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The Role of Bone Morphogenetic Protein 4 in Inner Ear Development and Function

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Abstract

Bone Morphogenetic Protein 4 (BMP4) is a member of the TGF- β superfamily and is known to be important for the normal development of many tissues and organs, including the inner ear. *Bmp4* homozygous null mice die as embryos, but *Bmp4* heterozygous null (*Bmp4*^{+/-}) mice are viable and some adults exhibit a circling phenotype, suggestive of an inner ear defect. To understand the role of BMP4 in inner ear development and function, we have begun to study C57BL/6 *Bmp4*^{+/-} mice. Quantitative testing of the vestibulo-collic reflex, which helps maintain head stability, demonstrated that *Bmp4*^{+/-} mice that exhibit circling behavior have a poor response in the yaw axis, consistent with semicircular canal dysfunction. Although the hair cells of the ampullae were grossly normal, the stereocilia were greatly reduced in number. Auditory brainstem responses showed that *Bmp4*^{+/-} mice have elevated hearing thresholds and immunohistochemical staining demonstrated decreased numbers of neuronal processes in the organ of Corti. Thus *Bmp4*^{+/-} mice have structural and functional deficits in the inner ear.

Keywords

Hair Cells; Neuronal Processes; Stereocilia; Semicircular Canals; Mouse; Vestibulo-Collic Reflex (VCR); Auditory Brainstem Response (ABR)

Introduction

The transforming growth factor β (TGF- β) superfamily consists of signaling molecules that are involved in many cellular processes in the development of both vertebrates and invertebrates (Nakayama et al., 2000). Bone morphogenetic proteins (BMPs) are members of TGF- β superfamily that have been found to play important roles in the organogenesis of almost all tissues, including the inner ear (Jones et al., 1991; Hogan et al., 1996; Nakayama et al.,

2000; DeRobertis and Kuroda, 2004; Wijgerde et al., 2005; Goldstein et al., 2005). Specifically, BMPs and their antagonists are involved in establishing the anteroposterior and dorsoventral axes of the inner ear (Chang et al., 1999; Gerlach et al., 2000). In addition, local application of either the BMP antagonists noggin or DAN, or retinoic acid (which represses *Bmp4* transcription) to chick embryo otocysts impairs development of the semicircular canals, whereas simultaneous exposure to recombinant BMP4 rescues semicircular canal development (Chang et al. 1999; Gerlach et al., 2000; Thompson et al., 2003; Gerlach-Bank et al., 2004; Barald and Kelley, 2004). Li et al., 2005, used a chick otocyst culture system to demonstrate that both hair cell and supporting cell numbers were significantly reduced when BMP4 signaling was blocked. They also showed that the number of hair cells increased significantly when cultured otocysts were treated with exogenous BMP4.

Bmp4 mRNA is expressed as early as E9.0 in the developing mouse otic placode and continues to be expressed in the sensory epithelium and mesenchyme surrounding the cochlea until P1 (Morsli et al., 1998). Most *Bmp4* homozygous null mouse embryos die at gastrulation, but *Bmp4* heterozygous (*Bmp4*^{+/-}) embryos can survive to adulthood and approximately 7% of these mice exhibit a circling behavior, consistent with an inner ear defect (Winnier et al., 1995; Dunn et al., 1997). Here we characterize the auditory and vestibular phenotypes of *Bmp4*^{+/-} mice. Defects in both auditory and vestibular function were observed, and were associated with cellular and tissue-specific abnormalities.

Materials and Methods

Mice

Breeding pairs (C57BL/6 *Bmp4*^{+/-} × wild type C57BL/6) were obtained from the Jackson Laboratory (Bar Harbor, ME). In all the studies, “aged matched” indicates *Bmp4*^{+/-} and wild type mice within 1 week of age. Animal care and handling procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Genotyping

All adult mice and embryos were genotyped for the neomycin resistance cassette, which was used to generate the *Bmp4*^{+/-} mice (Winnier et al., 1995), by polymerase chain reaction (PCR) according to the protocol from Jackson Laboratory, http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=701.

Vestibular Function Testing

The vestibulo-collic reflex (VCR) was measured as previously described (Takemura and King, 2005). Briefly, the body of the mouse was immobilized with a restraint jacket, leaving its head free to move. A small wire loop (search coil) was attached to the mouse's head using Skin-Bond® (a removable adhesive) and centered within an alternating electromagnetic field. The mouse and restraint device were rigidly attached to a servo controlled rotational table. Motion of the search coil within the magnetic field induced current in the coil which could be transformed into a head position in space signal. Head position on the body was computed by subtracting head position in space (measured by the coil) and body position in space (measured as turntable position). Rotational accelerations were applied about the yaw (vertical) and pitch (horizontal) axes at 3 different amplitudes-large (90°/s), medium (50°/s) and small (30°/s). The rotational stimulus consisted of a sum of sines pattern that included frequencies from 0.18 Hz to 15.56 Hz. Up to five *Bmp4*^{+/-} circling mice, four *Bmp4*^{+/-} non-circling mice and nine wild type mice ranging in age from 8–42 weeks were tested in the dark. The data were analyzed using Spike2 (Cambridge Electronic Design, Cambridge, UK) and MATLAB software (The MathWorks Inc, Natick, MA). VCR is measured in terms of gain, which is defined as the ratio of the head angular velocity to body angular velocity (Takemura and King, 2005). In a normal

functioning vestibular system, when the body is rotated, the VCR would act to counter-rotate the head. A perfect gain would be achieved when the head angular velocity is equal in amplitude to body angular velocity but opposite in direction to the body rotation. If there is reduced vestibular function, the head rotation velocity may be reduced with respect to body angular velocity resulting in a lower VCR gain. VCR data were normalized as a ratio of average *Bmp4*^{+/-} gain divided by average wild type gain.

Auditory Brainstem Responses (ABR)

Hearing was evaluated by ABR analyses as previously described (Beyer et al., 2000) with minor modifications. Mice were anesthetized by intraperitoneal injection of a mixture of xylazine 7 mg/kg, ketamine 65 mg/kg and acepromazine 2 mg/kg. The sound stimulus consisted of 15 ms tone bursts (1 ms rise/fall time, 10/s) at 6, 12 and 24 kHz. All recordings were done in an electrically and acoustically shielded chamber (Acoustic Systems, Austin, TX). Tucker Davis Technologies System II hardware and Sig Gen/Biosig software (TDT, Alachua, FL) were used to present stimuli and record responses. A Beyer driver (Beyer Dynamic Inc., Farmingdale, NY) was used to deliver tones. Eleven *Bmp4*^{+/-} mice that exhibited the circling phenotype, 4 *Bmp4*^{+/-} mice that did not exhibit the circling phenotype and 12 wild type mice were tested. The mice were tested once at an age ranging from 4 weeks to 12 weeks.

Histochemical Analysis

Phalloidin Staining—The inner ears of adult *Bmp4*^{+/-} and wild type mice were dissected and processed for phalloidin staining as previously described (Beyer et al., 2000). Briefly, tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS, permeabilized in 0.3% Triton-X 100 and incubated with phalloidin Texas Red (Invitrogen, Carlsbad, CA) 1:100 in PBS for 30 minutes. Whole mounts of the sensory epithelia were mounted on slides with Vectashield (Vector Laboratories Inc, Burlingame, CA). Tissues were analyzed and photographed on a Leica DMRB epifluorescent microscope using x10, x40 oil and x100 oil objectives and a standard TRITC filter. Eight *Bmp4*^{+/-} mice (6 circlers and 2 non circlers) and 4 wild type mice were analyzed.

Myosin and Neurofilament Staining—The primary antibodies used were anti-myosin VIIa rabbit polyclonal (Proteus Biosciences, Ramona, CA, cat# 25-6790) and anti-neurofilament 200kD mouse monoclonal (Sigma, cat# N0142) and the secondary antibodies were fluorescein goat anti-rabbit IgG (Invitrogen, cat # F-2765) and alexa fluor 594 goat anti-mouse IgG (Invitrogen, cat # A-21125). The inner ears of adult *Bmp4*^{+/-} and wild type mice were dissected and processed as previously described (Hasson et al., 1997). Tissues were incubated with the primary antibodies (1:200 in PBS/0.1% BSA/1% donkey serum) for 1 hour and with secondary antibodies (1:200 in PBS/0.1% BSA/1% donkey serum) for 30 minutes at room temperature. Tissues were mounted on glass slides with Vectashield. Twelve *Bmp4*^{+/-} mice (7 circlers and 5 non circlers) and 9 wild type mice were analyzed for neurofilament expression and 11 *Bmp4*^{+/-} mice (6 circlers and 5 non circlers) and 8 wild type mice were analyzed for myosin VIIa expression. Outer hair cells (OHCs), inner hair cells (IHCs) and neuronal processes from 100 μ m segments of organs of Corti, located about two-thirds into the first turn starting at the apex as indicated in the boxed regions in Fig. 4, were quantified using Metamorph software. The confocal images that were taken for Figs. 4A and 4B are low magnification single plane images, and those for 4C and 4D are higher magnified z-stack projected images consisting of 14 sections of 1 μ m thickness. Four *Bmp4*^{+/-} mice (2 circling and 2 non-circling) and 4 wild type mice were analyzed.

Isolation of otocyst RNA and real-time RT-PCR analysis of Neurogenin 1

Bmp4^{+/-} and wild type embryos age E14.5 were obtained from timed pregnancies, using noon on the day of the vaginal plug as day 0.5 of gestation. Genotyping of embryos was done as described above. Timed pregnant females were euthanized, embryos were removed and the otocysts were microdissected and placed in Trizol (Invitrogen) for isolation of RNA. cDNA was synthesized with random hexamers using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was then preamplified for 14 cycles using Neurogenin1 assay mix (Mm00440466_s1; Taqman Gene Expression Assay, Applied Biosystems) according to the protocol for Taqman PreAmp Master Mix kit (Early Access) (Applied Biosystems) with the substitution of Power SYBR Green for Taqman PreAmp Master Mix. Real time PCR was then performed using Neurogenin1 assay mix and β -actin assay mix (Mm00607939_s1; Taqman Gene Expression Assay, Applied Biosystems) according to Taqman Gene Expression Assay Protocol. Amplifications were performed in an Applied Biosystems 7500 real time pcr instrument. For both neurogenin 1 and actin, standard curves were run within each assay using cDNA from 1, 10 and 100 ng of starting RNA. The Applied Biosystems software used the cycle threshold data from these standards to quantify the input cDNAs for the experimental samples. Neurogenin1 expression was normalized to β -actin expression. Otocysts from 8 *Bmp4*^{+/-} and 8 wild type embryos were analyzed, derived from the matings of 2 *Bmp4*^{+/-} males with 2 wild type females.

Results

Behavioral analysis shows that *Bmp4* heterozygous null mice spend the majority of time circling

Bmp4^{+/-} mice were difficult to breed, limiting the number of mice that could be studied. The average litter from mating a wild type C57BL/6 mouse with a C57BL/6 *Bmp4*^{+/-} mouse yielded only 1 *Bmp4*^{+/-} pup, and only 10% of heterozygous pups exhibited circling behavior, which generally became apparent by two weeks of age. To characterize the circling phenotype of *Bmp4*^{+/-} mice, two circlers and one non-circler (based upon casual observation) were videotaped overnight using an infra-red camera to monitor their movements. Circling behavior, believed to be vestibular in origin, was examined in darkness to exclude any possible visual system involvement in the behavior. One hour of each mouse's circling behavior was analyzed and categorized as clockwise, counter-clockwise or not circling (either stationary or exhibiting normal foraging behavior). One mutant spent 50% of the time circling in a clockwise direction, 17% in a counterclockwise direction and 33% not circling. The second mutant spent 65% of the time circling in a clockwise direction, 1% in a counterclockwise direction and 34% not circling. The lack of circling phenotype in the non-circler *Bmp4*^{+/-} mouse was confirmed by analyzing 6 hours of the videotape.

The Vestibulo-Collic Reflex shows that the circling mutant mice have poor responses in the yaw axis

The VCR is a neural reflex using neck muscles to stabilize the head on the body. This reflex requires the vestibular organs in the inner ear. Rotation around the yaw axis stimulates the horizontal semicircular canals and rotation around the pitch axis stimulates the vertical semicircular canals and otoliths, the saccule and utricle. The VCR responses of the *Bmp4*^{+/-} circling mice relative to those of the wild type mice are shown in Figure 1A, and of *Bmp4*^{+/-} non-circling mice are shown in Figure 1B. The VCR gain was almost constant across all frequencies during rotations at all three amplitudes, demonstrating the consistency and linearity of the response. *Bmp4*^{+/-} circling mice have low gain ratios with yaw axis rotation indicating that these mutant mice have poorer head stability than the wild type mice. This poor response from the *Bmp4*^{+/-} circling mice is consistent with horizontal semicircular canal dysfunction. In the pitch axis, the *Bmp4*^{+/-} circling mice have as good or better head stability than the wild

type mice, as the gain ratios are ≥ 1 . In contrast, *Bmp4*^{+/-} non-circling mice have gain ratios of ≥ 1 in both the yaw and pitch axes, indicative of normal VCR function.

Auditory Brainstem Responses demonstrate that both circling and non-circling *Bmp4* heterozygous mice are partially deaf

Hearing thresholds to tone stimuli were assessed by ABR testing. Figure 2 shows the ABR thresholds for each mouse at 6, 12 and 24 kHz. The mice are divided into those 9 weeks or younger and those older than 9 weeks, to better account for age-related hearing loss that is associated with the C57BL/6 background. Most *Bmp4*^{+/-} circlers have elevated thresholds across all frequencies (7 out of 11 mice at 6 kHz and 12 kHz; 8 out of 11 mice at 24 kHz), consistent with a partial hearing loss. *Bmp4*^{+/-} non-circlers have elevated thresholds similar to those of circlers. Table 1 shows the threshold means $\pm$ SD at each frequency for the wild type mice and all *Bmp4*^{+/-} mice.

ABR testing was done on only the left ear according to standard protocol due to the limited number of *Bmp4*^{+/-} mice and concern of possible anesthesia-related death. However, following sacrifice, both ears of the mice were examined histologically, yielding similar findings.

Stereocilia are abnormal in the ampullae of *Bmp4*^{+/-} mice

Paint filling studies of the semicircular canals of 16 *Bmp4*^{+/-} embryos ranging in age from E15 to E18 showed that there were no gross morphological abnormalities (data not shown). Because we could not prospectively determine which, if any, of these embryos would have exhibited a circling phenotype, inner ears from four adult circling mice were also analyzed by paint filling, and again no gross abnormalities were found (data not shown). We then analyzed the hair cells and stereocilia of wild type and mutant mice.

Phalloidin staining of whole mounts of auditory and vestibular tissues (organ of Corti, saccule, utricle and ampullae) from wild type mice demonstrated the normal appearance of stereocilia (Fig. 3). For example, in the organ of Corti we observed 3 rows of outer hair cells with the characteristic V shaped stereocilia bundles and 1 row of inner hair cells with stereocilia. In the *Bmp4*^{+/-} circling mice, the stereocilia appeared normal in the organ of Corti, saccule and utricle, whereas the stereocilia of the ampullae were much fewer in number. A similar decrease was observed in 4 of 6 circling mice, and the other 2 circlers showed a patchy decrease in the number of stereocilia. In contrast, non-circling *Bmp4*^{+/-} mice had normal-appearing stereocilia in their auditory and vestibular tissues (2 mice examined, data not shown).

Neuronal processes innervating the cochlea of both circling and non-circling *Bmp4*^{+/-} mice are reduced in number

To evaluate the presence of neuronal processes, whole mounts were immunostained for neurofilament and costained for myosin VIIA to identify hair cells. The normal complement of hair cells and neuronal processes was present in auditory and vestibular tissues in wild type mice; for example, wild type organ of Corti is shown in Fig. 4A and C. In the *Bmp4*^{+/-} mice (both circling and non-circling), the hair cells in the auditory and vestibular tissues also appeared normal. However, the number of neuronal processes in the organ of Corti was decreased (Fig. 4B and D). This abnormality was apparent in 2 of 4 circler and 2 of 4 non-circler heterozygotes. Fig. 4E shows the means $\pm$ SD for numbers of hair cells and neuronal processes in 100 μ m segments of the organ of Corti for wild type and *Bmp4*^{+/-} mice. In contrast, the saccule, utricle and ampullae of *Bmp4*^{+/-} mice had normal numbers of neuronal processes (data not shown).

Neurogenin 1 expression is not altered in *Bmp4*^{+/-} otocysts

Neurogenin 1 (Neurog1) is a proneural gene that is essential for the development of sensory neurons in the inner ear (Ma et al., 2000), and hence is a potential *Bmp4* target gene. Real time RT-PCR was used to evaluate the expression of Neurog1 in *Bmp4*^{+/-} and wild type otocysts from day 14.5 embryos. As shown in Figure 5, Neurog1 expression was similar in the mutant and wild type otocysts.

Discussion

Several lines of evidence implicate BMP4 as an important signaling molecule in inner ear development. Here we confirm the circling phenotype of a subset of *Bmp4*^{+/-} mice, and show that these mice have both functional and anatomical inner ear defects.

We found that *Bmp4*^{+/-} circling mice have an abnormal VCR in the yaw axis, which would suggest abnormalities in the horizontal semicircular canals. This result is consistent with our finding of decreased numbers of stereocilia in the ampullae of these mice. Although all three ampullae were affected equally, the pitch axis VCR was normal. We postulate that this is because the utricle and saccule were normal in the *Bmp4*^{+/-} mice, since a pitch axis VCR can be produced by these structures as well as the vertical canals. In the embryonic vestibular system, *Bmp4* is expressed primarily in the developing ampullae, not the utricle and saccule, which again is consistent with the observed defects.

ABR thresholds in the *Bmp4*^{+/-} mice were approximately 20–30 dB elevated compared to those in control mice, indicating partial deafness. We did not find abnormalities in the hair cells or stereocilia in the cochlea, but the number of neuronal processes in the organ of Corti was decreased, and this may contribute to the auditory phenotype. Thus, *BMP4*^{+/-} mice have different abnormalities in their auditory and vestibular systems, fewer neuronal processes in the former and abnormal stereocilia in the latter, implying that the role of BMP4 differs in these two systems. We postulate that this might be explained by differences in the expression of BMP4, other BMPs, BMP antagonists, or downstream effectors. Other differences in the auditory and vestibular systems are known, such as their dependence on different neurotrophins. For example, at later stages in inner ear development, brain-derived neurotrophic factor is necessary for maintaining the survival of vestibular neurons and neurotrophin-3 is necessary for cochlear neuronal survival (Bianchi et al., 1998; Wyatt et al., 1998).

Bmp4 is expressed in the developing neural tube and plays a role in neurogenesis, along with numerous other genes such as the transcription factors Neurog1 and Neurogenic Differentiation Factor 1 (Neurod1) (Ma et al., 2000; Kim et al., 2001). Neurog1 null mice have a complete loss of sensory neurons and Neurod1 null mice are deaf and have very few neurons in the cochlea and ampullae (Ma et al., 2000; Kim et al., 2001). However, studies by Raft, et al., 2004, suggest that BMP4 may inhibit expression of Neurog1 and Neurod1 in the developing inner ear, the opposite of what would be predicted if deficiencies of these proteins were downstream mediators of the effects of *Bmp4* haploinsufficiency. We have investigated the expression of Neurog1 in the developing otocysts and found no difference in expression in *Bmp4*^{+/-} embryos compared to control embryos at embryonic day 14.5.

Bmp2, -4 and -7 bind to the same two types of transmembrane serine/threonine kinase receptors (type 1-BMPR-IA/BMPR-IB and type 2-BMPR-II/ACTR-IIB), activate the same Smad proteins, and have at least some functional redundancy. In the chick, *Bmp2* is expressed in the regions that are destined to become the semicircular canals, and in the mouse, *Bmp7* is expressed in the nonsensory regions of the inner ear as well as the sensory region of the cochlear duct (Oh et al., 1996; Chang et al., 2002). *Bmp4* is expressed in the sensory epithelia and in

the mesenchyme surrounding the cochlea in the chick and mouse (Oh et al., 1996; Morsli et al., 1998). *Bmp2* null mice die as embryos and *Bmp7* null mice die shortly after birth, and there are no published reports of auditory or vestibular defects in these heterozygous mutants, as seen in *Bmp4*^{+/-} mice (Dudley et al., 1995; Luo et al., 1995; Zhang and Bradley, 1996). Since BMP4 and BMP7 are co-expressed in the same regions of the developing mouse inner ear, it is possible that BMP7 partially compensates for the deficiency of BMP4 expression in *Bmp4*^{+/-} mice. Studies have shown that BMP4 can substitute for the loss of BMP7 in kidney development (Oxburgh et al., 2005).

Bone morphogenetic proteins are involved in the development of the central and peripheral nervous systems (CNS and PNS), where a number of BMP4 target genes have been identified. In the spinal cord, BMPs define the patterning of the dorsoventral axis of the CNS by regulating the expression of proneural genes such as *Msx1* and *Mash1* which are transcription factors that also are expressed in the inner ear (Tiveron et al., 2003; Liu and Niswander, 2005; Pujades et al., 2006). *Mash1* homozygous null mouse embryos have loss of inner ear efferents at the usual position but show ectopic neurons that do not innervate the inner ear (Tiveron et al., 2003). *Mash1* deficient mice have reductions of neuronal processes in the inner ear similar to the *Bmp4*^{+/-} mice and *Mash1* may be regulated by BMP4. Other genes such as Inhibitors of Differentiation (Ids) and *Math1* also are targets of BMP4 and are expressed in the developing inner ear; however, mouse mutants deficient in these genes show hair cell abnormalities that are not found in *Bmp4*^{+/-} mice (Bermingham et al., 1999; Jones et al., 2006). BMPs regulate many genes in many different cellular processes. It is probable that a combination of many different target gene deficiencies is responsible for the inner ear phenotype seen in *Bmp4*^{+/-} mice. BMPs also may function by inducing members of the WNT and Sonic Hedgehog pathways, which in turn may negatively regulate BMPs and thereby alter organogenesis (Liu and Niswander, 2005).

Several other mutant mice that exhibit a circling phenotype and hearing defects have abnormal hair cell stereocilia. The Jerker mouse, Ames waltzer, Shaker 1, waltzer, Shaker 2 and Snell's waltzer have mutations identified in *espin*, *protocadherin15*, *myosin VIIa*, *cadherin23*, *myosin XVa* and *myosin VI* respectively (Wang et al., 1998; Liang et al., 1999; Anderson et al., 2000; Zheng et al., 2000; Holme and Steel, 2002; Karolyi et al., 2003; Siemens et al., 2004). All of these mutant mice have disorganized stereocilia in the hair cells of the organ of Corti, but the Jerker mouse and Ames waltzer mouse also have shortened stereocilia in the vestibular tissues (Zheng et al., 2000; Raphael et al., 2001). However, none of these phenotypes matches the stereocilia defects in *Bmp4*^{+/-} mice, and there are no published data showing interaction of BMP4 with any of the genes described above.

Humans with loss-of-function mutations in *BMP4* have not been reported. However, DFNA23, a novel locus for autosomal dominant nonsyndromic hearing loss, has been mapped to Chromosome 14q21-q22, which overlaps with the location of *BMP4* on Chromosome 14q22-q23 (Salam et al., 2000). A number of other interesting candidate genes are located in this region, decreasing the likelihood that the DFNA23 gene is *BMP4*. Investigation of the role of BMP4 in inner ear development and function is hampered by the low survival rate of *Bmp4*^{+/-} mice and the low penetrance of the circling phenotype. Critical questions, such as the timing of the onset of the abnormalities, as well as the identification of key BMP4 target genes, will be aided by the use of alternative strategies such as the creation of mouse models with tissue-specific loss of BMP4.

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Figure 1. Vestibulo-collic reflex is reduced in the yaw axis in circling *Bmp4*^{+/-} mice, but is normal in non-circling *Bmp4*^{+/-} mice

Ratio of mean gain of *Bmp4*^{+/-} mice to that of wild type mice measured in response to yaw (dashed lines) and pitch (solid lines) axis rotations of small, medium and large amplitudes. (A) Circling mice: Pitch axis, large, *Bmp4*^{+/-} n=4, wild type n=5; medium, *Bmp4*^{+/-} n=4; wild type n=4; small, *Bmp4*^{+/-} n=4; wild type n=4. Yaw axis, large, *Bmp4*^{+/-} n=2, wild type n=5; medium, *Bmp4*^{+/-} n=2; wild type n=5; small, *Bmp4*^{+/-} n=3; wild type n=4. (B) Non-circling mice: Pitch and Yaw axes, large, medium and small amplitudes, *Bmp4*^{+/-} n=4, wild type n=4.

Figure 2. Auditory Brainstem Response thresholds are reduced in *Bmp4*^{+/-} mice
 ABR thresholds for *Bmp4*^{+/-} and wild type mice were measured at frequencies of 6, 12 and 24 kHz. Each symbol represents 1 mouse.

Figure 3. Stereocilia are reduced in number in ampullae of *Bmp4*^{+/-} mice

Whole mount of the organ of Corti or vestibular epithelia stained with phalloidin and viewed by epifluorescence. In the organ of Corti, utricle and saccule, the stereocilia are similar in *Bmp4*^{+/-} and wild type mice. In ampulla, the stereocilia are fewer in number in *Bmp4*^{+/-} mice compared to wild type mice. Scale bar, 10 μ m.

Figure 4. Reduced neuronal processes in organ of Corti of *Bmp4*^{+/-} mice

Whole mount of the organ of Corti stained with antibodies to myosin VIIa (green) and neurofilament 200kD (red) and viewed by confocal microscopy. In the organ of Corti there is a reduction of neuronal processes in the *Bmp4*^{+/-} mice compared to wild type mice. (C) and (D) represent the boxed regions in (A) and (B). Scale bars: A, B 100 μm; C–D 5 μm. Boxed regions in (A) and (B) are located two-thirds into the first turn starting from the apex. In (E), hair cells (OHCs and IHCs) and neuronal processes between the outer and inner hair cells were quantified for 4 *Bmp4*^{+/-} mice with decreased neuronal processes and 4 wild type mice. P value was determined by two-tailed *t* test.

Figure 5. Neurogenin 1 expression is unaltered in *Bmp4*^{+/-} otocysts

Real time RT-PCR for Neurogenin 1 normalized to β -actin in E14.5 otocysts. Median expression levels indicated by bars. *Bmp4*^{+/-} n=8, wild type n=8.

Table 1Auditory brainstem response thresholds in wild type and *Bmp4*^{+/-} mice

A.				
Frequency		Mean (dB SPL)	SD	P value
6 kHz	Wild type (n=12)	31.8	8.04	0.0002
	<i>Bmp4</i> ^{+/-} (n=15)	55.6	17.2	
12 kHz	Wild type (n=12)	24.5	5.80	0.002
	<i>Bmp4</i> ^{+/-} (n=15)	45.2	19.3	
24 kHz	Wild type (n=12)	39.2	18.2	0.044
	<i>Bmp4</i> ^{+/-} (n=15)	58.5	26.9	
B.				
Age	Wild type	<i>Bmp4</i> ^{+/-} circlers	<i>Bmp4</i> ^{+/-} non-circlers	
4 wks	n=2	n=2	n=1	
6 wks	n=2	n=1		
7 wks		n=1		
8 wks	n=2	n=2		
8.5 wks			n=1	
9 wks	n=2		n=1	
9.5 wks		n=1		
10 wks	n=2	n=3		
12 wks	n=2	n=1	n=1	

Mean $\pm$ SD ABR thresholds for all wild type mice and all *Bmp4*^{+/-} mice in Figure 1. P values were determined by two-tailed *t* test.

Age and number of all wild type mice and all *Bmp4*^{+/-} mice in Figure 1.