Research Article

The Stress Response in the Non-sensory Cells of the Cochlea Under Pathological Conditions—Possible Role in Mediating Noise Vulnerability

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ABSTRACT

Various stressors, such as loud sounds and the effects of aging, impair the function and viability of the cochlear sensory cells, the hair cells. Stressors trigger pathophysiological changes in the cochlear non-sensory cells as well. We have here studied the stress response mounted in the lateral wall of the cochlea during acute noise stress and during age-related chronic stress. We have used the activation of JNK/c-Jun, ERK, and NF-κB pathways as a readout of the stress response, and the expression of the FoxO3 transcription factor as a possible additional player in cellular stress. In the aging cochlea, NF-κB transcriptional activity was strongly induced in the stria vascularis of the lateral wall. This induction was linked with the atrophy of the stria vascularis, suggesting a role for NF-κB signaling in mediating age-related stria degeneration. Acutely following noise exposure, the JNK/c-Jun, ERK, and NF-κB pathways were activated in the spiral ligament of the lateral wall of CBA/Ca mice. This activation was concomitant with the morphological transformation of macrophages, suggesting that the upregulation of stress signaling leads to macrophage activation. In contrast, C57BL/6J mice lacked these responses. Only the combination of noise exposure and a systemic stressor, lipopolysaccharide, exceeded the threshold for the activation of stress signaling in the lateral wall of C57BL/6J mice. In

addition, we found that, at the young adult age, outer hair cells of CBA/Ca mice are much more vulnerable to loud sounds compared to these cells of C57BL/6J mice. These results suggest that the differential stress response in the lateral wall of the two mouse strains underlies, in part, the differential noise vulnerability of their outer hair cells. Together, we propose that the molecular stress response in the lateral wall modulates the outcome of the stressed cochlea.

Keywords: aging, auditory, lateral wall, LPS, noise, stress signaling

INTRODUCTION

Loud sounds cause the dysfunction and damage of the sensory hair cells of the organ of Corti of the cochlea. Hair cell loss is often an acute response to noise exposure (Wang et al. 2002). Supporting cells of the organ of Corti are more resistant to loud sounds and they regulate hair cell survival and death. Supporting cells are also responsible for wound healing at the surface of the sensory epithelium, at the sites of lost hair cells (reviewed in Francis and Cunningham 2017). Besides the cells of the organ of Corti, non-sensory cells of the stria vascularis and spiral ligament of the lateral wall respond to loud sounds. Spiral ligament fibrocytes show signs of histopathology and a part of them are lost following intense noise exposure (Wang et al. 2002; Hirose and Liberman 2003; Hirose et al. 2005; Ohlemiller and

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Gagnon 2007). In addition, loud sounds trigger the accumulation of inflammatory cells in the cochlea, particularly in its lateral wall, suggesting that the activation of cytokines and other inflammatory mediators is an important response to trauma (Hirose et al. 2005). These considerations bring about the question of the relationship between the sensory and non-sensory compartments of the cochlea. Following noise exposure, is the molecular stress response initially mounted in the organ of Corti and then conveyed to the lateral wall or do these compartments independently sense noise? What is the impact of the lateral wall stress response on hair cell loss and hearing impairment? What is the contribution of systemic stressors to the stress response of the lateral wall, considering that blood-borne substances can enter the lateral wall via its dense microvasculature? A large network of stress signaling pathways is acutely and often transiently activated in stressed tissues. Understanding the activation pattern of these pathways in the cochlea might provide novel insights into the mechanisms of noise-induced hearing loss.

Noise vulnerability is highly variable among mouse strains (reviewed by Ohlemiller et al. 2016). Part of this variation is due to early-onset presbycusis that characterizes strains such as the C57BL/6J (B6) strain. These mice show initial signs of elevated hearing thresholds already at the age of 3 months. Inter-strain differences are also evident in the effect of loud sounds on the endocochlear potential (EP). EP is based on the endolymph ionic concentration and is required for hair cell mechanotransduction (reviewed by Fettiplace 2017). EP is generated by the marginal cells of the stria vascularis, by their potassium secretion into the endolymph (reviewed by Nin et al. 2016). In response to a comparable noise level, there is a prominent, acute EP reduction in CBA/Ca (CBA) mice, while B6 mice are much less affected. In addition, noise-exposed CBA mice show pathological changes in their lateral wall, in contrast to B6 mice (Wang et al. 2002; Hirose and Liberman 2003; Hirose et al. 2005; Ohlemiller and Gagnon 2007).

Stress signaling pathways are activated in response to acute and chronic stressors. This activation is cell type-specific, such that cells activate different stress signaling pathways depending on the cells they interact with and on their intrinsic state. Activation of stress signaling can be beneficial or detrimental; there is often a threshold level of a stressor that determines if a stress pathway has pro-survival or pro-death effects (Fulda et al. 2010). Mitogen-activated protein kinase (MAPK) pathways include the c-Jun N-terminal kinase (JNK)/c-Jun and extracellular signal-regulated kinase (ERK) pathways. Both are activated in the stressed organ of Corti and inhibition of their activation has been shown to regulate hair cell loss

(Pirvola et al. 2000; Wang et al. 2003; Kurioka et al. 2015; Anttonen et al. 2016). Activation of the transcription factor NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) might also be part of the cochlear stress response (Masuda et al. 2006; Tahera et al. 2006; Adams et al. 2009), similar to the FoxO3 (Forkhead box O3) transcription factor that has been localized to the organ of Corti (Gilels et al. 2013; Han et al. 2016). Previous studies on stress signaling in the cochlea have concentrated on the organ of Corti. We have in the current work focused on the lateral wall cells, by studying the activation of stress pathways (1) following noise exposure, (2) following systemic challenge, (3) following systemic plus noise challenge, and for comparison of acute and chronic stress, (4) during aging. The current results on the stress biology in the cochlea might have translative significance regarding the development of protective therapies.

METHODS

Mice and Noise Exposure

C57BL/6JCrI (B6, Charles River Laboratories), CBA/CaOlaHsd (CBA, Envigo), and the transgenic *NF- κ B/LacZ* reporter mouse (Bhakar et al. 2002) were used in this study. The mice used in the experiments were 8 weeks old to avoid the early-onset presbycusis that characterizes the B6 strain. Both sexes were used. The animal experiments were conducted following relevant guidelines for animal work and approved by the National Animal Experiment Board.

In the *NF- κ B/LacZ* reporter mouse, the *NF- κ B*-responsive promoter drives transcription of the *LacZ* gene. *LacZ* activation can be monitored by X-Gal histochemistry. The reporter mouse line was maintained in the B6 background. For studying NF- κ B activity in the CBA background, CBA F1 hybrid reporter mice were used in the experiments. It has been previously shown that the two characteristics of noise-exposed CBA mice, the acute EP drop and the lateral wall pathology, are inherited in an autosomal dominant manner from CBA background to B6 F1 hybrids (Ohlemiller and Gagnon 2007). Eight-week-old reporter mice were exposed to octave-band noise (8 to 16 kHz) presented at 105 dB sound pressure level (SPL) for 2 h. Their cochleas were analyzed at 12 h post-exposure. Total numbers of specimens analyzed: B6 $n=6/3$ cochleas/mice; CBA 6/3. For aging studies, 10-month-old B6 mice were used ($n=6/3$ cochleas/mice). Both cochleas of all individuals were stained for X-Gal, and one of the cochleas was processed for paraffin sections and the other for whole mount specimens.

B6 and CBA mice were sacrificed 0 h, 2 h, 12 h, or 6 days after exposure to octave-band noise (8 to 16 kHz) presented at 105 dB SPL for 2 h. Exposures were performed in a self-built sound chamber (40 × 44 × 82 cm). Sound was produced with two active speakers (8130A Digital Bi-Amplifier Monitoring System, Genelec) mounted side by side 2 cm above a laboratory animal cage for rodents, which was subdivided into four individual cages (9 × 16 × 9 cm). Mice were able to turn and move in these cages. Speakers were connected with NuForce icon μ DAC2 to a laptop playing the sound continuously.

Total numbers of specimens analyzed in the acute (0, 2, 12 h post-exposure) experiments: B6 $n=11/6$ cochleas/mice for 0 h, 4/4 for 2 h, and 6/3 for 12 h; CBA $n=14/7$ for 0 h, 4/4 for 2 h, and 6/3 for 12 h. Cochleas were embedded in paraffin and consecutive sections were used to analyze p-c-Jun, p-ERK, and FoxO3 expression. Whole mount specimens prepared at 12 h post-exposure were used for macrophage counts (see below). Whole mount specimens were prepared for outer hair cell (OHC) counts 6 days after noise exposure. Total numbers of specimens analyzed: B6 $n=12/6$; CBA $n=12/7$ cochleas/mice. Postnatal day 4 (P4) B6 mice were used to study the specificity of the p-ERK antibody in the organ of Corti.

Paraffin Sections and Immunohistochemistry

Cochleas were perilymphatically fixed with 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4, and immersed in this fixative overnight at +4 °C. They were then decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 7.5, overnight. Cochleas were embedded into paraffin (Histoplast IM, Thermo Scientific) and cut in a midmodiolar plane to 5- μ m-thick sections. After deparaffinization, epitopes were unmasked by microwave heating (900 W) in 10 mM citrate buffer, pH 6.0, for 16 min. Sections were blocked for 1 h with 10 % goat serum (Jackson ImmunoResearch) in Tris-buffered saline (TBS), pH 7.4, containing 0.25 % Triton-X-100 (TBS-T). Incubation with primary antibodies diluted in TBS-T was performed for 48 h at +4 °C. The following rabbit primary antibodies were used: phospho-c-Jun Serine 73 (p-c-Jun, #3270; Anttonen et al. 2016), phospho-p44/42 (ERK1/2 (p-ERK, #4370; Kurioka et al. 2015), forkhead family of transcription factor 3a (FoxO3, #12829; Gilels et al. 2013; Han et al. 2016), acetylated tubulin (#5335; Kirjavainen et al. 2015) (all from Cell Signaling Technology), and ionized Ca^{2+} -binding adapter molecule (Iba1, #019-19741, Wako; Okano et al. 2008). Detection was done with the Vectastain Elite ABC kit and diaminobenzidine substrate (DAB Detection kit, both from Vector Laboratories). Counterstaining was done with 2.5 % methyl green.

Whole Mount Specimens and Immunostaining

Cochleas for whole mount specimens were fixed with 4 % PFA, decalcified with 0.5 M EDTA overnight, and cut in a midmodiolar plane in half, such that the spiraling organ of Corti was separated into four pieces. The organ of Corti, stria vascularis, and spiral ligament were separated and dissected clean from the surrounding bone. The tectorial membrane was removed. A frequency map was prepared from each cochlea using the Measure Line.class ImageJ plugin (available from Eaton-Peabody Laboratories Histology Resources) and the calculations according to Müller et al. (2005).

Whole mount specimens were blocked for 1 h with 10 % goat serum in TBS-T, followed by a 48-h-long incubation at +4 °C with appropriate primary antibodies in TBS-T. The following primary antibodies were used: Myosin7a (Myo7, #25-6790, Proteus Biosciences; Anttonen et al. 2016) and Iba1. Secondary antibodies conjugated to Alexa 594 (Invitrogen) were used for fluorescence detection. After antibody incubations, F-actin filaments were visualized using Oregon Green 514-conjugated phalloidin diluted in TBS-T (Invitrogen). Nuclei were stained with DAPI. In the spiral ligament whole mount specimens, Iba1 expression was detected with the Vectastain Elite ABC kit and the diaminobenzidine substrate. ProLong Gold anti-fade reagent was used for mounting (Invitrogen).

X-Gal Staining for NF- κ B Transcriptional Activity

Cochleas were perilymphatically fixed with 4 % PFA and immersed in this fixative for 1.5 h. X-Gal incubation was performed at +37 °C overnight in PBS containing 5 mM K-ferrocyanide, 5 mM ferricyanide, 3 mM MgCl_2 , 0.02 % deoxycholate, 0.02 % NP-40, and X-galactose (X-Gal, Promega) (1 mg/ml). Cochleas were post-fixed with 4 % PFA. After decalcification, specimens were processed for paraffin sections or whole mount specimens. Paraffin sections were counterstained with 0.1 % Nuclear Fast Red. In experiments with X-Gal staining together with Iba1 or p-c-Jun immunohistochemistry, no counterstaining was done.

Intraperitoneal LPS Injection

The *E. coli* endotoxin lipopolysaccharide (LPS) (Sigma Aldrich) was injected i.p. at the concentration of 1 mg/kg (100 μ l per mouse, diluted in PBS) (Hirose et al. 2014). Animals were sacrificed 3 h after injection (B6 $n=4/3$ cochleas/mice; CBA 3/3). Another group of animals was exposed to noise (as above) 2 h after LPS injection. These animals were killed immediately after noise exposure (B6 5/4 cochleas/mice; CBA 4/3).

Image Analysis

Paraffin sections and whole mount specimens were analyzed with bright-field optics under the BX61 microscope equipped with UPlanApo $\times 10$, $\times 40$, and $\times 60$ objectives. Images were acquired through the DP73 CCD color camera and CellSens software (all from Olympus). Whole mount specimens of the organ of Corti were imaged under epifluorescence illumination using the Axio Imager.M2 microscope equipped with PlanApo $\times 10$ and $\times 40$ objectives and Apotome 2 structured illumination slider (all from Zeiss). Images were acquired with the black and white CMOS camera (Hamamatsu ORCA Flash 4.0 V2) and ZEN 2 software (Zeiss). Camera settings to obtain images from immunohistochemistry specimens were always the same. Adobe Photoshop CC 2017 was used to prepare figure plates.

Cell Counts and Statistical Analysis

Whole mount specimens were double-labeled with the Myo7 antibody and phalloidin to mark hair cells. DAPI labeling was included to mark nuclei. Lack of both the nucleus and the positively stained cell body was used as the criterion for hair cell absence. The basal most hook region of the cochlea was excluded from the counts due to challenges in its preparation. In paraffin sections from the cochleas of *NF- κ B/LacZ* reporter mice, the proportion of X-Gal-positive spiral ganglion (SG) neurons was counted in the apical and basal coil of six cochleas from six mice. At least three adjacent sections per cochlea were used for counting.

Quantification of macrophages was made in whole mount preparations of the lateral wall of CBA and B6 mice, using Iba1 as a cell type-specific marker. This analysis was performed in the basal part of the cochlea with a $\times 10$ objective. The area for counting was $300 \times 400 \mu\text{m}$ wide, covering a region of the spiral ligament behind the stria vascularis. Preparations from this region are flat and thin that helps quantification. Two non-overlapping regions per cochlea were counted from the basal part of the cochlea. The regions corresponded to the frequency regions 32 and 45 kHz. Macrophages with different morphologies were quantified. Examples of a macrophage with completely withdrawn processes and a macrophage with a ramified morphology are shown Fig. 7d (insets). Total numbers of Iba1-positive cells were counted as well as the percentage of Iba1-positive cells with completely withdrawn processes. Quantification was performed in control (CBA $n = 4/3$ cochleas/mice; B6 $n = 4/3$) and noise-exposed (12 h after 105 dB SPL for 2 h, CBA $n = 5/3$ cochleas/mice; B6 $n = 6/3$) specimens.

Statistical analyses of OHC loss and macrophage numbers were done with OriginPro 8.6. In Figs. 1 and 7, histograms show mean $\pm$ standard deviation

(SD). The medians of experimental groups in Fig. 1 were compared with the Mann-Whitney U test. Comparison between the experimental groups in Fig. 7 was done with two-way ANOVA using Tukey test for means comparison. Normality was tested with the Shapiro-Wilk test. The p value < 0.05 was considered statistically significant with 95 % confidence intervals.

RESULTS

Differential Outer Hair Cell Loss in CBA and B6 Mice Following Noise Exposure

The inbred CBA and B6 mouse strains differ in their response to noise. CBA mice show a distinct EP decrease acutely following noise exposure, in contrast to B6 mice. In addition, the lateral wall of CBA, but not B6 mice shows noise-induced histopathology (Wang et al. 2002; Hirose and Liberman 2003; Ohlemiller and Gagnon 2007). To find out whether also hair cell vulnerability differs between the two strains, we exposed young adults (2 months) to octave-band noise (8 to 16 kHz) presented at 105 dB SPL for 2 h. B6 mice are characterized by early-onset presbycusis, but it is not yet evident at this age. In the cochlear whole mount specimens analyzed at 6 days post-exposure, quantification revealed a four-fold greater average loss of OHCs in CBA compared to B6 mice (CBA mean $\pm$ SD 457 ± 169 , $n = 12/7$ cochleas/mice; B6 mean $\pm$ SD 84 ± 45 , $n = 12/6$) (Fig. 1a). The hair cell loss counts were done with DAPI, phalloidin, and Myo7 staining from whole mount preparations. CBA mice showed near-total OHC loss from the middle, 32-kHz region to the basal most, 64-kHz region of the cochlea (Fig. 1b, d). They also showed scattered OHC loss in the 25- to 32-kHz region. B6 mice displayed only sporadic OHC loss in the 25- to 45-kHz region (Fig. 1c, e). In the 45- to 64-kHz region, OHC loss was more extensive, but also in there, the loss was less severe than in CBA mice. Inner hair cells (IHCs) that are more resistant to noise compared to OHCs were intact in both strains (Fig. 1b–d). These results on the differential noise vulnerability of OHCs of CBA and B6 mice correspond to previous findings on the differences in the acute EP decrease and lateral wall cell pathology (Wang et al. 2002; Hirose and Liberman 2003; Ohlemiller and Gagnon 2007).

Acute c-Jun Stress Response in the Noise-Exposed Cochlea

JNK/c-Jun activation has been shown to mediate noise-induced OHC death (Pirvola et al. 2000; Anttonen et al. 2016). Therefore, we asked whether

FIG. 1. Noise-induced outer hair cell loss in CBA and B6 mice. OHC loss was quantified 6 days after noise exposure (105 dB SPL for 2 h) in whole mount specimens covering the whole cochlear length, except for the basal most hook region. **a** Mean $\pm$ SD OHC loss was 457 ± 168 and 84 ± 45 in CBA and B6 cochleas, respectively (CBA $n = 12/7$ and B6 $n = 12/6$ cochleas/mice). The difference is highly significant (Mann-Whitney U test $U = 0$, medians for CBA = 424 and for B6 = 72, $p = 0.00011$). **b**, **c** Frequency maps of CBA and B6 cochleas stained for Myo7, a hair cell marker. Boxed areas

correspond to the higher magnification views in **d** and **e**. **d**, **e** Views from the 45-kHz region, showing double-labeling for Myo7 (red) and phalloidin (green). Prominent OHC loss is seen in CBA, but not B6 cochleas. In addition to the hair cell stereociliary bundles, phalloidin marks supporting cell scars that replace lost OHCs (arrow in **d**). The IHC row and the 1–3 rows of OHCs are marked in **e**. SPL sound pressure level, dat days after trauma, OHC outer hair cell, Myo7 myosin7a, phall phalloidin, IHC inner hair cell. Scale bar shown in **c** (**b**, **c** = 230 μ m, **d**, **e** = 20 μ m)

JNK/c-Jun activation correlates with the differential OHC loss found between CBA and B6 mice (Fig. 1). We used JNK-mediated N-terminal (Ser73) phosphorylation of c-Jun as a readout of c-Jun activation (Herdegen et al. 1998). We have termed this activation as the c-Jun stress response (Anttonen et al. 2016). Figure 2 depicts the cochlear compartments analyzed in the present study. c-Jun phosphorylation was largely absent from the non-exposed organ of Corti (data not shown; Anttonen et al. 2016), but was induced immediately after exposure to 105 dB SPL for 2 h. This induction in the organ of Corti was seen in both strains. Different supporting cell types and a part of IHCs showed the stress response, whereas the response was absent from OHCs, the vulnerable cells (Fig. 3a, e). Based on this expression pattern, it has been suggested that activated JNK/c-Jun signaling mediates OHC death in a paracrine manner (Anttonen et al. 2016). c-Jun phosphorylation was rapidly suppressed, evidenced at 2 h post-exposure when only one type of supporting cells, the Deiters' cells, showed the expression. Most of this expression had disappeared by 12 h post-exposure (data not shown; Anttonen et al. 2016). The pattern of c-Jun phosphorylation in the organ of Corti was similar in

CBA and B6 mice. In both strains, the response was restricted to the basal part of cochleas, corresponding to the region of OHC loss.

As opposed to the organ of Corti, c-Jun was differentially phosphorylated in the lateral wall of CBA and B6 mice. In the CBA lateral wall, c-Jun was activated in the root cells (Fig. 3b) and type 3 fibrocytes (data not shown) immediately after 2 h of noise at 105 dB SPL. This activation appeared to progress through the gap junctional syncytium in the spiral ligament: it was found in the type 1 and 2 fibrocytes at 2 h post-exposure, being concomitantly downregulated in the root cells (Fig. 3b–d). The c-Jun stress response was still present in the spiral ligament at 12 h post-exposure, but had disappeared by 24 h post-exposure (data not shown; Anttonen et al. 2016). In the stria vascularis, the c-Jun phosphorylation was observed only in scattered basal and intermediate cells at 2 h post-exposure. Similar as in the organ of Corti, c-Jun phosphorylation was restricted to the basal half of CBA cochleas. In contrast, loud sounds did not trigger c-Jun phosphorylation in the lateral wall of B6 mice, with the exception of a few positive root cells (Fig. 3f–h). Thus, the lateral wall cells of noise-exposed B6 mice

Fig. 2. A hematoxylin and eosin-stained cross-section from the upper basal turn of a non-treated cochlea. The view depicts the major cochlear compartments and cell types. In the organ of Corti, outer hair cells (OHCs) and inner hair cell (IHC) are shown with short and long arrows, respectively. Pillar cells are marked with arrowheads and Deiters' cells underneath OHCs with stars. Interdentary cells (ID) are located at the surface of the spiral limbus (SL). The cell layers of the stria vascularis (SV) are shown in the inset: InM intermediate cells, Ca capillary, MC marginal cells, BC basal cells. The spiral ligament contains the root cells (RC) and type 1–4 fibrocytes (Fb). The outer sulcus cells (OS) detach easily from the lateral wall surface during tissue processing, as seen in this view. Scale bar 25 μ m for the larger view and 10 μ m for the inset

have a striking resistance to the induction of the c-Jun stress response.

Systemic LPS Potentiates the Noise-Induced c-Jun Stress Response in the Lateral Wall

As the lateral wall cells of B6 mice did not mount a c-Jun stress response, a possibility was that loud sounds do not affect these cells. Another possibility was that these cells lack the capacity for c-Jun activation. To study this, we triggered cellular stress systemically, taking benefit of the dense microvasculature in the lateral wall, particularly in the stria vascularis (reviewed by Shi 2016). We used LPS as the stressor and hypothesized that LPS itself or its effectors trigger a stress response close to capillaries. We applied a low LPS concentration (1 mg/kg) that is known to compromise vascular permeability, yet without severe cochlear damage (Hirose et al. 2014; Koo et al. 2015). LPS treatment triggered acute c-Jun phosphorylation

in the capillary endothelial cells of the lateral wall of both B6 and CBA mice, evidenced at 3 h post-injection. In addition, marginal, intermediate, and a subset of basal cells of the stria vascularis showed c-Jun phosphorylation (Fig. 3i–k). This response was seen throughout the length of the cochlear duct (base to apex). It was present at 12 h post-injection, but disappeared by 24 h post-injection (data not shown).

As the results with LPS alone did not answer to the question of the ability of the spiral ligament of B6 mice to raise the c-Jun stress response, we studied whether the combined LPS and noise (105 dB SPL for 2 h) challenge triggers this response. We found c-Jun phosphorylation in fibrocytes of both CBA and B6 mice following this insult (Fig. 3l–o). In CBA mice, the c-Jun stress response was stronger and broader than the response to noise alone, based on the findings that the response following the combined challenge was concomitantly detected in the root cells and the type 1–4 fibrocytes in the basal part of the cochlea (Fig. 3l). These findings are consistent with previous data showing that LPS exacerbates the effects of aminoglycosides in the cochlea (Hirose et al. 2014; Koo et al. 2015). The fact that LPS plus noise triggered c-Jun phosphorylation in the fibrocytes of B6 mice as well indicates that these mice can mount the c-Jun stress response when the stressor level is high enough.

Constitutive FoxO3 Expression in the Root Cells and Interdentary Cells of the Cochlea

Oxidative damage is part of the acute response of the organ of Corti to acoustic overstimulation (reviewed by Henderson et al. 2006). Besides the organ of Corti, oxidative stress targets the spiral ligament (Yamaguchi et al. 2014). The transcription factor FoxO3 is known to antagonize oxidative stress in other tissues (reviewed by Storz 2011) and to interact with JNK/c-Jun signaling (Sykes et al. 2011). In the cochlea, FoxO3 has been localized to the organ of Corti (Gilels et al. 2013; Han et al. 2016). We wondered whether FoxO3 is expressed in the lateral wall, specifically in the CBA lateral wall that shows noise-induced c-Jun activation (Figs. 3b–d, f–h and 4a). In non-exposed cochleas, FoxO3 was localized to the nuclei and cytoplasm of root cells. These cells were marked by the acetylated tubulin antibody (Fig. 4b–e) (Jagger et al. 2010). Fibrocytes lacked FoxO3 expression (Fig. 4c, e). In addition to root cells, FoxO3 was expressed in the interdentary cells of the spiral limbus (Fig. 4g, h). This expression pattern was similar in the CBA and B6 cochleas. The pattern was not affected by noise exposure, based on the analysis made acutely after 2 h of noise at 105 dB SPL (Fig. 4d–h). Thus, rather than overlapping with JNK/c-Jun signaling, FoxO3 is constitutively expressed in two cell populations lining the endolymph and possessing high metabolic activity (Jagger and Forge 2013; Shodo et al. 2017).

FIG. 3. The c-Jun stress response in the noise-exposed and LPS-treated CBA and B6 cochleas, revealed by c-Jun phosphorylation (p-c-Jun) immunohistochemistry on paraffin sections. Sections are from the basal part of cochleas. Mice were exposed to 105 dB SPL for 2 h, and analysis was performed 0 and 2 h post-exposure. **a** In the organ of Corti of the CBA cochlea, the stress response is seen immediately after exposure in supporting cells and IHCs (*long arrow*), but not in OHCs (*short arrows*). **b–d** In the lateral wall of the CBA cochlea, root cells show transient c-Jun phosphorylation immediately (**b, c**) after noise. The activation in the fibrocytes and the basal cells of the stria vascularis is followed shortly thereafter (2 h) (**d**). **e** The stress response in the organ of Corti of B6 cochleas is comparable to CBA cochleas. **f–h** Lateral wall cells of B6 cochleas do not show the c-Jun stress response. **i–o** The c-Jun stress response in CBA and B6 mice following two experimental paradigms: (1) injection of lipopolysac-

charide (LPS) (1 mg/kg) and analysis 3 h post-injection, (2) LPS injection followed by noise (105 dB SPL for 2 h) 2 h after injection, and analysis immediately thereafter. In B6 mice, LPS alone triggers the c-Jun stress response in the capillary endothelial cells (*arrow* in **j**) and in the marginal, intermediate, and basal cells of the stria vascularis (**i, j**). Also endothelial cells (*arrow* in **k**) of the spiral ligament capillaries are stained for p-c-Jun. In both CBA (**l**) and B6 (**m–o**) mice, the LPS-plus-noise paradigm triggers widespread c-Jun phosphorylation in the lateral wall of the basal part of the cochlea. Note that p-c-Jun staining in the organ of Corti is similar in the LPS + noise and noise-only paradigms (**a, e, l–m**). SPL sound pressure level, OC organ of Corti, hat hours after trauma, RC root cells, Fb1 fibrocyte type 1, SV stria vascularis, LPS lipopolysaccharide. Scale bar shown in **m** (**a–h, j–k, n–o** = 15 μ m, **i, l, m** = 50 μ m)

Acute ERK Activation in the Noise-Exposed Cochlea

The antibody against p-ERK generated a signal in pillar cells, one type of supporting cells of the organ of Corti. Pillar cells possess a strong cytoskeleton and

p-ERK staining accumulated to this structure (Fig. 5a–c). The staining was seen in all cochlear turns and both in non-exposed and exposed cochleas. It was absent from pillar cells at the first postnatal week, just before the maturation of the cytoskeleton (Fig. 5d).

FIG. 4. Constitutive expression of the FoxO3 transcription factor in the root cells of the lateral wall and in the interdental cells of the spiral limbus of CBA and B6 cochleas. Immunohistochemistry for p-c-Jun, acetylated tubulin, and FoxO3 in paraffin sections of non-exposed and noise-exposed (105 dB SPL for 2 h) cochleas. **a** The c-Jun stress response is triggered in the nuclei of root cells acutely upon noise exposure. **b** Acetylated tubulin staining marks the contour of the finger-like processes of root cells. **c, d** FoxO3 is expressed in the root cells of control (non-exposed) animals (**c**) and noise exposure

does not alter this expression (**d**). **e, f** In B6 mice, FoxO3 expression in the root cells is comparable to this expression in CBA mice, both in control (**e**) and noise-exposed (**f**) mice. **g, h** The interdental cells of spiral limbus show constitutive FoxO3 expression in both mouse strains, shown here in control (**g**) and noise-exposed (**h**) CBA mice. *hat* hours after trauma, *Ac. tubulin* acetylated tubulin, *RC* root cells, *ctrl* control, *ID* interdental cells. Scale bar shown in **b** (**a–f** = 20 μ m, **g, h** = 13 μ m)

Therefore, p-ERK staining in pillar cells is likely to be non-specific, consistent with the fact that some other polyclonal antibodies have been shown to non-specifically bind to the dense cytoskeleton of pillar cells (e.g., Laos et al. 2017). Acutely after exposure to 105 dB SPL for 2 h, ERK phosphorylation was induced in the nuclear region of Deiters' cells, another type of supporting cells. This noise-induced expression was restricted to the basal most region of the cochlea, a region that was narrower than the p-c-Jun-positive region. Similar to c-Jun activation, ERK activation gradually disappeared from Deiters' cells, evidenced at 12 h post-exposure (data not shown). Noise-induced p-ERK induction was seen in the organ of Corti of both CBA and B6 mice (Fig. 5b, c).

In the lateral wall of CBA mice, noise exposure triggered acute p-ERK expression in fibrocytes, assessed at 2 h post-exposure (Fig. 5e, f). This expression was clearly broader than that of p-c-Jun as it included all four types of fibrocytes (Fig. 5f). At 12 h post-exposure, ERK phosphorylation was maintained in scattered fibrocytes only, most notably in the type 3 fibrocytes (Fig. 5g). As opposed to p-c-Jun, p-ERK was

not found in the root cells (Fig. 5f). Notably, the lateral wall of B6 mice lacked ERK activation at all post-exposure time points studied (Fig. 5h). Thus, similar as c-Jun activation, ERK activation was induced in the lateral wall of noise-exposed CBA, but not B6 cochlea.

Transcriptional NF- κ B Activity in the Cochlea

We next studied whether acoustic stress activates NF- κ B in the cochlea. This transcription factor is activated upon stress in various cells and tissues, and has been shown to interact with MAPK pathways (reviewed by Papa et al. 2004). We used a well-characterized *NF- κ B/LacZ* reporter line (B6 background) where activation of the reporter construct can be monitored by X-Gal histochemistry (Bhakar et al. 2002). We backcrossed the reporter mice with the CBA strain, and used the CBA F1 hybrids to represent CBA mice, based on prior data suggesting that the CBA phenotype is inherited in an autosomal dominant manner (Ohlemiller and Gagnon 2007). We found that constitutive NF- κ B transcriptional activity was comparable in non-exposed cochleas of the two types of

FIG. 5. ERK phosphorylation in noise-exposed CBA and B6 cochleas. ERK phosphorylation (p-ERK) is revealed by immunohistochemistry in paraffin cross-sections. Mice were challenged with noise at 105 dB SPL for 2 h, and the analysis was done 2 or 12 h post-exposure. **a–c** The cytoplasm of pillar cells of the adult organ of Corti shows p-ERK staining that appears non-specific (see text for details). The long arrow marks IHCs, short arrows OHCs, arrowheads pillar cells, and stars Deiters' cells. The view is from the middle coil of a noise-exposed CBA cochlea (**a**). Note that ERK phosphorylation in Deiters' cells does not reach the middle part of the cochlea (**a**). However, ERK phosphorylation is acutely induced in the nucleus and cytoplasm of Deiters' cells of the basal most part of the cochlea

(**b**, **c**). The nuclei of hair cells lack p-ERK staining. The same expression pattern is seen in noise-exposed CBA (**b**) and B6 (**c**) mice. **d** At P4, p-ERK immunostaining is seen in the basal, synaptic area of hair cells, but not in the structurally immature pillar cells (arrowheads). **e–h** ERK phosphorylation is not seen in the lateral wall of control (non-exposed) CBA cochleas (**e**), but is acutely and transiently induced in fibrocytes upon noise, assessed at 2 (**f**) and 12 h (**g**) post-exposure. Only the type 3 fibrocytes (arrow in **g**) have maintained ERK phosphorylation at this later time point. **h** In the lateral wall of B6 cochleas, ERK activation is not observed after noise. SPL sound pressure level, hat hours after trauma, ctrl control. Scale bar shown in **h** (**a–d** = 18 μ m, **e–h** = 50 μ m)

mice. NF- κ B was active in a subset of spiral ganglion (SG) neurons, evidenced both in whole mount specimens and paraffin cross-sections (Fig. 6a, b). Quantification revealed that a larger portion of SG neurons were stained for *LacZ* in the apical than the basal part of the cochlea (mean $\pm$ SD 11.3 ± 2.3 % and 3.2 ± 0.6 %, respectively, $n = 6/6$ cochleas/mice). Also, interdental cells of the spiral limbus were stained (Fig. 6a–d, e'). Notably, hair cells and supporting cells of the organ of Corti (Fig. 6a–e) and cells of the lateral wall (data not shown) lacked *LacZ* staining. We then examined NF- κ B transcriptional activity at 12 h after noise exposure (105 dB SPL for 2 h). The staining pattern remained unaltered in the SG and spiral limbus of both CBA and B6 mice (Fig. 6c, d). However, loud sounds induced distinct *LacZ* staining in the spiral ligament fibrocytes in the basal part of CBA cochleas (Fig. 6e, h). This induction was very weak in B6 cochleas (Fig. 6i). Thus, NF- κ B transcriptional activity is induced in the lateral wall as a part of the stress response and this induction occurs in a strain-specific manner.

NF- κ B activity has been associated to the immune system function (Bonizzi and Karin 2004). The lateral wall of the cochlea contains, in addition to mesenchymal fibrocytes, bone marrow-derived macrophages. These latter cells have been detected in normal conditions and particularly after trauma (Fredelius and Rask-Andersen 1990; Warchol 1997; Hirose et al. 2005; Okano et al. 2008). We used Iba1 as a marker for macrophages. In the spiral ligament of noise-exposed CBA mice, *LacZ* activity did not colocalize with Iba1 staining, but it did colocalize with p-c-Jun in a subset of cells (Fig. 6f, g). This analysis was performed at 12 h post-exposure (105 dB SPL for 2 h) when the macrophage population represents mostly resident cells (Hirose et al. 2005). Thus, these results confirm our other results that it is the fibrocytes in the lateral wall of noise-exposed CBA mice where NF- κ B is transcriptionally active.

Morphological Transformation of the Lateral Wall Macrophages upon Noise Exposure

Analysis of Iba1 staining in the lateral wall of CBA and B6 mice at 12 h after noise exposure (105 dB SPL for 2 h)

FIG. 6. NF-κB transcriptional activity in the cochlea studied with the *NF-κB/LacZ* reporter mouse. *LacZ* staining was revealed by X-Gal histochemistry. Non-exposed (control) and noise-exposed (105 dB SPL for 2 h) cochleas were prepared for whole mount specimens (a–d, h–i) and paraffin cross-sections (e–g). Analysis was done 12 h post-exposure. a–d Constitutive *LacZ* activity is seen in the interdental cells and a subpopulation of SG neurons. This staining is comparable in non-exposed (a, b) and exposed (c, d) specimens and in CBA (d) and B6 (a–c) mice. Note the abundance of positive neurons in the apical (a) compared to the basal (b–d) SG and the negative organ of Corti. e, e' In the lateral wall of noise-exposed CBA mice, a part of

fibrocytes show *LacZ* staining (e). Boxed area shows the spiral limbus of the same specimen. NF-κB transcriptional activity is located to the interdental cells (e'). f, g In the lateral wall of noise-exposed CBA mice, *LacZ* staining does not colocalize with Iba1 immunoreactivity (f), in contrast to p-c-Jun immunoreactivity (g). h, i Whole mount specimens show *LacZ* staining in fibrocytes of noise-exposed CBA mice (h), whereas the staining in B6 mice is minimal (i). *ctrl* control, X-Gal X-galactosidase, OC organ of Corti, ID interdental cells, SG spiral ganglion, SPL sound pressure level, hat hours after trauma, SL spiral limbus, Fb fibrocytes. Scale bar shown in d (a–d, h, i = 50 μm, e = 30 μm, e', f, g = 18 μm)

revealed differences in the morphology and amount of macrophages. Noise-exposed B6 mice showed Iba1-positive cell profiles with a small cell body and several long ramifications, typical to the surveillant status of macrophages. In CBA mice, noise exposure triggered morphological transformation of macrophages to a rounded cell body and withdrawn processes that has been considered to represent the activated, pro-inflammatory status of macrophages (Fig. 7a–c) (Jassam et al. 2017). A comparable change from a ramified to a withdrawn morphology has been shown in the macrophages of the basilar membrane in old B6 mice (Frye et al. 2017). Quantitative analysis showed that the portion of Iba1-positive cells with completely withdrawn processes was significantly higher in the lateral wall of CBA

compared to B6 mice after noise exposure (CBA mean $\pm$ SD 23.5 ± 5.7 %, $n = 5/3$ cochleas/mice; B6 mean $\pm$ SD 0.8 ± 1.0 % $n = 6/3$) (Fig. 7d). Comparison of the ratio of macrophages with withdrawn ramifications between mouse lines and between noise-exposed and control groups with two-way ANOVA revealed a significant difference in both cases (mouse lines $F = 58.290$, $DF = 1$, two-tailed p value = 1.524×10^{-6} ; noise vs. control $F = 56.204$, $DF = 1$, two-tailed p value = 1.774×10^{-6}). Significant differences were detected between CBA noise and B6 noise groups ($p = 0$) and between CBA noise and CBA control groups ($p = 3.372 \times 10^{-8}$). In B6 mice, the amount of macrophages with ramifications did not change after noise exposure (B6 noise and B6 control $p = 0.934$). Despite these differences in morphology, total amount of

FIG. 7. The macrophage marker Iba1 reveals a differential morphology of the spiral ligament macrophages in noise-exposed B6 and CBA mice. Images are from the lateral wall whole mount specimens where the stria vascularis and the bony capsule have been removed. All images are from the basal part of the cochlea. **a, b** After noise exposure (12 h after 105 dB SPL for 2 h), Iba1-positive cells of the B6 lateral wall show a ramified morphology (**a**), while part of these cells from CBA mice totally lack ramifications (arrow in **b**). **c** In the non-exposed (control) CBA lateral wall, Iba1-positive cells display a ramified morphology. **d** Histogram shows the mean $\pm$ SD percentage of Iba1-positive macrophages with totally withdrawn branches (see inset). The amount of these cells is significantly higher

in CBA compared to B6 mice after noise exposure (two-way ANOVA, means comparison with Tukey test B6: median = 0.9 % $n = 6/3$ cochleas/mice, CBA: median = 24.1 % $n = 5/3$, $p = 0$). **e** Mean $\pm$ SD of total numbers of macrophages is not significantly changed when non-exposed and noise-exposed mice are compared. Total number of Iba1-positive cells is greater in B6 compared to CBA mice, but the difference is statistically significant only between the noise-exposed groups (two-way ANOVA, means comparison with Tukey test for the noise-exposed B6 mice: median = 91 $n = 6/3$ cochleas/mice, and noise-exposed CBA mice: median = 40 $n = 5/3$, $p = 0.0262$). SPL sound pressure level, hat hours after trauma, ctrl control. Scale bar shown in **c** (**a–c** = 20 μ m, insets in **d** = 10 μ m)

Iba1-positive cells in the lateral wall was higher in B6 compared to CBA mice (noise-exposed B6 mean $\pm$ SD 99 ± 43.1 , $n = 6/3$ cochleas/mice; noise-exposed CBA mean $\pm$ SD 43 ± 10.0 , $n = 5/3$, $p = 0.026$, two-way ANOVA mouse lines $F = 12.789$, $DF = 1$, two-tailed p value = 0.003) (Fig. 7e). Together, the lateral wall of CBA mice activates stress signaling upon noise and displays concomitant morphological transformation of macrophages.

NF- κ B Transcriptional Activity in the Stria Vascularis During Aging

NF- κ B activity has been linked to aging in other tissues (Tilstra et al. 2011). We used NF- κ B transcriptional activity as a readout of age-related chronic stress in the cochlea and utilized as a model the B6 mouse strain that

shows an early-onset aging phenotype (Ohlemiller et al. 2016). Ten-month-old mice showed the loss of a large part of OHCs. The highly degenerated organ of Corti lacked *LacZ* staining, with the exception of occasional, likely migratory cells (Fig. 8a, i, data not shown). The majority of SG neurons were lost as well, but the constitutive NF- κ B activity was maintained in a part of remaining neurons (Fig. 8a, i–k). Constitutive NF- κ B activity was also maintained in a subset of interdental cells of the spiral limbus (Fig. 8a–m). The stria vascularis of aging cochleas was highly atrophic (Fig. 8a, b). In addition to the thinning of the cross-sectional width of the stria (Fig. 8b, c) (Hequembourg and Liberman 2001), its melanocyte-like cells were enlarged and showed increased melanin content, as compared to 2-month-old mice (Fig. 8c–g) (Spicer and Schulte 2005). Notably, marginal cells and a subset of melanocyte-like cells of the

FIG. 8. NF- κ B transcriptional activity in the cochlea of aging mice. Paraffin sections (**a–b**, **e**, **j–m**) and whole mount preparations (**c–d**, **f–i**) of cochleas of NF- κ B/LacZ reporter mice were exposed to X-gal histochemical staining. Specimens are from 10-month-old B6 mice. **a–d** NF- κ B activity is induced in the aging stria vascularis. The stria vascularis from the upper basal coil shows distinct atrophy that correlates with strong NF- κ B activity in the marginal cells. Note the absence of NF- κ B activity in the organ of Corti (arrow in **a**). The marginal cells are marked with an arrowhead in the higher magnification image (**d**). A part of pigment-containing melanocyte cells (brown) show LacZ staining, shown in a higher magnification view (arrow in **d**). Note the enlarged size of the pigment cells in the aging stria vascularis (**c–d**). **e–g** Two-month-old stria vascularis does

not show LacZ activity and its melanocyte cells are not prominent. **h** In addition to the stria vascularis, NF- κ B activity is upregulated in the spiral ligament fibrocytes of the aging cochlea. **i** Most outer hair cells are lost in the aged organ of Corti. The amount of LacZ-positive interstitial cells and SG neurons is decreased. **j–k** The amount of SG neurons is reduced in the basal part (**j**) of the aging cochlea, in contrast to the apical part (**k**). **l–m** A part of the interstitial cells display LacZ staining in the spiral limbus, evident both in the basal and apical part of the aging cochlea. X-Gal X-galactosidase, mo months, ID interstitial cells, SG spiral ganglion, SV stria vascularis, Fb fibrocytes, OC organ of Corti. Scale bar shown in **l** (**a**, **c**, **f**, **h**, **i** = 50 μ m, **b**, **d**, **e**, **g** = 16 μ m, **j**, **k** = 18 μ m, **l**, **m** = 10 μ m)

stria vascularis as well as fibrocytes of the spiral ligament showed distinct LacZ activity in the aging cochlea (Fig. 8b–d, h). These results show that the induction of NF- κ B transcriptional activity is a part of the chronic stress response in the aging cochlea, specifically in its lateral wall.

DISCUSSION

In this paper, we have characterized molecular stress responses mounted in the cochlea acutely upon noise exposure and during aging. We show distinct differences in the noise-induced stress response in the

lateral wall of two mouse strains. We discuss these results taking into account the reported inter-strain differences in the acute EP reduction following noise and the possibility that the EP drop and activation of stress signaling are interrelated. In addition, we discuss the emerging role of the lateral wall as a major site of inflammation in the cochlea and the possibility that activated stress signaling regulates pro-inflammatory events.

We have used activation of two MAPK pathways, the JNK/c-Jun and ERK pathways, as a readout of the noise-induced stress response. These pathways were activated in the organ of Corti of the basal half of the cochlea, the region affected by our noise exposure paradigm. Both pathways were activated in an acute and transient

manner. JNK/c-Jun was activated in supporting cells, but not in the vulnerable OHCs, consistent with previous data (Anttonen et al. 2016). We also found ERK activation in one type of supporting cells, the Deiters' cells. This noise-induced stress response in the organ of Corti was comparable in CBA and B6 mice. Thus, it did not correlate with our other findings showing that, at the age of 2 months, noise-induced OHC loss was much more severe in CBA mice. The question then arose as to the possible role of the other compartment of the cochlea, the lateral wall, in exacerbating the noise vulnerability of OHCs of CBA mice.

The lateral wall of noise-exposed CBA mice showed acute activation of c-Jun and ERK signaling, whereas these responses were absent from B6 mice. Similarly, we found NF- κ B transcriptional activity in the lateral wall of noise-exposed CBA, but not B6 mice. One possibility is that stress signaling mounted in the organ of Corti is transmitted along the gap junctional network to the lateral wall and that this communication between the compartments is less efficient in B6 cochleas. However, evidence is lacking for qualitative or quantitative differences in the cochlear gap junctional network between mouse strains. In accordance, we did not see obvious differences in connexin 26 expression between CBA and B6 cochleas (AH, UP, unpublished observations). Potassium is considered to recycle from the organ of Corti through the spiral ligament gap junctional network to the stria vascularis, to be secreted into the endolymph for the maintenance of EP (Kikuchi et al. 2000; Wangemann 2002; Nin et al. 2016). Based on this knowledge, heavier potassium flow along the recycling route might trigger the stress response in the CBA lateral wall. However, CBA mice showed exacerbated OHC loss following noise, a fact that it is likely to reduce rather than increase potassium flow. Thus, the possibility that potassium transport accounts for the stress response in the CBA lateral wall is less plausible. The most likely explanation is that loud sounds directly act on the lateral wall cells and that differences in genetic factors in these cells trigger the activation of the stress response. Based on the findings that the combined systemic (LPS) and noise challenge was strong enough to elicit the stress response in B6 mice, genetic factors in the lateral wall cells are likely to determine the threshold level for cell-extrinsic stressors above which stress signaling is activated.

Our findings of noise-induced acute stress response in the lateral wall of CBA, but not B6 mice correspond to previous studies showing an acute EP drop in noise-exposed CBA mice exclusively (Wang et al. 2002; Hirose and Liberman 2003; Ohlemiller and Gagnon 2007; Ohlemiller et al. 2018). The question is then whether the acute stress response/EP drop axis is linked with the observed heightened noise vulnerability of OHCs of CBA mice. It has been shown that a large-scale EP drop,

achieved by genetically impairing the structure and function of the stria vascularis, compromises OHC survival (Liu et al. 2016). Our unpublished results (AH, UP) as well as published data (Wang et al. 2002; Anttonen et al. 2016) show that most part of OHC loss occurs rapidly after noise exposure. Thus, based on the temporal correspondence, the stress response may contribute to the extent OHC loss in CBA mice. However, this possibility is still to be directly shown by relevant mutant mouse models. It is to be noted that a recent study suggested that lateral wall pathology drives OHC loss particularly in young adult CBA mice, which were also used in the current study, while older CBA mice are less affected (Ohlemiller et al. 2018).

We evaluated NF- κ B transcriptional activity in the adult cochlea using the transgenic *NF- κ B/LacZ* reporter mouse line (Bhakar et al. 2002). NF- κ B subunits and their cytoplasmic vs. nuclear expression have been previously localized to the cochlea, yet with variable results (Masuda et al. 2006; Tahera et al. 2006; Adams et al. 2009). The present results show constitutive NF- κ B transcriptional activity in both CBA and B6 cochleas: in interdental cells of the spiral limbus and in a part of SG neurons. Similar to the adult SG neurons, the adult forebrain neurons display constitutive NF- κ B transcriptional activity (Bhakar et al. 2002). Interestingly, this activity appears to be absent from most other peripheral ganglia, such as the dorsal root ganglion (Gushchina et al. 2009). NF- κ B signaling is a potent regulator of injury in many tissues. In neurons, it has been suggested to carry both pro-survival and pro-death functions. We found that the constitutive NF- κ B activity was maintained in the cochlea following noise and, additionally, that this activity was induced in the lateral wall fibrocytes. Notably, this noise-induced NF- κ B activation was prominent in CBA, but very weak in B6 mice. Thus, these results show that NF- κ B belongs to the cochlear stress signaling network activated upon noise and they add to our other findings on inter-strain differences in the lateral wall stress response.

What could be the local role of the stress response in the lateral wall of noise-exposed cochleas? Levels of pro-inflammatory cytokines have been shown to increase acutely following noise exposure in the lateral wall (Fujioka et al. 2006; Vethanayagam et al. 2016). Combining to the present results, this cytokine increase coincides with the activation of the stress response. Our results show that fibrocytes serve as a major site of the stress response, and thus, these cells could be responsible for cytokine production. Activated stress signaling is known to upregulate cytokine production in other cell types (reviewed by Bonizzi and Karin 2004; Hoffmann and Baltimore 2006; Waetzig et al. 2005). It has been previously shown that, in the lateral wall of CBA mice, a part of fibrocytes degenerate following noise exposure (Wang et al. 2002; Hirose and Liberman 2003; Hirose

et al. 2005; Ohlemiller and Gagnon 2007). This cellular degeneration might be triggered by the stress response and cytokine secretion, either in a paracrine or autocrine fashion. We found that the Iba1-positive macrophages in the noise-exposed CBA mice were transformed to a morphology that characterizes activated macrophages (reviewed by Jassam et al. 2017). This event might be linked with the stress response. One possible function of activated macrophages is phagocytosis of the debris of dying fibrocytes. Notably, in contrast to CBA mice, fibrocyte degeneration has not been found in the noise-exposed B6 mice (Ohlemiller and Gagnon 2007). These previous data are in accordance with the current findings that the stress response and macrophage morphological transformation are absent from the lateral wall of noise-exposed B6 mice. However, further studies are needed to dissect in detail the temporal sequence of lateral wall pathology in different mouse strains. Together, the current data suggest that, in addition to the possible effect on the extent of OHC loss following loud sounds, the lateral wall stress response might locally regulate cell pathology.

One characteristic feature of cochlear aging is the degeneration of the stria vascularis. It is currently unclear whether the age-related pathologies of the three cochlear compartments—the organ of Corti, SG, and stria vascularis—are independent of each other or if they share common influences and mechanisms (reviewed by Ohlemiller 2009; Schuknecht et al. 1974). We found prominent stria atrophy along aging and it was associated with strong induction of NF- κ B transcriptional activity. This induction was not seen in the degenerating organ of Corti nor in SG neurons of the aging cochlea. However, a subpopulation of SG neurons showed constitutive NF- κ B activity, suggesting that NF- κ B signaling has dual functions, regulating homeostasis in the SG and the aging process in the stria vascularis. The fact that NF- κ B activation was induced exclusively in the lateral wall during aging suggests that the molecular mechanisms driving age-related pathologies are at least partially different between the cochlear compartments.

In conclusion, stress signaling pathways are mounted in the cochlear lateral wall acutely and transiently upon noise exposure and chronically during aging. The noise-induced lateral wall stress response differs between mouse strains, correlating with the differences in OHC vulnerability and with previously shown differences in lateral wall pathology. However, the causal relationship between these events is still to be directly shown. Our study also shows constitutive expression of the FoxO3 transcription factor in two non-sensory cell populations of the cochlea, the interdental cells and root cells. These are metabolically highly active cells and, based on their luminal location, are likely to be involved in ionic homeostasis. As FoxO3 is implicated in other tissues in the maintenance of homeostasis following oxidative

imbalances (reviewed by Storz 2011), it may well play this role in the two cochlear non-sensory cell populations. Finally, our study shows that a systemic stressor can trigger and modulate the molecular stress response in the lateral wall. This result implies that blood-borne substances have the potential to modulate the cochlear stress response and perhaps this knowledge could be applied in protective interventions.

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COMPLIANCE WITH ETHICAL STANDARDS

The animal experiments were conducted following relevant guidelines for animal work and approved by the National Animal Experiment Board.

Conflict of Interest The authors declare that they have no conflict of interest.

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