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Title

Increased susceptibility to acoustic trauma in a mouse model of non-syndromic sensorineural deafness, DFNB91.

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Abstract

Inactivating mutations of *SERPINB6* in humans result in progressive hearing loss starting in early adulthood (DFNB91). We have previously shown that C57BL/6J mice lacking the orthologous gene, *Serpinb6a*, exhibit progressive hearing loss, which is associated with progressive loss of distinct cell types in the organ of Corti beginning with outer hair cells. However, deafness in these animals occurs much earlier than expected, possibly because C57BL/6J mice also carry an age-related hearing loss mutation in the cadherin 23 gene (*Cdh23*^{ahl}) that causes late onset hearing loss. The CBA/CaH strain of mice does not carry *Cdh23*$^{ah/ahl}$ and may represent a better model of the human DFNB91 patients. Here we show that transfer of the mutant *Serpinb6a* allele onto the *Cdh23* normal CBA/CaH background markedly delays onset of hearing loss, more closely phenocopying DFNB91, without altering the pattern of cellular loss. Young, pre-symptomatic mice of this genotype exposed to acoustic trauma exhibit permanent hearing loss, compared to controls, associated with the disappearance of outer hair cells. We conclude that Serpinb6 helps to maintain hearing by protecting hair cells from stress.

Introduction

Acquired hearing loss results from defects in any of the specialised cell types of the inner ear, and may be caused either by genetic or environmental factors such as ageing, infections, ototoxic drugs or traumatic noise. Noise-induced hearing loss (NIHL) comprises both a temporary and a permanent component. Temporary hearing loss recovers in hours or days

and is thought to be due to breakage and subsequent repair of tip links between stereocilia (Zhao *et al.*, 1996). However, repeated exposure to sound intensities that generate temporary hearing loss (Wang & Ren, 2012), or exposure to very high intensity noise (Wang & Ren, 2012), can lead to permanent elevation of hearing thresholds. Permanent hearing loss results from death of hair cells, which do not regenerate spontaneously in mammals (Cotanche & Kaiser, 2010).

Given the inaccessibility of the inner ear, rodent models are extremely useful for understanding the molecular pathology of human deafness, although different strains of mice have different susceptibility to hearing disorders. For example, CBA/CaJ mice retain effective hearing throughout life, whereas C57BL/6J mice exhibit age-related hearing loss due to tip link malfunction (Zheng *et al.*, 1999). C57BL/6J mice also exhibit increased susceptibility to noise-induced hearing loss (Harding *et al.*, 2005).

Rodent models are also used to define pathological pathways of human genetic hearing loss. For example, the autosomal recessive, non-syndromic hearing loss gene, DFNB91, was first identified in a consanguineous family and mapped to the gene encoding protease inhibitor SERPINB6 (Sirmaci *et al.*, 2010), before being identified in other individuals with hearing loss (Kim *et al.*, 2015). Audiological assessments and self-reported histories indicate that the mutation results in progressive hearing loss beginning in adolescence, with impairment evident first at the higher frequencies before spreading to lower frequencies. Although SERPINB6 was demonstrated in the organ of Corti, no cellular or molecular pathological mechanism for DFNB91-associated hearing loss was identified.

We have produced a mouse model of human DFNB91, by replacing the orthologous mouse gene (*Serpinb6a*) with a green fluorescent protein (GFP) gene on the C57BL/6J background (Scarff *et al.*, 2003). By following GFP we showed that Serpinb6 is expressed in the neuroepithelium, lateral wall and spiral limbus, with highest levels in hair cells. *Serpinb6a* null mice develop normally, however they exhibit severe sensorineural hearing loss across all frequencies (4-32 kHz) by 6 weeks of age, and are almost completely deaf by 10 weeks (Tan *et al.*, 2013). The cochlear pathology begins with loss of outer hair cells, followed by specific fibrocyte subpopulations. This progression is much more aggressive than that observed in the DFNB91 patients, who retain some hearing ability, at least at lower frequencies, well into middle age (Sirmaci *et al.*, 2010; Kim *et al.*, 2015).

C57BL/6J mice carry an age-related hearing loss (ahl) mutation in the cadherin 23 gene (*Cdh23*) that causes progressive, age-related hearing loss (Zheng *et al.*, 1999), and $Cdh23^{ahl/ahl}$ is known to interact with other hearing loss gene mutations to increase their

severity. Given the co-localization of CDH23 and Serpinb6 in hair cells, we therefore propose that *Cdh23*$^{ahl/ahl}$ exacerbates the effects of *Serpinb6a* deletion in C57BL/6J mice. Here we describe the effects of *Serpinb6* mutation on CBA/CaH mice in which *Cdh23* is normal. These new mice display a phenotype that closely matches the human DFNB91 patients. In keeping with the idea that Serpinb6 is a protective factor, pre-symptomatic *Serpinb6a* null mice display increased sensitivity to NIHL.

Materials & Methods

Mice

The production and maintenance of *Serpinb6a*$^{tm1.1Pib/tm1.1Pib}$ (*Serpinb6a*$^{-/-}$) mice was described previously (Scarff *et al.*, 2003). Wild type CBA/CaH mice for backcrossing were purchased from the Animal Resource Centre (Canning Vale, Western Australia). Heterozygous mice were backcrossed for 10 generations and the F10 generation was then in-crossed to produce wild type (WT) and *Serpinb6a*$^{-/-}$ (KO) mice for analysis. WT and KO mice were maintained by homozygous breeding and all experimental cohorts were within 4 generations of the F10 in-cross.

The animal experiments were carried out according to all relevant Australian laws as overseen by the Monash University Animal Ethics Committee (application IDs: MARP/2012/018, MARP/2014/034, MARP/2017/001 and MARP/2017/166).

Testing of auditory function

Mice were anaesthetised with an intra-peritoneal injection of ketamine (75 mg/kg body weight, Parnell Laboratories, Australia) and xylazil (7.5 mg/kg body weight, Troy Laboratories, Australia). Body temperature was maintained at 37ºC using a deltaphase isothermal pad (Braintree Scientific). Electrodes were inserted sub-cutaneously at the snout (reference), behind the measured ear (active) and on the abdominal flank close to the thigh (ground). Computer-generated pure tones at 4, 8, 16 and 32 kHz were channelled to a loudspeaker positioned 10 cm from the measured ear. Tone stimuli lasted 5 ms with a linear rise-fall period of 0.5 ms, presented at a rate of 30 per second. Mice recovered from anaesthetic in a heated recovery box, before being returned to their original housing.

The response for each stimulus was averaged across 500 repetitions rejecting artefacts of 20 μV and higher, with a sampling rate of 20,000 per second. Responses recorded in the electrodes were amplified 10^5 fold and bandpass filtered between 150 Hz (24 dB per octave) – 3 kHz (6 dB per octave). We began recording at 90 dB SPL intensity, decreasing the

stimulus by 20 dB SPL initially to obtain a visual estimation of threshold. Approaching threshold, stimulus intensity was varied in steps of 5 dB SPL to more accurately arrive at the hearing threshold. We repeated responses at or 5 dB above or below threshold for consistency.

Responses were plotted using Igor Pro 6.0 software. For each frequency, the same investigator traced the decline in Wave II with decreasing stimulus intensity to reach the threshold. A threshold was determined when the amplitude of Wave II exceeded 0.25 μV. Wave II (elicited from the cochlear nucleus) was selected for threshold measurements because it gave the most robust response in our system. While Wave I (elicited from the auditory nerve) was observed, it could not be tracked reliably down to threshold. This limited interpretation of the results to inference relating to auditory brainstem sensitivity, and precluded specific comment on auditory nerve function.

Acoustic Trauma

We used a sine-wave signal generator (BWD, Australia, Model 141) and a custom-built amplifier to generate a 10 kHz pure tone. Mice were anaesthetised as described above and placed inside a reverberant chamber lined with an isothermal heat pad to maintain their temperature at 37 ºC. Acoustic stimuli were calibrated at the pinnae of the mice as described (Tan *et al.*, 2010). For acoustic trauma, mice were exposed to a 110 dB SPL pure tone for 1 h on 3 consecutive days. When examining temporal changes of Serpinb6, mice were exposed to a single dose of 130 dB SPL pure tone for 1 h and then left to recover for 1, 3 or 7 days.

Experimental cohorts

In the ageing study, auditory brainstem responses (ABRs) were measured for age-matched cohorts of female mice at 6, 10, 20 and 50 weeks of age. WT cohorts were 8 mice (16 ears) at 6 and 10 weeks, 6 mice (12 ears) at 20 weeks, and 5 mice (10 ears) at 50 weeks. KO cohorts were of 11 mice (22 ears) at 6 weeks, 10 mice (20 ears) at 10 weeks, 8 mice (16 ears) at 20 weeks, and 7 mice (14 ears) at 50 weeks.

The acoustic trauma study used mixed gender cohorts of 11 WT or 10 KO mice (22 and 20 ears, respectively). Hearing was tested less than 5 days before the animals were exposed to acoustic trauma. ABRs were then repeated at 3 days and 2 weeks after the last acoustic trauma. A separate mixed gender cohort of 6 KO mice (12 ears) was used as control and their hearing was tested at the beginning and end of the experimental period, without exposure to acoustic trauma.

Tissue preparation and histological analysis

Mice were humanely killed with a lethal injection of intra-peritoneal sodium pentobarbiturate followed by trans-cardiac perfusion with phosphate-buffered saline (PBS) and the inner ears were rapidly removed. Both middle ear ossicles and membranes covering the round window were dissected away and cochleae were fixed for 2 h for immunohistochemistry or overnight for stain-based histology with 4% paraformaldehyde dissolved in PBS. Tissues were then decalcified in 10% ethylenediaminetetraacetic acid in PBS. Once decalcified, they were incubated overnight in 30% sucrose dissolved in PBS before embedding along the modiolar axis in OCT compound (Sakura, Tokyo, Japan). 10 μm sections cut along the mid-modiolar plane were mounted on SuperFrost Plus microscopic slides (Menzel, Germany) before staining with haematoxylin and eosin (H&E) or 0.1% thionin in 0.25% acetic acid.

H&E or thionin stained sections were examined with a Zeiss Axioplan 2 microscope. The middle turn (representing the region of approximately 8-22 kHz) was used for analysis because of the following advantages: clearer boundaries of the Rosenthal's canal, better morphology of the organ of Corti and broader lateral wall which facilitates recognition of the different fibrocytes. A minimum of 6 representative sections, separated by a distance of at least 40 μm, from 1 cochlea per animal were used for cell counts. Identification and quantification of cell types was as previously described (Tan *et al.*, 2013). Counts were obtained from 6 WT and 4 KO cochleae at 6 weeks of age, 8 WT and 9 KO cochleae at 20 weeks of age, and 7 WT and 9 KO cochleae at 60 weeks of age

Immunohistochemistry to detect Serpinb6 was performed in frozen sections as previously described (Tan *et al.*, 2013).

Statistics

Statistical analyses were carried out using IBM SPSS Statistics version 26. Analysis of ageing and noise-induced hearing loss data utilised a mixed linear model, where animal was the grouping factor and frequency was nested within ear (each ear was considered a repeated measure), all repeated over time. The model assumed a compound symmetry covariance matrix, i.e. equal variance and covariance (correlation between variables) over time. The model did not improve by assuming diagonal covariance, which assumes difference variance at each time but constant covariance. The significance threshold for pairwise comparisons between genotypes within each age and frequency was 0.05.

Histological counts were analysed in GraphPad Prism version 8.4.3 for OSX as pairwise comparisons (WT vs KO) at each time point using Student's t-test. These p-values were then

adjusted for multiple comparisons using the Holm-Sidak method. Data in the Results are expressed as mean ± standard deviation. Parameters plotted in the figures are mean and 95% confidence intervals.

Results

Removal of *Cdh23*$^{ahl/ahl}$ delays progressive hearing loss in *Serpinb6a* null mice

To remove the potentially confounding genetic interaction between *Cdh23*$^{ahl/ahl}$ and *Serpinb6a*$^{-/-}$ in C57BL/6J mice we transferred the *Serpinb6a* null allele onto the CBA/CaH genetic background through 10 rounds of backcrossing. We measured the hearing thresholds of these CBA/CaH *Serpinb6a*$^{-/-}$ (KO) mice in response to pure tones at 4, 8, 16 and 32 kHz. An example is illustrated in Figure 1.

The removal of *Cdh23*$^{ahl/ahl}$ through the backcrossing resulted in a marked improvement in the hearing thresholds of KO mice and significantly delayed their hearing loss. At 6 weeks of age, KO mice retained good ABR thresholds from 4-16 kHz that were indistinguishable from those of wild type (WT) mice. They displayed moderate hearing loss at 32 kHz, with thresholds increasing by 29 dB (WT = 43 ± 13 dB, KO = 72 ± 13 dB) (Figure 2). By contrast, C57BL/6J *Serpinb6a*$^{-/-}$ mice displayed severe hearing loss at 6 weeks of age with thresholds ≥ 90 dB across all 4 frequencies (Tan *et al.*, 2013).

The mixed linear model revealed a significant effect of genotype ($F = 39.283$, df(1,14.711), $p < 0.001$) and age ($F = 24.786$, df(2,273.483), $p < 0.001$), as well as a significant interaction between these ($F = 11.660$, df(2,273.483), $p < 0.001$), indicating that thresholds deteriorated less over time in the wild-type animals. At 20 weeks of age, we observed a decline in hearing in KO mice extending to 16 kHz, where they were 32 dB higher than those of WT mice. Hearing at 32 kHz continued to decline by 10 dB in KO mice (82 ± 9 dB) (Figure 2). At 50 weeks of age, hearing was significantly worse at all frequencies. Thresholds in KO mice were elevated by 35 dB at 4 kHz and 32 dB at 8 kHz compared to WT mice, while thresholds continued to deteriorate at 16 kHz (48 dB) and 32 kHz (37 dB) (Figure 2). Our data indicate that the absence of *Cdh23*$^{ahl/ahl}$ in CBA/CaH *Serpinb6a*$^{-/-}$ mice delays hearing loss, or conversely, mutation of *Cdh23* accelerates hearing loss in individuals lacking Serpinb6. Relative to human lifespan, the onset and gradual decline in hearing ability in these mice closely resembles the human DFNB91 patients.

As anticipated, the animals with the poorer thresholds had lower amplitude responses to high intensity acoustic simulation, as evidenced by the ABR's Wave II amplitudes in response to

90 dB SPL (Figure 3). At 6 weeks of age, there were no differences in the Wave II amplitude between WT and KO mice at the best hearing frequency region of 8-16 kHz (Figure 3), however there was a significant reduction in KO mice at 4 kHz and 32 kHz. The ABR Wave II amplitudes of KO mice steadily declined as the mice aged, with significant decreases at 8 and 16 kHz appearing from 20 weeks of age onwards (Figure 3). Wave II was elicited at the expected response latency (Figure 3), and was analysed in more detail at 8 and 16 kHz (Figure 3), within the best frequency range of the mouse. At 6 and 20 weeks of age, there were no significant differences in the Wave II latency. However, in older mice, the latency of Wave II was slightly longer in KO mice at 16 kHz (WT: 2.00 ± 0.05 ms, KO: 2.29 ± 0.04 ms).

OHC are most susceptible to lack of Serpinb6

We next investigated the structural changes within the cochlea that result from the loss of Serpinb6a in mice. Cochleae were collected from 4-9 mice of each genotype at 6, 20 and 60 weeks of age, then stained in order to enumerate various cell types, focussing on the middle turn of the cochlea corresponding to the region of approximately 8-22 kHz. The tunnel of Corti was well preserved in KO mice up to 20 weeks (Figure 4). In older mice, the most striking features observed were the collapse of the organ of Corti and loss of type IV fibrocytes (Figure 4). Consistent with the hearing tests, these defects developed progressively between 6 and 20 weeks and were more severe by 60 weeks (Figure 4). No change could be seen in type I or type II fibrocytes at a histological level (Figure 5).

To identify more subtle changes and to pinpoint specific cell types affected, we performed a quantitative assessment of hair cells and type I-IV fibrocytes in age-matched wild type and KO mice. We began by focussing on the organ of Corti, which contains the hair cells required to detect the sound stimulus. The earliest and greatest effect was seen in the outer hair cells (OHC) (Figure 6). Sections from wild type mice contained the normal complement of 3 OHC per section across all time points. OHC loss in sections from KO mice was apparent by 6 weeks (1.8 ± 0.5) and increased by 20 weeks (1.2 ± 0.4). OHC had almost completely disappeared by 60 weeks (0.4 ± 0.2). By contrast, inner hair cells (IHC) were lost later. There was a normal complement of a single IHC at 6 weeks, and evidence of some loss by 20 weeks (0.8 ± 0.1). Most IHC were lost by 60 weeks (0.4 ± 0.2) (Figure 6).

The endocochlear potential depends on high concentrations of K^+ in endolymph. The ionic composition of endolymph is critical to the correct functioning of the hair cells, is maintained by K^+ recycling carried out by different populations of fibrocytes within the spiral ligament.

These fibrocytes also express Serpinb6 (Tan *et al.*, 2013). As such, we focussed next on the effects of *Serpinb6a* deletion on fibrocyte populations. We found that type IV fibrocytes were the first to be affected, with 70% lost between 6 and 20 weeks of age (Figure 6). Other fibrocyte populations were not significantly affected (Figure 6).

Taken together, both hearing measurements and histological data support the hypothesis that the CBA/CaH strain is more suitable than C57BL/6J for analysing the role of Serpinb6 in hearing because the *Cdh23*$^{ahl/ahl}$ mutation in the C57BL/6J strain exacerbates the onset of hearing loss in *Serpinb6a*$^{-/-}$ mice.

Serpinb6$^{-/-}$ mice are more susceptible to acoustic trauma

Serpinb6 is a member of the intracellular, clade B serpins that function as protective factors under conditions of cellular stress. Because OHC in the organ of Corti express Serpinb6 and we have shown that they are the most susceptible to loss of Serpinb6, a likely stressor is acoustic trauma. The good residual hearing at 20 weeks of age in our CBA/CaH *Serpinb6*$^{-/-}$ mice from 4 to 16 kHz (4 kHz: 44 ± 5 dB SPL; 8 kHz: 28 ± 5 dB SPL; 16 kHz: 16 ± 5 dB SPL) allows us to measure acoustic trauma producing a threshold shift up to 40 dB. To test this, we exposed cohorts of 10 -11 WT or KO mice to an acoustic trauma (AT) protocol of 1 h at 110 dB SPL (10 kHz) for 3 consecutive days. Hearing tests were performed a week before AT, as well as 3 days and 2 weeks after the last acoustic exposure. These times correlate with temporary and permanent changes to auditory sensitivity after an AT event. ABR thresholds prior to acoustic exposure showed that the KO mice were already exhibiting increased thresholds of 22 dB SPL at 32 kHz (Figure 7), and therefore threshold shifts were not analysed at this frequency after exposure.

The AT produced temporary threshold shifts in WT mice of 9 dB at 4 kHz, and 18 dB at 16 kHz 3 days after exposure. KO mice experienced similar threshold shifts of 14 dB at 4 kHz and 23 dB at 16 kHz (Figure 7). Additionally, the KO mice experienced hearing loss of 18 dB at 8 kHz. At 2 weeks post-AT, threshold changes in WT mice had essentially subsided to pre-AT values. By contrast, the hearing of KO mice was damaged by AT, with sustained threshold shifts relative to WT mice of 16 dB at 4 kHz, 9 dB at 8 kHz and 28 dB at 16 kHz (Figure 7).

To exclude the possibility that threshold changes in AT-exposed CBA/CaH *Serpinb6*$^{-/-}$ mice could be attributed partially to age-related hearing loss, we measured threshold changes in a separate cohort of control KO mice that were not exposed to acoustic trauma. Within the experimental period of 3 weeks, there were no significant changes in thresholds. This

confirmed that acoustic trauma was the cause of the threshold elevation in AT-exposed KO mice (Figure 7).

When we examined the organ of Corti in AT-exposed WT and KO mice at the end of the experiment, we did not notice any significant difference in the IHC count but found fewer OHC in AT-exposed KO mice (Figure 8). After acoustic trauma, the mean number of OHC in WT mice was 2.9 ± 0.1 whereas KO mice had only 0.8 ± 0.4 OHC, a 3-fold change ($p < 0.01$). By contrast, control KO mice showed a higher number of OHC (1.6 ± 0.4), which is significantly greater than that in AT-exposed KO mice ($p < 0.01$). These data demonstrate that KO mice are more vulnerable to acoustic trauma, highlighting a protective role of Serpinb6 in acoustic stress.

Serpinb6 is upregulated after acoustic trauma

To examine whether Serpinb6 expression in the inner ear changes after acoustic exposure, we exposed WT mice to a 10 kHz pure tone at 130 dB sound intensity for 1 h and used immunohistochemistry to qualitatively monitor expression levels at different time points up to 1 week. We focussed our examinations on the organ of Corti as this region expresses the highest levels of Serpinb6 (Tan *et al.*, 2013) and is the most susceptible to NIHL. In 4 separate experiments, Serpinb6 protein consistently increased in the sensory epithelium after acoustic trauma between 1 and 7 days post-exposure (Figure 9), although the amount of upregulation could not be quantitated. The stria vascularis also appeared to be oedematous at 1 day post-AT, consistent with the effects of acoustic trauma. By contrast, there were no comparable changes in the expression of Serpinb6 in mesenchymal stem cells localised in the marrow of the temporal bone. The immuno-labelling in the tectorial membrane is non-specific (Tan *et al.*, 2013). These qualitative findings support the histochemistry and electrophysiology evidence demonstrating an important role for Serpinb6 in protecting hearing from age-related stress or acoustic trauma.

Discussion

DFNB91 is an autosomal recessive, non-syndromic hearing loss first identified in 2010 in a consanguineous family (Sirmaci *et al.*, 2010), and since described in sporadic cases of progressive hearing loss (Kim *et al.*, 2015). The mutations in these patients comprise premature stop codons and splicing defects in *SERPINB6*, resulting in a non-functional protein. We have previously reported a mouse model of DFNB91, generated by inserting the enhanced GFP into the mouse *Serpinb6a* gene to create a knockout allele (Scarff *et al.*,

2003). C57BL/6J mice carrying this allele display progressive hearing loss beginning at high frequencies as early as 3 weeks old, eventually reaching complete deafness across all frequencies by 10 weeks of age (Tan *et al.*, 2013). However, these mice display much more severe hearing loss than is observed in human patients, where hearing loss is first apparent in adolescence and hearing is preserved, at lower frequencies, at least into middle age (Sirmaci *et al.*, 2010; Kim *et al.*, 2015).

Different strains of mice are known to exhibit age-related hearing loss (Zheng *et al.*, 1999). In particular, the C57BL/6J strain carries the *ahl* allele (Johnson *et al.*, 1997), a mutation in *Cdh23* (Noben-Trauth *et al.*, 2003) that results in age-related hearing loss. Thus the combination of $Cdh23^{ahl/ahl}$ and $Serpinb6^{-/-}$ alleles may be causing the accelerated hearing loss in C57BL/6J mice. Here we have shown that transferring the $Serpinb6a^{-/-}$ allele from C57BL/6J onto the CBA/CaH background, which does not carry $Cdh23^{ahl/ahl}$ (Zheng *et al.*, 1999) and maintains good hearing through its life (Spongr *et al.*, 1997), produces a much slower disease that effectively phenocopies human patients. The mice exhibit mild, high frequency hearing loss at 6 weeks that slowly increases in severity and affects lower frequencies. Importantly, most animals retain some hearing ability even at 1 year of age.

The pattern of cell loss in CBA/CaH $Serpinb6^{-/-}$ mice, reported here, matches that of the C57BL/6J mice reported earlier (Tan *et al.*, 2013). This is important, as it has previously been reported that the C57BL/6J and CBA/CaH strains exhibit different damage patterns after acoustic trauma, with CBA-related strains (CBA/N and CBA/CaJ) showing strial defects while C57BL/6J mice show damage predominantly within the organ of Corti (Ohlemiller & Gagnon, 2007; Park *et al.*, 2013). The fact that $Serpinb6^{-/-}$ mice display a primary injury to OHC, rather than the stria, on both CBA/CaH and C57BL/6J strains suggests that this is a direct result of the mutation, rather than an increased susceptibility to the strain-based injury patterns.

Beyond hair cells, type IV fibrocytes were also significantly lost as KO mice aged. Loss of type IV fibrocytes has previously been identified as a major contributor to hearing loss in ageing C57BL/6J mice. This loss was found to be more widespread than the loss of OHC, suggesting that the fibrocyte degeneration preceded OHC loss (Hequembourg & Liberman, 2001). Similarly, analysis of noise-damaged CBA/CaJ mouse cochleae indicates a more severe effect on type IV fibrocytes than IHC and OHC. The loss of type IV fibrocytes extends basally beyond the frequency range of the noise stimulus and occurs at stimulus levels that do not affect hair cells (Wang *et al.*, 2002).

In contrast to these previous observations of increased sensitivity of type IV fibrocytes, the primary lesion identified in mice lacking *Serpinb6* is the loss of OHC. The location and critical function of CDH23 (mutated in C57BL/6J mice) to hair cells may explain the observation that OHC are the first cell type to be affected by loss of Serpinb6. Death of hair cells in C57BL/6J mice is caused by the induction of intrinsic cell death pathways (Someya *et al.*, 2009). As a member of the clade B serpin family of protease inhibitors, the function of Serpinb6 is to protect the cell from damage by endogenous proteases during periods of cell stress (Bird, 1998).

The nature of the stress in hair cells is likely to be the ionic (particularly K^+) fluxes induced during sound detection. Sound triggers the deflection of stereocilia in hair cells, opening mechanically-gated transduction channels to allow influx of K^+ from the potassium-rich endolymph into the hair cells. The resulting depolarisation triggers the release of neurotransmitters from the base of these hair cells, initiating activity in the auditory nerve. To maintain ionic homeostasis, several ion channels in these hair cells remove the K^+ and channel it along the epithelial cell network into fibrocytes with the final goal of recycling potassium back into the endolymph (Spicer & Schulte, 1996; Wangemann, 2006; Zdebik *et al.*, 2009). Serpinb6 is expressed in both inner and outer hair cells of the organ of Corti, as well as fibrocytes that are involved in potassium recycling.

This explanation is supported by our observation that the KO mice are more susceptible to acoustic trauma than their WT littermates. A larger sound impulse places an increased stress on the hair cells. The lack of protective Serpinb6 lowers the threshold between temporary and permanent hearing loss and increases the susceptibility of these mice to permanent hearing loss. This suggests that the underlying pathology of the age-related hearing loss in KO mice and patients is accumulation of sub-acute acoustic traumas over time. Such links between age-related and sound-induced hearing loss have also been identified in strains of mice carrying the *Cdh23*$^{ahl/ahl}$ and *ahl2* alleles (Ohlemiller *et al.*, 2000).

Interestingly, the expression of Serpinb6 increases in hair cells and surrounding supporting cells after acoustic trauma. This up-regulation could be required to counteract target proteases inadvertently released when cochlear homeostasis is disrupted by acoustic trauma. Other clade B serpins, such as the closely related Serpinb9, also act as stress response genes under conditions where their target proteases are upregulated or released into the cytoplasm (Hirst *et al.*, 2003). A similar response has also been reported in the cochlea for osmotic stress protein 94 (Yamamoto *et al.*, 2009) and heat shock protein 70 in the utricle (Baker *et al.*, 2015).

Using CBA/CaH *Serpinb6*$^{-/-}$ mice as our novel model of DFNB91 deafness syndrome, this study has demonstrated a significant role of Serpinb6 in ameliorating NIHL. Although numerous deafness-associated genes have been identified in humans, very few have been causally linked to NIHL susceptibility (Clifford *et al.*, 2016). A decrease in levels of protective serpins due to haploinsufficiency has previously been identified as predisposing patients and animal models to disease in other settings such as angioedema (Han *et al.*, 2002; Jaradat *et al.*, 2016) and thrombosis (Picard *et al.*, 2006). The NCBI variation database lists 309 nonsynonymous substitutions in human *SERPINB6*, including 17 frame shifts and 14 nonsense mutations. The frequency of *SERPINB6* heterozygosity within the human population as a whole is therefore significantly higher than the frequency of homozygous and compound-heterozygous null patients that have been reported to date. These heterozygous individuals may represent a population at high risk for noise-induced hearing loss.

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Conflict of Interest Statement

The authors declare no competing interests.

Data Sharing

The data that support the findings of this study are available on request from the corresponding author.

Author Contributions

All authors designed research; JT and DK performed research; JT, DK and SJO analysed data; all authors wrote and edited the paper.

Abbreviations

ABR: auditory brainstem response

AT: acoustic trauma

dB: decibels

IHC: inner hair cell/s

NIHL: noise-induced hearing loss

OHC: outer hair cell/s

SPL: sound pressure level

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Figure Captions

Figure 1. The auditory brainstem response (ABR). A representative ABR recording at 16 kHz for a wild type CBA/CaH mouse at 6 weeks of age. Threshold is indicated with an arrow. The measurement at threshold was repeated and both traces are overlaid. Waves I, II and III are identified. The amplitude of Wave II was measured between the maximum positive peak and the preceding minimum negative peak (double headed arrow).

Figure 2. Age-related hearing loss in CBA/CaH *Serpinb6*$^{-/-}$ mice. Wildtype CBA/CaH (WT, grey triangles) or *Serpinb6*$^{-/-}$ (KO, white circles) mice were aged up to 1 year with hearing tests performed at 6, 20 or 50 weeks of age. Auditory brainstem responses to pure tones at 4, 8, 16 and 32 kHz were recorded in anaesthetised mice to determine hearing thresholds. Data are presented as scatter plots with mean and 95% confidence interval indicated. Significant ($p < 0.05$) pairwise comparisons are indicated with an asterisk.

Figure 3. Age-related loss of auditory sensitivity in CBA/CaH *Serpinb6*$^{-/-}$ mice. Wildtype CBA/CaH (WT, grey triangles) or *Serpinb6*$^{-/-}$ (KO, white circles) mice were aged up to 1 year with hearing tests performed at 6, 20 or 50 weeks of age. Auditory brainstem responses to pure tones at 4, 8, 16 and 32 kHz were recorded in anaesthetised mice and Wave II amplitudes were determined at 90 dB SPL (left). Wave II latency (right) was determined to 90 dB SPL stimulus at 8 and 16 kHz. Data are presented as scatter plots with mean and 95% confidence interval indicated. Significant ($p < 0.05$) pairwise comparisons are indicated with an asterisk.

Figure 4. Histological changes in ageing CBA/CaH *Serpinb6*$^{-/-}$ mice. Representative images from stained sections of cochleae extracted from wild type CBA/CaH (WT) or *Serpinb6*$^{-/-}$ (KO) at the indicated ages. Sections are focussed on the organ of Corti (A) or the type IV fibrocytes (B). Arrowheads indicate inner hair cells and arrows indicate outer hair cells. Scale bars represent 20 μm.

Figure 5. Type I and II fibrocytes are unaffected in ageing CBA/CaH *Serpinb6*$^{-/-}$ mice. Representative images from stained sections of cochleae extracted from wild type CBA/CaH

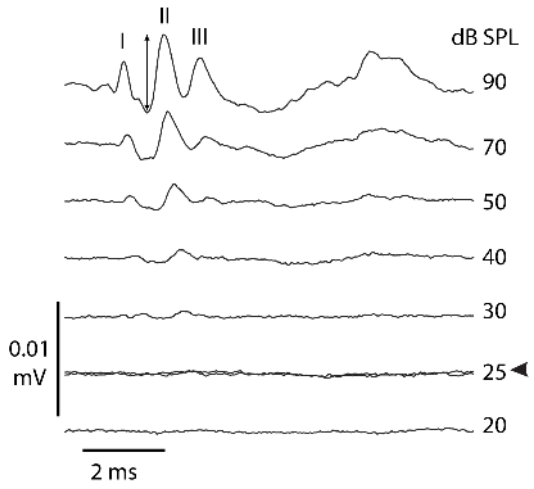
(WT) or *Serpinb6*$^{-/-}$ (KO) at the indicated ages. Sections are focussed on the type I (A) or type II fibrocytes (B). Scale bars represent 20 μm.

Figure 6. Age-related loss of cochlear cell types in CBA/CaH *Serpinb6*$^{-/-}$ mice. 4-9 cochleae were collected from wildtype CBA/CaH (WT, grey triangles) or *Serpinb6*$^{-/-}$ (KO, white circles) mice at 6, 20 or 50 weeks of age, processed for histology and specific cell types enumerated in 6-13 sections of each cochlea. Data are presented as scatter plots with mean and 95% confidence interval indicated. Statistical significance was determined by pairwise comparisons using Student's t-test and multiplicity adjusted p values were calculated for each cell type using the Holm-Sidak method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 7. CBA/CaH *Serpinb6*$^{-/-}$ mice are susceptible to hearing loss caused by acoustic trauma. 10 week old wildtype CBA/CaH (WT, grey triangles) or *Serpinb6*$^{-/-}$ (KO, white circles) mice were exposed to 1 h 110 dB SPL sound at 10 kHz for 3 consecutive days. An additional group of KO mice had hearing tests but no sound exposure (KO ctrl, black diamonds). Auditory brainstem responses to pure tones at 4, 8, 16 and 32 kHz were recorded in anaesthetised mice to determine hearing thresholds. Hearing tests were performed prior to acoustic trauma, 3 days after and 14 days after the final trauma. Data are presented as scatter plots with mean and 95% confidence interval indicated. Significant ($p < 0.05$) pairwise comparisons are indicated with an asterisk.

Figure 8. CBA/CaH *Serpinb6*$^{-/-}$ mice show greater loss of outer hair cells after acoustic trauma. 10 week old wildtype CBA/CaH (WT AT) or *Serpinb6*$^{-/-}$ (KO AT) mice were exposed to 1 h 110 dB SPL sound at 10 kHz for 3 consecutive days. An additional group of KO mice had hearing tests but no sound exposure (KO ctrl). 3-9 cochleae were collected from WT (grey triangles), KO (white circles) or KO ctrl (black diamonds) mice 2 weeks after the final sound exposure and processed for histology. (A-C) Representative images of one turn of the cochlea. Scale bars represent 50 μm. (D-F) Higher magnification view of the region in A-C indicated by the dashed box highlighting the organ of Corti. Arrowheads indicate inner hair cells and arrows indicate outer hair cells. Scale bars represent 20 μm. (G) Inner hair cells and (H) outer hair cells were enumerated in 6-13 sections of each cochlea.

Figure 9. Serpinb6 expression is up-regulated after acoustic trauma. Wildtype CBA/CaH mice were exposed to 1 h 130 dB SPL sound at 10 kHz and then humanely killed 1, 3 or 7 days later. Cochleae were collected and cryosections through the middle turn were stained for Serpinb6 using a polyclonal antiserum (red) and nuclei stained with DAPI (blue). (Left) Images of the organ of Corti indicate that Serpinb6 expression is higher in both the inner hair cells (arrow heads) and outer hair cells (arrows) 1 day after acoustic trauma, before returning to base line by 7 days. (Right) Expression of Serpinb6 in myeloid cells does not vary through the time course. BM, bone marrow; SV, stria vascularis. Scale bars represent 20 μm.



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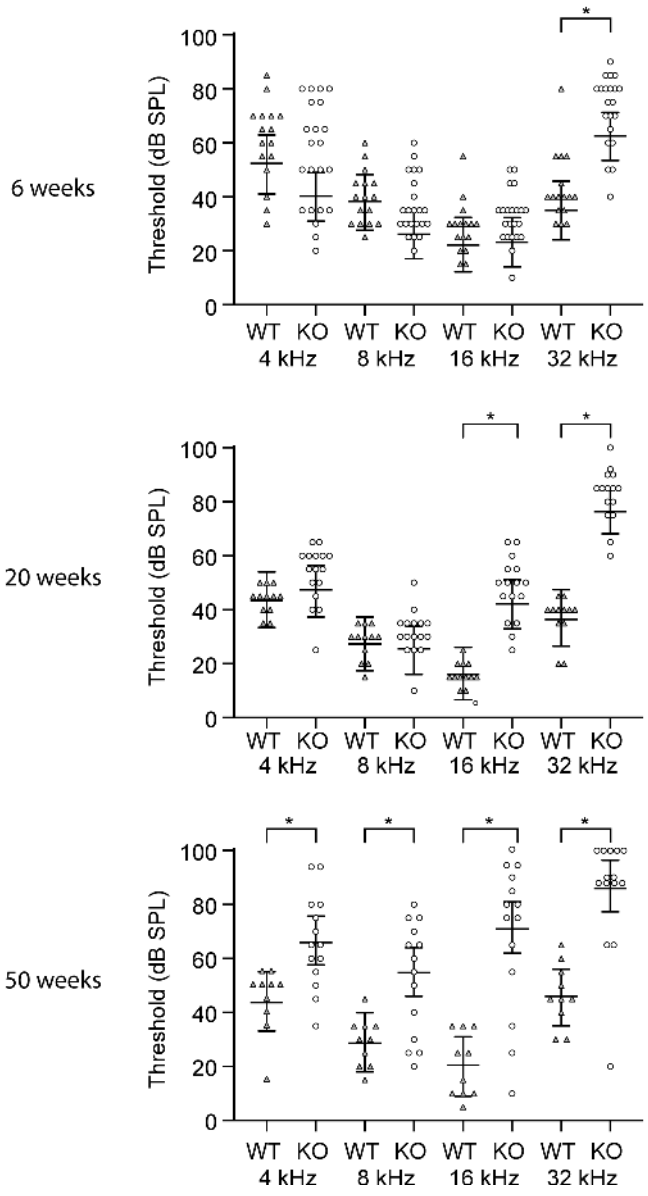

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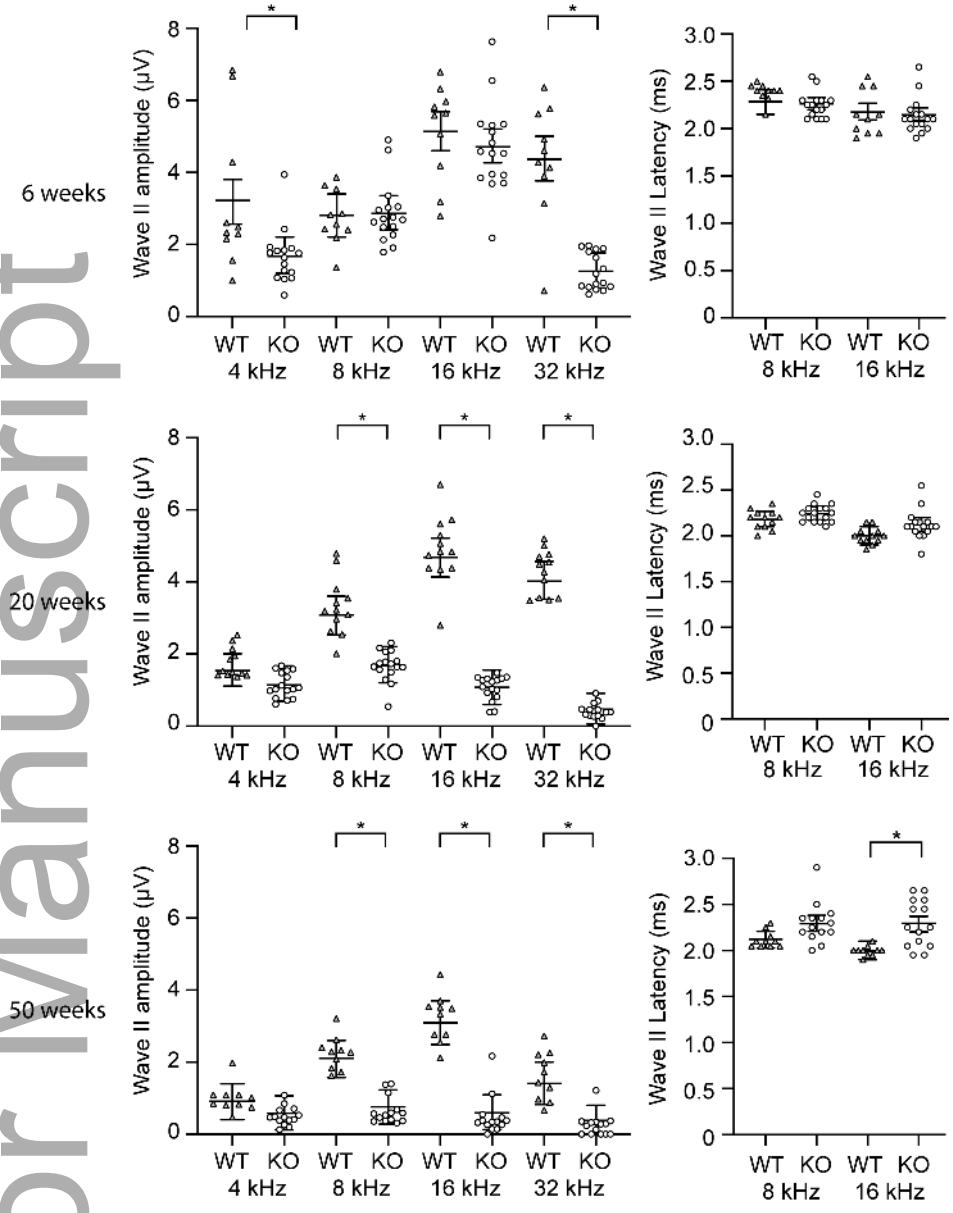

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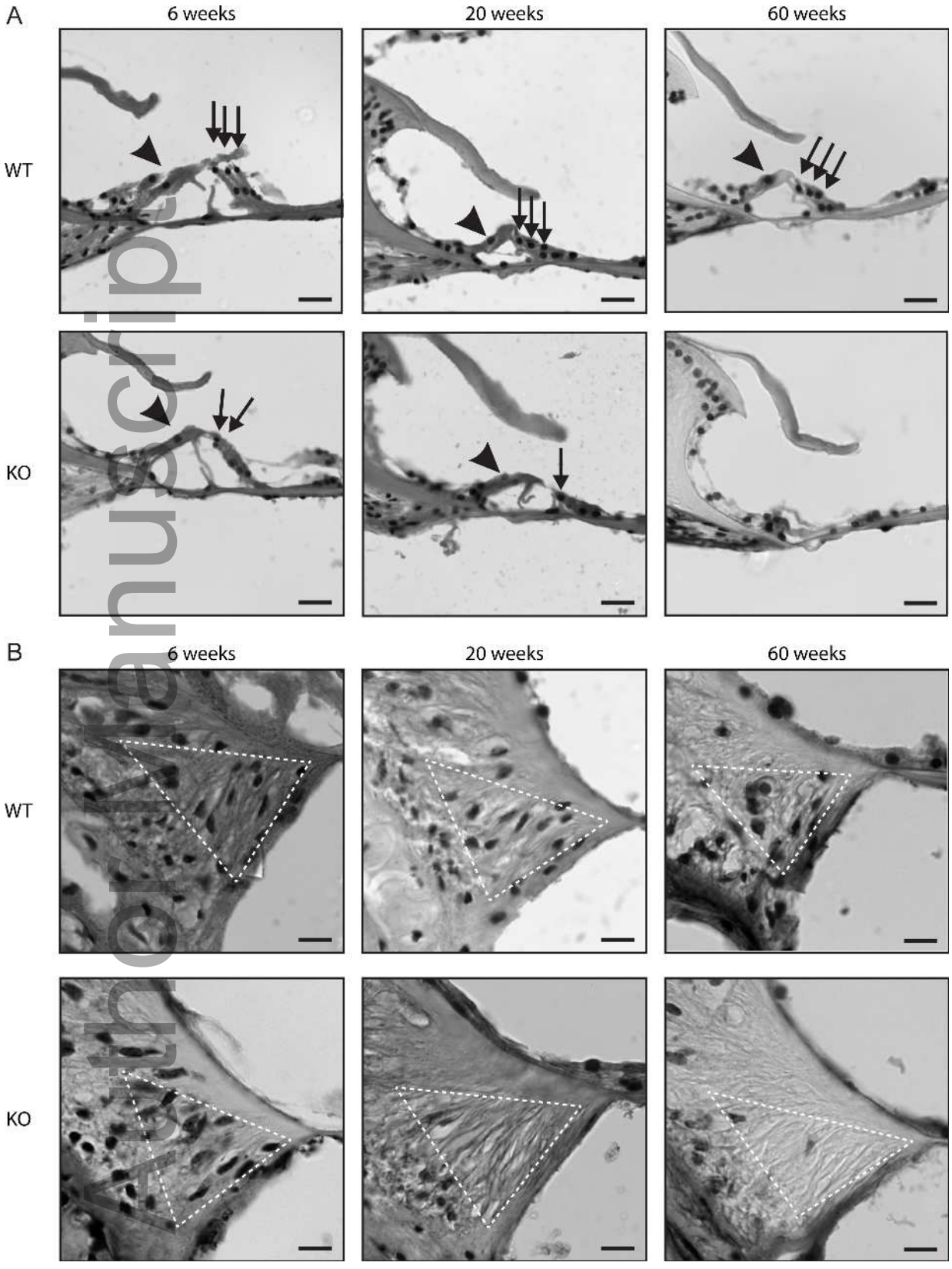

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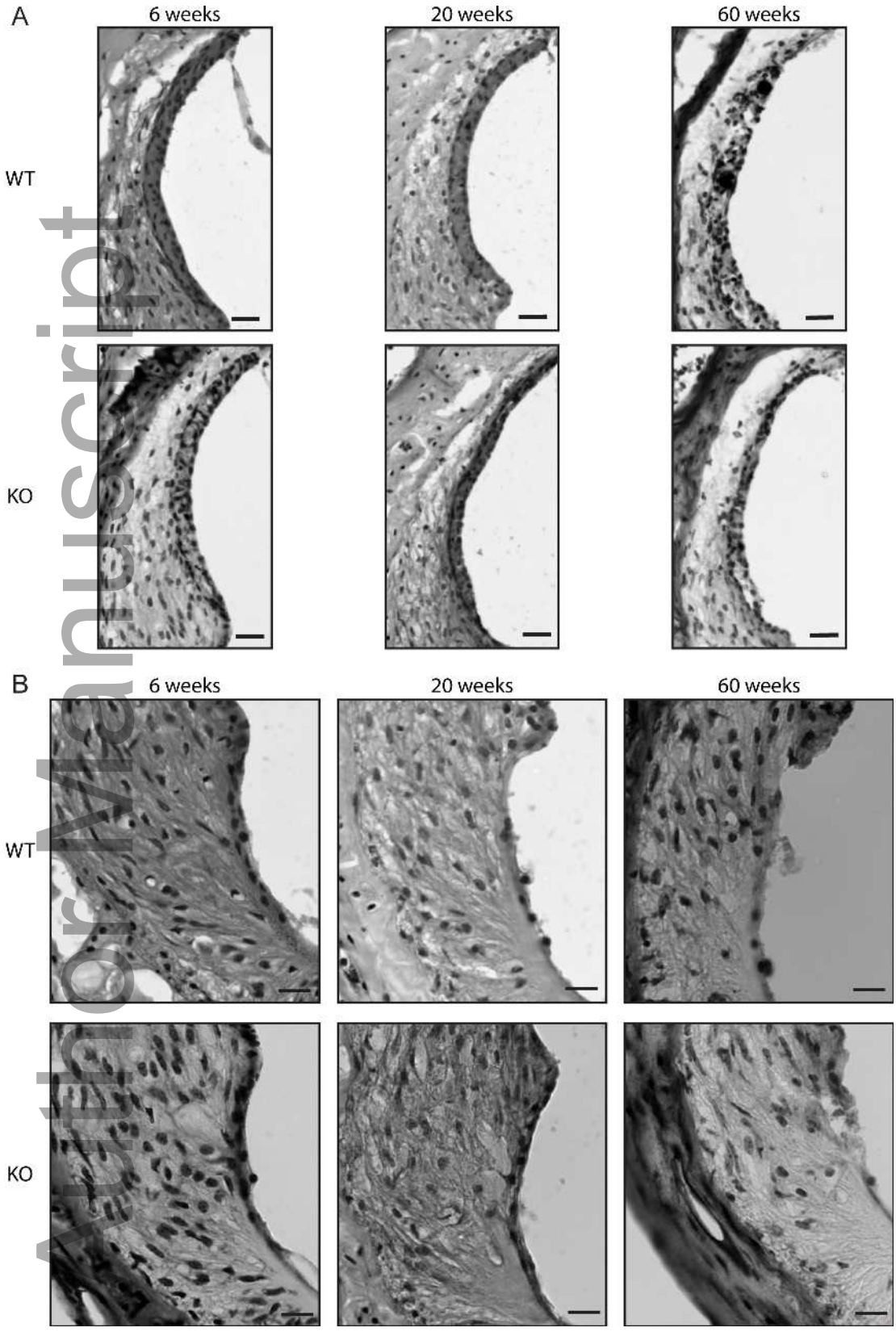

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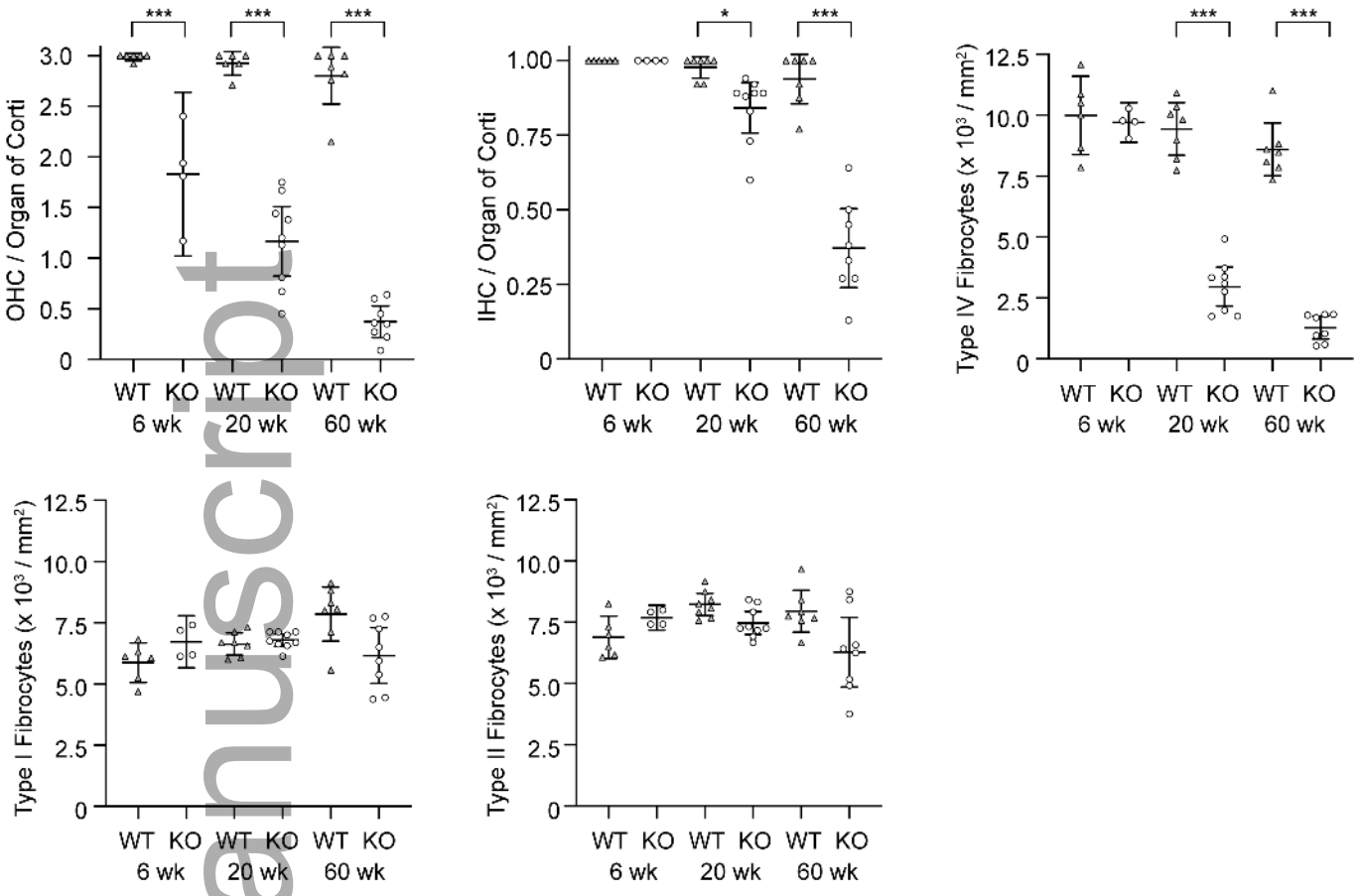

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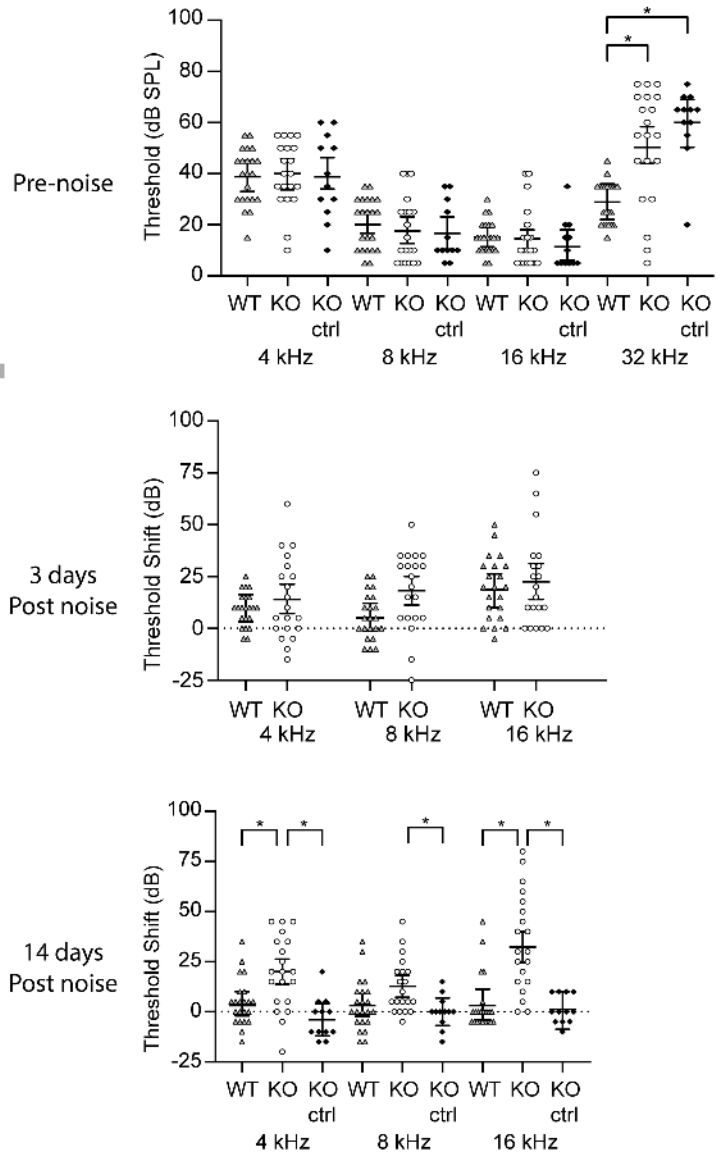

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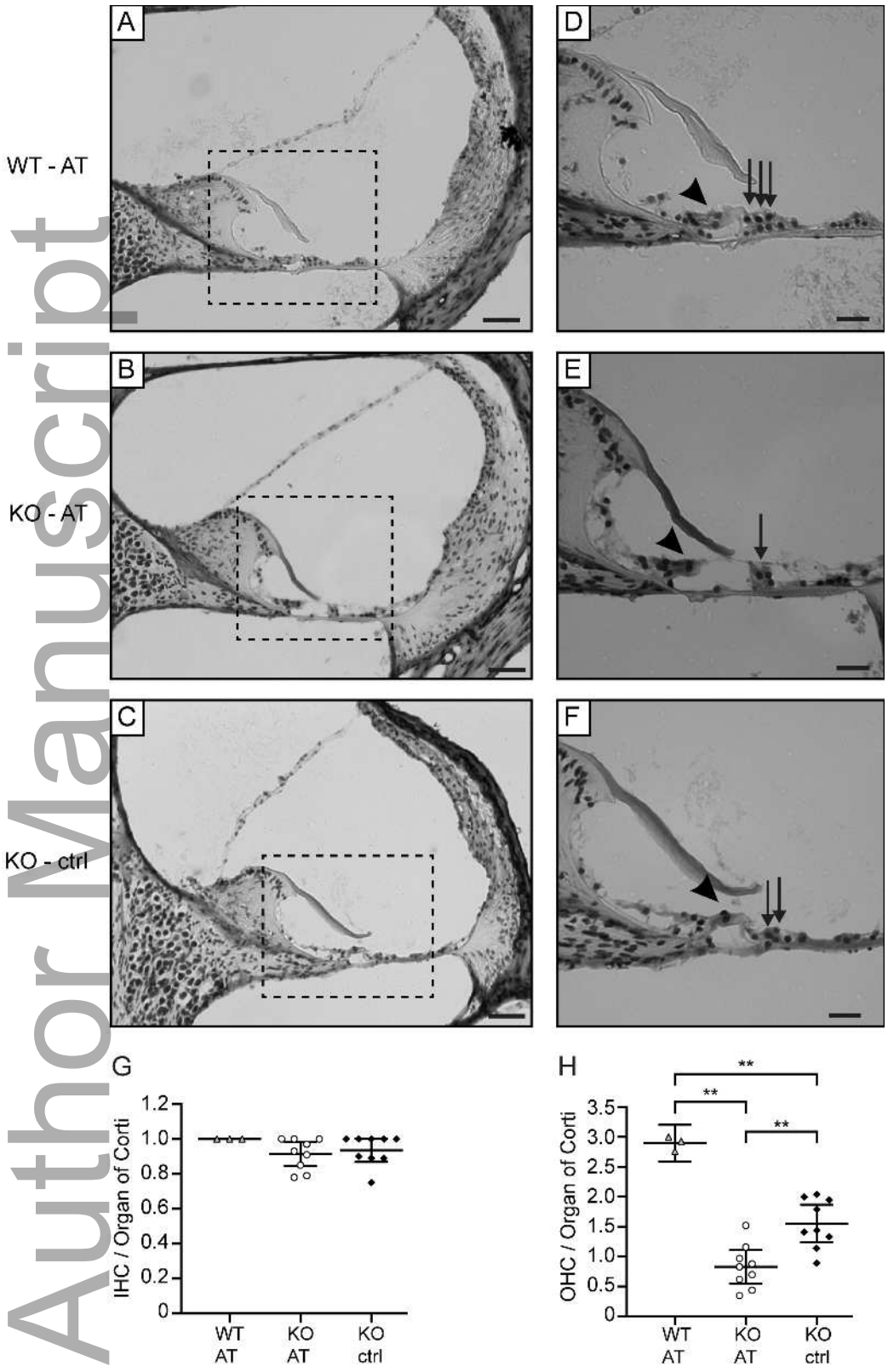

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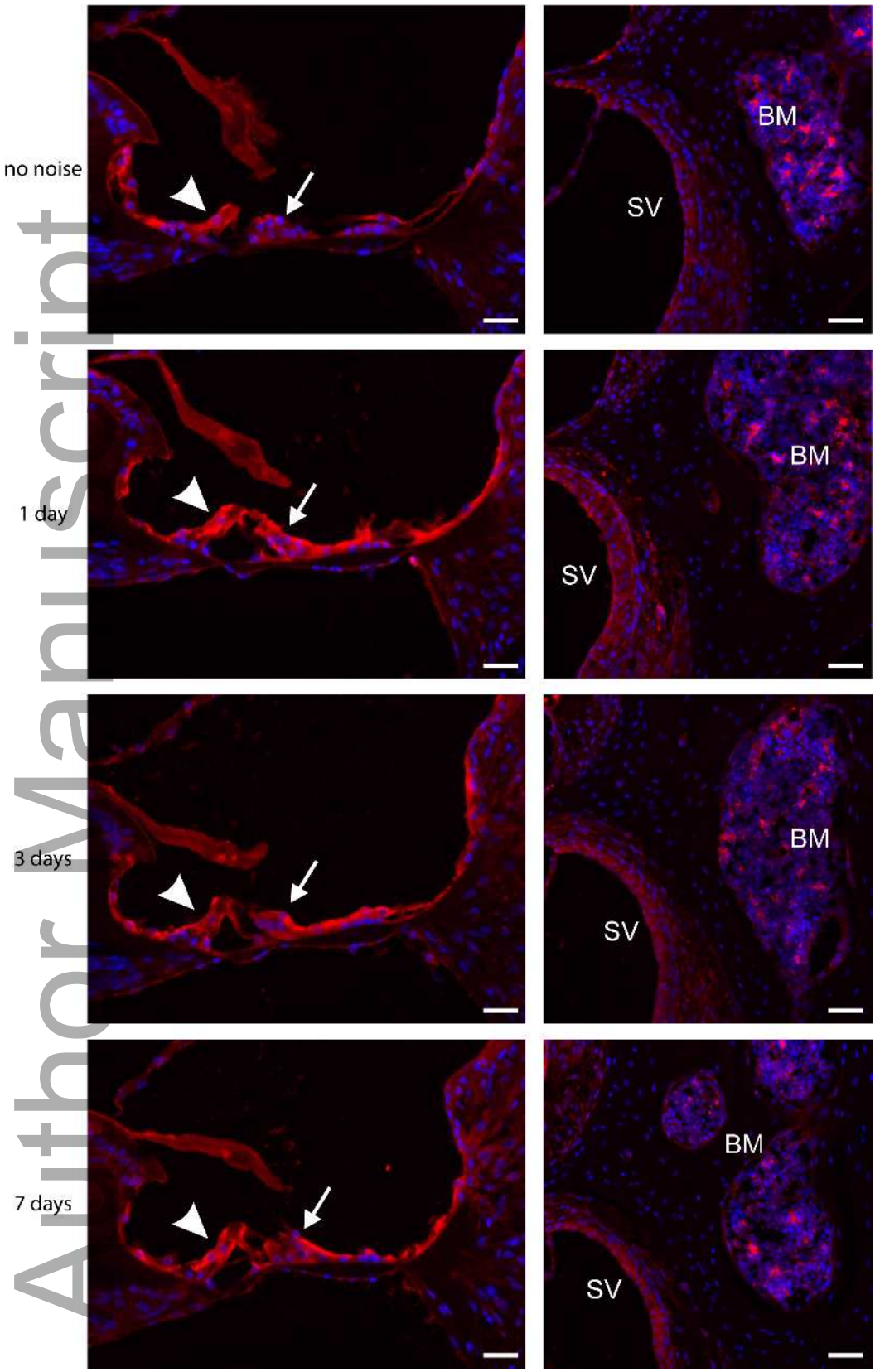

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