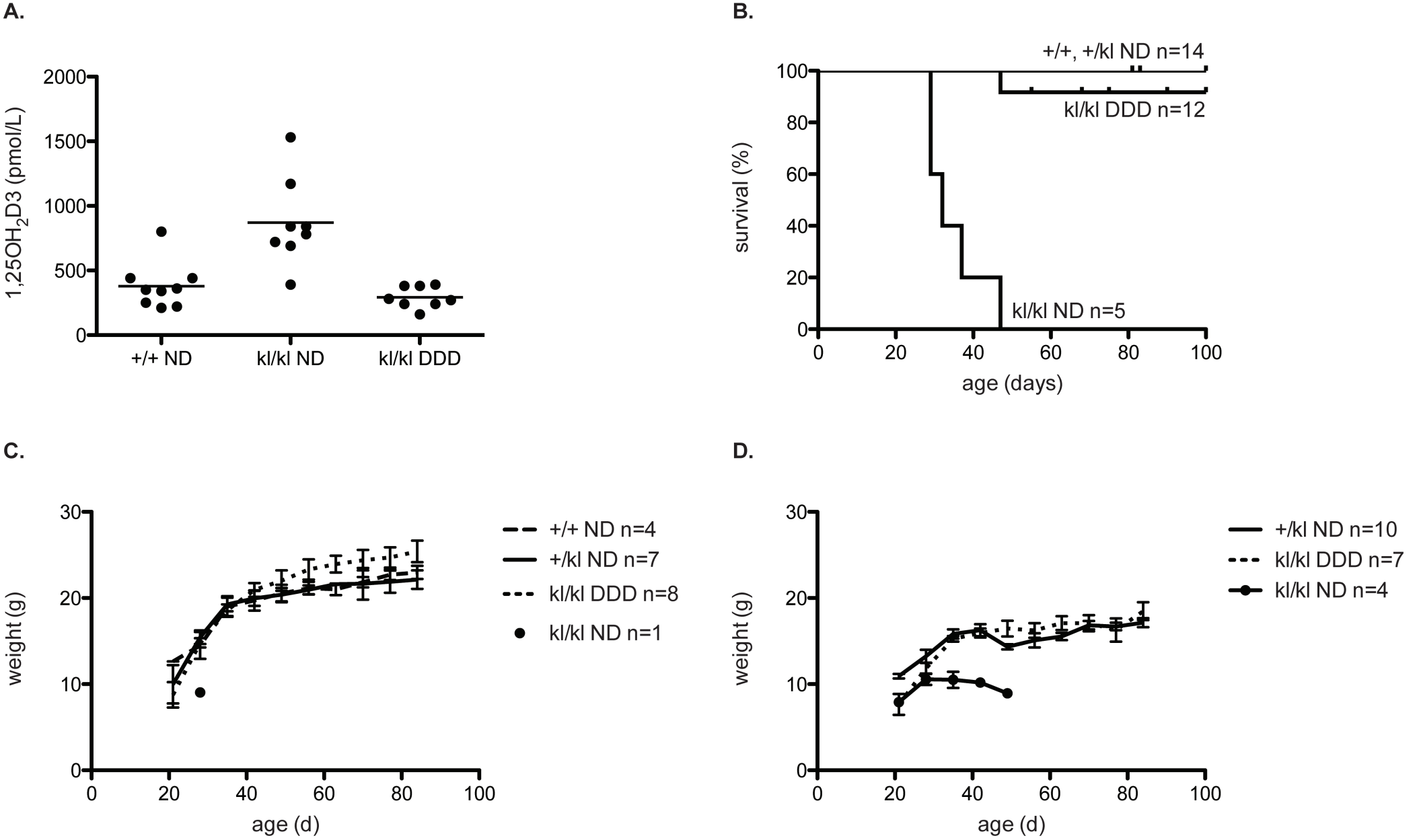
ABR threshold shifts between *+/+* and *kl/kl* mice fed ND or DDD.

Frequency (Hz)	Comparison[a]	Mean threshold shift (dB)	p-value[b]
Click	+/+ ND v kl/kl ND	17	**0.02**
	+/+ ND v kl/kl DDD	6	0.52
4000	+/+ ND v kl/kl ND	14	**0.04**
	+/+ ND v kl/kl DDD	8	0.30
8000	+/+ ND v kl/kl ND	18	**0.02**
	+/+ ND v kl/kl DDD	4	0.67
16000	+/+ ND v kl/kl ND	14	**0.05**
	+/+ ND v kl/kl DDD	6	0.50
32000	+/+ ND v kl/kl ND	16	**0.02**
	+/+ ND v kl/kl DDD	7	0.67

[a] *+/+* ND n=8; *kl/kl* ND n=11; *kl/kl* DDD n=9

[b] Significance of group mean differences was assessed using Student's t-test. Significant p-values are indicated by bold type.


A.
p<0.05
sound pressure level (dB)
100
80
60
40
20
0
+/+ ND
kl/kl ND
kl/kl DDD
B.
sound pressure level (dB)
100
80
60
40
20
0
0
10000
20000
30000
40000
frequency (Hz)
+/+ ND n=8
kl/kl ND n=11
kl/kl DDD n=9
*p≤0.05 +/+ vs kl/kl ND
C.
Middle Turn
Hair Cells
Rosenthal's canal
+/+
kl/kl


A.
1,25OH2D3 (pmol/L)
2000
1500
1000
500
0
+/+ ND
kl/kl ND
kl/kl DDD
B.
+/+, +/kl ND n=14
kl/kl DDD n=12
kl/kl ND n=5
survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
age (days)
C.
weight (g)
30
20
10
0
0
20
40
60
80
100
age (d)
+/+ ND n=4
+/kl ND n=7
kl/kl DDD n=8
kl/kl ND n=1
D.
weight (g)
30
20
10
0
0
20
40
60
80
100
age (d)
+/kl ND n=10
kl/kl DDD n=7
kl/kl ND n=4