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Neuronal erythropoietin overexpression is protective against kanamycin-induced hearing loss in mice

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Highlights

- The protective effect of erythropoietin against aminoglycoside-induced hearing loss was studied in mice
- Neuronal erythropoietin overexpression protected against aminoglycoside-induced hearing loss
- Neuronal erythropoietin overexpression reduced hair cell and spiral ganglion neuron loss

Abstract

Aminoglycosides have detrimental effects on the hair cells of the inner ear, yet these agents indisputably are one of the cornerstones in antibiotic therapy. Hence, there is a demand for strategies to prevent aminoglycoside-induced ototoxicity, which are not available today. In vitro data suggests that the pleiotropic growth factor erythropoietin (EPO) is neuroprotective against aminoglycoside-induced hair cell loss.Here, we use a mouse model with EPO-overexpression in neuronal tissue to evaluate whether EPO could also in vivo protect from aminoglycosideinduced hearing loss. Auditory brainstem response (ABR) thresholds were measured in 12-weeks-old mice before and after treatment with kanamycin for 15 days, which resulted in both C57BL/6 and EPO-transgenic animals in a high-frequency hearing loss. However, ABR threshold shifts in EPOtransgenic mice were significantly lower than in C57BL/6 mice (mean difference in ABR threshold shift 13.6 dB at 32 kHz, 95 % CI 3.8 to 23.4 dB, p = 0.003). Correspondingly, quantification of hair cells and spiral ganglion neurons by immunofluorescence revealed that EPO-transgenic mice had a significantly lower hair cell and spiral ganglion neuron loss than C57BL/6 mice. In conclusion, neuronal overexpression of EPO is protective against aminoglycoside-induce hearing loss, which is in accordance with its known neuroprotective effects in other organs, such as the eye or the brain.

Keywords: EPO; neuroprotection; hair cells; spiral ganglion neurons; kanamycin; aminoglycoside-induced hearing loss; ototoxicity; cochleotoxicity; ABR

1. INTRODUCTION

Ototoxicity (i.e., cochleo-vestibular toxicity) is one of the most common causes of sensorineural hearing loss and bilateral vestibulopathy [1]. Over 100 medications are known to be ototoxic, e.g. aminoglycosides, anti-neoplastic agents, salicylates, loop diuretics, sildenafil, or organo- and heavy metals [2–4]. Aminoglycosides are oligosaccharides that act as broad-spectrum antibiotics used in the treatment of severe sepsis or pulmonary *Pseudomonas* infections in cystic fibrosis patients [3]. However, ototoxicity and nephrotoxicity after systemic administration often limit the duration of

aminoglycoside therapy. These adverse effects occur in 10 to 20 % of patients and are dependent on the dose, aminoglycoside agent and length of administration [5, 6]. Because of this, strategies to prevent or reverse aminoglycoside-induced ototoxicity are of great interest.

One of the best-studied preventive strategies against aminoglycoside-induced ototoxicity is pharmacological protection, including the application of antioxidants such as acetylsalicylic acid, iron chelators and anti-free radical agents – yet, none of these compounds have reached routine clinical application [7, 8]. However, even though protection from aminoglycoside-induced ototoxicity has been demonstrated in animals and seems to be feasible in the near future, there is currently no clinically applicable solution to overcome this issue.

Erythropoietin (EPO) is primarily known as a hematopoietic growth factor and is routinely used to treat anemia in chronic kidney disease. Advances in understanding EPO's ability to inhibit cell death along with the discovery of EPO and its receptor (EpoR) in the nervous system have led to research identifying its neuroprotective potential. In fact, multiple mechanisms contribute to the neuroprotective effects of EPO, including inhibition of apoptosis, reduction of inflammatory reactions and direct antioxidant effects [8, 9]. In animal models, EPO was protective against light-induced retinal degeneration and otoprotective in new-born hypoxic-ischemic encephalopathy [10, 11]. In clinical trials, EPO was found to improve the neurodevelopmental outcome in preterm infants and to improve neurological outcomes in adults with middle cerebral artery stroke [12, 13].

EPO and the EpoR are present in various cell types of the inner ear including a majority of spiral ganglion cells and hair cells [14, 15]. Additionally, functional effects of EPO on several cell types of the inner ear could be demonstrated, supporting the finding of the presence of EpoR. EPO was partially protective against gentamicin- and ischemia-induced cochlear hair cell loss in cochlear explant cultures [15, 16]. EPO was also protective against cisplatin-induced hearing loss as well as hearing loss after acoustic overstimulation [17, 18]. Moreover, administration of EPO increased hearing preservation after cochlear implantation in guinea pigs [19]. Also, EPO significantly decreased cochlear hair cell loss and auditory brainstem response (ABR) thresholds in a mouse model of progressive hearing loss [20]. Lastly, the onset of age-related hearing loss was inhibited in a transgenic mouse overexpressing EPO exclusively in neuronal tissues [21].

Although EPO was shown to exert protective effects against aminoglycoside-induced hair cell damage *in vitro* [15], it is currently unknown whether this is reflected on a functional level, i.e. if EPO can prevent aminoglycoside-induced hearing loss. Since both auditory hair cells and spiral ganglion cells are affected by aminoglycosides, and EpoR was detected in both cell types, targeting EPO and the EpoR may be a promising approach to counteract the cochleotoxic effects of aminoglycosides on the inner ear. In this study, we hypothesized that EPO has a protective effect against aminoglycoside-induced hearing loss *in vivo*.

2. MATERIALS AND METHODS

2.1 Animals

All animal experiments were performed according to Swiss Animal Welfare laws and were carried out according to an animal research protocol approved by the local veterinary authorities (permission number ZH269/16, Kantonales Veterinäramt, Zurich, Switzerland). Female mice aged 12 weeks were used. C57BL/6J-Crl1 wild-type mice (hereinafter referred to as C57BL/6) were purchased from Charles River. Female EPO-transgenic mice TgN(PDGFBEPO)322ZbZ (hereinafter referred to as tg21) were bred in-house [12]. Tg21 mice have a fourfold increase of EPO protein levels restricted to neuronal tissue while the hematocrit, EPO plasma levels and white blood cell count are at normal levels [12]. All animals were kept in individually ventilated cages at an in-house animal facility with unlimited access to a standard chow diet and water under a standard 12 h light/dark cycle.

2.2 Kanamycin administration

An established protocol to induce ototoxic damage in mice by use of the aminoglycoside kanamycin was applied (Fig. 1A) [22]. Kanamycin sulfate containing 784 µg kanamycin base per mg was purchased from Sigma Aldrich (#60615, lot 076M4071V; Buchs, Switzerland.). Kanamycin was dissolved in physiological saline to obtain the desired concentration of 40 mg/ml so that a dose of 800 mg of kanamycin base/kg body weight was obtained by injecting 0.02 ml/g body weight. Mice

received body-weight-adjusted dosages of kanamycin subcutaneously (s.c.) twice a day at intervals of 12 ± 1 h for 15 days.

2.3 Auditory brainstem response measurements

ABR measurements were carried out before and 4 weeks after the first day of kanamycin administration as it has been shown ABR thresholds reach a plateau 3 to 4 weeks after ototoxic injury by kanamycin [22]. Animals were anaesthetized with a mixture of xylazine (12 mg/kg) and ketamine (80 mg/kg), both applied intraperitoneally. ABR responses were recorded with a TDT System 3 auditory-evoked potential workstation running BioSig software (Tucker-Davis Technologies, Alachua, FL, USA). Stainless-steel needle electrodes were placed subcutaneously over the vertex (non-inverting input, channel 1), posterior to the stimulated ear (inverting input, reference) and the lower back near the base of the tail (ground). ABRs were elicited with clicks and tone bursts at 4, 8, 12, 16, 24 and 32 kHz (80 to 0 decibel [dB] sound pressure level [SPL] in 5-dB intervals, 5-ms duration, 2-ms rise/fall time, rate 21/s). Tone bursts and clicks were generated digitally using a multifunction processor (RX6; Tucker-Davis Technologies; 200 kHz sampling rate) and fed to a programmable attenuator (PA5; Tucker-Davis Technologies), an electrostatic speaker driver (ED1; Tucker-Davis Technologies), and an electrostatic loudspeaker (EC1; Tucker-Davis Technologies). The electrode outputs were delivered to a pre-amplifier/base station (RA4LI and RA4PA; Tucker-Davis Technologies). Prior to each ABR measurement, the system was calibrated for click and pure tone stimuli. The ABR threshold was defined as the lowest stimulus that allowed a reliable elicitation of a positive waveform in the evoked response tracing.

2.4 Immunohistochemistry

After the second ABR measurement, mice were sacrificed by cervical dislocation during anesthesia, the temporal bones were harvested and fixed in 4 % high-quality, methanol-free formaldehyde (15710-S, Lucerna-Chem AG, Luzern, Switzerland) in 10 mM phosphate-buffered saline (PBS) pH 7.4 for 4 hours at room temperature. Then, the temporal bones were decalcified with 120 mM EDTA

in 10 mM PBS for 5 days, during which the EDTA solution was changed daily. The right cochleae were used for spiral ganglion histology and the left cochleae were used for hair cell quantification.

For hair cell quantification, the cochlea was micro-dissected into the three cochlear turns, and any remains of the otic capsule, spiral ligament and Reissner's membrane were removed [23]. After applying a blocking solution (1 % Triton X-100, 1 % bovine serum albumin, 10 % normal goat serum, diluted in 10 mM PBS pH 7.4) for 30 minutes, the cochlear turns were stained with Alexa Fluor 568-conjugated phalloidin (1:400; A12380, Thermo Fischer, Waltham, MA, USA) for 20 minutes and mounted with an aqueous mounting medium (Vectashield, H-1200, Vector Laboratories). All steps were performed at room temperature.

For spiral ganglion histology, the specimens were embedded in paraffin. Then, 4-µm sections were cut and collected on SuperFrost Plus slides (Fisher Scientific AG, Reinach, Switzerland). Sections were de-paraffinized in Histo-Clear (National Diagnostics, Atlanta, USA), rehydrated in a graded series of ethanol solutions (100 %, 95 %, 70 %, and 50 %), and rinsed in tap water. Heat-induced epitope retrieval (HIER) was performed in sodium citrate (10 mM) at pH 6.0 for 10 minutes in a microwave at maximum intensity. Next, unspecific binding was blocked with 1 % normal horse serum (NHS) for 15 minutes. Then, the sections were incubated with a neuron-specific primary monoclonal mouse antibody against β-III Tubulin (TuJ1), diluted 1:100 (MAB1195, R&D Systems, Minneapolis, MN, USA). After washing for 5 minutes in PBS, the sections were incubated with a secondary, biotinylated antibody raised in donkey and directed against mouse (1:400; 715-065-151, Milan Analytica AG). After washing for 5 minutes, the sections were then incubated with avidin-biotin-HRP complex (Vectastain ABC HRP Kit, Vector Laboratories, Burlingame, CA, USA). The sections were washed again for 5 minutes in PBS and visualization was performed with Alexa Fluor 488-conjugated streptavidin (1:400; 016-540-084, Milan Analytica AG, Rheinfelden, Switzerland). Finally, the sections were mounted with an aqueous mounting medium containing DAPI (Vectashield, H-1200, Vector Laboratories). All incubation steps were performed at room temperature.

Images were acquired using a Leica SP5 microscope (Leica Camera AG, Wetzlar, Germany) using a 20x immersion objective. Confocal Z-stacks were projected into a single image by a maximal intensity projection using open source Fiji software (version 1.0 for Mac OS X) [24].

2.5 Quantification of spiral ganglion neurons and hair cells

For each cochlear turn, the total number of F-actin-positive hair cells was counted in three to four randomly selected 200-µm fields for each of the three cochlear turns. Counting of hair cells was performed on three to four mice of each group.

Three to four non-consecutive midmodiolar sections of three animals of each group were used for spiral ganglion neuron quantification. First, the area of the spiral ganglion (i.e., Rosenthal's canal) was measured. Next, the amount of TuJ1-positive ($TuJ1^+$) cells with a clearly distinguishable DAPI-stained nucleus were counted in the respective area. Area measurements and counting were performed by using Fiji software.

2.6 Statistical analysis

Statistical analyses were performed using Prism for Apple Macintosh, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical tests used, error bars, n values, and type of replicates for experiments are defined in the respective figure legends. The significance level was set to $p < 0.05$.

3. RESULTS

3.1 Drug administration

All mice tolerated the kanamycin treatment regimen well. During the treatment, none of the mice lost more than 20 % of their initial body weight obtained before the first injection. However, two animals died during kanamycin administration, and one animal died during the early recovery phase from anesthesia after the second ABR measurement, accounting for the final sample sizes. A *post mortem* examination was performed by a certified animal pathologist of the Laboratory for Animal Model Pathology of the University of Zurich (Switzerland) on the two mice that died during kanamycin administration. Both of these deaths could be ascribed to bleeding secondary to the injection procedure. The first mouse (tg21) was found to have suffered from a cardiogenic shock due to

iatrogenic hemopericardium. The second mouse (C57BL/6) died from excessive subcutaneous bleeding following an injection. It was suspected that bleeding might be secondary to the kanamycin-related necrosuppurative inflammation observed at the injection sites. Alteration of clotting times might also be speculated as a possible contributing factor [25]. In both mice examined, histological examination revealed minimal bilateral renal tubular degeneration, which, based on the low severity, was unlikely to have had an impact on the health of the mice.

3.2 Neuronal EPO-overexpression is protective against kanamycin-induced hearing loss as assessed by auditory brainstem measurements

Before treatment, ABR waveforms in response to click stimuli were elicited at similar thresholds in all groups (Fig. 2A). After treatment, C57BL/6 control animals injected with saline exhibited stable thresholds whereas in both C57BL/6 and tg21 animals injected with kanamycin, ABR waveforms were elicited with higher thresholds at around 50 dB SPL in C57BL/6 animals and at 40 dB SPL in tg21 animals (Fig. 2B). Comparing ABR thresholds before and after treatment, C57BL/6 mice treated with saline showed no statistically significant ABR threshold differences in response to click and tone burst stimuli (Fig. 3A). In C57BL/6 mice treated with kanamycin, we found statistically significant differences before and after treatment for the click ABR and all tone burst ABRs except at 4 kHz (Fig. 3B). There was a predominantly high-frequency hearing loss with the highest threshold difference at 32 kHz (mean difference 26.4 dB, 95 % confidence interval [CI] 16.6 to 35.5 dB, $p < 0.0001$). In tg21 mice treated with kanamycin, there was a mild high-frequency hearing loss with only tone burst ABRs at 24 kHz and 32 kHz resulting in statistically significant differences (Fig. 3C; mean difference 11.9 dB, 95 % CI 0.9 to 22.9 dB, $p = 0.026$ at 24 kHz and mean difference 13.7 dB, 95 % CI 2.7 to 24.6 dB, $p = 0.006$ at 32 kHz).

We then compared ABR threshold shifts among the three groups of animals. ABR threshold shifts of C57BL/6 mice either treated with saline or kanamycin were statistically significantly different for the click ABR and all tone burst ABRs except at the lowest frequency tested, i.e., 4 kHz (Fig. 3D). For these two groups, the greatest difference in threshold shifts was observed at 32 kHz (mean difference 23.6 dB, 95 % CI 12.4 to 34.8 dB, $p < 0.0001$). Moreover, comparing ABR threshold shifts of C57BL/6

mice and tg21 mice both treated with kanamycin were significantly different for the click ABR and all tone burst ABRs except at 4 and 8 kHz. Again, the greatest difference in threshold shift was observed at 32 kHz (mean difference 13.6 dB, 95 % CI 3.8 to 23.4 dB, p = 0.003). However, the differences were smaller than between C57BL/6 mice either treated with saline or kanamycin. No statistically significant differences between click and tone burst ABR threshold shifts of C57BL/6 mice treated with saline and tg21 mice treated with kanamycin were found.

Next, we analyzed ABR wave latencies (I to V) and interwave latencies in click ABR waveforms at 80 dB SPL. No statistically significant differences were observed among the latencies of the three groups, comparing pre- and post-treatment latencies for each wave (Fig. 3E). Furthermore, no statistically significant differences were observed among the interwave latencies I-III, III-V, I-IV and I-V of the three groups comparing pre- and post-treatment interwave latencies (Fig. 3F).

3.3 Neuronal EPO-overexpression is protective against kanamycin-induced outer hair cell and spiral ganglion neuron loss

In order to evaluate the histomorphological damage of the cochlea underlying the ABR threshold elevation observed, the number of cochlear hair cells and the density of SGNs were analyzed by immunofluorescence (Fig. 4).

To quantify the amount of surviving cochlear hair cells, whole mount samples were stained with Alexa Fluor 568-conjugated phalloidin labelling filamentous actin (F-actin), which is present in stereocilia, the cuticular plate and the infracuticular F-actin network [26]. Quantifying the number of outer hair cells (OHCs), we found a loss of OHCs predominantly in the basal turn in C57BL/6 and tg21 animals treated with kanamycin. In the basal turn, C57BL/6 mice treated with saline had a statistically significantly higher number of OHCs than C57BL/6 and tg21 mice treated with kanamycin (Fig. 5A; mean difference 42.8 OHCs/200µm, 95 % CI 36.8 to 48.7 OHCs/200µm, $p < 0.0001$ and mean difference 13.2 OHCs/200µm, 95 % CI 7.3 to 19.2 OHCs/200µm, $p < 0.0001$, respectively). Moreover, tg21 mice treated with kanamycin also exhibited a statistically significantly higher number of OHCs in the basal turn than C57BL/6 mice treated with kanamycin (mean difference 29.6 OHCs/200µm, 95 % CI 23.6 to 35.5 OHCs/200µm, $p < 0.0001$). In the middle turn, C57BL/6 animals treated with

saline and tg21 animals treated with kanamycin showed a statistically significantly higher number of OHCs than C57BL/6 animals treated with kanamycin (mean difference 20.8 OHCs/200µm, 95 % CI 14.9 to 26.8 OHCs/200µm, $p < 0.0001$ and mean difference 15.7 OHCs/200µm, 95 % CI 9.8 to 21.7 OHCs/200µm, $p < 0.0001$), but no statistically significant difference was found between the first two groups, i.e. C57BL/6 animals treated with saline and tg21 animals treated with kanamycin. No statistically significant difference was found among the number of OHCs of the apical turn. In contrast to the OHC loss, no loss of inner hair cells (IHCs) was found in the basal, middle or apical cochlear turn in any of the three groups (Fig. 5B).

Next, we quantified the amount of $TuJ1^+$ SGNs per mm^2 of Rosenthal's canal (i.e., SGN density). In the basal turn, C57BL/6 mice treated with saline had a statistically significantly higher SGN density than C57BL/6 mice treated with kanamycin (Fig. 5C; mean difference 1031 $TuJ1^+$ cells/mm^2, 95 % CI 548 to 1514 $TuJ1^+$ cells/mm^2, $p < 0.0001$). The Tg21 mice treated with kanamycin also exhibited a statistically significantly lower SGN density than C57BL/6 mice treated with saline (mean difference 646 $TuJ1^+$ cells/mm^2, 95 % CI 163 to 1129 $TuJ1^+$ cells/mm^2, $p = 0.006$). In the middle turn, C57BL/6 mice treated with saline as well as tg21 mice treated with kanamycin had a statistically significantly higher SGN density than C57BL/6 mice treated with kanamycin (mean difference 619 $TuJ1^+$ cells/mm^2, 95 % CI 136 to 1102 $TuJ1^+$ cells/mm^2, $p = 0.009$ and 596 $TuJ1^+$ cells/mm^2, 95 % CI 113 to 1079, $p = 0.012$, respectively). No statistically significant difference was found between the SGN density of C57BL/6 mice treated with saline and tg21 mice treated with kanamycin. Furthermore, no statistically significant difference was found among the SGN densities of the apical turn.

4. DISCUSSION

Ototoxicity is one of the most common causes of sensorineural hearing loss and bilateral vestibulopathy, but effective strategies to prevent ototoxic side effects of medications are not yet available. *In vitro* data suggest that the pleiotropic growth factor EPO is neuroprotective against aminoglycoside-induced hair cell loss [15]. Therefore, we hypothesized that EPO may also prevent aminoglycoside-induced hearing loss *in vivo*. In the present study, we have determined that EPO

overexpression restricted to neuronal tissue in tg21 mice protects against kanamycin-induced hearing loss. Using ABR as the primary outcome, we found significantly lower threshold shifts in tg21 mice after treatment with kanamycin for 15 days as compared to wild-type C57BL/6 mice. Furthermore, tg21 showed less OHC and SGN loss as compared to wild-type C57BL/6 mice.

In this study, we used an established mouse model of ototoxicity based on administering kanamycin for 15 days. Click and tone burst ABRs were obtained both before as well as 4 weeks after treatment. Moreover, histology was performed after the last measurement. Most importantly, we demonstrate that the characteristic aminoglycoside-induced high-frequency hearing loss caused by the kanamycin treatment is substantially lower in tg21 mice than in wild-type C57BL/6 mice. While the latter experienced a threshold shift of up to 20 dB in the lower frequencies and up to 30 dB in the higher frequencies, tg21 mice only suffered from a 15-dB threshold shift at the highest frequencies. The threshold shifts of the C57BL/6 mice are in accordance with the published data on this mouse model of ototoxicity [22].

In accordance with the functional results, we found an OHC and SGN loss predominantly in the basal turn of the cochlea. In aminoglycoside-induced hearing loss, hair cell and spiral ganglion loss are the characteristic histopathological findings, which are not related and may occur independently [27, 28]. Hair cell and spiral ganglion loss starts in the basal turn, and of the two types of hair cells, the OHCs are affected first [29]. This is in line with the histological results of our study. In C57BL/6 mice treated with kanamycin, around 40 % of the SGNs of the basal turn were lost compared to C57BL/6 mice treated with saline. In contrast, tg21 mice treated with kanamycin only showed a 25 % loss of SGNs. Regarding OHCs, less than 40 % of OHCs in C57BL/6 mice treated with kanamycin survived compared to C57BL/6 mice treated with saline, while around 80 % of OHCs survived in tg21 mice treated with kanamycin. We observed slightly higher survival of OHCs compared to the published original data on the mouse model of ototoxicity used in the present study [22]. One reason may be the older age of the animals used in our study, rendering the mice less susceptible to ototoxic damage [30, 31]. Our results are in agreement with the ABR threshold shifts obtained, which were also slightly smaller than originally published for this mouse model [22]. The

EPO-mediated protection of neurosensory cells in the inner ear against kanamycin is in line with other studies demonstrating a beneficial effect of EPO against deleterious impacts on the inner ear. Early *in vitro* data showed a partial protection against gentamicin- and ischemia-induced cochlear hair cell loss [15, 16]. Initial studies reporting functional data regarding hearing ability were able to demonstrate a protective effect of EPO against cisplatin-induced ototoxicity [17]. While the latter study showed a full protective effect of EPO administered 24 hours before and 30 minutes after cisplatin injection, in our study the tg21 animal still exhibited a slight high-frequency hearing loss after the kanamycin treatment. Moreover, in a mouse model of progressive hearing loss, EPO administered every second day for 7 weeks significantly decreased ABR threshold shifts by 20 to 30 dB [20]. Our data show a comparable threshold shift decrease of around 15 dB in the higher frequencies. However, these comparisons should be interpreted with caution since these reported studies (i) investigated slightly different paradigms than we did and (ii) used direct administration of EPO rather than an animal model overexpressing EPO, as in our study. Yet, we previously showed for the tg21 mouse that development of age-related hearing loss was significantly reduced as compared to C57BL/6 mice, which was also reflected by a reduced loss of SGN and hair cells [21].

The current study was not set up to address the molecular mechanisms underlying the protective effect of neuronal EPO overexpression on aminoglycoside-induced hearing loss directly because many previously published studies have convincingly explained the protective mechanisms of EPO signaling. First, the presence of EpoR that is crucial for an effect of EPO on neurosensory cells of the inner ear has been confirmed by immunofluorescence and later by mRNA detection in spiral ganglion cells as well as hair cells [14, 15]. In tg21 mice, the presence of the EpoR has been confirmed by PCR in the organ of Corti, in SGN and the stria vascularis [21]. Binding of EPO to the EpoR activates several intercellular pathways such as the Jak2/STAT5 signaling cascade, the PI3K/AKT/mTOR pathway, Wnt signaling and the Ras/MAPK pathway, all of which, among other effects, are counteracting apoptosis and have been shown to play a role in Epo-mediated (neuro-)protection and aminoglycoside-induced hair cell death [32–39]. Apoptosis is the major mechanism of hair cell loss in aminoglycoside ototoxicity [1]. Within the hair cell, one of the most important factors promoting apoptosis is the generation of reactive oxygen species, exposing hair

cells to oxidative stress [40]. Interestingly, it has been shown that EPO itself has antioxidant properties as it is able to prevent oxidative damage directly [41]. Regarding SGNs, EPO has been demonstrated to promote neurite outgrowth and to increase survival of isolated SGNs [42, 43]. Finally, it has been shown that after hypoxic injury, there is an increased expression of EPO as well as angiogenic genes [44]. In summary, EPO in the inner ear may protect hair cells and neurons of the spiral ganglion against damage by direct protection against oxidative stress as well as by inhibiting apoptosis.

A limitation of our study might be the use of a model of neuronal EPO overexpression rather than the direct application of EPO. Yet, the latter paradigm is itself associated with several difficulties. It is well known that EPO poorly crosses the blood-brain barrier as well as the blood-retina barrier. Even though perilymph sampling after systemic administration of EPO has revealed a slightly higher bioavailability in the inner ear than in the CSF [19], only 1 % of the total EPO dose applied systemically reaches the CSF [10, 45]. To overcome the problem of low EPO concentrations in the inner ear, local drug delivery strategies may be administered, such as intratympanic or intracochlear delivery [46].

In this study, we demonstrated that overexpression of EPO restricted to neuronal tissue successfully reduces aminoglycoside-induced hearing loss. Our novel finding that EPO may prevent aminoglycoside-induced hearing loss could facilitate new treatments, such as the administration of EPO or analogous agents acting on EPO-associated intracellular signaling pathways. That such a therapeutic strategy might indeed be successful is suggested by the observations that administration of EPO was associated with improved hearing ability in patients on long-term hemodialysis [47]. However, a direct influence of EPO on neuronal structures of the auditory system was not considered; rather, anemia was hypothesized to be a major contributing factor in hearing disorders in patients with end-stage renal failure. Nevertheless, this issue needs further investigation because another study found decreased high-frequency hearing loss if patients with chronic renal failure and low hematocrit were treated with EPO [48]. The authors speculated that this is mainly due to improved oxygen supply of the inner ear, but a direct effect of EPO on the cochlea was also not investigated. However, the use of EPO as an otoprotective agent may be hindered by unacceptable side effects,

in particular because the doses applied for tissue protection exceed hematopoiesis stimulating doses by a tenfold factor [49]. If administered systemically, EPO increases thromboembolic events, has a vasoconstrictive effect and may activate the EpoR on cancer cells [50–52]. This dilemma could be handled with either EPO analogues exerting anti-apoptotic effects without activation of the hematopoietic system or, as stated above, local drug delivery to the inner ear [46, 50].

5. CONCLUSION

We provide evidence that neuronal overexpression of EPO is protective against aminoglycoside-induced hair cell and spiral ganglion cell loss as shown by histology and consequently preserves hearing, as demonstrated by click and tone burst ABR. Therefore, identification of EPO and its associated intracellular pathways as targets protecting against aminoglycoside-induced hair cell death may facilitate the development of novel therapies against hearing loss induced by aminoglycosides or other ototoxic medications.

6. CONTRIBUTIONS

DB, AE, JV and AMN developed the concept and designed experiments; LH assisted in setting up the ABR measurements; DB performed all the experiments and collected the data; MMG assisted with anesthesia and TH assisted with hair cell quantification; DB analyzed the data; DB, JV and AMN interpreted the data; DB prepared the figures; DB drafted the manuscript; MG generously donated the tg21 mice; all authors contributed to revising the article for intellectual content and approved the final manuscript.

CONFLICTS OF INTEREST

The authors have no conflict of interests to declare.

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FIGURE LEGENDS

Figure 1. Schematic diagram describing the experimental procedure and timeline. Wild-type C57BL/6 mice (n = 5) treated with saline for 15 days served as negative control group and wild-type C57BL/6 mice (n = 9) treated with kanamycin for 15 days served as positive control group. EPO-transgenic mice (tg21; n = 8) treated with kanamycin for 15 days constituted the treatment group. Click and tone burst evoked auditory brainstem responses (ABR) were obtained before and 28 days after the start of the treatment. After the second ABR measurement, mice were sacrificed and histological studies were performed.

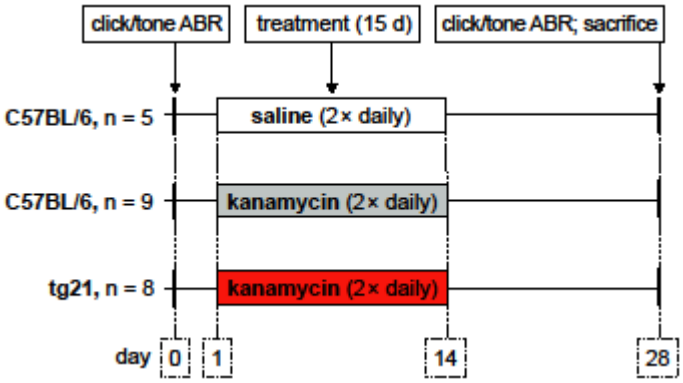

Figure 2. Representative auditory brainstem response recordings in response to click stimuli before (A) and after (B) treatment. The roman numerals label waves I, II, III, IV and V of the auditory brainstem response.

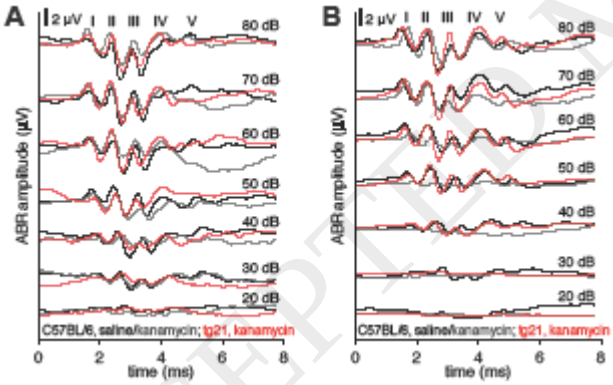

Figure 3. Analysis of auditory function. **(A – C)** Auditory brainstem response thresholds in response to click and tone burst stimuli before and after treatment in the negative control group (A), the positive control group (B) and the treatment group (C). The mean ± SD is shown. A one-way ANOVA with a post-hoc comparison of preselected pairs applying the Sidak correction for multiple comparisons was used. **(D)** Comparison of auditory threshold shifts among the three groups. **(E)** Auditory brainstem response wave latencies in response to a click stimulus at 80 dB SPL. No significant differences were observed among the latencies before and after treatment. **(F)** Auditory brainstem response interwave latencies in response to a click stimulus at 80 dB SPL. No significant differences were observed among the interwave latencies before and after treatment. For D – F, the mean ± SEM is shown. A two-way ANOVA and the Tukey's range test as post-hoc multiple comparison procedure was used.

* p < 0.05, ** p < 0.01, *** p < 0.001, **** < 0.0001, the color of the asterisks indicates the group used for comparison with the group of C57BL/6 animals treated with kanamycin; ABR, auditory brainstem response; dB, decibel; SPL, sound pressure level.

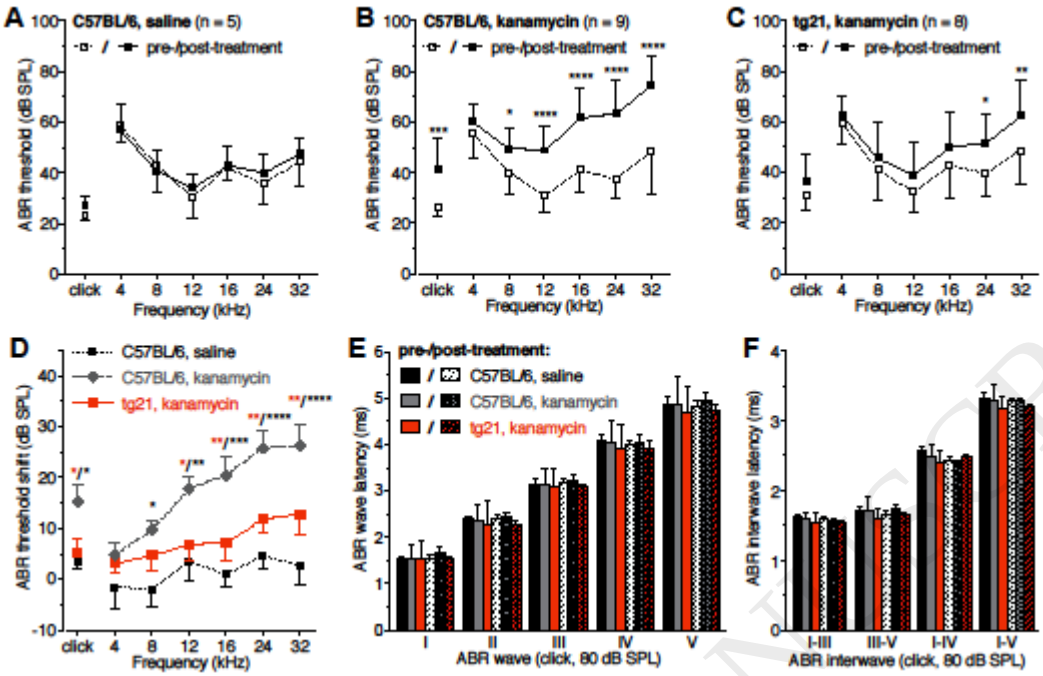

Figure 4. Immunohistological analysis of spiral ganglion neurons and hair cells 28 days after the start of the treatment. **(A – C'')** Representative confocal images of the basal (A – A''), middle (B – B'') and apical (C – C'') turn of cochlear whole mount samples immunostained for F-actin (red) labelling the single row of inner hair cells and the three rows of outer hair cells. Scale bar: A (for A – C''), 25 µm. **(D – D'')** Representative confocal images of the basal spiral ganglion in midmodiolar cross sections immunostained for TuJ1 as a neuronal marker (green) and DAPI labelling cell nuclei (blue). Scale bar: D (for D – D''), 50 µm.

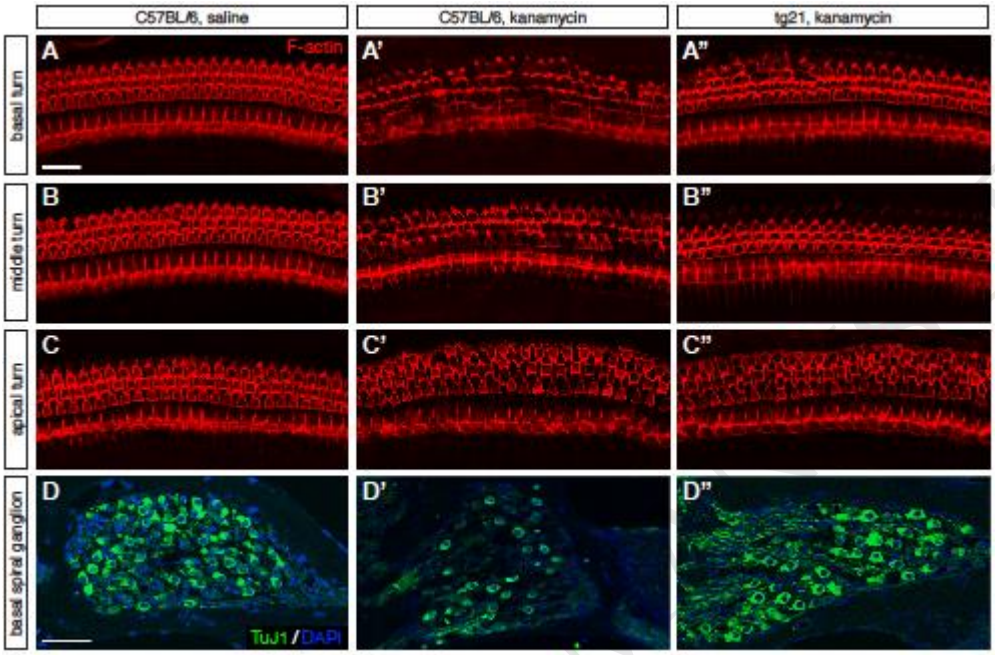

Figure 5. Quantification of intracochlear neurosensory cells by immunofluorescence microscopy analysis. **(A)** Number of outer hair cells per 200 µm cochlear length of the basal, middle and apical turn. **(B)** Number of inner hair cells per 200 µm cochlear length of the basal, middle and apical turn. **(C)** Spiral ganglion neuronal density expressed as TuJ1-positive **(**TuJ1^{+}) cells per mm^{2} of Rosenthal's canal in the basal, middle and apical turn. The mean ± SEM is shown. A two-way ANOVA and the Tukey's range test as post-hoc multiple comparison procedure was used. * $p < 0.05$, ** $p < 0.01$, **** < 0.0001; IHCs, inner hair cells; OHCs, outer hair cells.