Abnormal Mesenchymal Differentiation in the Superior Semicircular Canal of Brn4/Pou3f4 Knockout Mice

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Objective: To examine the developmental time course of the mutant phenotype and cellular mechanisms that result in malformations of the superior semicircular canal (SSCC) in Brn4 knockout mice. Mutations in the Brn4/Pou3f4 gene result in characteristic inner ear abnormalities in mutant mouse pedigrees, and the findings in these mice are similar to those in human X-linked deafness type III.

Design: Mutant and control mice were killed at various neonatal time points to assess the development of the SSCC. Measurements of SSCC diameter were made on paint-perfused specimens at postnatal day (P) 0, P7, P10, and P14. Histologic evaluation of the SSCC was made on hematoxylin-eosin–stained sections at P10.

Results: A dysmorphic constriction of the superior arc of the SSCC in Brn4 knockout mice was initially detectable at P14. Interestingly, the mutant SSCC is indistinguishable from control mice at earlier neonatal time points. In mutant neonates, there is persistence of immature woven bone with high cellularity surrounding the perilymphatic space of the SSCC. These findings are not present in control animal specimens, which demonstrate appropriate lamellar bony architecture.

Conclusions: In Brn4 knockout mice, constriction of the SSCC with narrowing of the bony labyrinth develops in the postnatal period at approximately P14. The persistence of immature bone in affected mice indicates that signaling abnormalities disrupt normal mesenchymal differentiation in the SSCC.


Normal inner ear development results from a complex series of morphogenetic changes that occur in response to interactions between epithelial and mesenchymal embryonic tissues. The molecular signaling events underlying normal inner ear development have yet to be fully elucidated. The recent development of genetic approaches provides the means to study the underlying pathogenesis of hereditary auditory dysfunction in animal models of human congenital malformations.

X-linked deafness type 3 results from mutations in the POU3F4 gene, a member of the POU domain family of transcription factors. POU domain genes play critical roles in the development of the central nervous system and the inner ear as well as other organ systems. Linkage analysis has localized the POU3F4 gene to the Xq13-21.1 region. The Brn4 gene (also called Pou3f4) is the murine ortholog of the human POU3F4 gene. This gene is expressed early in the development of the nervous system and is the only gene in the POU domain family expressed in the mesenchyme of the developing inner ear. The gene has been...
localized by immunocytochemical analysis to the mesenchymal cells of the developing otic capsule and plays a role in the regulation of normal mesenchymal remodeling, epithelial-mesenchymal interactions, and mesenchymal-mesenchymal interactions, which are vital for normal labyrinthine development.30,31

Mutations in Brn4 result in a number of characteristic behavioral abnormalities in mutant mouse pedigrees, including hearing loss, vertical head bobbing, changes in gait, and reduced whisker mobility.30,32 A number of studies have evaluated auditory function as well as middle and inner ear structure in Brn4 knockout mice. Evaluation of the auditory brainstem response in mutant mice has demonstrated severe hearing loss and evidence of cochlear dys-function.31 Abnormal fibrocyte structure within the spiral ligament of Brn4 knockout mice has been hypothesized to result in an abnormal endolymphatic potential, which may explain these auditory brainstem response results. Knockout alleles of the Brn4 gene have demonstrated multiple malformations of tissues derived from the otic mesenchyme, including hypoplasia of the temporal bone, enlarged internal auditory meatus, dysplastic fibrocytes, and malformed stapes.30,31 Other morphologic abnormalities identified in knockout mice have included cochlear hypoplasia, a reduction in the number of cochlear turns, and endolymphatic hydrops.

Vestibular abnormalities of Brn4 knockout mice give rise to the vertical head-bobbing phenotype detected in the mutant mice. The vertical nature of the aberrant head motion prompted evaluation of the superior semicircular canal (SSCC, which lies in the vertical plane) by perfusion studies by Phippard et al.30,32 Findings included a constriction of the bony labyrinth surrounding the canal with a reduction in the thickness of the bone in this area in adult mutant mice. This was not observed in the posterior semicircular canal and was only rarely observed in the horizontal canal.

The first objective of the present study was to delineate the timing of the development of the SSCC constriction by analyzing the canal diameter of Brn4 knockout and control mice at different postnatal time points using paint perfusion analyses. The second goal was to investigate the histologic findings within the mesenchymal compartment of mutant and control animal SSCCs at a time point preceding the constriction. We postulated that the vestibular abnormalities observed in Brn4 knockout mice were the result of aberrant mesenchymal differentiation in the developing SSCC.

METHODS

Mouse pedigrees containing a null mutation of the Brn4 gene have been described.30 CD1 and wild-type littermate mice served as controls. Normal and Brn4 knockout mice were killed at several defined postnatal time points to assess the development of the SSCC.

PAINT PERFUSION ANALYSIS

In preparation for paint perfusion analyses, Brn4 knockout and control mice were anesthetized and killed by cervical dislocation. Temporal bone preparations were made on postnatal day (P) 0 (9 controls, 8 mutants), P7 (9 controls, 12 mutants), P10 (5 controls, 20 mutants), and P14 (14 controls, 10 mutants). These whole-mount preparations were obtained by bisecting the head in the sagittal plane, dissecting out the temporal bone, and placing the specimens in Bodian fixative (7% ethanol, 15% water, 5% formalin, and 5% glacial acetic acid) for 24 to 48 hours. Preparations were then dehydrated through a graded series of ethanol and cleared in methyl salicylate. Inner ears were perfused through the oval window with a suspension of 1% gloss paint in methyl salicylate using a 33 gauge standard Hamilton needle. These paint fills visualized the bony labyrinth within the temporal bones of these mice.

HISTOLOGIC ANALYSIS

Anesthetized mutant and control animals (6 of each) were fixed by cardiac perfusion using 4% paraformaldehyde in phosphate-buffered saline on P10. Preparations were obtained by bisecting the head in the sagittal plane, dissecting out the temporal bone, and placing the specimens in 4% paraformaldehyde for overnight immersion fixation.

Specimens were washed 3 times using phosphate-buffered saline and decalcified in 0.1M EDTA for 2 to 3 days. After decalcification, temporal bone specimens were serially dehydrated in alcohol, embedded in paraffin, and sectioned (8 μm) in the coronal plane (cross section through the SSCC) in preparation for staining with hematoxylin-eosin.

DATA ANALYSIS

Measurements of SSCC diameter were made on paint-perfused specimens and reported in micrometers as mean ± standard error of the mean (SEM). On sections stained with hematoxylin-eosin, the narrowest point of the SSCC perilymphatic space was identified for each sample. To account for the natural variability that may exist between specimens, a fixed point anterior to the common crus that could be consistently located was identified in all samples. Multiple cross-sectional area measurements were made at these points on the hematoxylin-eosin–stained sections by tracing and analysis using the National Institutes of Health Image J program (version 1.32; available at http://rsb.info.nih.gov/ij/). The mean cross-sectional area of the narrowest and fixed points was reported in square micrometers ± SEM.

Statistical analysis was performed using a 2-tailed t test with P < .01 used for statistical significance. All animal protocols used during this research were approved by the institutional animal care and use committee at The Children's Hospital of Philadelphia in accordance with regulations established by the National Institutes of Health.

RESULTS

SSCC DIAMETER

Although there was a trend of decreasing SSCC diameter in Brn4 knockout mice with increasing developmental age, no statistically significant difference was found between mutant mice and controls at P0, P7, and P10. The mean ± SEM diameters of the SSCC in P0, P7, and P10 mutant mice were 194.8 ± 4.2 μm, 149.5 ± 10.3 μm, and 126.1 ± 8.3 μm, respectively. The mean diameters of the SSCC in P0, P7, and P10 control mice were 197.4 ± 4.4 μm, 168.2 ± 4.2 μm, and 143.0 ± 8.1 μm, respectively.
A discrete constriction of the superior arc of the SSCC was present in P14 Brn4 knockout mice but not controls. The mean±SEM diameter of the narrowest portion of the SSCC was significantly smaller in P14 mutant mice (78.3±10.1 µm) than in controls (171.4±4.1 µm) (P<.001) (Figure 1 and Figure 2).

SSCC PERILYMPHATIC CROSS-SECTIONAL AREA ANALYSIS

The mean±SEM cross-sectional area of the perilymphatic space at the narrowest point of the SSCC was significantly smaller in mutant mice (16.3 × 10^3±1.3 × 10^3 µm²) than in controls (21.8 × 10^3±0.8 × 10^3 µm²) (P<.01). The mean cross-sectional area of the perilymphatic space at the fixed point of the SSCC was not significantly different in mutant mice (56.2 × 10^3±6.5 × 10^3 µm²) and controls (53.3 × 10^3±12.9×10^3 µm²) (Figure 3).

MORPHOLOGIC ANALYSIS

Evaluation of hematoxylin-eosin–stained sections demonstrated a deficiency of mature lamellar bone formation surrounding the perilymphatic space in the SSCC of mutant mice. There was persistence of immature woven bone with high cellularity throughout the

Figure 1. Paint perfusion analyses demonstrate a trend of decreasing superior semicircular canal (SSCC) diameter from postnatal day (P) 0 through P14 in Brn4 knockout mice. Results at P14 demonstrate a statistically significant difference in the diameter of the SSCC in mutant mice compared with controls. Error bars indicate standard error of the mean; asterisk indicates P<.001.

Figure 2. Medial view of paint-perfused superior semicircular canals (SSCCs) of postnatal day (P) 0 mice (A and B) and P14 mice (C and D). Control mice are shown in panels A and C; the Brn4 knockout mice in panels B and D. The arrow in panel B indicates the normal superior arc of the SSCC in a mutant mouse at P0. The arrow in panel D indicates the position of the SSCC constriction at P14 in a mutant mouse. Note the absence of this narrowing in the mutant mouse at P0 and in the controls. The scale bar represents 500 µm.
SSCC. These findings were not present in control animal specimens, which demonstrated appropriate lamellar bony architecture surrounding the perilymphatic space (Figure 4).

The findings in this study demonstrate that the Brn4/Pou3f4 gene inactivation results in aberrant differentiation of the bone surrounding the SSCC and a characteristic constriction in the superior arc of this canal. Our first goal was to determine the time course of the SSCC constriction. We observed a trend of decreasing SSCC diameter from P0 through P14, which was significantly different from the control group at P14. Although there was no statistical difference in the canal diameter at P10, cross-sectional area measurements of the perilymphatic space on histologic sections were significantly smaller in Brn4 knockout animals than in controls. These findings suggest that the observed narrowing of the SSCC may be due to deficiencies in mesenchymal remodeling that occur postnatally.

Once the developmental time course of the SSCC constriction was delineated, we evaluated the morphologic changes in the mutant SSCC at a time point immediately preceding the constriction. In control animal specimens, there was appropriate lamellar bony architecture surrounding the perilymphatic space. Histologic evaluation of Brn4 knockout mice demonstrated a deficiency of lamellar bone formation. Instead there was persistence of immature woven bone with high cellularity found throughout the SSCC.

Our observations are consistent with other temporal bone defects seen in Brn4 knockout mice that are associated with osteogenic abnormalities. Phippard et al demonstrated that adult mutant mice have thinning of the
stapes footplate, an enlarged internal auditory canal, and a reduction in the thickness of bone surrounding the SSCC. Fibrocytes within the spiral ligament of mutant mice were found to be dysplastic with a reduced number of cytoplasmic extensions and mitochondria.\textsuperscript{8,10,11} This finding was consistently present throughout all turns of the cochlea and suggests that Brn4 is essential for normal fibrocyte differentiation. Because Brn4 is expressed exclusively within the mesenchymal tissues of the developing inner ear, we hypothesize that its gene product plays a critical role in mesenchymal cell differentiation. Mutation of the Brn4 gene may alter osteoprogenitor cell differentiation, which may explain the abnormal bone structure seen in mutant mice.

In conclusion, we have demonstrated that a constriction of the SSCC in Brn4 knockout mice results from narrowing of the perilymphatic space. The persistence of immature bone in the SSCC in mutant mice suggests that there are signaling abnormalities preventing normal differentiation of the canal. These findings may help us further understand the pathologic mechanisms underlying X-linked deafness type 3 in humans.

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