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Mice with conditional deletion of Cx26 exhibit no vestibular phenotype despite secondary loss of Cx30 in the vestibular end organs

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Abstract

Connexins are components of gap junctions which facilitate transfer of small molecules between cells. One member of the connexin family, Connexin 26 (Cx26), is prevalent in gap junctions in sensory epithelia of the inner ear. Mutations of *GJB2*, the gene encoding Cx26, cause significant hearing loss in humans. The vestibular system, however, does not usually show significant functional deficits in humans with this mutation. Mouse models for loss of Cx26 function demonstrate hearing loss and cochlear pathology but the extent of vestibular dysfunction and organ pathology are less well characterized. To understand the vestibular effects of Cx26 mutations, we evaluated vestibular function and histology of the vestibular sensory epithelia in a conditional knockout (CKO) mouse with Cx26 loss of function. Transgenic C57BL/6 mice, in which cre-Sox10 drives excision of the Cx26 gene from non-sensory cells flanking the sensory epithelium of the inner ear (*Gjb2*-CKO), were compared to age-matched wild types. Animals were sacrificed at ages between 4 and 40 weeks and their cochlear and vestibular sensory organs harvested for histological examination. Cx26 immunoreactivity was prominent in the peripheral vestibular system and the cochlea of wild type mice, but absent in the *Gjb2*-CKO specimens. The hair cell population in the cochleae of the *Gjb2*-CKO mice was severely depleted but in the vestibular organs it was intact, despite absence of Cx26 expression. The vestibular organs appeared normal at the latest time point examined, 40 weeks. To determine whether compensation by another connexin explains survival of the normal vestibular sensory epithelium, we evaluated the presence of Cx30 in the *Gjb2*-CKO mouse. We found that Cx30 labeling was normal in the cochlea, but it was decreased or absent in the vestibular system. The vestibular phenotype of the mutants was not different from wild-types as determined by time on the rotarod, head stability tests and physiological responses to vestibular stimulation. Thus presence of Cx30 in the cochlea does not compensate for Cx26 loss, and the absence of both connexins from vestibular sensory epithelia is no more injurious than the absence of one of them. Further studies to uncover the

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physiological foundation for this difference between the cochlea and the vestibular organs may help in designing treatments for *GJB2* mutations.

Keywords

Vestibular end organ; Connexin 26; Connexin 30; Balance; GJB2; mouse

1. Introduction

The inner ear provides sensory information about orientation in space, postural control and sound. Several mutations lead to deficits in both vestibular and auditory parts of the inner ear (Ahituv et al., 2000) but sometimes hearing loss occurs without an associated vestibular phenotype (Jones et al., 2014). One such example is the *GJB2* mutation, the most common deafness mutation, responsible for up to 50 percent of the non-syndromic sensorineural hearing loss in several populations (Dodson et al., 2011; Schrijver, 2004). The *GJB2* gene encodes connexin 26 (Cx26), a component of the gap junction channels that enable neighboring cells to interchange signaling molecules (Kasai et al., 2010; Koval et al., 2014). The role of connexin proteins of the gap junction in the inner ear is not completely understood, but it is hypothesized that these gap junctions are responsible for potassium recycling and homeostasis of endolymphatic fluid (Forge et al., 2002; Forge et al., 1999; Wangemann, 2002). In mechanosensory hair cells, mechanically gated cation channels on stereocilia bundles are opened by bundle deflection, which permits a large influx of potassium from the endolymph into the cell, initiating membrane depolarization. To avoid potentially toxic concentrations of potassium within the epithelium, potassium must be removed from the sensory region. Transport of this excess potassium (which subsequently may be returned to the endolymph) is thought to be facilitated by gap junctions in supporting cells as well as cells in the transitional epithelium immediately surrounding the sensory region (Wangemann, 2002). Absence of gap junctions, or their failure to perform these essential functions, may explain the progressive hair cell loss and deafness resulting from *GJB2* mutations.

The most prevalent connexins in both the cochlea and vestibular organs are Cx26 and connexin 30 (Cx30) (Forge et al., 2002). Considering the functional deficits in the cochlea when connexin genes are mutated, vestibular impairment might also be predicted. Nevertheless, in human cases with a genetic mutation of Cx26 or Cx30 there is a much lower incidence of vestibular dysfunction than hearing loss (Dodson et al., 2011; Kasai et al., 2010; Todt et al., 2005).

Mouse models for hereditary inner ear disease have been used as research tools for understanding the pathophysiology of genetic inner ear disease and designing therapies (Akil et al., 2012; Probst et al., 1999). Studies of mouse mutants modeling connexin mutations may shed light on the structure and function of the vestibular end organs and the dependence of vestibular function on specific connexins. Several transgenic mice generated to mimic human *GJB2/6* mutations have been useful for investigating the role of connexins in the cochlea (Ahmad et al., 2007; Chang et al., 2008; Chen et al., 2014a; Chen et al.,

2014b; Cohen-Salmon et al., 2002); here we use a conditional knockout mouse (CKO) to investigate the consequence of Cx26 loss of function in the vestibular system.

2. Materials and Methods

2. 1. Generation of conditional knock out animals

Conditional knock out animals were generated using Cx26loxP mice, which were provided by Prof. Willecke (Cohen-Salmon et al., 2002). These mice were on a C57BL/6 background. Sox 10-Cre mice were provided by Prof. Richardson (Matsuoka et al., 2005). These mice express Cre recombinase under the Sox10 promotor. In the vestibular organ, Sox 10 is expected to be expressed in the supporting cells (Wakaoka et al., 2013). These two mouse lines were outcrossed repeatedly to wild type mice to generate a double transgenic mouse (*Gjb2*-CKO).

2. 2. Experimental animals and genotyping

We used male and female *Gjb2*-CKO mice and age matched wild types from the same colony for this experiment. The animals were on C57BL/6 genetic background. These mice develop progressive age-related hearing loss beginning about 3-6 months of age (Spongr et al., 1997) but the vestibular system is unaffected. A total of 30 animals were used (*Gjb2*-CKO: 16, wild type: 14). Genotypes of all animals were confirmed prior to evaluation. These animals were sacrificed for histological examination at ages of 4 (n=5), 8 (n=9), 12 (n=10) and more than 20 (n=6) weeks. In total, 48 ears were dissected for histologic analysis. Twenty five animals (13 *Gjb2*-CKO and 12 wild type mice) were used for the evaluation of vestibular function, of which 18 were used for the rotarod with or without centrifuge testing, and the other 7 for evaluation of head stability. Animals were housed and bred in the animal care facility at the University of Michigan. All animal use and care procedures were approved by the University Committee on the Use and Care of Animals of the University of Michigan.

2. 3. Immunocytochemistry

Animals were placed under deep anesthesia with ketamine (120 mg/kg, i.p.) and xylazine (7 mg/kg, i.p.) and euthanized by decapitation. Temporal bones were dissected immediately and fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) for 1 h. After a PBS rinse, the organ of Corti and the vestibular sensory epithelia were permeabilized in 0.3% Triton X-100 in PBS for 10 min., then incubated with blocking buffer (5% normal goat serum in PBS) for 30 min. to block non-specific binding of secondary antibodies. Samples were then reacted with primary antibodies. The following primary antibodies, dilutions and incubation conditions were used: rabbit antibodies specific for Cx26 and Cx30 (Invitrogen 51-2800 and 71-2200, 1:200 dilution in blocking buffer, overnight at 4 °C); rabbit anti-Myosin VIIa (Proteus 25-6791, 1:200 dilution in blocking buffer, 1 h, room conditions); mouse anti-neurofilament (Millipore/Chemicon AB9563, 1:200 dilution in blocking buffer, 1 h, room conditions). The specificity of the connexin antibodies to Cx26 or Cx30 has been demonstrated (Nagy et al., 2001).

After rinsing off the primary antibody, tissues were incubated with a fluorescence-labeled secondary antibody (Alexa Fluor 488 or 594; Invitrogen/Molecular Probes, Carlsbad, CA) for 30 min. and rinsed with PBS before mounting on microscope slides. To label F-actin, permeabilized dissected tissues were incubated with Alexa Fluor 488-conjugated phalloidin (1:400 dilution in PBS, 30 min, room temperature). Stained tissues were mounted on glass slides with Fluoro-Gel mounting media (Electron Microscopy Sciences, Hatfield, PA).

For epi-fluorescence analysis, we used a Leica DMRB epi-fluorescence microscope (Leica, Eaton, PA) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and SPOT-RT software Ver.5.0. For confocal microscopy analysis, we used Olympus FV 500 Confocal Microscope (Olympus, Center Valley, PA). Photographs taken with these microscopes were cropped and labeled with Adobe Photoshop and Illustrator software (Adobe System, San Jose, CA).

2. 4. Counting and data analysis

We assessed hair cell density, in wild type and mutant ears in the two macular vestibular organs: the utricle and the saccule. We selected these two organs because their relatively flat shape allows a visualization of the entire sensory epithelium. Using epi-fluorescence, we obtained digital images (40x objective lens) from two different areas (1600×1200 pixels, 5 pixels/ μm) in each end organ. One area is the central part which covers about 1/4 of the utricular macula, and contains both striolar and extra-striolar regions (it is difficult to distinguish between the areas in these preparations). The other area is the peripheral part of the utricular macula composed mostly of extra-striolar sensory epithelium. We counted all vestibular hair cells in the area based on MyosinVIIa staining, type I and type II (Huwé et al., 2015), and determined the proportion of type I hair cells by counting calyx nerve endings. Calyx nerve endings were identified by a neurofilament-positive ring-shaped pattern surrounding the hair cells. Dimorphic afferents could not be distinguished from pure calyx nerve endings with this counting method. Counting was performed using tpsDig software (Version 2.12; F. James Rohlf, Ecology & Evolution, SUNY at Stony Brook, NY).

2. 5. Vestibular function

Vestibular function was tested at 12 weeks (wild type=6; *Gjb2*-CKO=6). Additional rotarod test of older animals (wild type=3, *Gjb2*-CKO=3) was performed to evaluate age-related changes of these mice. The ages of these older animals were 20, 24 and 40 weeks. We checked for spontaneous spinning behavior and examined the righting reflex, which tests common signs of vestibular dysfunction including failure to right in air or spinning while dropping (Allam et al., 2012). We also tested whether mice spin while held in air and grabbed by the tail. Then we evaluated performance in the rotarod. Inability to stay on the rotating rod for a normal amount of time does not necessarily indicate a peripheral vestibular pathology; however, normal performance on the rotarod can be used to rule out functional disability due to a vestibular lesion. Before measuring time on the rod, animals were trained by placing them on a non-accelerating spinning rod (motor speed of 5 RPM) until they could remain on the rod for 30 second on three consecutive days. Once the experimental measurements started, the amount of time animals were able to stay on the accelerating (0.1

RPM/s) spinning rod was measured for three consecutive sessions and these values were averaged for each animal.

An additional test was used to indirectly assess vestibular function of the animals. There is ample direct evidence of neuronal connections between the vestibular nucleus of the brainstem and centers regulating physiological and emotional states (Eiken et al., 2005; Horii et al., 1995; Nobel et al., 2010) as well as evidence that mutations affecting the vestibular organs can influence anxiety related behavioral patterns as well as locomotion (Darlington et al., 2009; Khan et al., 2004; Shefer et al., 2010). In addition, vestibular stimuli similar to those that induce motion sickness in humans also can provoke physiological shifts such as lowering core body temperature in humans and other mammals (Cheung et al., 2011; Holmes et al., 2002; Murakami et al., 2002; Nobel et al., 2012). Since stressing the mouse by a strong fluctuating acceleration, perceived by the normal vestibular system, reduces body temperature, we tested whether the body temperature change is altered due to loss of Cx26. For this “centrifuge test” (or, off vertical axis rotational test), animals were placed in a 50 ml tube in which a longitudinal bar was placed to constrain the animal's position. This tube was attached to a rotating arm (25.4 cm radius), operated by an automatic controller (Fig. 1). The plane of rotation is inclined 30° from earth-horizontal to induce additional fluctuations in both magnitude and direction of acceleration experienced by the animal. The arm was accelerated to 1.4 cycles per second (cps), then alternated speed between 1.4 and 2.4 cps (2 G and 6 G), maintaining each speed for 10 seconds. After 2 min., the arm slowly decelerated and the mouse was removed from the tube. Body temperature was taken by rectal probe before spinning, immediately after spinning and at 5 min. intervals up to 25 min. after spinning.

2. 6. Evaluation of head stability

The most common test of vestibular function is measurement of the vestibulo-ocular reflex (VOR). This measurement is technically difficult to implement in mice so we developed an alternative approach and measured the vestibulo-collic reflex (VCR). The VCR is a 3-neuron reflex arc that links the vestibular periphery to the neck musculature. The VOR stabilizes eye position in space whereas the VCR stabilizes head position in space. Vestibular function is assessed as the effectiveness of the VCR in stabilizing head position in space. Three wild type and 4 *Gjb2*-CKO mice were implanted with small titanium head-posts. The posts were glued to the mouse's skull using bone cement under isofluorane anesthesia in a 30-min procedure. Following recovery from surgery (~3 days), the mice were tested on 3 successive days. A small, lightweight (~0.6 g) inertial measurement unit (IMU) was mounted on the surgically implanted head-post to measure linear head acceleration and angular velocity in 3-dimensions. The mice were placed in a 1 square foot container, in complete darkness and in a sound proof booth to eliminate visual and auditory cues to head orientation. They were permitted to move freely for 5 min. Linear acceleration and angular velocity were continuously sampled (1 kHz rate) and the samples collected during the 5-min interval were stored for off-line analysis. The data were analyzed to compute the average magnitudes of linear head acceleration and angular velocity over 100 ms epochs during the 5-min experiment. Previous studies have shown that animals with vestibular dysfunction are unable to stabilize head position when they move about the container; typically they

demonstrate greater mean and variance of angular head velocity and linear head acceleration (i.e., less head stability) than normal animals (Godin et al., 2015).

2. 7. Statistical analysis

Group differences in hair cell counts and calyx nerve endings were evaluated by student T-test and comparisons of functional vestibular measures were evaluated by repeated measures ANOVA in R; $p < 0.05$ was considered significant.

3. Results

3. 1. Localization of Cx26 in the vestibular organs of wild type and *Gjb2*-CKO mice

Immunocytochemistry of 8 week old mice revealed large differences between wild type and *Gjb2*-CKO in the distribution of Cx26 gap junctions in the vestibular organs and the cochlea (Fig. 2). In the cochlea of wild type animals, robust and well-defined puncta-like Cx26 immunoreactivity was detected in the spiral limbus, in non-sensory cells surrounding the inner hair cells, and in the area lateral to the outer hair cell region (Fig. 2A). In addition, weak immunoreactivity was also seen between non-sensory cells beneath the focal plane of hair cells in the organ of Corti, where only supporting cells reside (supplementary Fig. 1B). Staining was restricted to non-sensory cells of the cochlea and appeared as puncta around the circumference of the cells (Fig. 2A). In contrast, epi-fluorescence analysis of cochleae obtained from *Gjb2*-CKO mice was completely negative for staining with the same antibody and protocol (Fig. 2B), demonstrating a lack of Cx26 in these cochleae.

In the vestibular end-organs of wild type mice, Cx26 was seen both in the sensory epithelium (Fig. 2C) and in the surrounding transitional epithelium (Supplementary Fig. 2). In the sensory epithelium, puncta were seen in focal planes below hair cells where only supporting cell bodies are present, consistent with their hypothesized role in communication between supporting cells. Puncta also were seen in more apical planes, where hair cells and supporting cells are present. In some cases the puncta seemed to be at interfaces between two supporting cells, but the possibility that puncta were at a hair cell – supporting cell junction could not be ruled out by epi-fluorescence alone (Fig. 2C, 2E). We conclude that in wild type mice the puncta seen in focal planes where hair cells reside likely represent staining in junctions between supporting cells that are also present at these relatively apical levels of the tissue (Fig. 2G, 2H). In contrast, epi-fluorescence for Cx26 in *Gjb2*-CKO mouse ears showed that both vestibular end-organs were completely devoid of puncta in the sensory epithelium (Fig. 2D, 2F). However, in the transitional epithelium Cx26 puncta were present and their density did not decrease compared to wild type mice (Supplementary Fig. 2). It therefore appears that the *Gjb2*-CKO mouse lacks Cx26 in the sensory epithelium area but the transitional epithelium is not affected.

3. 2. Cx30 (Gjb6) distribution in *Gjb2*-CKO mice

Heteromeric junctions with contribution of both connexins (26 and 30) have been demonstrated and the possibility for substitution of one for the other has been suggested (Lautermann et al., 2001; Lautermann et al., 1998; Sun et al., 2009). In light of hypothesized interactions between Cx26 and Cx30, we evaluated the expression of Cx30 in the Cx26

deprived inner ear by epi-fluorescence. In the cochleae of wild type mice, Cx30 puncta were localized in the spiral limbus area and medial to the inner hair cell area (Fig. 3A) and in pillar and Deiters cells (not shown). The cochleae of *Gjb2*-CKO mice appeared to have a similar distribution and intensity of Cx30 to the wild type cochleae (Fig. 3B). Cx30 puncta were also identified within the sensory epithelium of the vestibular organs (Fig. 3 C, E and G). However, in contrast to the cochleae, the vestibular organs of the *Gjb2*-CKO mouse showed a marked reduction in Cx30 puncta (Fig. 3D, F and H). In the utricle and ampullae, puncta were absent, but in the saccule a few puncta were detected. Thus, we determined that in the cochlea of the *Gjb2*-CKO mouse Cx30 expression is not affected by deletion of Cx26 whereas in vestibular organs, absence of *Gjb2*-CKO leads to reduction or elimination of Cx30.

3. 3. Vestibular hair cell distribution and morphology in the *Gjb2*-CKO mice

Presence of hair cells in the *Gjb2*-CKO mouse vestibular epithelia (8 weeks old) was assessed using a combined staining with phalloidin (predominantly showing the stereocilia bundle and cell-cell adherens junctions) and antibody to Myosin VIIa (predominantly localized to hair cell cytoplasm) on whole-mounts (Fig. 4). Qualitative assessment of the sensory epithelium revealed that the hair cell density in the *Gjb2*-CKO mouse (Fig. 4A, B) was similar to wild type mice (compare to Fig. 2 C, E). The morphology of these cells appeared normal, with no pathological changes in the organization of stereocilia bundles (Fig. 2 D, F). At low magnification, in the utricle, saccule and ampullae, Myosin VIIa positive cells were uniformly distributed throughout the sensory epithelium, showing no obvious gaps or scars, therefore suggesting little or no loss of hair cells (Fig. 4 C, D, and Supplementary Fig. 3). Quantitative analysis of Myosin VIIa-positive cells of *Gjb2*-CKO mice revealed no statistically significant differences from wild type mice in either utricle or saccule (Fig. 4E).

In addition to the presence of hair cells and the general organization of the tissue, we also assessed for the presence of calyx nerve endings in the vestibular sensory epithelium of mutant mice, with epi-fluorescence using antibody for neurofilament (Fig. 5). In both utricle and saccule, calyx nerve endings were observed in the center and in the peripheral portion of each organ, in both *Gjb2*-CKO and wild type mice. Overall counts of calyx nerve endings in the *Gjb2*-CKO mice were not statistically different from wild type mice in either vestibular organ (Fig. 5E). In addition, we found no age-related changes in vestibular organs of these *Gjb2*-CKO mice. In older animals (20 - 40 weeks old), there were no quantitative difference in hair cell survival and the number of calyx nerve endings between mutant and wild-type mice (Supplementary Fig. 4).

3. 4. Vestibular evaluation of the *Gjb2*-CKO mice

For younger mice, an individual's time on the rotarod often varied by a factor of more than 2 (Fig. 6A), but there was no consistent ordering that would indicate a general training effect ($F_{2,20}=0.35$, $p=0.71$). The *Gjb2*-CKO group included a subject that performed relatively poorly; however, its best time was within the range of times recorded for other mice. In general, there was a broad overlap of times for the two genotypes, and no significant effect

of genotype ($F_{1,10}=0.01$, $p=0.93$). There also was no effect of genotype on variation among trials within individuals ($F_{2,20}=0.67$, $p=0.52$).

For older mice, an individual's time on the rotarod varied by a factor of at least 1.5 (Fig. 6B), but again, there was no consistent ordering indicative of a general training effect ($F_{4,16}=0.30$, $p=0.88$). Both genotypes include individuals that performed well on at least one trial, and both genotypes include an individual that performed more poorly. Analysis of the distribution of times among the genotypes indicated no significant effect of genotype ($F_{1,4}=0.12$, $p=0.75$), and no significant effect of genotype on variation among trials within individuals ($F_{4,16}=1.39$, $p=0.28$). There were some older mice that did not perform as well as most of the younger mice, but this also did not differ between genotypes (1/3 for each). These mice with poor performance were the oldest mice (10 month old) in both genotype groups.

The centrifuge test demonstrated a consistent pattern of temperature decrease immediately after spinning followed by a gradual recovery (Fig. 6C). This pattern of temperature changes was highly significant ($F_{5,50}=17.6$, $p<0.0001$) and did not differ between genotypes ($F_{5,50}=0.34$, $p=0.89$). The slight difference in average temperature between genotypes also was not significant ($F_{1,10}=0.99$, $p=0.34$).

To further test vestibular function we measured the effectiveness of the VCR to maintain head stability. The test determined that there was no difference in the magnitude and variance of either mean head angular velocity (ordinate) or mean head linear acceleration (abscissa) between wild type or *Gjb2*-CKO mice (Fig. 7, Supplementary Table 1). These results suggest that there is no major vestibular deficit in the *Gjb2*-CKO mice at the ages tested.

4. Discussion

4. 1. Vestibular End Organs in Connexin Mutations

The severe cochlear pathology and hearing deficits in the *Gjb2*-CKO mouse studied here have been previously documented (Takada et al., 2014). Other mouse models with Cx26 mutations have also revealed severe cochlear dysfunction and pathologies (Cohen-Salmon et al., 2002; Kudo et al., 2003; Sun et al., 2009). Our current study also shows that vestibular phenotype is absent in these mutants up to at least 10 months; this lack of vestibular symptoms well into maturity is comparable to most cases of human Cx26 disease (Denoyelle et al., 1999). We show that Cx26 is present in both auditory and vestibular systems in wild-type mice, and is lacking from the sensory epithelia of both systems in the *Gjb2*-CKO mice. The lack of a vestibular phenotype in the mutants despite absence of Cx26 is difficult to explain, as are the strikingly different consequences of eliminating Cx26 expression in the auditory and the vestibular systems of the inner ear. In contrast to the organ of Corti, the vestibular end organs of the *Gjb2*-CKO mice exhibited neither hair cell loss nor any other marked pathology, and no apparent deficit in vestibular function despite the loss of Cx26. Comparing our data to findings obtained with other connexin mutants may shed some light on the consequences of these mutations in the auditory and vestibular compartments of the inner ear.

Loss of Cx30 caused a phenotype restricted to the saccule among vestibular organs at tested ages (p17 to 1 year), and did not appear to influence expression of Cx26 in any of the vestibular end-organs (Qu et al., 2007). This led Qu et al. to propose that the saccule is more susceptible than other vestibular organs to the loss of connexins because the saccule is located closer to the cochlea than any other vestibular organs, increasing its exposure to cochlear endolymph. Although this is a plausible hypothesis, it is not supported by our data, which show a lack of vestibular pathology all vestibular organs of Cx26 CKO mice despite a severe cochlear phenotype. In addition, our data demonstrate that one effect of the loss of Cx26 is the near total elimination of Cx30 expression in the utricle and ampullae as well as the saccule. Thus, it remains unclear why the saccule was affected by the loss of Cx30 whereas the other vestibular end organs had no hair cell degeneration or any other visible pathology. Future experiments using conditional deletion technology for each of the connexins will likely continue to advance our understanding of the role each connexin plays in the inner ear, during development and in the mature ear, and may lead to an explanation for the disparate effects of loss of Cx26 and Cx30 on auditory and vestibular systems. More conclusive evidence for absence of gap junctions in vestibular hair cells can be obtained using dye transfer experiments, where injection of a tracer into supporting cells has been found to spread to neighboring supporting cells but not to hair cells (personal communication with Drs. R.R. Taylor, D.J. Jagger and A. Forge, UCL, London, UK)(Chang et al., 2008).

4. 2. Possibility of Compensation by Other Connexins

A previous study found that Cx26 over-expression compensated for the deleterious effects of Cx30 mutation in the cochlea (Ahmad et al., 2007); therefore, we posited a reciprocal relationship where one connexin could compensate for the lack of another throughout the vestibular system but not in the cochlea. Our results reject both parts of this conjecture. When Cx26 is mutated, Cx30 is not available in the vestibular organs to compensate for the deleterious effects of the Cx26 mutation, so another molecule must be playing that role. In the cochlea, where Cx30 is available in Cx26 mutants, Cx30 is not effective in counteracting the effects of the mutation.

Our results do not rule out the possibility that another connexin plays the role we predicted for Cx30. Other candidates for compensation include connexins 29, 31, 43 (Cohen-Salmon et al., 2004; Eiberger et al., 2006; Forge et al., 2013; Forge et al., 2003b; Jagger et al., 2014; Lopez-Bigas et al., 2002; Suzuki et al., 2003; Tang et al., 2006; Xia et al., 1998). The extent of molecular compensation by other members of the connexin family of proteins and their identity will have to be determined using our mouse and other connexin loss of function models. Our finding that the loss of both Cx26 and Cx30 in the vestibular end organs was not associated with vestibular dysfunction and pathology suggests that the role of these connexins in the vestibular system may be more complex than previously appreciated.

The presence of connexins in the transitional epithelium of the Cx26 CKO mice may contribute to the normal vestibular architecture and function in these animals (Supplementary Fig. 2). It is possible that connexins in the transitional epithelium are sufficient for recycling potassium and perform other homeostatic roles needed for

maintaining the vestibular hair cells. The presumed role of dark cells of the transitional epithelium in potassium homeostasis would support this possibility (Masuda et al., 2001; Wangemann, 2002). Further analysis using transgenic mice with a cre line that deletes connexin-based gap junctions in the transitional epithelium may shed light on the role of connexins in this non-sensory region of the vestibular epithelium. In addition, transgenic lines that delete various combinations of connexins may help elucidate the role of these proteins in the sensory epithelium proper.

4. 3. The difference between cochlea and vestibular systems: Mechanistic Speculations

The difference in levels of hair cell death between cochlea and vestibular organs might be related to sensitivity to their physiologic environment. Differential susceptibility of specific hair cell types to toxic agents insults has been documented. For instance, outer hair cells are more vulnerable than inner hair cells to certain ototoxic drugs (Amarjargal et al., 2009) and to noise exposure (Stebbins et al., 1979; Yang et al., 2004). Several mouse mutations modeling human hereditary inner ear disease have demonstrated that a specific hair cell type is more sensitive than others to the genetic changes (Liberman et al., 2006; Nakano et al., 2012; Seal et al., 2008). In the vestibular system, type I hair cells are more vulnerable than type II hair cells to ototoxicity and in certain mutant mice with a vestibular phenotype, type I vestibular hair cells are selectively involved while type II hair cells are spared (Udagawa et al., 2012). In a similar fashion, survival of vestibular hair cells despite the loss of their auditory counterparts may be due to reduced sensitivity to changes in potassium recycling or to a lesser effect of the loss of Cx26 and Cx30 on homeostasis in the vestibular end organs than in the cochlea.

Although the potassium recycling pathway in the cochlea is well characterized (Helling et al., 2005; Marcus et al., 2002; Wangemann, 2002; Zdebik et al., 2009), its vestibular counterpart is not fully understood. Differences between the two inner ear compartments in potassium recycling have been demonstrated (Kharkovets et al., 2000; Marcus et al., 2002; Wangemann, 2002). These differences suggest that other molecules may be responsible for the potassium recycling in the vestibular end organs. Putative molecules may include the inward rectifier Kir4.1 channels (Jagger et al., 2010; Udagawa et al., 2012) or pH controllers in the hair bundles of vestibular hair cells (Hill et al., 2006). These differences may explain why Cx26 mutations that affect hearing cause little or no vestibular deficit.

One main difference between cochlear and vestibular hair cells is the calyx nerve ending, where potassium may accumulate between the type I hair cell and the calyx (Contini et al., 2012; Lim et al., 2011; Soto et al., 2002). One possibility is that the calyx removes excess potassium from the space between the two cells, limiting the exposure of type I vestibular hair cells to high potassium levels. If the damaging potassium is mostly in the extracellular fluid (rather than in hair cells) this mechanism could indeed be protective. However, the contrary argument is that the calyx may trap the potassium that exits the hair cell, limiting its diffusion into extracellular fluid, potentially increasing the risk of potassium exposure. If true, this would suggest that both the hair cell and the nerve have enhanced tolerance for high potassium levels, enabling them to cope with such potentially damaging conditions.

4. 4. Why Does Loss of Cx26 Lead to Loss of Cx30 in the *Gjb2*-CKO Mouse Vestibular System?

Our epi-fluorescence analysis in wild type mice revealed the presence of Cx30 puncta in both the vestibular and cochlear sensory epithelium, and loss of Cx26 resulted in loss of Cx30 in the vestibular end organs but not in the cochlea. The underlying pathway for the secondary loss of Cx30 puncta with loss of Cx26 in the vestibular system is enigmatic. It could be a result of lack of gene expression, or blocks on transcription or translation. An alternative explanation for this difference between the cochlea and the vestibular organs may be caused by differences in the contribution of each connexin to the formation of gap junction hexamers (Forge et al., 2003b). Previous work suggests that there are two kinds of gap junction hexamers (two hexamers from each cell dock to form a gap junction), homomeric (composed of same connexin subtypes in the same junctional plaque) and heteromeric (composed of different connexin subtypes in the same plaque) (Koval et al., 2014). Studies in the inner ear have revealed the existence of heteromeric hexamers as well as homomeric hexamers composed of Cx26 and Cx30 (Forge et al., 2003a; Jagger et al., 2014). We speculate that in the vestibular epithelium the majority of junctions are heteromeric which is why loss of Cx26 leads to loss of Cx30, whereas the homomeric gap junctions in the cochlea composed only of Cx30 are unaffected by loss of Cx26. If the auditory system is a model for the vestibular system, then gap junctions formed by other connexins may be present. The difference in the functional phenotype could then reflect a difference in the sufficiency of the gap junctions remaining after Cx26 deletion. Cx30 junctions may be inadequate in number or capacity to meet the demands arising from Cx26 loss in the cochlea, but the yet unidentified junctions may suffice in the vestibular system.

The lack of a marked vestibular phenotype despite the absence of both connexins (26 and 30) on the vestibular epithelium of the *Gjb2*-CKO mouse raises two questions: what could be the roles of Cx26 and Cx30 in the vestibular system and what molecules compensate for their loss? At present we can only speculate about these questions. One possibility is that functional deficits in vestibular function are too subtle to measure with the means we currently have. Subtle functions of connexins have been noted in other systems such as cardiomyocytes, uterine smooth muscle and tracheal cilia, where synchronization of excitation has been linked to gap junction functions (Doring et al., 2006; Saffitz et al., 2000; Sanderson et al., 1988). Alternatively, the role of these connexins is related to regulation of repair in an epithelial tissue (Forge et al., 2013). As for the identity of molecules that may compensate for the loss of connexin 26 and 30, it will likely be necessary to design and produce a more complex mouse model that will have a deletion in one of the other candidate connexins, such as 29, 31, or 43, in addition to the Cx26 and Cx30 mutations.

4. 5. Clinical Implication of the Current Results

Commonalities of development and sensory cell function suggest that hearing loss might often be associated with vestibular dysfunction; however, the influence of deafness genes on the vestibular system is variable, with some genes influencing both systems and others only hearing (Eppsteiner et al., 2011; Jones et al., 2014). In *GJB2* patients an abnormal vestibular phenotype is sometimes found (Dodson et al., 2011; Eppsteiner et al., 2011; Kasai et al., 2010; Todt et al., 2005; Zagolski, 2007) but in most cases it is absent (Cohn et al., 1999;

Denoyelle et al., 1999; Shinjo et al., 2007). Our current results, showing hair cell survival as well as normal vestibular function with loss of Cx26 and 30 in the vestibular system may shed light on the function of these connexins in the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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In *Gjb2*-CKO mice designed to exclude Cx26 in the cochlea Cx26 is absent also in the vestibular system

Loss of Cx26 resulted in secondary loss of Cx30 in the vestibular system

Despite the loss of both connexins, vestibular structure and function appear intact

Figure 1. Apparatus of “centrifuge test”
 (A). 25.4 cm aluminum arms. (B). Vented 50 ml conical tube to hold the mouse. (C). Acrylic cylinder to hold the tube. (D). Motor. (E). Controller, determines arm speed and timing of changes in speed. (F). Base plate is inclined 30 degrees. (G) Power supply.

Figure 2. Epi-fluorescence and confocal fluorescence images (phalloidin green and Cx26 red) of the cochlea and vestibular end organs (C~F: presumed extra-striolar region) in *Gjb2*-CKO and wild type mice showing Cx26 puncta

Images A, C, E and G are wild type whereas B, D and F are *Gjb2*-CKO, in 8 weeks old mice. (A). Cx26 puncta are seen in the cochlea of a wild type mouse in non-sensory cells located medial to the inner hair cells. (B). In the same location in *Gjb2*-CKO ears Cx26 puncta are absent. (C). In the utricle Cx26 puncta are localized in the area of the sensory epithelium in wild type mice, with a distribution pattern that surrounds the hair cells. (D). In a *Gjb2*-CKO utricle Cx26 puncta are absent, whereas hair cells are present. (E-F). In the

sacculle Cx26 puncta are found in the wild type ear and absent in the mutant. (G). Stacked confocal microscope image show Cx26 puncta in areas near the luminal membrane of a utricle of wild type mouse. (H) Stacked confocal microscope images set shows Cx26 puncta in areas of the epithelium near the basement membrane of a wild type mouse ear. (I). Schematic illustrating a cross section of the vestibular sensory epithelium, demarcating (dotted line) the focal planes of the optical sections used to generate images G and H (Yellow: Afferent nerve ending). Images in G are from place above the dotted line in I and in H are below the line. Scale bars indicate 20 μm .

Figure 3. Epi-fluorescence images (phalloidin green and Cx30 red) of the cochlea and vestibular end organs (C-F: presumed extra-striolar region) in *Gjb2*-CKO and wild type mice showing Cx30 puncta

Images A, C, E and G are wild type whereas B, D, F and H are *Gjb2*-CKO, in 8 week old mice. (A-B). Cx30 puncta are seen in the cochlea of a wild type and *Gjb2*-CKO mouse along the apical membrane of non-sensory cells located medial to the inner hair cells. (C). In the utricle Cx30 puncta are localized in the area of the sensory epithelium in a wild type mouse, with a similar distribution pattern of Cx26 puncta (compare to Fig. 2C). (D). In a *Gjb2*-CKO utricle Cx30 puncta are absent. (E-F). In the saccule, Cx30 puncta are found in

the wild type ear and are decreased in the mutant. (G-H). In the ampulla, Cx30 puncta are found in the wild type ear and absent in the mutant. Hair cells in the vestibular organs of *Gjb2*-CKO mice appear normal in shape in distribution despite the absence of Cx30 and Cx26 puncta. Scale bars indicate 20 μ m.

Figure 4. Epi-fluorescence images of vestibular end organs in *Gjb2*-CKO showing density of Myosin VIIa-positive cells at the age of 8 weeks

Two-color merged images with Myosin VIIa (green) and phalloidin (red) staining in the utricle (A) and saccule (B) of *Gjb2*-CKO mice show that Myosin VIIa-positive cells appear normally and evenly distributed throughout the end organs, without areas of cell loss. Stereocilia also appear normal. Lower magnification monochrome epi-fluorescence images of the utricle (C) and saccule (D) show that Myosin-VIIa positive cells are homogenously distributed throughout the sensory epithelium. Quantitative analysis of Myosin VIIa-positive

cells (E) indicates that there is no statistical difference between *Gjb2*-CKO and wild type mice in utricle ($p = 0.22$) or saccule ($p = 0.94$). Scale bars indicate 20 μm .

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Figure 5. Epi-fluorescence images of vestibular end organs in *Gjb2*-CKO showing density of calyx nerve endings at age of 8 weeks

Epi-fluorescence images of sensory epithelium of the utricle (A, C) and saccule (B, D) of *Gjb2*-CKO mice, showing neurofilament staining alone (green, A and B) or in combination with Myosin VIIa (red, C and D). Calyx nerve endings in both utricle and saccule of *Gjb2*-CKO mice are identified by a ring shape surrounding the hair cell. Quantitative analysis of calyx nerve endings (E) shows no statistical difference between the *Gjb2*-CKO and wild type mice in utricle ($p = 0.77$) or saccule ($p = 0.40$). Scale bars indicate 20 μ m.

Figure 6. Vestibular function tests using rotarod and centrifuge

Duration of staying on the rotarod (A and B) for younger mice (A) as well as older mice (B) shows no statistical difference between *Gjb2*-CKO and wild type animals. In the centrifuge test (C) there is a temperature drop after the spinning in both *Gjb2*-CKO and wild type animals, however body temperatures do not show significant difference between groups.

Figure 7. Vestibular function test based on head stability

Scatter plots of mouse head movement data show that there is no difference in either the magnitude or variance of mean head angular velocity (ordinate) or mean head linear acceleration (abscissa) between wild type (right panel) or *Gjb2*-CKO mice (left panel). Each point is a mean value obtained over a 100 ms time interval while the mouse freely moved about the test chamber for 5 mins. The green box represents ± 3 standard deviations from the mean velocity and acceleration (red + symbol). Data were collected over 3 separate test days. The mean linear acceleration includes gravitational acceleration (~ 980 cm/sec²).