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RESEARCH ARTICLE

Early transtympanic administration of rhBDNF exerts a multifaceted neuroprotective effect against cisplatin-induced hearing loss

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Abstract

Background and Purpose: Cisplatin-induced sensorineural hearing loss is a significant clinical challenge. Although the potential effects of brain-derived neurotrophic factor (BDNF) have previously been investigated in some ototoxicity models, its efficacy in cisplatin-induced hearing loss remains uncertain. This study aimed to investigate the therapeutic potential of recombinant human BDNF (rhBDNF) in protecting cells against cisplatin-induced ototoxicity.

Experimental Approach: Using an *in vivo* model of cisplatin-induced hearing loss, we investigated the beneficial effects of transtympanic administration of rhBDNF in a thermogel solution on hearing function and cochlear injury, using electrophysiological, morphological, immunofluorescence and molecular analyses.

Key Results: Our data showed that local rhBDNF treatment counteracted hearing loss in rats receiving cisplatin by preserving synaptic connections in the cochlear epithelium and protecting hair cells (HCs) and spiral ganglion neurons (SGNs) against cisplatin-induced cell death. Specifically, rhBDNF maintains the balance of its receptor levels (pTrkB and p75), boosting TrkB-CREB pro-survival signalling and reducing caspase 3-dependent apoptosis in the cochlea. Additionally, it activates antioxidant mechanisms while inhibiting inflammation and promoting vascular repair.

Abbreviations: 3-NT, 3-nitrotyrosine; ABRs, auditory brainstem responses; CtBP2, C-terminal-binding protein 2; HCs, hair cells; IHCs, inner hair cells; NF200, neurofilament 200; OHCs, outer hair cells; rhBDNF, human recombinant BDNF; RT, room temperature; SGNs, spiral ganglion neurons; SIRT1, sirtuin 1 isoform; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; VEGFA, vascular endothelial growth factor A.

Anna Pisani and Rolando Rolesi contributed equally.

Claudio Grassi and Anna Rita Fetoni share the senior authorship.

Conclusion and Implications: Collectively, we demonstrated that early transtympanic treatment with rhBDNF plays a multifaceted protective role against cisplatin-induced ototoxicity, thus holding promise as a novel potential approach to preserve hearing in adult and paediatric patients undergoing cisplatin-based chemotherapy.

KEYWORDS
cisplatin, ototoxicity, rhBDNF, sensorineural hearing loss

1 | INTRODUCTION

Neurotrophins are a family of neurotrophic factors that play crucial roles in neuronal survival and growth, supporting the differentiation and maturation of neurons in the nervous system (Bathina & Das, 2015). Among neurotrophins, brain-derived neurotrophic factor (**BDNF**) and **neurotrophin-3** have been recognized as key players in the development of the inner ear (Pisani et al., 2023), specifically of the cochlea, and for the maintenance of hearing function throughout life (Fritzsch et al., 2004). The expression of BDNF and its high-affinity receptor, tropomyosin receptor kinase B (**TrkB**), and the low-affinity receptor, **p75^{NTR}**, has been shown in rodents and humans in the inner ear (Wiechers et al., 1999), specifically in cochlear spiral ganglion neurons (SGNs). A fine-tuned balance between TrkB-dependent signalling that promotes cell survival and p75^{NTR}-dependent activation of apoptosis in SGNs has been found to be crucial for the maintenance of normal hearing function (Pisani et al., 2023; Tan & Shepherd, 2006). Considering their functions, BDNF and its receptors have been widely investigated for their potential role in cochlear degeneration and as potential neuroprotective targets against cochlear injuries of different origins (Leake et al., 2020; Ramekers et al., 2012). Both *in vitro* and *in vivo* studies have shown that BDNF can exert beneficial effects on the cochlear neuronal compartment, increasing afferent fibres and synapse number and improving hearing function after noise or ototoxic-induced hearing loss (Leake et al., 2011; Schmidt et al., 2018; Wan et al., 2014; Wise et al., 2005). Ototoxicity is a common adverse event associated with **cisplatin** chemotherapy (Landier, 2016), frequently observed in both adult and paediatric patients (Chattaraj et al., 2023; Fetoni, Ruggiero, et al., 2016; Meijer et al., 2022). Although the exact molecular mechanisms underlying cisplatin ototoxic effects are not fully understood, there is evidence to support the important contributions of DNA damage with the subsequent activation of the caspase-dependent apoptosis signal and the accumulation of reactive oxygen species (ROS), which exert direct cytotoxic effects, and inflammation (Fetoni et al., 2022; Ramkumar et al., 2021; Tang et al., 2021). Pharmacological agents have thus been tested to target these key pathophysiological mechanisms with the aim to protect/recover the function and structure of the primary cochlear structures affected by cisplatin: the organ of Corti, SGNs and *stria vascularis* (Fetoni et al., 2015; Fetoni, Eramo, et al., 2014; Paciello et al., 2020; Van Ruijven et al., 2005). To date, only one drug (sodium thiosulfate) has been approved. Indeed, it has been demonstrated to counteract cisplatin-induced hearing loss in experimental models (Wang et al., 2003) and reduce the risk of ototoxicity associated with cisplatin in paediatric patients (Dhillon, 2023). However, concerns about the potential interference of systemic adjuvant therapies, such as the use of antioxidants and ROS scavengers, with cisplatin's therapeutic efficacy remain to be fully addressed (Chattaraj et al., 2023; Fetoni et al., 2022; Paciello et al., 2020; Wang et al., 2003). In this scenario, BDNF treatment has shown promising efficacy, protecting auditory neurons *in vitro* (Zheng & Gao, 1996), whereas only a few studies have been conducted in *in vivo* models (Blakley et al., 2020; Meen et al., 2009), evaluating the effects of BDNF treatment in rescuing hearing function after the overt onset of cisplatin-induced ototoxicity, and did not investigate the potential mechanisms of the treatment in protecting hearing function and inner ear structure.

Therefore, we investigated the ability of transtympanic administration of human recombinant BDNF (rhBDNF) to protect hearing function and cochlear cells from cisplatin-induced ototoxicity. Our experiments investigated the underlying mechanisms of action and the protective effects of rhBDNF formulation on hair cells (HCs), SGNs and *stria vascularis* by using an experimental setting with

What is already known

- BDNF exerts neuroprotective effects on spiral ganglion neurons.

What does this study add

- Transtympanic administration of rhBDNF exerts a multifaceted protective effect against cisplatin-induced ototoxicity.

What is the clinical significance

- Early rhBDNF treatment is a potential approach to preserve hearing in patients undergoing cisplatin-based chemotherapy.

concomitant administration of cisplatin and rhBDNF, an approach that could be potentially translated into clinical practice with patients undergoing cisplatin chemotherapy.

2 | METHODS

2.1 | Experimental animal model

Two-month-old male Wistar rats from the Catholic University Laboratories were used in this study.

Female rats were not used because the ovarian cyclicity of sex hormones and glucocorticoids in females can potentially increase stress responses and inflammatory reactions, which could interfere with the interpretation of the effects of BDNF (Herman et al., 2016; Novais et al., 2017).

Animals were housed two per cage in the Catholic University animal facility at a controlled temperature (22–23°C) and constant humidity (60%–75%), under a 12-h light/dark cycle, with food (Mucedola 4RF21, Italy) and water *ad libitum*.

First, to select the best rhBDNF dosage against cisplatin ototoxicity, we performed a dose–response curve in 12 animals and evaluated auditory function. We treated animals with cisplatin (12 mg·kg^{-1}, i.p.) and transtympanically injected one ear with increasing rhBDNF concentrations (3, 5 and 10 μg·μl^{-1}) and the other ear with the vehicle solution. The volume of rhBDNF and vehicle solution injected was 30 μl.

Then, in our experimental setting, we used a total of n = 58 animals, randomly divided as follows: (1) control group ('Ctrl' group, n = 23); (2) animals (n = 5) subjected to transtympanic injection procedure (without any solution) in the right ear ('Sham' group) and treated with the vehicle in the left ears ('Vehicle' group); (3) animals (n = 7) receiving cisplatin intraperitoneally (i.p., 12 mg·kg^{-1}) ('Cis' group) and transtympanically injected with vehicle in the right ear ('Cis + vehicle' group); (4) animals (n = 23) treated with cisplatin, receiving vehicle in the left ears ('Cis + vehicle' group), and with rhBDNF (30 μl; 5 μg·μl^{-1}) in the right ear ('Cis + rhBDNF' group). Experimental procedures are summarized in Figure 1.

2.2 | Cisplatin and rhBDNF treatment

Cisplatin solution was prepared as described previously (Fetoni et al., 2015; Fetoni, Eramo, et al., 2014; Paciello et al., 2020). A single dose of 12 mg·kg^{-1} was selected as the optimal dose to achieve the desired ototoxicity level while maintaining an acceptable mortality rate (Chen et al., 2019; Waissbluth et al., 2012), and was delivered i.p. at a rate of 8 ml·hr^{-1} with an infusion pump (Axon Instruments, Foster City, CA, USA) for approximately 30 min. Following treatment, the rats were hydrated daily with saline solution (subcutaneous injection) to avoid dehydration. rhBDNF (5 mg/kg) was transtympanically injected into anaesthetized rats (**Ketamine** 87.5 mg/kg + **Xylazine** 12.5 mg/kg). rhBDNF was suspended in a thermogel vehicle solution (Engleder et al., 2014; Zhang et al., 2015). Transtympanic injection was performed as previously described (Fetoni, Lattanzi, et al., 2014; Paciello et al., 2018), and 30 μl of either the rhBDNF suspension or vehicle was injected at a constant rate (2 μl/s) into the round window niche area. All procedures were performed using aseptic techniques under aseptic conditions. The rhBDNF and vehicle formulations were supplied by Dompé Farmaceutici SpA.

2.3 | Surface plasmon resonance on sensor chip NTA

All SPR experiments were conducted using a Biacore X100 instrument (Cytiva, Washington, DC, USA) at 25°C, and a Biacore Sensor Chip CM5 (Cytiva) was used to capture histidine-tagged molecules for

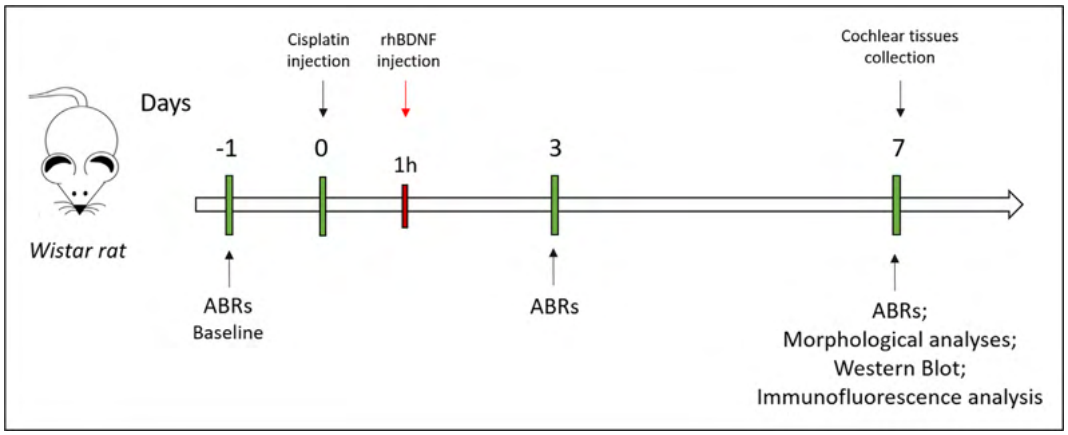

FIGURE 1 **Experimental design and protocol.** Male Wistar rats were used in this study. Baseline hearing thresholds were evaluated by using auditory brainstem responses (ABR) the day before (−1) cisplatin treatment. One hour (1 h) after cisplatin injection, rhBDNF was administered by the transtympanic route in the right ear. Functional analyses were performed after 3 and 7 days. Cochlear tissues were collected after 7 days from the onset of treatments to perform experimental analyses.

subsequent SPR interaction analysis. To this aim, 20 μg·ml^{-1} of anti-Histidine antibodies (Abcam, Cambridge UK, Cat# ab137839, RRID: AB_2732046) were immobilized onto a CM5 sensor chip using standard amine-coupling chemistry. Then, recombinant mouse TrkB his-tagged protein (mTrkB, Abcam Cat# ab208479) or recombinant mouse p75 NGF receptor his-tagged protein (mp75R, Abcam Cat# ab276954) were injected over the specific antibody surfaces, allowing their capture. This procedure allowed for the recognition of the recombinant protein of interest in an oriented way. A sensor chip coated with anti-Histidine antibodies alone was used as a negative control and for blank subtraction. Increasing concentrations (from 6.25 to 100 nM) of recombinant human BDNF (rh-BDNF) in 10-mM Hepes pH 7.4 containing 0.15-mM NaCl, 50-μM EDTA and 0.005% Surfactant P20 (running buffer) were injected over the rm-TrkB and rm-p75R or control surfaces for 4 min and allowing dissociation for 5 min to determinate the kinetic parameters of its interaction with captured receptors. After each run, the sensor chip was regenerated by injection of 2 M NaCl. Kinetic parameters were calculated from the sensorgram overlays by using the nonlinear fitting single-site model software package BIA evaluation (version 3.2, Cytiva). Only sensorgrams whose fitting gave χ^2 values close to 10 were used.

2.4 | Auditory brainstem responses (ABRs)

In all animals, ABRs were evaluated bilaterally at different frequencies before treatment (at day −1, baseline) and at 3 and 7 days after cisplatin treatment and/or rhBDNF administration. Rats were mildly anaesthetized (ketamine, 35 mg·kg^{-1} and medetomidine-domitor, 0.25 mg·kg^{-1}), and three stainless steel electrodes were placed subcutaneously into the right mastoid (active), vertex (reference), and left mastoid (ground). Measurements were performed using a TDT System 3 (Tucker–Davis Technologies, Alachua, FL, United States). Tone bursts of 6, 12, 16, 20, 24 and 32 kHz (1-ms rise/fall time, 10-ms total duration, 20/s repetition rate) were used. Responses were filtered (0.3–3 kHz), digitized, and averaged across a minimum of 550 discrete samples for each frequency and sound level. The threshold values were determined as the minimum stimulus level (in dB) at which a consistent waveform-based onset could be observed, indicating repeatability. Considering the well-known interindividual variability of cisplatin susceptibility observed both experimentally and in clinical evaluations (Fetoni et al., 2022; Wang et al., 2003), we used a model of cisplatin systemic administration and rhBDNF local delivery in one ear, thus obtaining functional ABR data from both treated (cisplatin + rhBDNF) and untreated (cisplatin + vehicle) ears from the same animal. Indeed, in our model, each animal acted as its own control, thus avoiding the high interindividual variability of the cisplatin susceptibility rate as a confounding factor. To further strengthen the electrophysiological data, we compared not only the ABR threshold shifts, but also the latency and amplitude of ABR waves I and II (Church et al., 2012; Fetoni et al., 2013).

2.5 | Immunofluorescence and immunohistochemical analyses

Immunofluorescence analyses were performed on formaldehyde-fixed cochlear cryosections and whole-mount cochlear preparations. Cochlear samples were extracted and processed as previously described (Fetoni, Eramo, et al., 2018). Samples were incubated overnight at 4°C with primary antibodies, including anti-CtBP2-IgG1 (Cat# 612044, RRID:AB_399431), anti-**GluA2** (Cat# ZRB1008, RRID:AB_3552219), anti-NF200 (Cat# N4142, RRID:AB_477272), TrkB (Cat# ab18987, RRID:AB_444716), **VEGF-A** (Cat# bs-4572R, RRID:AB_11070754), anti-cleaved-**caspase 3** (Cat# 9664, RRID:AB_2070042) and **TNF-α (Cat# sc-52746, RRID:AB_630341)**. Samples were washed in PBS and incubated at RT for 2 h in the dark with labelled-conjugated donkey anti-rabbit and/or anti-mouse secondary antibodies [Alexa Fluor 488 (anti-rabbit: Cat# A-21206, RRID:AB_2535792) or 546 (anti-rabbit: Cat# A10040, RRID:AB_2534016; anti-mouse: Cat# A10036, RRID:AB_11180613)]. To obtain the HC count, F-actin staining was performed by incubating samples with ActinGreen 488 Ready Probes Reagent. Samples were counterstained with **DAPI** for 20 min in the dark at RT and mounted with ProLong™ mounting medium. Further details for the immunofluorescence and immunohistochemical procedures are provided in Table S1.

A confocal microscope system (Nikon Ti-E, Confocal Head A1 MP, Tokyo, Japan) was used to capture images of the immunolabelled specimens using a 20 × or 60 × objective lens.

Haematoxylin and eosin (H&E) staining was performed using the H&E Staining Kit (ab245880, Abcam, Table S1). The count of paired ribbon synapses and SGNs was performed using ImageJ 1.43u. To ensure accurate analysis, control experiments were conducted by excluding the primary antibody during tissue processing (data not shown). Tissues from all groups were processed together to minimize variability related to antibody penetration, incubation time, post-sectioning time and tissue condition.

2.6 | Western blot and dot blot analyses

Total protein lysates of cochlear cells were obtained from five cochleae per group using a previously published protocol (Fetoni, Eramo, et al., 2016). For dot blots, 5 μl of lysate was carefully applied to a nitrocellulose membrane (0.2 μm, Amersham) pre-wetted with TBST. Equal protein loading was ensured by staining the membrane with Ponceau S (Paciello et al., 2023, 2024). The membranes were processed as described previously (Paciello et al., 2024) and then incubated overnight at 4°C with the following primary antibodies: caspase 3 cleaved (Cat# AB3623, RRID:AB_91556), caspase 3 (Cat# sc-7272, RRID:AB_626803), p75 (Cat# ab52987, RRID:AB_881682), NF-κB (Cat# 8242, RRID:AB_10859369), pTrkB (Cat# 4619, RRID:AB_10235585), TrkB (Cat# ab18987, RRID:AB_444716), pCREB (Cat# 06-519, RRID:AB_310153), **CREB** (Cat# MA1-083, RRID:AB_558523), SOD1 (Cat#

ab20926, RRID:AB_445919), SOD2 (Cat# 06-984, RRID:AB_310325), **SIRT1** (Cat# ab110304, RRID:AB_10864359), TNF−α (Cat# sc-52746, RRID:AB_630341), 3-NT (Cat# 06-284, RRID:AB_310089), **GSH** (Cat# ab19534, RRID:AB_880243) and **4-HNE** (Cat# HNE11-S, RRID:AB_2629282). After three 10-min rinses in TBST, the membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated mouse (Cat# 7076, RRID: AB_330924) or rabbit (Cat# 7074, RRID: AB_2099233) secondary antibodies. Equal protein loading among the individual lanes was confirmed by re-probing the membranes with anti-GAPDH (Cat# ab8245, RRID: AB_2107448) or anti-β-tubulin antibodies (Cat# 2146, RRID:AB_2210545). Protein expression was evaluated and documented using UVItec Cambridge Alliance. Further details for the western blot procedure are provided in Table S1. The western blot and immunohistochemical procedures adhere with the recommendations made by the British Journal of Pharmacology (Alexander, Christopoulos, et al., 2023; Alexander, Reid, et al., 2018).

2.7 | Data and statistical analysis

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018) and with the ARRIVE guidelines (Kilkenny et al., 2010).

We used online random number generator (https://www.graphpad.com/quickcalcs/randomize1/) to allocate experimental units to control and treatment groups. All analyses were performed in a blinded manner. The sample size was determined after a power analysis to obtain a statistical power of 80% at an α level of 0.05, according to the results of prior pilot data sets or studies, including ours, which use similar methods or paradigms. For each analysis, the group size (specified in the figure legends) was at least $n = 5$, and it refers to independent values used for statistical analyses. Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for statistical comparisons. Data were first assessed for normality (Shapiro–Wilk test) and equal variance (Brown–Forsythe test). For multiple group comparisons, analysis of variance (ANOVA) was employed, followed by Bonferroni correction to compare means. Post hoc comparisons were run only if F achieved $P < 0.05$ for interactions and there was no significant variance inhomogeneity. Statistical significance was set at $P < 0.05$ for all analyses. Data are presented as mean ± standard error of the mean (SEM). For the number of ribbon synapses, OHCs and SGNs, each raw value has been normalized to the mean of the control values (100%).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Fabbro, 2023; Alexander, Fabbro, et al., 2023; Alexander, Kelly, et al., 2023; Alexander, Mathie, et al., 2023).

3 | RESULTS

3.1 | rhBDNF administration counteracts cisplatin-induced hearing loss.

Surface plasmon resonance analysis revealed that the affinity constants of the interaction between rhBDNF and TrkB or p75 were 0.17 nM (K_D) and 1.8 nM (K_D), respectively, whereas steady-state binding levels affinity values were 59 nM (K_{Deq}) and 3.14 nM (K_{Deq}), respectively (Figure S1). Having demonstrated the ability of rhBDNF to bind both BDNF receptors, we then optimized rhBDNF dosage and time points for hearing function evaluation in our model of cisplatin-induced ototoxicity, choosing the best rhBDNF dosage based on the results from a pilot study conducted on $n = 12$ animals to obtain a dose–response curve. Our results demonstrated that the best protection was achieved at a dose of 5 $\mu g \cdot \mu l^{-1}$ (Figure S2); thus, we selected this dosage in our experimental setting. Additionally, no significant differences in ABR measurements were found between Sham and Vehicle animals (not treated with cisplatin), indicating the absence of any alteration in hearing function due to the administration of the vehicle alone (Figure S3A,B). Similarly, no worsening of auditory thresholds was observed when the Cis and Cis + vehicle groups were compared (Figure S3C,D). Because the Ctrl *versus* Sham groups, as well as the Cis *versus* Cis + vehicle groups, showed comparable ABR thresholds, we decided to consider the Ctrl and Cis + vehicle groups as a reference for the following analyses.

Once the best protective dose of rhBDNF and the safety of the vehicle injection were established, we performed an experimental analysis (Figure 1). Auditory function evaluation revealed that rhBDNF at a dosage of 5 $\mu g \cdot \mu l^{-1}$ exerted a significant protection of approximately 10 dB in the mid-high frequency region after only 3 days, compared with a threshold shift of about 20–25 dB caused by cisplatin treatment (Figure 2a). At day 7, rhBDNF animals showed a threshold shift of approximately 10 dB and about 15–20 dB in low, mid, and high frequencies respectively, compared with the hearing impairment of 25–30 dB observed in the Cis + vehicle group (Figure 2b). Latency and amplitude evaluation showed that cisplatin treatment significantly affected neuronal transmission, increasing latency and decreasing the amplitude of ABR wave I (Figure 2c,d) and wave II (Figure 2e,f). Notably, the latency and amplitude of ABR waves I and II recorded at 24 kHz (in which we observed the strongest protective effect of rhBDNF) in the Cis + rhBDNF and Ctrl groups were perfectly superimposable in this experimental setting, demonstrating that rhBDNF treatment significantly attenuated the increase in ABR latency and the decrease in amplitude caused by cisplatin, suggesting a neuroprotective effect.

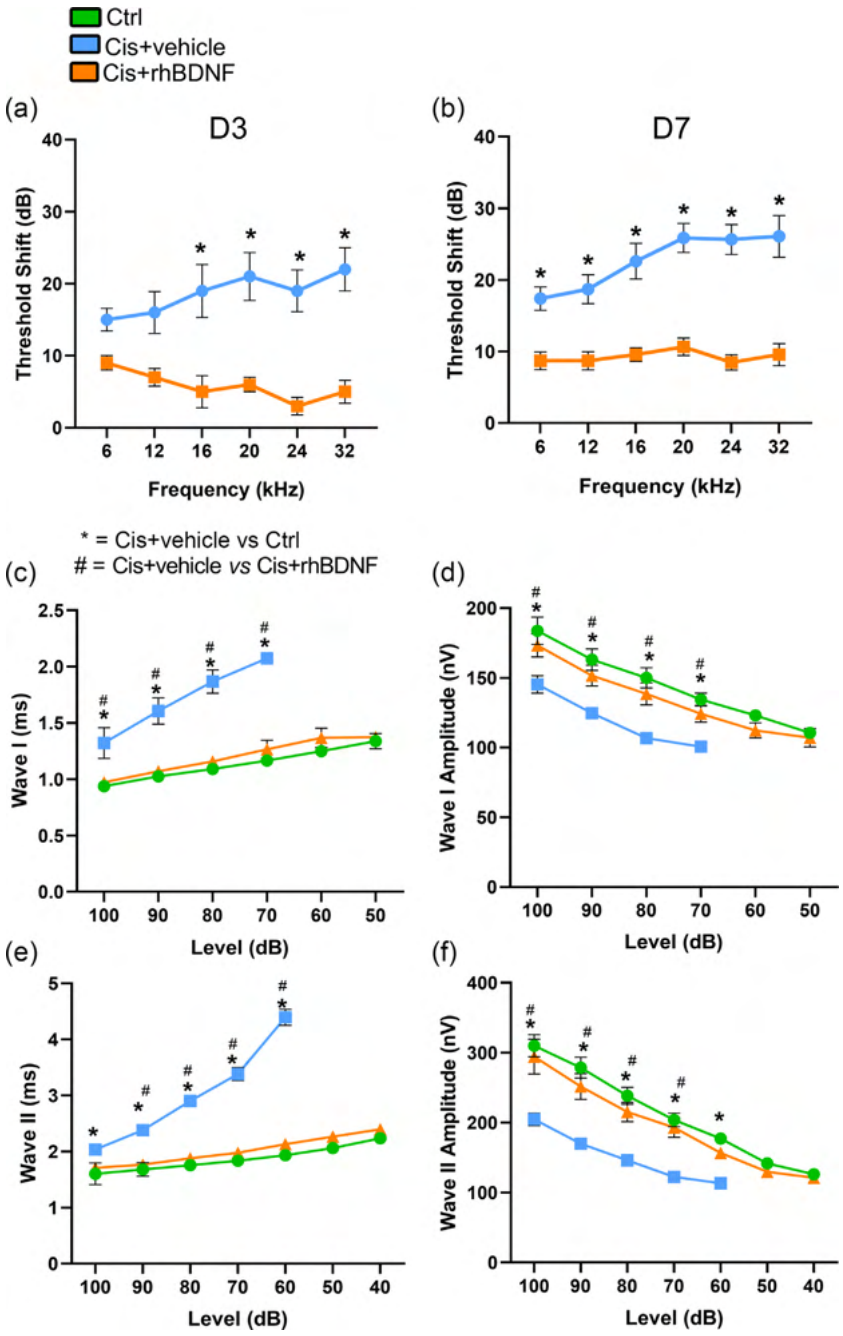

FIGURE 2 **Protective effects of rhBDNF treatment against cisplatin ototoxicity.** (a,b) Auditory threshold shift values (mean ± SEM) recorded in Cis + vehicle and Cis + rhBDNF rats, measured on day 3 (D3; n = 5) and day 7 (D7; n = 23). Notably, at D3, 5-μg·μl^{-1} rhBDNF (30 μl) showed an early protective effect against cisplatin. Significant protection of rhBDNF was also observed at D7 (b), spanning all frequencies analysed. (c–f) Latency–intensity and amplitude–intensity curves (mean ± SEM) of ABR waves I (c,d) and II (e,f) at 24 kHz, measured at D7 (two-way ANOVA;# refers to the statistical comparison between Cis+vehicle and Cis+rhBDNF; * refers to the statistical comparisons between Ctrl and Cis +vehicle).). *P < 0.05; #P < 0.05.P

3.2 | Neuroprotective effect of rhBDNF treatment against cisplatin-induced synaptopathy and neuropathy

To gain more insight into the striking functional protective effects of rhBDNF treatment, we studied inner hair cell (IHC)/neuronal afferent fibre synapses by performing immunofluorescence analyses. Staining with anti-CtBP2 allowed for the labelling of presynaptic compartments in IHCs, whereas anti-GluA2 was used for the identification of postsynaptic puncta on neuronal afferent fibres. In surface preparations of the organ of Corti of control rats, we clearly identified juxtaposed presynaptic and postsynaptic puncta (Figure 3a,a1), whereas, in the Cis + vehicle group, the number of paired ribbon synapses significantly decreased by approximately 70% compared with that in the control group (Figure 3b,b1,d). Notably, rhBDNF treatment was able to significantly protect against synaptopathy caused by cisplatin, as the number of paired ribbon synapses was approximately 50% higher in the Cis + rhBDNF group than in the Cis + vehicle group (Figure 3c,c1,d).

BDNF can induce the regrowth of nerve fibres towards neurotrophin-secreting cells to drive the re-innervation of the sensory area in the inner ear (Shibata et al., 2010). To better understand the protective effects exerted on hearing function and ribbon synapses, we evaluated the effect of rhBDNF on afferent fibres.

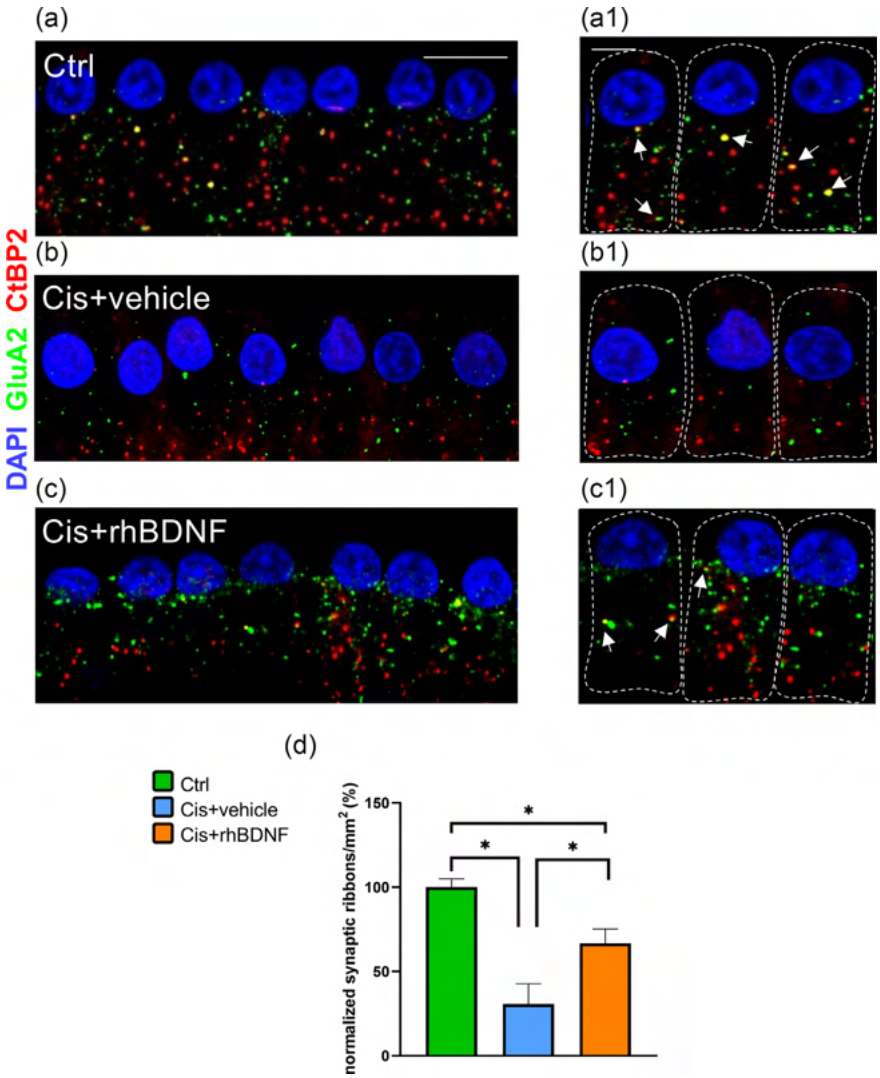

FIGURE 3 **Protective effect of rhBDNF exogenous administration against synaptic damage caused by cisplatin treatment.** (a–c) Representative images of micro-dissected whole-mount cochlear specimens stained for the presynaptic marker CtBP2 (red channel) and postsynaptic marker GluA2 (green channel) in the inner hair cell (IHC) area of the cochlear middle turn. DAPI staining (blue) was used to identify the cell nuclei. (a1–c1) High magnifications of three OHC defined by dotted lines. In the control group (a,a1), presynaptic ribbons and postsynaptic receptors were clearly detected (white arrows in a1 indicate paired synaptic ribbons). In the Cis + vehicle group (b,b1), a marked decrease in synaptic contacts was observed. After rhBDNF administration (c,c1), an increase in synaptic contacts was observed. (d) Histograms (mean ± SEM) showing the number of paired synaptic ribbons expressed as a percentage (n = 6 cochleae for each group; one-way ANOVA). Scale bar in (a): 10 μm; scale bar in (a1): 2.5 μm. Asterisks indicate significant differences among groups (* *P* < 0.05).

Immunofluorescence analysis targeting neurofilament 200 (NF200), a well-established marker of neurofilaments, and the subsequent fluorescence intensity spectrum analysis (Figure 4a1–c1 and Figure 4f1–h1) revealed that cisplatin administration significantly decreased the number and thickness of neural fibres between outer hair cells (OHCs) and SGNs (Figure 4b,d,e), as well as between IHCs and SGNs (Figure 4g,i,j), as compared with control animals (Figure 4a,f). rhBDNF treatment protected neural fibres (Figure 4c,h), thus demonstrating its effectiveness against cisplatin-induced synaptopathy by maintaining afferent fibres capable of establishing synaptic contacts with HCs.

OHC viability in the organ of Corti was analysed by F-actin staining (Figure 5a–c). In the control rats, the OHC surface exhibited a well-organized arrangement of three rows of OHCs (Figure 5a). No significant differences were observed in the apical turn among groups (Figure 5d). However, in both the middle and basal turns, cisplatin administration induced a substantial loss of approximately 40% of OHCs compared with the control group (Figure 5d). Interestingly, treatment with rhBDNF almost completely counteracted cisplatin-mediated damage in these cells (Figure 5c) in both the middle and basal turns (Figure 5d). H&E staining of cochlear cryosections (Figure 5e–h) revealed that Rosenthal's canal was densely packed with SGNs in control rats (Figure 5f). A marked reduction in SGNs was detectable in all cochlear turns, with 40% of SGN loss in the middle and basal turns and approximately 25% in the apical turn (Figure 5i). rhBDNF treatment significantly counteracted cisplatin-mediated neurotoxicity in SGNs, with the neuronal density being approximately 30% higher in the Cis + rhBDNF group than in the Cis + vehicle group (Figure 5i), demonstrating that neurotrophin-induced neuronal protection was associated with significant preservation of SGNs.

At the molecular level, cisplatin-induced cochlear cell damage was associated with a significant increase in cleaved caspase 3, a marker of apoptosis (Crowley & Waterhouse, 2016). Consistent with our morphological analyses, cleaved-caspase 3 levels were significantly reduced in Cis + rhBDNF rats compared with those in the Cis + vehicle group, confirming the widespread protective effect of this neurotrophic factor against cisplatin-induced cochlear cell death (Figure 5j,k). Moreover, to localize cleaved-caspase 3 levels in different cochlear structures (hair cells and SGNs), we performed immunofluorescence analyses on surface preparations of the organ of Corti

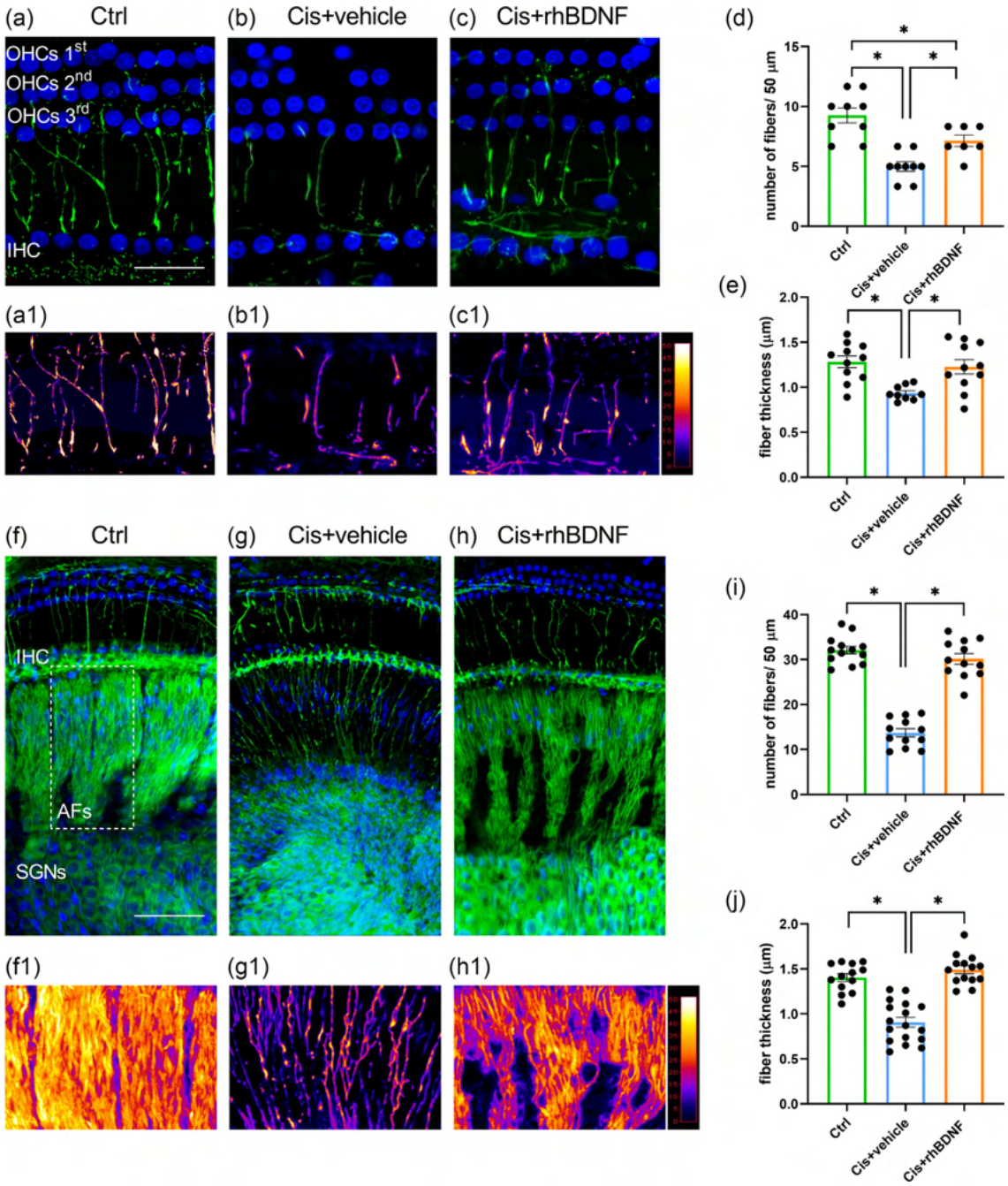

FIGURE 4 **rhBDNF treatment restores primary afferent fibre alterations induced by cisplatin.** (a–c) and (f–h) Representative images of the surface preparations of the organ of Corti stained with NF-200 (green fluorescence) and DAPI (blue fluorescence). Images show afferent fibres extending from spiral ganglion neurons (SGNs) to outer hair cells (OHCs; a–c) and inner hair cells (IHCs; f–h). In the Ctrl group (a,f), we observed the normal organization of afferent fibres (AFs) in both OHC-SGN (a) and IHCs-SGN connections (f). In the Cis + vehicle groups (b,g), a decrease in primary afferent fibres (as shown by the decrease of neurofilament signal density-NF200) was observed. In the Cis + rhBDNF group, we observed an increase in NF200 fluorescence (c,h) compared with that in the Cis + vehicle group. (a1–c1) and (f1–h1) show the distribution of the fluorescence signal, analysed on a pseudo-colour rainbow scale from blue (lower intensity) to yellow (maximum intensity). (d,i) Graphs showing the number (mean ± SEM) of neural fibres reaching the OHCs (d, n = 5 cochleae for each group; one-way ANOVA) and IHCs (i, n = 5 cochleae for each group; one-way ANOVA) analysed in a segment of 50 µm. (e,j) Graphs (mean ± SEM) showing the fibre thickness of both IHC-SGN connections (e, n = 5 cochleae for each group; one-way ANOVA) and IHC-SGN connections (j, n = 5 cochleae for each group; one-way ANOVA). Asterisks indicate significant differences among groups (* *P* < 0.05).

and on cochlear cryosections. Results show a marked increase of cleaved-caspase 3 labelling mainly in the IHCs and SGNs of cisplatin-treated animals, and its decreased fluorescence intensity in samples from animals treated with rhBDNF (Figure S4), confirming the ability of the neurotrophin to counteract apoptotic cell death in the cochlear sensory-neuronal epithelium.

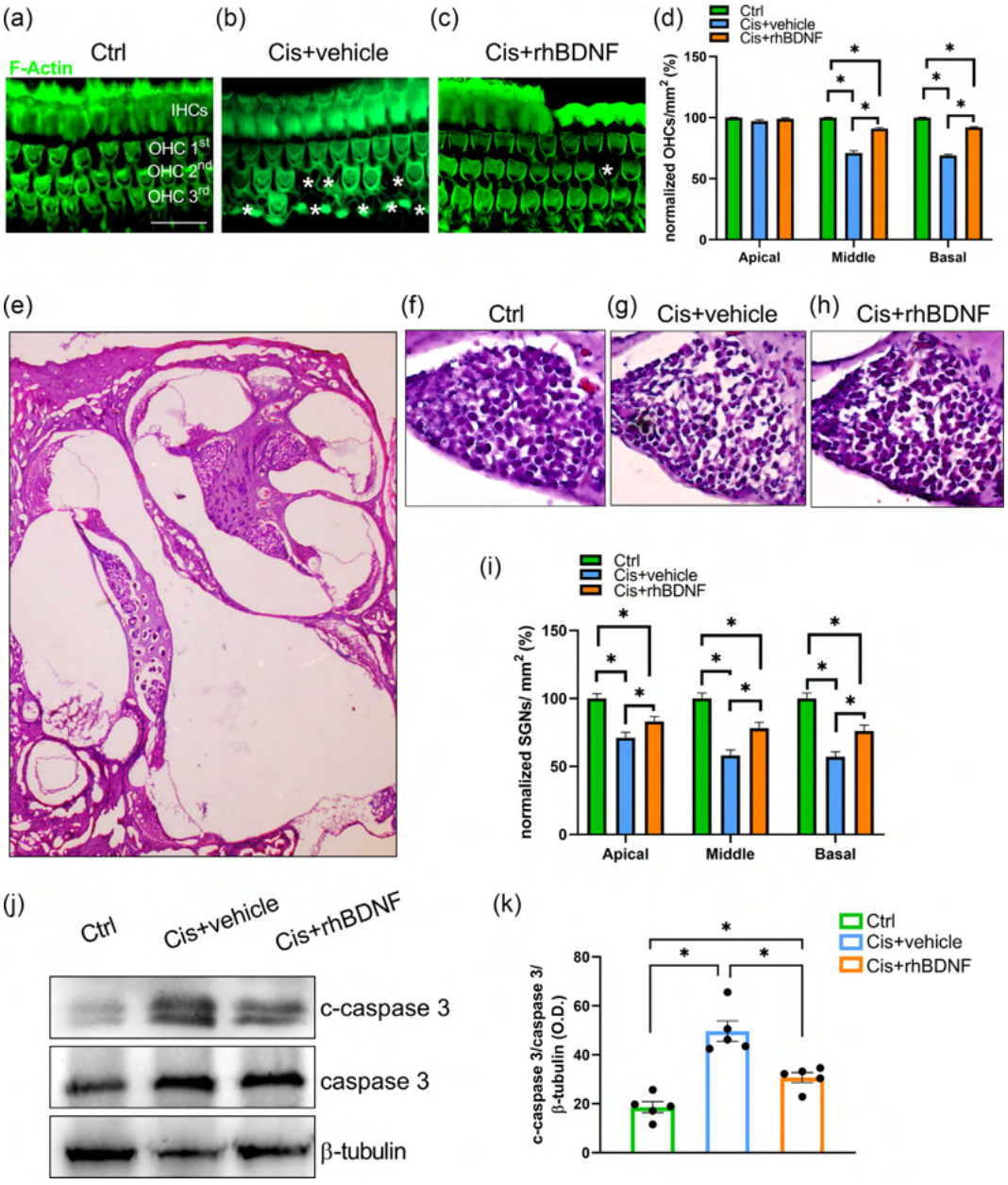

FIGURE 5 **rhBDNF protection against cochlear cell death induced by cisplatin treatment.** (a–c) Representative images of surface preparations of the organ of Corti showing F-actin distribution in the middle basal cochlear turn. Normal cochlear organization, represented by one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs), was observed in control animals (a). Severe OHC loss was observed in the Cis + vehicle group (b). White asterisks indicate missing OHCs (dark spots). rhBDNF treatment shows a protective effect against cisplatin-induced OHC death (c). Scale bar: 25 μm. (d) Cochleograms (mean ± SEM) showing the percentage of OHC survival in all cochlear turns (n = 5 cochleae for each group; one-way ANOVA). (e) Representative cochlear cryosections showing all cochlear turns stained with haematoxylin and eosin. (f–h) Representative images of spiral ganglion neurons (SGNs) in the Rosental's canal stained with haematoxylin and eosin in all experimental groups. (i) Histograms (mean ± SEM) illustrating SGN viability (n = 5 cochleae for each group; one-way ANOVA). (j) Representative Western blot images showing an increase in cleaved-caspase-3 (c-caspase 3) protein levels after cisplatin treatment and a decrease in its expression after rhBDNF transtympanic injection. (k) Bar graphs (mean ± SEM) show the results of densitometric analyses (n = 5 cochleae for each group; one-way ANOVA) normalized to the corresponding total protein levels (caspase 3 and GAPDH). Asterisks indicate significant differences among the groups (*P < 0.05).

3.3 | rhBDNF counteracts cisplatin-induced imbalance of pTrkB and p75 expression in cochlear cells

To mechanistically explain the protective effect of rhBDNF against cisplatin-induced cochlear damage, we investigated the expression of the BDNF receptors TrkB and p75 within the cochlea. TrkB was expressed in HCs and markedly expressed in SGN nuclei and their afferent fibres (Figure S5). p75 displayed a similar pattern, with its expression observed mainly in SGNs (Figure S5B,C). We then examined whether cisplatin administration and rhBDNF treatment could modulate both TrkB and p75 expression levels. In the cochlea, the basal level of TrkB observed in control specimens was not significantly altered in Cis + vehicle rats (Figure 6a,c), whereas p75 levels were significantly up-regulated in Cis + vehicle specimens compared with those in controls (Figure 6b,d). These results indicate that cisplatin administration induces dysregulation of the expression of BDNF receptors, leading to a possible imbalance in the p75-dependent activation of apoptosis. Notably, activation of the TrkB pathway was significantly decreased in rats receiving cisplatin compared with that in the control group, as demonstrated by the down-regulation of both TrkB and **CREB** phosphorylation (Figure 6e–h). Consistent with the protective effects of rhBDNF reported thus far, phosphorylation levels of TrkB and CREB were significantly increased in Cis + rhBDNF animals compared with those observed in the Cis + vehicle group and were comparable with those of the control group (Figure 6e–h). Significantly reduced p75 levels were observed in the Cis + rhBDNF group, similar to those observed in control animals (Figure 6b,d). These data demonstrate that rhBDNF treatment exerts its protective effects by maintaining the balance between TrkB and p75 levels and promoting the pro-survival TrkB signalling pathway.

3.4 | rhBDNF reduces cochlear oxidative stress and inflammation and boosts vascular repair

Considering that CREB is a redox-sensitive transcription factor able to modulate the transcription of several antioxidant genes (Barlow et al., 2006; Bedogni et al., 2003; Sayed et al., 2020), such as sirtuins, we studied sirtuin 1 isoform (**SIRT1**) expression in our samples. Consistent with our observations of pCREB expression, a marked

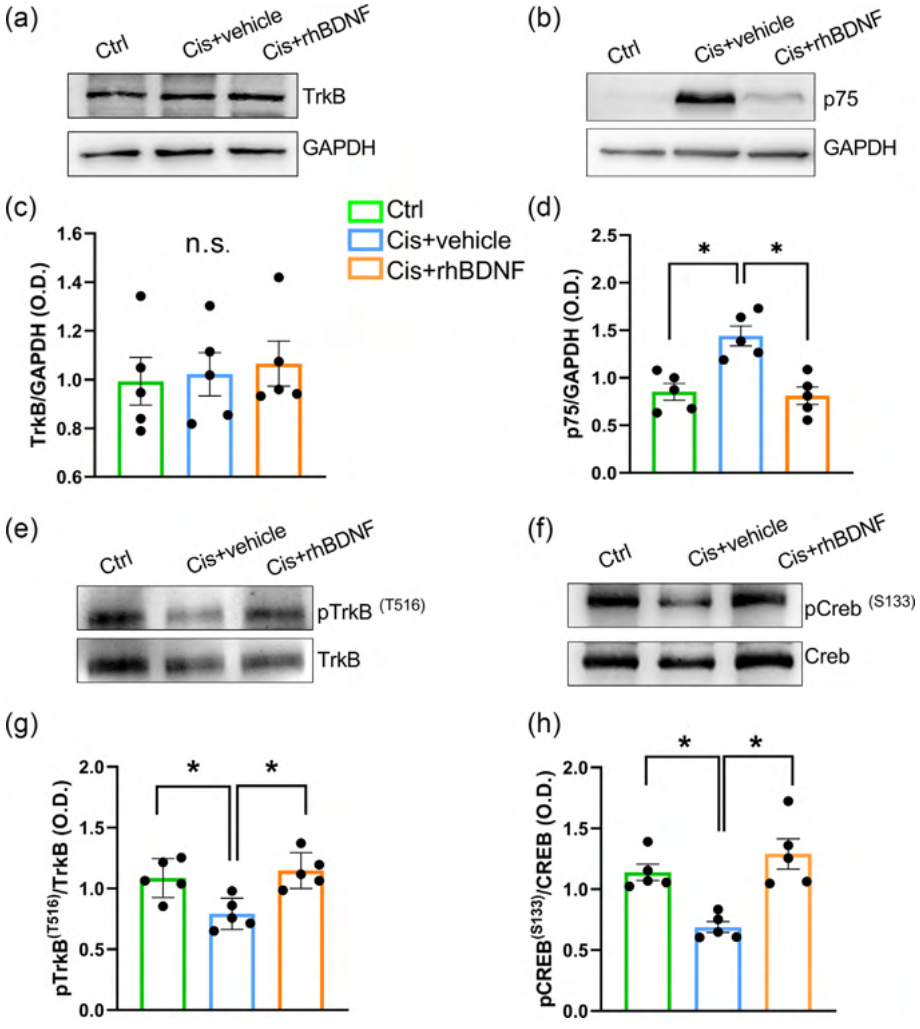

FIGURE 6 **rhBDNF administration maintains pTrkB/p75 balance and activates the TrkB/CREB signalling pathway.** Representative western blot bands showing TrkB (a), p75 (b), pTrkB (e) and pCREB (f) levels after cisplatin and rhBDNF administration. (c,d,g,h) Histograms (means ± SEM) showing densitometric analyses for TrkB (n = 5 cochleae for each group, one-way ANOVA), p75 (n = 5 cochleae for each group; one-way ANOVA); pTrkB (n = 5 cochleae for each group; one-way ANOVA) and for pCREB (n = 5 cochleae for each group; one-way ANOVA) normalized to the corresponding total protein levels (TrkB and CREB) or GAPDH. Asterisks indicate significant differences among the groups (*P < 0.05).

reduction in SIRT1 levels was detected following cisplatin exposure (Figure 7a,b). Interestingly, treatment with rhBDNF significantly increased SIRT1 compared with that observed in cisplatin-treated samples, and a complete recovery to the control level was observed (Figure 7a,b), suggesting a role for the BDNF/TrkB signalling pathway in regulating endogenous antioxidant mechanisms. We also evaluated oxidative stress levels using dot blot to detect protein tyrosine nitration (3-NT), a marker of nitro-oxidative stress, and 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation. Our analyses demonstrated an increase in 3-NT (Figure 7c,d) and 4-HNE (Figure 7e,f) levels following cisplatin treatment, which were observed at the control levels in the cochlea of rats receiving concomitant rhBDNF treatment. We analysed the endogenous antioxidant system, focusing on superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and glutathione. Specifically, we performed a western blot to detect SOD1 and SOD2 levels and a dot blot to analyse the level of glutathionylated proteins. Indeed, glutathionylation can be considered an indicator of oxidized glutathione (GSSG)/reduced glutathione (GSH) ratio (Fratelli et al., 2002), reflexing tissue redox balance. Although the levels of SODs and protein glutathionylation were down-regulated upon cisplatin administration, a significant increase, reaching control values, was observed in rhBDNF-treated rats, further confirming the role of this neurotrophic factor in maintaining cellular redox balance (Figure 7g–l).

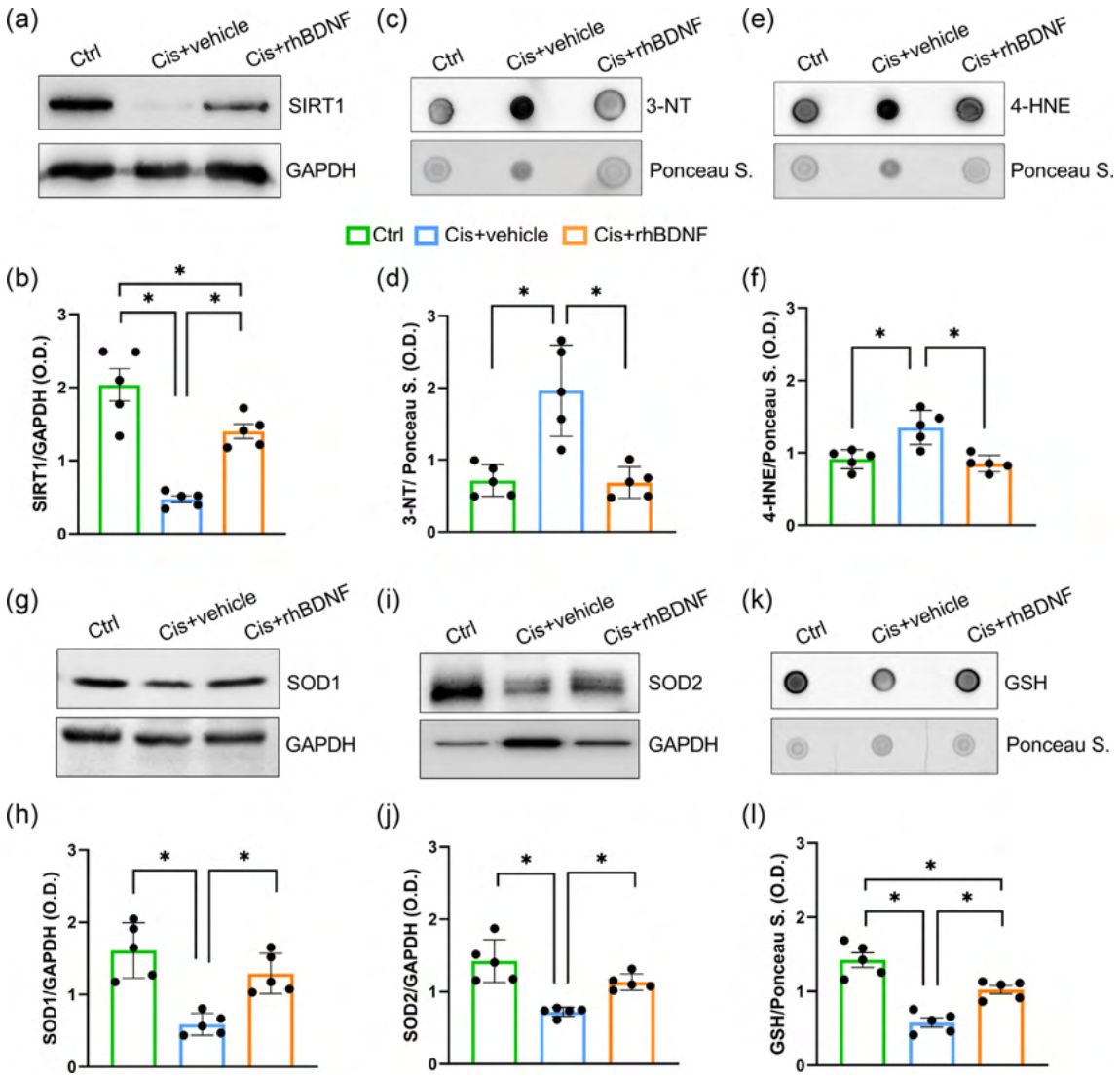

FIGURE 7 **Treatment with rhBDNF decreases oxidative stress and potentiates endogenous antioxidant defences.** (a) Representative western blots showing the expression of SIRT1 after cisplatin and rhBDNF administration. Histograms in (b) (means ± SEM) represent the results of densitometric analyses normalized to total protein levels (GAPDH) (n = 5 cochleae for each group; one-way ANOVA). (c–f) Dot blots showing the increased levels of oxidative stress and lipid peroxidation markers (3-NT and 4-HNE) in all experimental groups. Histograms (mean ± SEM) showing the results of densitometric analyses normalized to total protein levels detected by Ponceau staining (n = 5 cochleae for each group; one-way ANOVA). (g–l) Western blot and dot blot experiments evaluating the level of endogenous antioxidant enzymes including SOD1, SOD2, and glutathionylated proteins (GSH) in cochlear samples collected from all the experimental groups (n = 5 cochleae for each group; one-way ANOVA). Asterisks indicate significant differences among the groups (*P < 0.05).

Because inflammation is usually a consequence of oxidative tissue damage and ototoxic insults, we investigated the expression levels of NF-κB and TNF-α. Cisplatin administration induced strong up-regulation of these markers, whereas rhBDNF treatment maintained their expression to almost basal levels (Figure 8a–d). Moreover, given that inflammation derived from cisplatin-induced cochlear damage can affect the vascular integrity in the *stria vascularis* (Shi, 2011; Trune & Nguyen-Huynh, 2012; Zhang et al., 2020; Yu et al., 2022), immunofluorescence analyses for TNF-α were performed on *stria vascularis* whole mounts. Confocal images of the *stria vascularis* marginal cell monolayer and a 3D reconstruction of *z*-series acquired images of all experimental groups are shown in Figure 8. We focused specifically on the marginal cell layer, considering that these cells contain numerous mitochondria, they are very active metabolically and most susceptible to ROS generation (Shi & Nuttall, 2003). TNF-α labelling was faint in control samples (Figure 8e,e2), and marginal cells appeared to

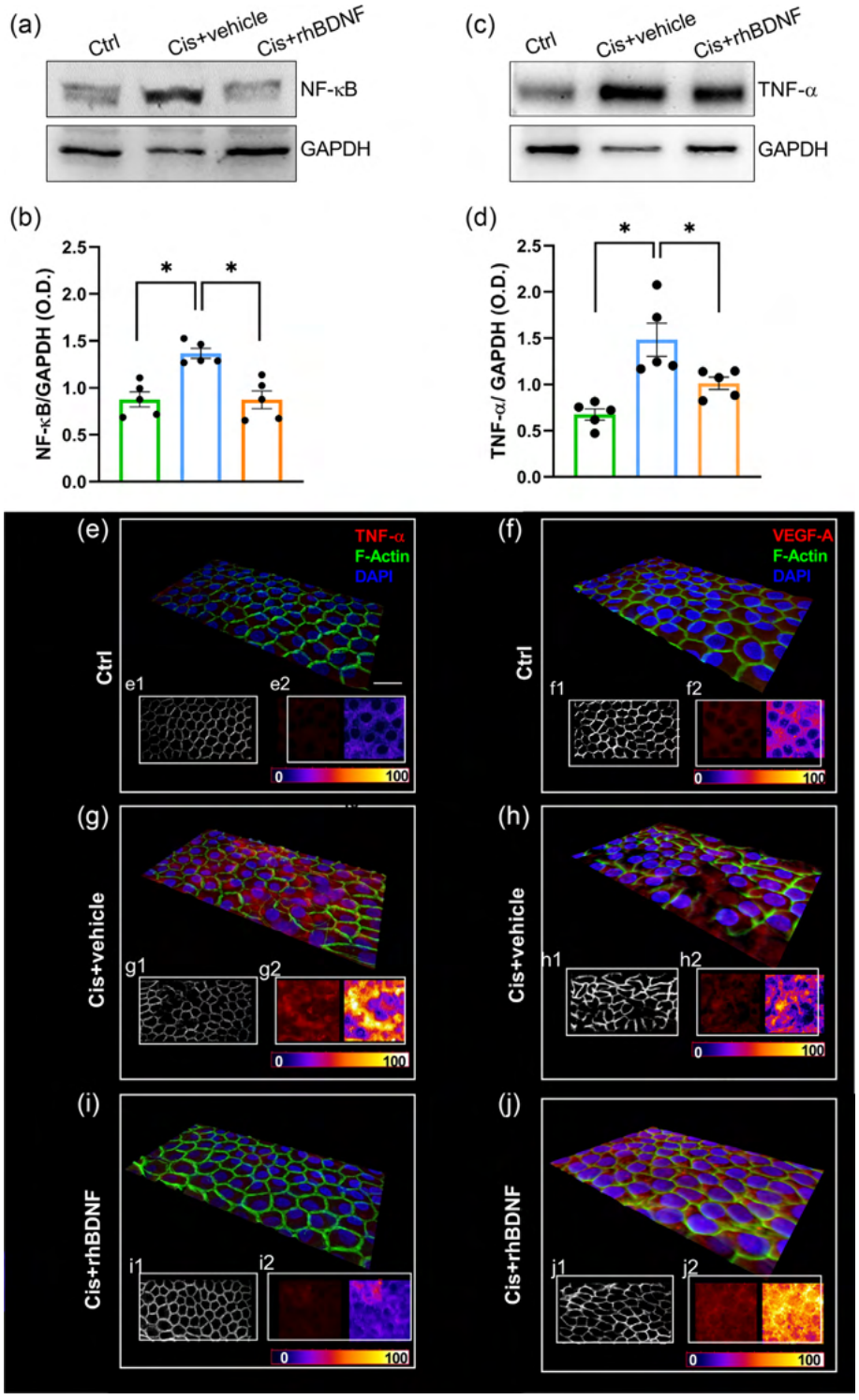

FIGURE 8 **rhBDNF local delivery attenuates cochlear pro-inflammatory marker expression and increases VEGFA expression in the marginal cell monolayer of *stria vascularis*.** (a,c) Representative western blot bands showing NF-κB (a) and TNF-α (c) levels after cisplatin and rhBDNF administration. (b,d) Histograms (means ± SEM) represent the results of densitometric analyses (n = 5 cochleae for each group; one-way ANOVA) normalized to the total protein levels (GAPDH). Asterisks refer to significant differences among groups (*P < 0.05). (e–j) Representative 3D reconstruction of confocal Z-stack images of TNF-α (e,g,i) and VEGFA (f,h,j) expression (red fluorescence) in *stria vascularis* whole mounts stained with F-actin (green fluorescence) and DAPI (blue fluorescence) showing marginal cell monolayers in all experimental groups. A 2D reconstruction of the marginal cell monolayer is shown in boxes (e1)–(j1). (e2–j2) Distribution of TNF-α and VEGFA fluorescence intensity signals in a pseudo-color rainbow scale from lower (blue, 0) to highest (yellow, 100) values.

form a continuous layer, as defined by F-actin staining (Figure 8e,e1). A marked increase in inflammatory markers was observed, together with a loss of the structural integrity of marginal cells, after cisplatin administration (Figure 8g,g1–g2). This was also confirmed by the optical density fluorescence signals reported on a pseudo-ccolor rainbow scale (compare the right panels in Figure 8e2,g2). Interestingly, in Cis + rhBDNF rats, rhBDNF not only reduced the expression of TNF-α (Figure 8i,i2), but also preserved the structural integrity and stability of marginal cells (Figure 8i1).

Prompted by these data, we also evaluated vascular endothelial growth factor A (VEGFA). Our results showed low, homogeneous VEGFA expression in control animals (Figure 8f,f2), together with an ordered marginal cell layer (Figure 8f1) that increased after cisplatin administration, specifically in the damaged area of the marginal cell monolayer (Figure 8h,h1,h2). In rhBDNF-treated rats, VEGFA was significantly up-regulated and localized to marginal cells (Figure 8j,j1,j2).

Collectively, these data indicate a role for rhBDNF in counteracting inflammation, promoting angiogenesis, and preserving *stria vascularis* integrity in the cochlea.

4 | DISCUSSION

In the present study, rhBDNF was delivered transtympanically 1 h after cisplatin administration to investigate whether treatment could counteract cisplatin-induced loss of hearing function and cochlear damage. We demonstrated, for the first time, that the striking functional protection of rhBDNF treatment is associated with multiple molecular and cellular effects: (1) neuroprotection against cisplatin-induced synaptopathy, (2) preservation of neuronal fibres, (3) protection of both HCs and SGNs against cisplatin-induced cell death, (4) maintenance of pTrkB and p75 balance in cochlear cells, (5) activation of the TrkB/CREB pro-survival signalling pathway, (6) attenuation of redox imbalance and inflammation and (7) stimulation of angiogenesis factors and vascular repair.

The different timing and faster effect of rhBDNF that we observed on auditory thresholds recorded over time in the mid-high frequencies can be explained by data indicating that BDNF has a tonotopic gradient in its physiological expression during adulthood, with higher levels in high-frequency basal cochlear regions (Adamson et al., 2002). Consistently, the same expression pattern has been described for its high-affinity receptor TrkB (Adamson et al., 2002; Pisani et al., 2023; Singer et al., 2014; Zuccotti et al., 2012), suggesting that rhBDNF treatment may target these cochlear regions more efficiently. These data are even more meaningful given that the high-frequency ABR threshold, wave latency and amplitude are the most affected by ototoxic drugs and are generally evaluated for early identification of ototoxicity (Chen et al., 2021; Dreschler et al., 1985; Fausti et al., 1993), thus indicating the ability of rhBDNF treatment to rapidly and strongly protect against cisplatin auditory damage. Studies have shown that significant synaptic loss precedes hair cell death and SGN degeneration in several animal models of hearing loss (Kujawa & Liberman, 2009; Sergeyenko et al., 2013), and this primary neuropathy has been recognized as a key factor contributing to sensorineural hearing loss (Rance et al., 2007; Zeng & Liu, 2006). From a cellular point of view, the striking effect of rhBDNF treatment against cisplatin-induced alteration of hearing function that we observed was associated with significant protection against cisplatin-induced synaptopathy, which was due to the maintenance of afferent fibres capable of establishing synaptic contacts with hair cells.

From a translational point of view, these results demonstrate that the ability of rhBDNF to preserve synapses between hair cells and SGNs can support combined approaches based on the use of cochlear implants associated with drug-based therapy to preserve cochlear neurons (Dabdoub & Nishimura, 2017). Indeed, in clinical settings, neuropathy and synaptopathy represent the highest limitation to cochlear implants, the only available treatment for deafness, causing poor post-implant outcomes (Seyyedi et al., 2014). Supplementation of neurotrophic factors, including BDNF, has been demonstrated to have a protective effect on SGN viability, enhancing cochlear implant outcomes (Bailey & Green, 2014; Konerding et al., 2017; Leake et al., 2019; Peter et al., 2022; Pfingst et al., 2017; Vink et al., 2022). In animal models, using growth factors as adjuvant therapy associated with cochlear implants has been shown to preserve cochlear neurons, synapses, and peripheral processes and to reduce the threshold of electrical stimulation (Pfingst et al., 2015). Thus, according to previous studies, our data suggest that combining the delivery of effective BDNF with cochlear implant devices could be an efficient approach.

Interestingly, in our experimental setting, we observed that cisplatin can also induce the alteration of BDNF receptor expression, leading to an imbalance in their cochlear expression compared to healthy animals, with the up-regulation of p75 and down-regulation of TrkB activity. Physiologically, the TrkB receptor mediates the pro-survival actions of BDNF, whereas p75 regulates neuronal survival by mediating the physiological elimination of approximately 50% of the neurons generated during embryogenesis (Oppenheim, 1991). Thus, the increased apoptosis of both HCs and SGNs that we observed after cisplatin administration can be explained, at least to some extent, by the up-regulation of p75 and down-regulation of TrkB phosphorylation that was induced in the cochlea by the chemotherapy agent. In contrast, treatment with rhBDNF prevented apoptosis by maintaining the cochlear balance of both receptors, as well as the activation of the pro-survival TrkB/CREB pathway at control levels. The effect of BDNF in blocking apoptotic neuronal cell death by activating the TrkB receptor has been well documented in both in vitro and in vivo studies (Han et al., 2000; Snider, 1994); as far as we are aware, this is the first demonstration that cisplatin can induce an imbalance of pTrkB and p75 cochlear levels and that transtympanic exogenous administration of rhBDNF in cisplatin-treated animals directly counteracts apoptosis by maintaining the balance of its receptor levels and down-regulating the caspase 3-dependent pathway in the cochlea.

Nowadays, several studies have strictly linked cisplatin-induced damage to increased oxidative stress (Fetoni et al., 2022; Paciello et al., 2020; Ramkumar et al., 2021). As part of TrkB signalling, CREB induces the deactivation of pro-apoptotic targets, as well as the

transcription of genes related to cell survival (Chowdhury et al., 2023), and is known to regulate ROS detoxification. CREB is also involved in the regulation of antioxidant enzymes, including SODs, ROS scavenging, and induction of mitochondrial biogenesis (Sayed et al., 2020), and studies support a complex interplay between CREB and SIRT1, as these factors can reciprocally control each other's functions, either directly or via an integrated signalling network (Noriega et al., 2011). In our experimental setting, treatment with rhBDNF increased SIRT1 levels, suggesting the potential involvement of the BDNF/TrkB signalling pathway in the regulation of antioxidant mechanisms in the cochlea. Indeed, we observed an increase in both SOD1 and SOD2 protein levels in the Cis + rhBDNF group compared with that in the Cis + vehicle group, along with a significant decrease in oxidative stress markers and lipid peroxidation levels. Moreover, it has been shown that the inflammatory response induced by cisplatin treatment in the cochlea involves the activation of the redox-sensitive transcription factor and key mediator of inflammation, NF-κB (Kim et al., 2011), which regulates the expression of pro-inflammatory cytokines such as **IL-1β**, TNF-α and **IL-6** (Paciello et al., 2020; Rybak et al., 2007; So et al., 2007, 2008). TNF-α can activate NF-κB, thereby creating a positive feedback loop that intensifies the inflammatory response (Barnes, 1997). In this study, we demonstrated that rhBDNF treatment can significantly reduce the expression of pro-inflammatory markers, such as TNF-α and NF-κB, which might be due to its action in inhibiting the imbalance of p75 expression.

Finally, our analyses on *stria vascularis* whole mounts, focused on the marginal cell layer, showed the ability of rhBDNF treatment not only to reduce neuroinflammation, but also to provide structural protection against vascular damage induced by cisplatin. Indeed, it is known that the marginal cell layer is particularly susceptible to ototoxic damage and that cisplatin can target **connexins** in the *stria vascularis*, probably altering tight junctions and, consequently, vascular integrity (Tong et al., 2013; Zhang et al., 2020). We have previously documented that connexin expression is crucial for maintaining *stria vascularis* integrity (Gentile et al., 2021; Paciello et al., 2022), and considering that oxidative stress can modulate connexin expression in cochlear tissues (Fetoni, Zorzi, et al., 2018), it can be hypothesized that crosstalk exists between ototoxic damage, ROS production, and vascular injury. Our data on the protective effect of rhBDNF on *stria vascularis* are supported by studies describing its ability to regulate angiogenic processes (Donovan et al., 2000; Hu et al., 2007). Indeed, in our study, we found that rhBDNF boosts **VEGFA** expression, and this ability of rhBDNF to stimulate angiogenic factors could have a dual effect, considering that neural tissue damage repair processes include angiogenesis and vascular repair (Ma et al., 2021).

In conclusion, the present study showed for the first time that transtympanic delivery of rhBDNF after (1 h) cisplatin administration results in striking neuroprotection against ototoxicity. From a translational point of view, such a schedule of administration with early transtympanic rhBDNF treatment holds promise in counteracting the side effects of cisplatin on auditory function, and this can open the way for the development of novel interventions to preserve hearing in adult and paediatric patients undergoing cisplatin-based chemotherapy.

AUTHOR CONTRIBUTIONS

Anna Pisani: Conceptualization (equal); formal analysis (lead); investigation (lead); methodology (lead). **Rolando Rolesi:** Investigation (equal); methodology (equal). **Veronica Mohamed-Hizam:** Investigation (equal); methodology (equal). **Raffaele Montuoro:** Investigation (equal); methodology (equal). **Gaetano Paludetti:** Funding acquisition (equal); supervision (equal); validation (equal). **Cristina Giorgio:** Data curation (equal); formal analysis (equal). **Pasquale Cocchiaro:** Data curation (equal); formal analysis (equal). **Laura Brandolini:** Conceptualization (equal); data curation (equal); formal analysis (equal). **Nicola Detta:** Investigation (equal). **Anna Sirico:** Investigation (equal). **Pier Giorgio Amendola:** Investigation (equal). **Rubina Novelli:** Writing—review and editing (equal). **Andrea Aramini:** Conceptualization (equal). **Marcello Allegretti:** Conceptualization (equal). **Fabiola Paciello:** Conceptualization (equal); writing—original draft (equal). **Claudio Grassi:** Resources (equal); supervision (equal). **Anna Rita Fetoni:** Conceptualization (equal); funding acquisition (equal); supervision (equal); validation (equal).

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CONFLICT OF INTEREST STATEMENT

Cristina Giorgio, Pasquale Cocchiaro, Laura Brandolini, Nicola Detta, Anna Sirico, Pier Giorgio Amendola, Rubina Novelli, Andrea Aramini and Marcello Allegretti are employees of Dompé Farmaceutici S.p.A., Italy. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical

research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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