# 17β-Estradiol Reduces Nitric Oxide Production in the Guinea Pig Cochlea

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## Abstract
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Intense noise exposure and the application of ototoxic substances result in increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as nitric oxide (NO). In order to reduce the free NO concentration in the inner ear under pathological conditions, the use of natural cytoprotective substances such as 17β-estradiol is a promising therapeutic concept. In male guinea pigs the organ of Corti and the lateral wall were isolated from the cochlea and afterwards incubated for 6 h in cell-culture medium. 17β-Estradiol was adjusted in 2 concentrations to organ cultures of the right ears (12 animals per concentration). The left ears were used as controls. The NO production was quantified in the supernatant by chemiluminescence after incubation. Depending on the concentration, 17β-estradiol reduced NO in the organ of Corti by 43% (p=0.015) and 46% (p=0.026), respectively. In the lateral wall, the NO concentration was reduced by 24%, but without statistical significance (p=0.86). However, when analyzing the association between the 2 cochlear regions for each animal separately, the NO concentrations were lower in nearly all 17β-estradiol-treated ears compared to controls. In order to demonstrate the flexibility of the organ culture system, the NO donor DETA NONOate and the nitric oxide synthase inhibitors L-NAME and L-NMMA were applied. The electron microscopic analysis revealed a well-preserved cochlear cell morphology after incubation. The ability of 17β-estradiol to influence the NO production preferentially in the organ of Corti might offer new therapeutic perspectives for inner ear protection.

## Introduction
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It has been known for some years that nitric oxide (NO) is not only a versatile key player in cochlear function, but also responsible for inner ear hearing disorders [1–3]. Based on different animal models, it is currently assumed that an increased NO production triggers various inner ear diseases such as acute noise trauma, sudden idiopathic hearing loss, acute tinnitus, and presbycusis [4–8]. Only glucocorticoids which include synthetic dexamethasone have been clinically used in inner ear therapy so far [9–11]. Based on this restricted repertoire of drugs and the limited knowledge about the underlying NO-dependent mechanisms in inner ear pathophysiology, it is of great medical and economic interest to develop new therapeutic strategies against inner ear distress. One strategy might be based on the utilization of the body's own natural substances, such as the sex gonadal steroid hormone 17β-estradiol.

In different approaches, it was shown that 17β-estradiol can influence NO production differently depending on the tissue. In the endothelium of blood vessels, a 17β-estradiol-dependent increase in the endothelial nitric oxide synthase (eNOS) expression or an upregulation of its activity was detected, both resulting in an increased NO concentration [12]. On the other hand, a 17β-estradiol-dependent downregulation of nitric oxide synthase activity was identified in the uterus and vagina of rabbits [13].

At the cellular level, an estrogen-mediated inhibition of $Ca^{2+}$ influx via L-type $Ca^{2+}$ channels was described for murine cardiomyoctes [14] and this attenuated glutamate-induced cell death in a ventral spinal motoneuron and neuroblastoma hybrid cell line (VSC 4.1) [15]. Thus, 17β-estradiol was found to fulfill multiple tasks in NO-regulated vertebrate physiology including neuroprotection [16–19]. In addition, clinical and experimental studies have shown that estradiol

plays an important role in inner ear adaptation in respect to sound perception, especially in females [20].

Despite its known gender-dependent alterations in auditory physiology [20], estradiol has more common, gender-independent functions in the inner ear. The 2 known estrogen receptors (ERα and ERβ) were found to be co-expressed in cell nuclei of different cell types in the inner ear in both male and female vertebrates. In the mouse model, both receptors were localized in the cochlea within the nuclei of stria vascularis, in the outer and inner hair cells and in the spiral ganglion cells [21]. Analyzing quantitative differences in immunohistochemical staining intensities, ERα was more dominant in young females than in young males. The expression of both receptors was clearly reduced in old animals of both sexes compared with young ones [21].

Besides receptor-dependent genomic effects, numerous nongenomic effects were identified in different systems summarized by Chow et al. [22]. These effects are faster than the genomic regulated processes and occur within minutes rather than hours [22]. The aim of this animal study was to clarify the still unknown question of whether the application of the neuroprotective agent estradiol can influence NO production within the vertebrate cochlea. Therefore, organ cultures of the organ of Corti and the lateral wall were incubated in culture medium and the NO production was determined in the supernatant by chemiluminescence. Any alterations in NO production and the presumed estrogen-dependent mechanisms are discussed below.

## Materials and Methods
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### Animals
Twenty-four healthy pigmented male guinea pigs (tricolor, Charles River, Sulzfeld, Germany) weighing 200–250 g with good Preyer's reflexes and no evidence of middle ear disease were used in the present study. Up to 6 animals were housed in 1 cage and maintained at a 12:12-h light:dark cycle in the university animal facility. All experiments were conducted in accordance with the German Prevention of Cruelty to Animals Act and were approved by the supervising authorities.

### Cochlea preparation and tissue incubation
Animals were killed by sodium pentobarbital (Narcoren®; Hallbergmoos, Germany; 448 mg/kg body weight) and the bullae were rapidly removed. Thereafter, the organ of Corti and lateral wall were completely removed from the cochlea and incubated separately in culture medium for 6 h at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$, which are the widely used conditions for cell cultures and organ cultures [23, 24]. The culture medium was a Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen; Karlsruhe, Germany), which was modified by adding 1% Glutamax (Invitrogen), 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria), 1% ml sodium pyruvate solution (PAA Laboratories) and 10% inactivated fetal calf serum (PAA Laboratories). Prior to incubation, a volumn of 150 μl 17β-estradiol (Sigma, St Louis, MI, USA) was admitted in 2 final concentrations to cultures of the organ of Corti or the lateral wall of the right ears. The cultures of the left ears were used as controls (group A). Animals of experimental group B (n = 12) received 17β-estradiol in a concentration of 2.5 ng/kg body weight (bw) corresponding to $10^{-8}$ M and animals of group C (n = 12) received 17β-estradiol in a concentration of 2.5 μg/kg bw corresponding to $10^{-5}$ M after recommendation by others [16].

After incubation, the supernatants were shock-frozen in liquid nitrogen and stored at −80°C until analysis.

### NO determination
The formation of $NO_2^-$, the oxidation product of NO, was determined in the supernatants using an NO analyzer (NOA 280i, Sievers Instruments Inc.; Boulder, CO, USA). The oxidation products of NO were measured using a reaction tank containing 2 ml of 0.1 M potassium iodide (Merck; Darmstadt, Germany) and 16 ml of glacial acetic acid (Roth; Karlsruhe, Germany). The reaction of NO with ozone led to the emission of red light and was in direct proportion to the $NO_2^-$ concentration in the samples analyzed. The NO concentration was determined for the organ of Corti and the lateral wall of untreated controls (group A) after adding a 17β-estradiol concentration of $10^{-8}$ M (group B) or a concentration of $10^{-5}$ M (group C).

The influence of NO donors and NOS inhibitors on the NO production in the system used was tested by incubating organ cultures with 3 different substances: the NO donor (Z)-1-2[2-aminoethyl)-N-(2-ammonioethyl1)amino]diazen-1-ium-1,2-diolate] (DETA NONOate, Alexis Biochemicals; Grünberg, Germany) given in 2 concentrations, the NOS-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Alexis Biochemicals), and the NOS-inhibitor N^G-monomethyl-L-arginine monoacetate (L-NMMA; Calbiochem Merck Biosciences; Schwalbach, Germany). In detail, the organ of Corti and the lateral wall of 4 ears in each case were incubated with 50 μM DETA and 500 μM DETA solutions demonstrating NO upregulation (positive controls) and with 500 μM L-NAME and 200 μM L-NMMA demonstrating NO downregulation (negative controls).

### Electron microscopy
Six hours after incubation in cell culture medium, the cochlear tissues were taken out and fixed for at least 12 h in a mixture of 4% freshly made paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid dissolved in 0.1 M sodium phosphate buffer (PBS, pH 7.4). Subsequently, the tissues were dehydrated and embedded in the hydrophilic methacrylate resin, London Resin (LR) White. After hardening the resin for 3 days at 60°C, the samples were cut with a microtome (Ultracut, Reichert-Jung/Leica) and placed on nickel grids. Staining was performed with uranyl acetate and the cell morphology was analyzed by a ZEISS 906 electron microscope (Oberkochen, Germany).

### Statistical approaches
We used a mixed linear model to analyze the association of estradiol treatment and $NO_2^-$ production in the organ of Corti and the lateral wall. Animal effect was considered as a random effect, thereby taking possible dependencies due to repeated use of animals into account. The mean values and the standard deviations (SD) are presented in the corresponding graphs and in the text; the p-values are included in the text. All tests were used in an explorative sense and therefore p-values of these tests must be considered descriptive. Statistical analyses were performed using SAS 9.2 (SAS Institute, Inc., Cary, NC, USA) and the mixed linear model was fitted using PROC MIXED.

We investigated the association of $NO_2^-$ in the organ of Corti and the lateral wall for each of the treatment groups. $NO_2^-$ values were in the "low" group if their values were closer to the minimum than to the maximum; otherwise they were in the "high" group. The measurements are displayed in a scatter plot below and the lines separating "low" and "high" are shown.

## Results
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### Cell morphology

The electron microscopic analysis revealed a well-preserved cell morphology after the 6 h incubation period (● **Fig. 1**). In the extracted tissue of the organ of Corti, the Hensen cells could be clearly identified because of their marginal position and their specific cellular morphology. The microscopic fine structure revealed an undamaged cell membrane and there was no evidence for apoptotic or necrotic processes or vacuolization (● **Fig. 1**). The partly-cut microvilli that protrude into the endolymph of the Scala media could be seen as well as the homologous tight junctions at the basolateral cell-side (● **Fig. 1**).

### Nitric oxide content in the cochlea

The $NO_2^-$ production was lowered by 17β-estradiol in cultures of both the organ of Corti and the lateral wall (● **Fig. 2**). In the organ of Corti, 17β-estradiol reduced the mean $NO_2^-$ production in a dose-dependent manner by 43 % or 46 %, respectively, compared with untreated controls. In the lateral wall, 17β-estradiol led to an $NO_2^-$ reduction of about 24 %.

In detail: In the organ of Corti, a mean $NO_2^-$ content of 293 nM ± 172 nM SD was measured in untreated animals (group A). After 17β-estradiol application, the average $NO_2^-$ content was reduced to 168 nM ± 147 nM SD (group B, estradiol $10^{-8}$ M) or to 158 nM ± 107 nM SD (group C, estradiol $10^{-5}$ M), respectively. This reduction was evident when comparing group A and group B (p = 0.015) and when comparing groups A and C (p = 0.026). The difference between groups B and C was within random variation (p = 0.857).

In the lateral wall, a mean $NO_2^-$ content of 489 nM ± 224 nM SD was determined for the untreated control group (group A). 17β-Estradiol reduced the $NO_2^-$ content to 373 nM ± 160 nM SD (group B, estradiol $10^{-8}$ M) or to 375 nM ± 226 nM SD (group C, estradiol $10^{-5}$ M). However, all differences were within random variaton (p = 0.131 comparing groups A and B, p = 0.125 comparing groups A and C).

The up- and downregulation of NO production within the cochlear tissues was demonstrated using the NO donor DETA and the 2 NOS inhibitors L-NAME and L-NMMA. When DETA was given at a low concentration (● **Fig. 3a**), a significant increase in NO production was detected in the organ of Corti (p = 0.039) compared with controls. No effect was seen in the lateral wall (p = 0.526). After application of the high DETA concentration, the NO production was clearly increased in the organ of Corti and lateral wall (p < 0.001). Comparing the low and high concentrations, significant differences were found for both the organ of Corti and the lateral wall (p < 0.001).

A downregulation of NO production was observed after application of L-NAME or L-NMMA (● **Fig. 3b**). In the organ of Corti, the NO production was statistically significantly reduced by L-NAME (p = 0.028) and by L-NMMA (p = 0.020). Also in cultures of the lateral wall, the NO production was reduced by L-NAME (p = 0.032) and by L-NMMA (p = 0.036). There was no statistically significant difference in the effect on NO production by L-NAME and L-NMMA in either the organ of Corti (p = 0.598) or the lateral wall (p = 0.809).

### Inter-animal variation of NO production

When comparing the individual $NO_2^-$ production of the organ of Corti and the lateral wall of all experimental groups, differences between 17β-estradiol-treated ears and controls became evident
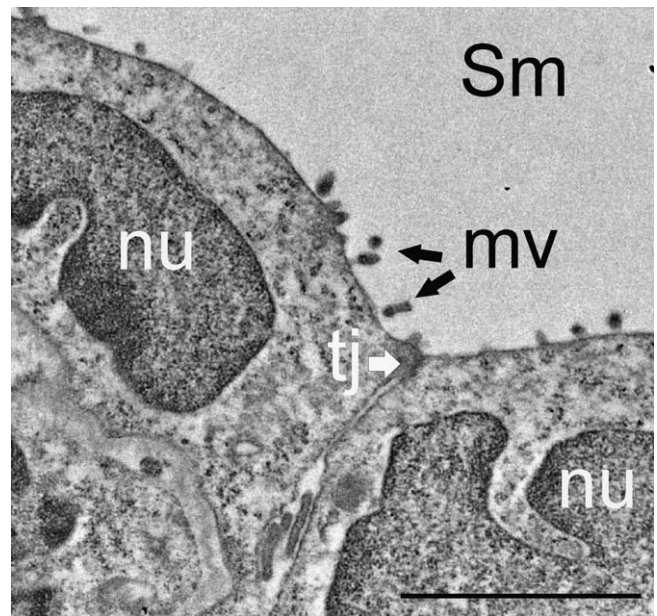


**Fig. 1**  Electron microscopic image of the Hensen cells. 6 h after incubation in culture medium, the cellular morphology is well-preserved without any evidence of necrotic processes. nu: nucleus; mv: microvilli; Sm: Scala media; tj: tight junction. Scale bar = 2 μm.
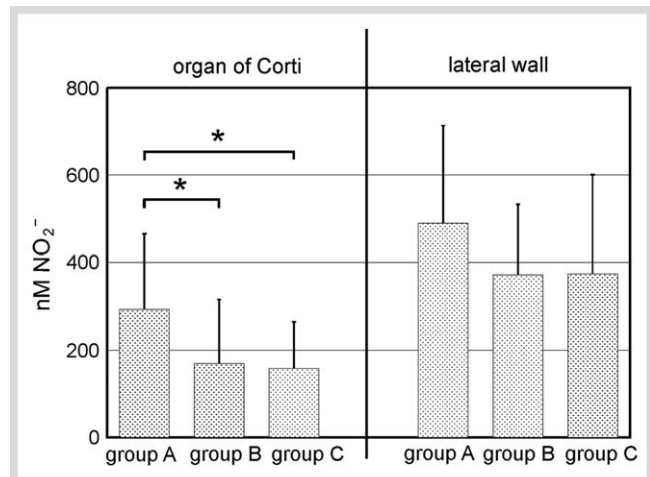


**Fig. 2**  Concentration of $NO_2^-$ in nM (Columns: mean ± SD) measured in the supernatant of cultures of the organ of Corti and the lateral wall. A statistically significant reduction of $NO_2^-$ production by 17β-estradiol was identified in the organ of Corti when comparing the control ears (group A) with group B and group C. Statistically significant changes are marked by the asterisks (*p < 0.05).

by dividing the amount of $NO_2^-$ production into ranges of low and high values (● **Fig. 4**). After application of 17β-estradiol, a reduced $NO_2^-$ production was identified in 21 out of 24 ears (87.5 %). In these ears, the $NO_2^-$ production was below 468 nM in the lateral wall and below 328 nM in the organ of Corti. A higher $NO_2^-$ production was only found in 3 ears (12.5 %). In controls, a reduced $NO_2^-$ production below these specified values was only found in 9 ears (39 %), whereas in 14 ears the $NO_2^-$ production was above these values (61 %).
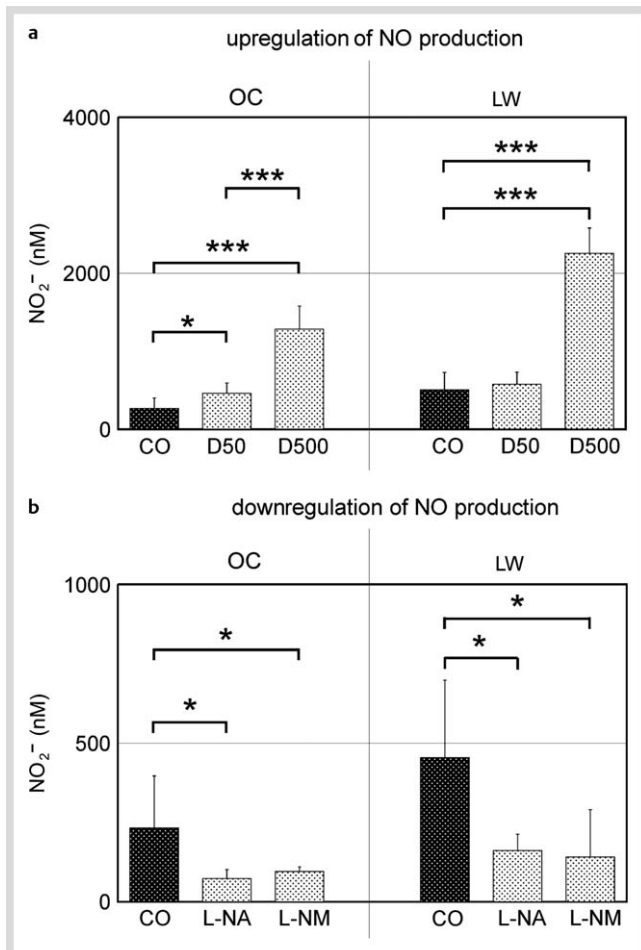
**Fig. 3** Up- and downregulation of NO production in cochlear organ cultures. **a** The NO production was clearly upregulated in the organ of Corti after DETA application using a concentrations of 50 μM and 500 μM and in the lateral wall using a concentration of 500 μM. **b** The application of L-NAME (L-NA) or L-NMMA (L-NM) reduced the NO production in the organ of Corti and in the lateral wall.
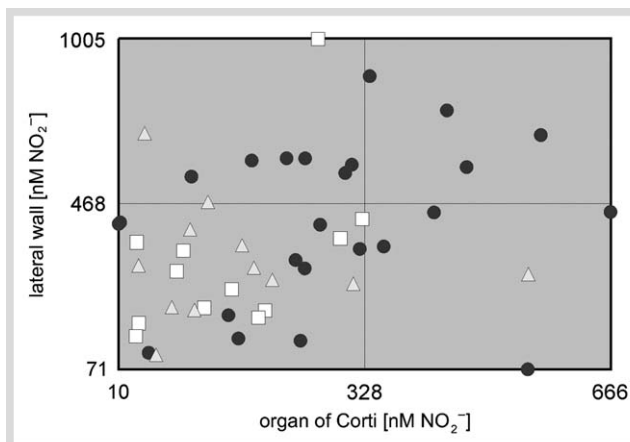


**Fig. 4** Scatter plot of the values of $NO_2^-$ production in the organ of Corti and the lateral wall for each control ear (solid circle) and 17β-estradiol-treated ears of group B (open squares) and group C (triangles). The values were separated by lines into a "low" group if their values were closer to the minimum than to the maximum and otherwise to a "high" group. Nearly all 17β-estradiol-treated ears exhibited lower values, with $NO_2^-$ production below 468 nM in the lateral wall and below 328 nM in the organ of Corti.

## Discussion and Conclusion

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In this experimental animal study, the influence of 17β-estradiol on NO production was tested in organ cultures of the guinea pig cochlea. In addition, the system was evaluated by the NO donor DETA and the NOS inhibitors L-NAME and L-NMMA. The basal mean NO production in cultures of the organ of Corti was clearly reduced by 17β-estradiol, whereas the average NO production in the lateral wall was only marginally lowered. When comparing the values of NO production in both regions within the same cochlea, a clear association becomes evident. Low values of NO production were predominately measured in the groups, which were treated by 17β-estradiol compared with controls. Thus, an NO-reducing effect of 17β-estradiol was clearly demonstrated for the organ of Corti. In both cochlear regions, an increase in NO production was achieved by DETA and a decrease by L-NAME and L-NMMA. Furthermore, it was also shown that no alterations in cellular morphology were seen in the electron microscope after a 6 h incubation period.

Analyzing the average values of the NO production in both the organ of Corti and the lateral wall, it became evident that there was a great variability in each group. Comparable variations in NO production were found in other experiments [25–27], which are based on the natural inter-animal differences in inner ear physiology, especially of guinea pigs [28]. Organ cultures are widely used in inner ear research to identify cellular physiological pathways [23, 24]. Earlier studies already demonstrated that explants of the stria vascularis and spiral ligament dissected from the guinea pig cochlea could be successfully cultivated for several weeks [29]. Unfortunately, the culture technique through extraction of tissues from the bony capsule leads to the loss of structural preservation thus hampering further morphological analyses or histological examinations. Despite this disadvantage, the present study confirmed the integrity of cellular morphology at the electron microscopic level.

17β-Estradiol is a naturally produced hormone and was shown to reduce the inner ear NO production predominantly in the organ of Corti. Comparable effects were demonstrated for other pharmacological substances. A noise-induced increase in NO production could be prevented in both the organ of Corti and the lateral wall when ascorbic acid was supplemented over a period of 7 days prior to noise exposure [25]. In addition, a reduction in tissue-related NO production within organ cultures of both the organ of Corti and the lateral wall was demonstrated by the application of doxycycline [30]. Doxycycline and its related minocycline both belong to the semi-synthetic second-generation tetracyclines, which possess anti-inflammatory effects that are completely distinct from their antimicrobial actions [31]. The comprehensive NO reduction was comparable to the effect of the nitric oxide synthase (NOS) inhibitors $N^G$-Nitro-L-arginine methyl ester (L-NAME) and NG-monomethyl-L-arginine monoacetate (L-NMMA) [30]. An increase in NO production was measured in the lateral wall after intratympanic application of gentamicin, an aminoglycoside, which is commonly used in the treatment of Ménière's disease [32]. This alteration was exclusively restricted to the lateral wall, whereas the NO production in the organ of Corti remained nearly unaltered [26]. Thus, the various findings demonstrate that NO production in the organ of Corti and the lateral wall can be differently influenced by various stimuli resulting in an increase or decrease respectively. The comparable analysis by the NO donor DETA or the NOS inhibitors L-NAME and L-NMMA, respectively, confirmed the ability to

selectively influence the NO production in cochlear tissues, the organ of Corti and the lateral wall.

The adequate regulation of NO amount is important for the precise controlling of physiological pathways [33]. Generally, the reduction of NO production by 17β-estradiol in the organ of Corti could be evoked by 2 mechanisms – a spatial-specific downregulation of the constitutively expressed NOS isoforms and/or a reduction of its activity via blockage of $Ca^{2+}$ transporters. To the best of our knowledge there is currently no report about the direct downregulation in the expression of eNOS or the neuronal nitric oxide synthase (nNOS) by 17β-estradiol. It is commonly known that the activity of the constitutive NOS isoforms, the eNOS and the nNOS, are dependent on intracellular $Ca^{2+}$ concentration. Therefore, a 17β-estradiol-mediated downregulation of NOS activity might exist in the organ of Corti as identified so far in the uterus and vagina in rabbits [13].

Two different $Ca^{2+}$ transporters are generally involved in the blockage process – the $Ca^{2+}$ channels and the $Na^{+}$-$Ca^{2+}$ exchanger. A 17β-estradiol-dependent blockage of L-type $Ca^{2+}$ channels was reported for murine cardiomyoctes [14] and VSC 4.1 cell line [15]. It is assumed that 17β-estradiol leads to a direct change of the pore conformation close to the external mouth of the L-type channels, a closure of the channels in their open state and/or modulation of channel function by estrogen-induced phosphorylation [34]. In the organ of Corti, $Ca^{2+}$ channels were identified in sensory and supporting cells with variable expression patterns [35–38]. In the lateral wall, $Ca^{2+}$ channels were detected so far in the marginal cells [37,39].

A 17β-estradiol-dependent influence on the $Na^{+}$-$Ca^{2+}$ exchanger was reported for mouse myocytes [40]. Furthermore, 17β-estradiol produced an increase in the $Na^{+}$-$Ca^{2+}$ exchanger-mediated inward current and a decrease in the outward current in rat cultured primary cortical neurons. The blockade resulted in a decreased $Ca^{2+}$ influx and increased $Ca^{2+}$ efflux [41]. In the vertebrate cochlea, the $Na^{+}$-$Ca^{2+}$ exchanger was identified in the outer hair cells [42].

From the present state of knowledge, it is evident that there is a quantitative divergence in the amount of calcium transport systems between the organ of Corti and the lateral wall. Because of that, it is hypothesized that the significant NO downregulation in the organ of Corti is primarily based on the higher number of $Ca^{2+}$ transporters, which became blocked by 17β-estradiol.

## Acknowledgements
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## Conflict of Interest
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The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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