Chronic cigarette smoke exposure drives spiral ganglion neuron loss in mice

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Tobacco use is associated with an increased risk of hearing loss in older individuals, suggesting cigarette smoke (CS) exposure may target the peripheral auditory organs. However, the effects of CS exposure on general cochlear anatomy have not previously been explored. Here we compare control and chronic CS exposed cochleae from adult mice to assess changes in structure and cell survival. Two-photon imaging techniques, including the imaging of second harmonic generation (SHG) and two-photon excitation fluorescence (TPEF) from native molecules, were used to probe the whole cochlear organ for changes. We found evidence for fibrillar collagen accumulation in the spiral ganglion and organ of Corti, consistent with fibrosis. Quantitative TPEF indicated that basal CS-exposed spiral ganglion neurons experienced greater oxidative stress than control neurons, which was confirmed by histological staining for lipid peroxidation products. Cell counts confirmed that the CS-exposed spiral ganglion also contained fewer basal neurons. Taken together, these data support the premise that CS exposure induces oxidative stress in cochlear cells. They also indicate that two-photon techniques may screen cochlear tissues for oxidative stress.

Tobacco use correlates with an increased risk of high-frequency hearing loss in older individuals1. Since an estimated thirty-six million Americans smoke cigarettes2, this correlation confers a large social and economic burden. However, the origins of this correlation are unclear. In the United States, smokers are more likely to hail from poorer socioeconomic groups, who are at additional risk for hearing loss3. Moreover, individuals who accept the known health risks incurred through smoking may be less likely to protect their hearing from other sources of damage, including noise. These observations raise the possibility that smoking may not directly impact the cochlea, but rather correlate with other risk factors for hearing loss.

Burning tobacco initiates a complex chemistry of reactive organic molecules and free radicals, which are immediately inhaled into the lungs and sinuses of exposed individuals4. These molecules may access the cochlea through the bloodstream, or alternatively, via the Eustachian tube, which opens into the nasal part of the pharynx during swallowing5. One such component, nicotine, binds to nicotinic acetylcholine receptors (nAChR) present throughout the nervous system4, including the cochlea4. While the effects of CS exposure on inner ear cells have not been explored, there is epidemiological evidence that passive CS inhalation increases the likelihood of Eustachian tube dysfunction6. For endothelial cells and lung, CS exposure drives an inflammatory response as a consequence of oxidation8. Later stages of inflammation include cell death and destruction of the extracellular matrix (ECM, reviewed in10). The combination of apoptosis, inflammation, and ECM degradation causes alveolar enlargement and a loss of elasticity11. These destructive processes culminate into chronic obstructive pulmonary disease (COPD,12).

While the use of animal models with long-term CS exposure has contributed significantly to our current understanding of CS effects on the lung, little is known about CS effects on cochlear cells. Mice exposed to CS for six months in a pulmonary project also provided inner ear tissue for the current study, an additional anatomical...
analysis of CS-specific effects. This chronic CS exposure is sufficient to induce airspace enlargement, similar to emphysema, in exposed mice\(^{13,14}\). We hypothesized that, as in the lung, chronic CS exposure induces oxidative stress in the cochlea. We used two-photon microscopy to rapidly screen CS-exposed cochleae for altered regions. Alterations in collagen fiber microstructure, including some associated with fibrosis\(^{15-17}\), can be detected by imaging second harmonic generation (SHG) with specialized two-photon microscopy\(^{18}\). The infrared wavelengths deeply penetrate tissue without loss of resolution, and may be used to probe entire tissue samples in whole mount, enabling the unbiased identification of altered regions without the use of expensive reagents. Other biological materials associated with metabolism display intrinsic fluorescence that may be quantified with two-photon excitation fluorescence (TPEF). In this report, we used these imaging techniques together with an analysis of oxidative stress, neuronal survival, and efferent innervation to assess effects of chronic CS exposure on the cochlea from adult C57BL/6J mice.

**Methods**

**Animal usage.** C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Cochleae from air (n = 8) exposed mice and cigarette smoke (CS, n = 10) exposed mice were obtained for this study. Mice were fed on regular diet and water ad libitum. Both male and female mice were used in this study. All experiments were performed within compliance of the US Department of Health and Human Services and were approved by the University of Rochester Medical Center Committee on Animal Resources.

**Cigarette smoke (CS) exposure.** Eight to ten-week-old mice were exposed to chronic treatment of CS using a Baumgartner-Jaeger CSM2082i cigarette smoking machine (CH Technologies, Westwood, NJ) for six months. Exposure was performed as previously described\(^{19}\). Treatments were performed at the Inhalation Core Facility at the University of Rochester. Mice were placed in individual wire cages housed in a closed plastic box which was connected to the smoking machine. CS was generated from 3R4F research cigarettes containing 10.9 mg of total particulate matter (TPM), 9.4 mg of tar, 0.726 mg of nicotine, and 11.9 mg of carbon monoxide per cigarette (University of Kentucky, Lexington, KY). Mice received a single 5-hour exposure once per day according to Federal Trade Commission protocol (1 puff/minute of 2-second duration and 35 mL volume). Mainstream CS was diluted along with filtered air and directed into the exposure chamber. The noise level experienced by the mice during this procedure was measured at 70 dB SPL. Real-time monitoring of CS exposure was performed using a MicroDust Pro-aerosol monitor (Casella CEL, Bedford, UK) and verified daily by thermogravimetric sampling immediately after exposure was completed. CS concentration was adjusted to a constant level of ~300 mg/m\(^3\) TPM. Control mice received identical treatments, but with unlit cigarettes.

**Two-photon microscopy.** Microdissected cochlear pieces were mapped using the MEEI Image plug-in prior to imaging. TPEF imaging was performed using an Olympus Fluoview 1000 AOM-MPM system equipped with a DeepSee laser system. Samples were illuminated by a Spectra-Physics MaiTai HP DeepSee Ti:Sapphire laser, with the following characteristics: excitation/emission centered at 755/420–460 for NADH fluorescence, and at 800/495–540 for elastin fluorescence. Elastin parameters were used to find tissue regions. An Olympus 25×, 0.95 N.A. (numerical aperture) objective was used to image the tissue, with the following excitation/emission: excitation fluorescence (TPEF). In this report, we used these imaging techniques together with an analysis of oxidative stress, neuronal survival, and efferent innervation to assess effects of chronic CS exposure on the cochlea from adult C57BL/6J mice.

**Immunostaining.** Standard practices of decalcification and sucrose cryoprotection were used to protect the morphology of cochlear structures. Freshly dissected cochlear tissues were immersed in 4% paraformaldehyde in PBS overnight, after removal of the stapes and puncture of the apical cochlear capsule. Cochleae were decalcified in 0.1 M EDTA at 4 °C on a rotating platform for 3–5 days, submersed in 30% sucrose, and embedded in OCT to present the 16 and 32 kHz regions in transverse sections. The tissue was snap-frozen in liquid nitrogen to preserve delicate structures, cryosectioned at 20 microns as serial sections, and dried onto Fisher SuperPlus slides. Sections were washed in PBS, blocked in PBS with 0.1% Triton and 5% donkey serum, and incubated overnight in primary antibody at 4 °C. Secondary antibody incubations were done at room temperature for one hour.

For whole mount staining, decalcified tissue was dissected as described\(^{21}\). Frequency mapping was done using an ImageJ plug-in developed by the Massachusetts Eye-Ear Institute’s Eaton-Peabody Laboratories (available at http://rsbweb.nih.gov/ij/). Dissected turns were washed in room temperature Dulbecco’s PBS (Gibco) and blocked for one hour in 1% Triton and 5% donkey serum in PBS. Primary antibody incubations of anti-synaptophysin (SYP) were performed at 37 °C for 20 hours, the tissue was washed in PBS, and secondary antibody incubation
was performed at 37 °C for an additional 2 hours. Whole mounts were placed between two 50 mm coverslips in Fluoromount-G (Southern Biotech).

Primary antibodies were anti-βIII tubulin, clone TUJ1 (1:500, Millipore, RRID: AB_2210524), anti-3-nitrotyrosine (3NT) (1:200, Enzo Life Sciences, Inc., RRID: AB_10539899), anti-4-hydroxynonenal (4HNE) (1:100, Gene Tex, RRID: AB_424345) and anti-synaptophysin (SYP) (1:200, OriGene Technologies, RRID:AB_2623575). Secondary antibodies were purchased from Jackson Labs: donkey anti-goat Alexa 647 (1:500; RRID:AB_2340438), donkey anti-mouse Alexa 488 (1:500, RRID: AB_2340849), donkey anti-rabbit Alexa 594 (1:500, RRID: AB_2340622). Sections and whole mount tissue were imaged on an Olympus FV 1000 laser scanning confocal microscope. The optical stack depth used for SGN quantification was 6 microns, which allowed for restricted counting of a single SGN cell layer.

Quantification and statistics. In this publication, spiral ganglion neurons were quantified in frozen sections, similar to24–29. Serial sections were obtained. Every other section was stained with anti-TUJ1 and DAPI to reveal neurons and nuclei respectively. Up to 5 sections were imaged for each turn, and only sections where the ganglion structure was intact, as assessed through bright-field images of these structures, were used in quantification. ImageJ (NIH) was used to project optical stacks for quantification of spiral ganglion neurons. Photoshop 5.1 was used to generate masks to measure the area of Rosenthal's canal. The area of these masks was measured using ImageJ 64. 4–6 cochleae per condition were processed for SGN quantification. 26 images of SGNs for each condition were blinded, randomized, and counted by a different individual. SGN density at the 16 kHz and 32 kHz turns was averaged for each cochlea, and these biological replicates were averaged. Statistical significance was determined by ANOVA and Student's two-tailed t-test using Bonferroni's adjustment in R (version 3.4.3).

For SYP quantification, CS-exposed and air-exposed cochleae were imaged at the 32 kHz region (n = 3 per condition). Confocal stacks were imported into the Amira FEI software suite with the XImagePAQ extension (RRID:SCR_007353). Deconvolution was performed using the iterative maximum likelihood estimation package. The stack was segmented and thresholded, and the Separate Objects function was used to reconstruct the individual SYP volumes. These were exported using the Connected Components function. Statistical significance was computed using the Wilcoxon rank sum test in R (version 3.4.3).

Results

Two-photon analysis reveals ECM remodeling and oxidative stress in CS-exposed cochleae.

We used two-photon analysis to find regions of the mouse cochlea that may be altered as a consequence of chronic (6 month) CS exposure. Collagen remodeling is a signature of CS exposure in the lung30. We used SHG on whole mount pieces of cochleae to identify potential alterations in microstructural properties of collagen I, such as changes in interfibrillar spacing (Fig. 1, amber,31). Figure 1a is exposure-matched to 1b, and 1c is matched to 1d. At 16 kHz, the fields were comparable with the exception that increased SHG signal was observed in the CS-exposed spiral ganglion (SG; Fig. 1b, cf. 1a, amber, white arrowhead at SG). Similar signal was detected in the SG at 32 kHz (Fig. 1d, cf. 1c, amber, white arrowhead at SG). Signal was also detected in the basilar membrane at 32 kHz (Fig. 1d, amber, white arrowhead at BM). Elastin, FAD, and NADH display intrinsic fluorescence which may be detected through two-photon excitation fluorescence (TPEF)32–34. Novel fluorescent deposits (810 nm excitation, <700 nm emission) were observed in the CS-exposed SG and organ of Corti in the 32 kHz turn (Fig. 1d, gray, arrows). Little SHG or TPEF signals were observed in sensory regions of whole mount control cochleae (Fig. 1a,c, amber and gray), although some signal was observed in bony tissues lateral to and far from the sensory region (Fig. 1c, amber, left side of image). These data suggest that CS exposure may have effects on cochlear ECM in sensory regions.

Oxidative stress is a frequent consequence of CS exposure in the lung35. NADH is an electron donor, and FAD is an oxidized product that accumulates with mitochondrial oxidation. More stringent TPEF parameters may be used to quantify intrinsic fluorescence consistent with NADH or with FAD, and their relative levels may then be computed as a heat plot to identify regions of oxidation stress36. We performed this analysis on unstained cochlear sections (Fig. 2, n = 3 per condition, 32 kHz). SGNs from control cochleae emit fluorescence consistent with FAD (excitation/emission 860 nm/495–540 nm) and NADH (755 nm/420–460 nm) in the same imaging plane (Fig. 2a). 2H, 2F). A heat map generated from these images reveals a few small, orange-red speckles, consistent with mitochondrial oxidation, among green and white cell bodies (Fig. 2c, purple arrows). Some speckles, originating from the FAD autofluorescence channel, are located within control SGNs and can be better appreciated in confocal projections (Fig. 2a, inset). CS-exposed cochlear sections from the same 32 kHz region display more and larger areas of orange-red speckles (Fig. 2e–g, purple arrow). The heat plot of CS-exposed SGNs displays less white and more green labeling, possibly because of lower overall NADH levels (cf. Fig. 2a,e and 2f). Moreover, confocal projections of individual CS-exposed neurons have more extensive FAD-like granulation (Fig. 2e, inset). Granulation is not observed in the NADH-like autofluorescence channel in either condition (Fig. 2b,f). These data suggest that basal SGNs experience oxidative stress from CS exposure. (Fig. 2a, cf. e).

In contrast to the SG, hair cells in the CS-exposed organ of Corti did not appear more oxidized in a similar heat plot representation (Fig. 2d, cf. with 2h). We did, however, observe apparent oxidative stress in regions of the stria vascularis (Fig. 2h, purple arrow). Strikingly, the spiral ligament showed less oxidation in the CS-exposed organ of Corti compared to controls (Fig. 2h, grey arrowhead). These results indicate that oxidative stress in CS-exposed tissues are cell type specific.

Oxidative stress and reduced SGN numbers in CS-exposed cochleae. To independently support the observation that SGNs are under oxidative stress from CS exposure, we tested an antibody to 4-hydroxy-2-nonenal (4-HNE), which binds to lipid peroxidation products, and an antibody to 3-nitrotyrosine...
(3NT), which detects accumulation of nitrated proteins, in sections of control and CS-exposed cochleae. Sections were counterstained for acetylated β-tubulin, which reveals neurons (Fig. 3a,d,g,j). Control SGN showed little evidence for either kind of damage (Fig. 3a–c,g–i), whereas the lipids in CS-exposed SGN were highly modified (Fig. 3e,k). In contrast, protein nitration was similar in both control and CS-exposed SGN (Fig. 3c,f,i,l), showing the specificity of lipid peroxidation. These data are consistent with our quantitative TPEF studies (Fig. 2), indicating that CS exposure increases oxidative stress in SGNs.

Sections through the basal SG (Fig. 2) suggest that high-frequency SGNs may be lost in CS-exposed cochleae. Accordingly, we quantified SGNs per unit area on sections stained with the antibody to acetylated β-tubulin. Typical images are presented for both the 16 kHz (Fig. 4a,b,e,f) and the 32 kHz regions (Fig. 4b,c,g,h). The two conditions were significantly different (Fig. 4i, p = 0.03, n = 4, 6, CS- and air-exposed cochleae respectively, two-way ANOVA). The results for the 32 kHz region were significantly different (Fig. 4i, p = 0.05, Student’s two tailed t-test with Bonferroni adjustment). Taken together, these data confirm that CS exposure drives oxidative stress in SGN, and that CS-exposure correlates with reduced SGN numbers in the basal cochlea.

**Increased volumes of efferent projections to HCs in the CS-exposed cochlea.** HCs express nicotinic acetylcholine receptors and their activities are modulated through cholinergic efferent signaling. We visualized efferent terminals in the 32 kHz region of air- and CS-exposed cochleae, using an antibody against synaptophysin (SYP). Three-dimensional modeling software was used to process the confocal images to obtain

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**Figure 1.** Extracellular matrix changes in the cochlea as a consequence of chronic CS exposure. (a) A whole mount preparation of the 16 kHz turn, from a mouse exposed to air, is imaged with second harmonic generation (SHG) to visualize fibrillar collagen (amber). Two-photon excitation fluorescence (TPEF) reveals intrinsic fluorescence from biomolecules such as elastin (gray). Scale bar; 200 microns. (b) A whole mount preparation of the 16 kHz turn, from a mouse exposed to CS, imaged with identical settings for SHG (amber) and TPEF (gray) as performed in (a). Arrowhead denotes changed SHG signal in spiral ganglion (SG). (c) A whole mount preparation of the 32 kHz turn from a mouse exposed to air, imaged for SHG (amber) and TPEF (gray). (d) A whole mount preparation of the 32 kHz turn from a mouse exposed to CS, imaged with identical settings for SHG (amber) and TPEF (gray) as performed in (c). White arrows highlight TPEF fluorescent accumulation in the spiral ganglion (SG) and inner hair cell region. White arrowheads (b,d) highlight SHG alterations in the medial SG and the basilar membrane (BM) of the organ of Corti. n = 3 cochleae per condition.
representations of separate efferent terminals (Fig. 5a,b, amber) and extract volumetric data (Fig. 5c). On average, efferent terminals were twice as large under CS-exposed OHCs (50.6 ± 7.9 µm³ for CS-exposed versus 26.6 ± 3.3 µm³ for air-exposed terminals). Efferent terminals were roughly eight times larger under CS-exposed IHCs (69.4 ± 12.1 µm³ for CS-exposed versus 8.3 ± 1.7 µm³ for air-exposed terminals). The distributions of these volumes were vastly different between conditions (Fig. 5c, n = 3 organs per condition, p < 2.2 × 10⁻¹⁶, Wilcoxon rank sum test, both for OHC terminals and IHC terminals). These data suggest that chronic CS exposure may be associated with alterations in cochlear efferent innervation.

Discussion

This report describes for the first time histological changes in the mammalian cochlea after chronic exposure to tobacco smoke. Using two-photon imaging in mouse tissue, we find evidence for collagen alteration, as well as increased levels of FAD-like autofluorescence, in the high-frequency SG after six months of CS exposure. In other systems, similar findings correlate with increased fibrosis15–17 and mitochondrial oxidation20 respectively. To further support these findings, we analyzed levels of lipid peroxidation products in SGN after chronic CS exposure and found that they were greatly increased. We also report significant losses of 32 kHz SGN after CS exposure. Finally, we show enlargement of CNS efferent terminals beneath cochlear hair cells, suggesting that after chronic CS exposure, CNS modulation of auditory cells may also be altered. These findings indicate that chronic inhalation of tobacco smoke, a common environmental toxin, has specific and potentially deleterious effects on cochlear cells and structures.

SHG/TPEF imaging has been used to quantify fibrosis in liver tissue17 and kidney tissue19, and assess cartilage degeneration16 and tumor progression31. Others have previously used SHG and TPEF imaging to investigate pathological changes to the cochlea. In one such study, intravital multiphoton microscopy was used to measure the volume of the scala media compartment in the apical turns of control and pendrin-deficient mice37. In another, little difference was observed between the SHG signals detected from the SG and basilar membranes of control and noise-exposed cochleae35. In contrast, we see significant divergence between CS-exposed and control tissues (Fig. 1). The altered levels of SHG signal observed here may also be compared to similar changes in chronic arthritis models34. We anticipate that this rapid, non-destructive, and powerful imaging technique shows great promise for future studies that screen for widespread changes in the cochlea.

Figure 2. Quantitative TPEF to assess mitochondrial oxidation. (a) A single optical section from the 32 kHz spiral ganglion, from an air-exposed mouse, is imaged with TPEF filtered to collect FAD signal. A purple arrow highlights a neuron with a bright granular FAD-like signal. Inset shows a higher power confocal projection of a similar neuron. (b) Same section as in (a), imaged with TPEF filtered to collect NADH-like signal. NADH-like signal is not granular in aspect. Scale bar: 50 microns. (c) Heat map computed from (a) and (b) using the formula (FAD/(FAD + NADH)) as described in Methods. Red indicates regions of relatively higher FAD-like signal, indicating oxidative stress, whereas the cooler blue and white colors indicate regions of lower FAD-like signal. (d) Similar heat map computed with identical data from the apical organ of Corti region. Scale bar: 50 microns. (e) A single optical section from the 32 kHz spiral ganglion, from a CS-exposed mouse, is imaged with TPEF filtered to collect FAD signal. Exposure matches (a). Purple arrow highlights a neuron with bright granular FAD-like signal. Inset shows a higher power confocal projection of a similar neuron with many granules. (f) Same section as in (a), imaged with TPEF filtered to collect NADH signal. Exposure matches (b). No granules are observed. (g) Heat map computed from (e) and (f) reveals regions of increased oxidative stress in the spiral ganglion, indicated by red and orange speckles. (h) Similar heat map analysis for apical organ of Corti from CS-exposed mouse reveals cellular variation in oxidation. Gray arrowhead indicates the less oxidized spiral ligament, whereas the purple arrow indicates the more oxidized stria vascularis. n = 3 cochleae per condition.
Increased oxidative stress, particularly in the form of lipid peroxidation products, is observed in many tissues after CS exposure, such as the lung\(^8\). We similarly see a dramatic and widespread increase in lipid peroxidation products in SGN after CS exposure (Fig. 3). This result supports our quantitative TPEF analysis of oxidative stress (Fig. 2). TPEF appears to be somewhat less sensitive for oxidative stress compared to antibodies for lipid peroxidation (Fig. 3). Lipid peroxidation may be due to the presence of various free radicals in tobacco smoke\(^4\).

**Figure 3.** Oxidative stress of SGN as a consequence of chronic CS exposure. Sections through control (a–c, g–i) and CS-exposed (d–f, j–l) SGN are probed with antibodies to βIII tubulin (a,d,g,j) to reveal neurons, 4-hydroxy-2-nonenal (4-HNE, b,e,h,k) to reveal peroxidation, and 3-nitrotyrosine (3-NT, c,f,l) to reveal nitrosylation. Sections through the 16 kHz (a–f) and 32 kHz (g–l) are shown. n = 3 cochleae per condition. Scale bar: 20 microns.
Non-nicotinic compounds in cigarettes activate antioxidant response genes in endothelial cells, correlating with CS extract toxicity for brain microvasculature. Microvascular pathology is also associated with different forms of hearing loss. Further experiments will be needed to determine if CS exposure drives a microvascular pathology in the inner ear.

It is also possible that chronic nicotine administration directly affects cochlear structures via the modulation of nicotinic acetylcholine receptors (nAChR). This idea is partly supported by our preliminary analysis of cholinergic efferent terminals, which are greatly enlarged in CS-exposed cochleae. Adult rodent SGNs express Chrna6, Chrna7, and Chrnb2 and receive cholinergic input from neurons of the lateral olivocochlear (LOC) nucleus while OHCs express Chrna9 and Chrna10 and receive cholinergic input from medial olivocochlear (MOC) neurons. Stimulation of LOC neurons enhances the amplitudes of cochlear neuronal responses, whereas ablation of LOC neurons depresses such amplitudes without affecting auditory thresholds. In this way, the CNS may regulate a “set point” of excitability for SGNs, wherein cholinergic stimulation increases excitability. In other systems, chronic nicotine administration can drive the up-regulation of functional nAChRs, especially those containing Chrnb2 subunits. If a similar mechanism exists in the cochlea, we speculate that some SGNs in chronically CS-exposed mice could have a lower threshold for activation, driving oxidative stress. Notably, a study that compared hearing loss in hunters who either smoked or chewed tobacco found that both groups had greater hearing loss than hunters who did not do either, suggesting a specific role for nicotine. Upregulation of Chrna9 and Chrn10 in OHCs might also explain the associated enlarged MOC terminals. Experiments that use only chronic nicotine administration will be helpful in dissecting its role in the cochlear changes described here.

This study may be considered opportunistic. Most studies of disease or environmental exposure concentrate on a single organ system. For example, the mice used here were part of a larger cohort from an investigation of COPD development in the mouse lung after chronic CS exposure. We note that in humans, chronic CS exposure is associated with a broad range of health problems in addition to COPD. Hearing loss, for example, has been linked to smoking in multiple studies. Thus, CS exposure via systemic oxidative stress may have repercussions on hearing loss while COPD/emphysema ensues. However, the mechanism of CS-induced hearing loss is not known. Prior to commencing this secondary analysis, we discussed aspects of the protocol that were applicable to hearing. As the CS- and air-exposed mice experienced the same levels of environmental noise and stress, with the sole difference being the presence of CS, we considered that a secondary study, on whether CS induces...
anatomical changes in the inner ear, would be interpretable. However, opportunistic studies are unfortunately limited in scope, as we were unable to perform functional studies to assess if CS-exposed mice have changes in their hearing thresholds or distortion product emissions, as reported for humans.

Future experiments could further confirm and extend the findings reported here. Functional hearing studies on CS-exposed animals, including studies of noise damage and recovery, will further illuminate the effects of smoking on the auditory system. It would be interesting to use SEM to examine the cochlear surface for changes in ECM, for example. Cochlear stiffness could also be assayed through cochlear gel analysis. One may also wish to investigate potential changes in cortical auditory regions, such as the MOC and LOC. We have not yet determined which component of CS drives the changes we report, whether it is nicotine, oxidizing radicals or some combination of the two. Nonetheless, this is the first study to analyze the anatomical effects of chronic CS inhalation on the cochlea in an animal model. Our findings support an interpretation that exposure to CS increases oxidative stress in the cochlea and correlates with a reduction in the numbers of high-frequency spiral ganglion neurons.

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Author Contributions
S.T.P. conceived of the project, urged the use of two-photon microscopy, performed experiments and provided analysis; R.P.D. and E.B.B. performed two photon microscopy, provided analysis and edited the manuscript; I.K.S. and I.R. developed the protocols for mouse C.O.P.D. induction, provided inner ear tissue and edited the manuscript; P.M.W. performed experiments, provided analysis and wrote the manuscript. Raw data files are available upon request.

Additional Information
Competing Interests: The authors declare no competing interests.

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