

Gna14-Associated Gq-IP₃R Signaling Contributes to the Transition to Sustained Calcium Dysregulation in Cisplatin Ototoxicity

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Research Article

Keywords:

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11072309/v1>

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Additional Declarations: No competing interests reported.

Abstract

Cisplatin ototoxicity is generally viewed as the progressive accumulation of cellular damage, but whether cochlear cells pass through a definable transition before irreversible injury remains unclear. Here, calcium imaging at defined time points over 72 h revealed a biphasic response in mouse cochlear explants. Early fluctuations in the calcium signal were followed by sustained elevation that persisted after cisplatin withdrawal. This transition was accompanied by mitochondrial oxidative stress, consistent with a shift from an early compensated phase toward sustained calcium dysregulation. The transition was accompanied by remodeling of ER-mitochondria contacts (MERCs) and mitochondrial structural damage. Time-resolved transcriptomics identified *Gna14* near the transition boundary, and *Gna14* silencing reduced hair-cell injury. Pharmacological experiments further implicated Gq-IP₃R signaling in the calcium phenotype. p-Coumaric acid (p-CA) induced thermal stabilization of *Gna14* and attenuated sustained calcium dysregulation, MERC remodeling, ER stress, and mitochondrial dysfunction. In a chronic mouse model, p-CA preserved auditory thresholds and cochlear cellular architecture without reducing cisplatin cytotoxicity in two tested nasopharyngeal carcinoma cell lines. These findings suggest that cisplatin ototoxicity contains a previously underappreciated transition from compensated calcium handling to a persistent organelle-stress state. Targeting this transition before overt cochlear injury may provide a temporal strategy for otoprotection. RNA-sequencing data are available in the Genome Sequence Archive under accession CRA050592.

1. Introduction

Cisplatin remains a cornerstone treatment for many solid tumors, but permanent sensorineural hearing loss remains a major dose-limiting adverse effect (Dasari and Tchounwou 2014; Kelland 2007; Florea and Büsselberg 2011; Wang et al. 2023). The cochlea is particularly vulnerable because sensory hair cells have limited regenerative capacity, so clinically relevant injury may result in persistent hearing impairment. Although sodium thiosulfate is approved for selected pediatric patients with localized tumors, no broadly applicable preventive treatment is established for adults with cisplatin-induced ototoxicity (Freyer et al. 2017; Kros and Steyger 2019; Li et al. 2023). An effective otoprotective strategy must therefore protect cochlear cells while preserving the antitumor activity of cisplatin.

Calcium homeostasis is a plausible point at which early cellular stress may become sustained cochlear injury. Ca²⁺ signals are required for hair-cell mechanotransduction, adaptation, synaptic transmission, and mitochondrial metabolism (Berridge et al. 2003; Chan and Rouse 2016; Fettiplace and Nam 2019). By contrast, prolonged intracellular Ca²⁺ elevation can promote oxidative stress, mitochondrial dysfunction, and apoptosis. Altered calcium handling has been implicated in cisplatin ototoxicity (Lu et al. 2019), but most studies have relied on measurements at isolated endpoints. This makes it difficult to distinguish a gradual accumulation of damage from a temporally defined loss of calcium-control capacity.

The temporal behavior of Ca^{2+} may therefore be as important as its absolute magnitude. A cell can initially buffer a toxic stimulus through ER, mitochondrial, and plasma-membrane calcium-handling systems, whereas failure of these compensatory mechanisms may mark a transition toward persistent injury. Whether such a transition occurs during cisplatin ototoxicity has not been clearly defined. In particular, it remains unclear whether cochlear Ca^{2+} homeostasis can remain compensated during early exposure and then enter a sustained dysregulated state that continues after the initiating drug is removed. Defining this trajectory could identify an earlier stage of injury before overt hair-cell loss and provide a temporal framework for mechanistic intervention.

To investigate this question, we followed intracellular Ca^{2+} over 72 h during cisplatin injury and integrated the resulting trajectory with time-resolved transcriptomic analysis. This phenotype-first approach prioritized *Gna14* near the transition to sustained Ca^{2+} elevation. *GNA14* is a member of the Gq family of heterotrimeric G-protein α subunits, which can couple receptor signaling to $\text{PLC}\beta$ activation, IP_3 production, and Ca^{2+} release from the ER (Hubbard and Hepler 2006; Zou et al. 2019). We therefore combined *Gna14* loss-of-function with pharmacological modulation of Gq- IP_3 R signaling to examine its functional relationship with the emerging calcium-injury phenotype.

We also examined the ER-mitochondria interface because it provides a spatial platform through which calcium signaling can be translated into mitochondrial stress. Mitochondria-ER contacts (MERCs), also referred to as mitochondria-associated membranes, coordinate local Ca^{2+} transfer, mitochondrial metabolism, lipid exchange, and stress signaling (Rizzuto et al. 2012; Csordás et al. 2018). Excessive or maladaptive ER-to-mitochondrial Ca^{2+} transfer can promote mitochondrial Ca^{2+} overload, oxidative stress, and apoptosis (Giorgi et al. 2018). Altered ER-mitochondria organization has been described in vulnerable cochlear hair cells during aging (Perkins et al. 2020), but whether MERC remodeling accompanies the transition in calcium homeostasis during cisplatin ototoxicity remains poorly understood.

Finally, we used p-coumaric acid (p-CA), a phenolic compound with reported antioxidant and cytoprotective activities (Pei et al. 2016; Chen et al. 2024). Previous work showed biochemical and histopathological protection by p-CA in a rat model of cisplatin ototoxicity (Ekinci Akdemir et al. 2018), but the temporal calcium response and its relationship with ER-mitochondria remodeling and *Gna14*-associated signaling have not been defined. We therefore used p-CA as a pharmacological probe of this injury framework. We examined whether p-CA could attenuate the sustained calcium phenotype together with ER-mitochondria remodeling, organelle stress, and cochlear injury. Target engagement was explored using molecular docking and a cellular thermal shift assay, and the protective phenotype was evaluated in a chronic mouse model. Because an otoprotective adjunct must not compromise cisplatin-mediated tumor toxicity, we also tested the effect of p-CA on cisplatin cytotoxicity in two nasopharyngeal carcinoma cell lines.

2. Materials and Methods

2.1. Animals and chronic ototoxicity model

All animal procedures were approved by the Institutional Animal Care and Use Committee of Fudan University (Approval No. IACUC-DWZX-2023-031) and followed the NIH Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice (5 weeks old; Jihui Laboratory Animal Care, Shanghai, China) were housed under specific-pathogen-free conditions on a 12-h light/dark cycle with free access to food and water. For the chronic model, mice were randomly assigned to four groups: Control, Cisplatin, Cis+p-CA (5 mg/kg), and Cis+p-CA (2 mg/kg). Baseline auditory brainstem responses (ABRs) were recorded two days before treatment. Each of three 10-day cycles consisted of cisplatin (4 mg/kg/d; Sigma-Aldrich, St. Louis, MO, USA) given intraperitoneally for three consecutive days, followed by seven days of recovery. Mice in the p-CA groups received p-CA (5 or 2 mg/kg; Sigma-Aldrich) intraperitoneally 2 h before each cisplatin injection; controls received saline. Final ABRs were measured on day 40, after which cochleae were collected.

2.2. Organotypic culture of postnatal cochleae

Cochlear explants were prepared from postnatal day 3 (P3) mice. Cochleae were dissected in ice-cold PBS, and the organ of Corti with the spiral ganglion was isolated and mounted on Cell-Tak-coated (BD Biosciences, San Jose, CA, USA) coverslips. Explants were cultured in DMEM/F12 (Gibco, Grand Island, NY, USA) with N2/B27 (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin–streptomycin at 37 °C in 10% CO₂.

2.3. Drug treatments

For dose–response experiments, explants were pretreated with p-CA (30, 60, 100, 150, or 200 µM) for 2 h before 24-h co-incubation with 30 µM cisplatin. For mechanistic experiments, explants were pretreated for 2 h with 100 µM p-CA, 50 µM carbachol (Sigma-Aldrich), 1 µM FR900359 (MedChemExpress, Monmouth Junction, NJ, USA; in DMSO, final DMSO < 0.1%), or 50 µM 2-APB (Sigma-Aldrich) before 30 µM cisplatin. For the pharmacological rescue experiment, explants were pretreated with 100 µM p-CA for 2 h and then co-treated with 50 µM carbachol during cisplatin exposure. siRNA experiments began 24 h after transfection.

2.4. Cell culture and viability

HEI-OC1 cells (RRID:CVCL_D899) were obtained from iCell Bioscience (Shanghai, China; Cat. No. iCell-m114). The supplier lists this product as STR-identified. Human 6-10B cells were obtained from FuHeng Biotechnology (Shanghai, China; Cat. No. FH1070), and CNE-2 cells from Sunncell Biotechnology (Wuhan, China; Cat. No. SNL-723). The Sunncell product record identifies CNE-2 as a nasopharyngeal carcinoma cell product but also notes documented HeLa cross-contamination; accordingly, the present

study uses it only as a commercially supplied in vitro tumor-line model and does not claim independently authenticated nasopharyngeal lineage. HEI-OC1 cells were grown in high-glucose DMEM with 10% FBS, without antibiotics, at 37 °C in 10% CO₂. The 6-10B and CNE-2 cells were grown in RPMI-1640 with 10% FBS at 37 °C in 10% CO₂; CNE-2 medium additionally contained 1% penicillin-streptomycin. All three cell lines tested negative for mycoplasma in our laboratory using the BeyoDirect Mycoplasma qPCR Detection Kit (Beyotime, C0303M). STR profiling was not repeated in our laboratory. For dose-response experiments, HEI-OC1 cells were pretreated with p-CA (20, 40, 60, or 80 µM) for 2 h before 30 µM cisplatin for 24 h. For tumor-line experiments, 6-10B and CNE-2 cells were treated with cisplatin (10 µM) alone or with p-CA (50 µM) for 24 h. Viability was measured by CCK-8 (Dojindo, Kumamoto, Japan) at 450 nm.

2.5. Immunofluorescence and confocal microscopy

Images were acquired at room temperature using a Leica STELLARIS STED confocal microscope operated in conventional confocal mode and equipped with an HC PL APO CS2 40×/1.30 oil-immersion objective and LAS X software (version 4.6.1.27508). DAPI, Alexa Fluor 488, Cy3/TMR red, and Alexa Fluor 647 were sequentially excited at 405, 499, 553, and 653 nm, respectively, with corresponding detection windows of 425–504, 504.1–556.1, 558.5–643.5, and 657.8–749.8 nm. Images were acquired at 1024 × 1024 pixels and 8-bit depth using unidirectional scanning at 600 Hz, with a pixel dwell time of 0.8625 µs and a pinhole setting of 0.91 Airy units. Line and frame averaging were each set to 1. Z-stacks were acquired at approximately 2 µm intervals and processed by maximum-intensity projection. Acquisition settings were kept identical across groups within each staining experiment. Fluorescence channels were pseudocolored for figure presentation where appropriate; displayed colors therefore did not necessarily correspond to the native fluorophore emission colors. Hair-cell counts, fiber density, and synapse numbers were quantified in ImageJ (v1.54g; NIH, Bethesda, MD, USA). Individual fluorescence channels and their corresponding merged images were derived from the same microscopic field. When brightness or contrast adjustments were used for presentation, they were applied uniformly to the entire image and did not selectively alter local image features.

2.6. Auditory brainstem responses

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and kept at 37 °C. ABRs were recorded on a TDT System III workstation (Tucker-Davis Technologies, Alachua, FL, USA), with electrodes at the vertex (active), ventral to the test ear (reference), and the contralateral thigh (ground). Clicks and tone bursts (4, 8, 16, 24, 32 kHz) were delivered, and thresholds were taken as the lowest level giving a reproducible waveform in 5-dB steps. The maximum stimulus level was 90 dB SPL. If no reproducible response was detected at this level, the result was recorded as no response (NR), rather than as a measured threshold of 90 dB SPL. Both ears were assessed in eight mice per group. NR

denotes a threshold beyond the measurable range and was retained as a censored observation rather than assigned a measured value of 90 dB SPL.

2.7. Reactive oxygen species and mitochondrial membrane potential

Mitochondrial superoxide was detected using MitoSOX Red (5 μ M; Thermo Fisher Scientific), and mitochondrial membrane potential was assessed using TMRM (20 nM; Thermo Fisher Scientific) in HEI-OC1 cells. MitoSOX was used in both cochlear explants and HEI-OC1 cells. Cells or explants were incubated with MitoSOX Red or TMRM for 30 min at 37 °C, washed, and imaged immediately using a Leica STELLARIS 8 confocal microscope. Fluorescence intensities were quantified using ImageJ. For the hair-cell mitochondrial membrane-potential assay shown in Fig. 6I, cochlear explants were stained using a JC-1 mitochondrial membrane potential assay kit (Beyotime, C2006) according to the manufacturer's instructions. Explants were incubated with freshly prepared JC-1 staining working solution for 20 min at 37 °C and washed twice with 1 $\times$ JC-1 staining buffer before imaging. JC-1 aggregates were detected in the red channel and JC-1 monomers in the green channel. Mitochondrial depolarization was assessed using the background-corrected JC-1 green-to-red fluorescence ratio. Images were acquired under identical settings across experimental groups and quantified using ImageJ. Five independent experiments were performed per group, with 10 cells quantified in each experiment; the experiment-level mean was used as the biological replicate.

2.8. Live-cell calcium imaging

Intracellular calcium was assessed using Fluo-4 AM (5 μ M; Beyotime, Shanghai, China). Explants were loaded for 30 min at 37 °C and imaged using a Leica STELLARIS 8 confocal microscope. Cisplatin (30 μ M) was applied for 24 h and then withdrawn, with measurements covering up to 72 h from the start of exposure. Independent explants were used at the specified sampling time points. Imaging covered the apical, middle, and basal turns (Fig. 1) or the middle turn (Fig. 5). Fluorescence was expressed as background-corrected F/F_0 , calculated as $(F_{\text{raw}} - F_{\text{bg}})/(F_{0\text{raw}} - F_{\text{bg}})$, where F_{raw} is the measured fluorescence, F_{bg} is the background signal, and $F_{0\text{raw}}$ is the baseline reference fluorescence. The early compensated and later decompensated phases describe the observed transition from fluctuating to sustained elevated fluorescence.

2.9. Transmission electron microscopy and morphometry

Cochleae from adult mice were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined on a Talos L120C microscope (Thermo Fisher Scientific). Contacts and mitochondrial morphology were scored in ImageJ by an investigator blinded to group, with

mitochondrial morphometry performed in cochlear hair-cell regions sampled at different section planes from 5 independent mice per group. An ER-mitochondria contact was defined as ER within 30 nm of the mitochondrial outer membrane. MERC coverage was the length of outer membrane within 30 nm of ER, divided by the total mitochondrial perimeter (%). Aspect ratio was the major-to-minor axis ratio, and cristae density the number of cristae per μm^2 . The TEM experiments in Figs. 4 and 6 used separate sets of five mice per group from the same modeling cohort; animals were not shared between these two TEM experiments.

2.10. RNA sequencing and bioinformatics

RNA sequencing was performed on control cochlear explants and cisplatin-treated explants collected at 0, 12, and 24 h after withdrawal following 24 h of cisplatin exposure, with three independent biological replicates per group. This sequencing experiment was treated as discovery-stage analysis rather than as a stand-alone confirmatory experiment. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA); quality was assessed using a NanoDrop 2000 and an Agilent 2100 Bioanalyzer. Libraries were prepared with the TruSeq Stranded mRNA LT kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina HiSeq 4000 platform (150-bp paired-end reads). Reads were trimmed with Trimmomatic. Differential expression was analyzed from the sample-level count data using DESeq2, which estimates dispersion across genes and applies negative-binomial generalized linear models; Benjamini-Hochberg false-discovery-rate control was used. Differentially expressed genes were defined as $|\log_2 \text{fold change}| > 1$ and $\text{FDR} < 0.01$. KEGG enrichment was assessed using the hypergeometric distribution, and additional enrichment analysis was performed with Metascape (version 3.5; accessed June 2026). A protein-protein interaction network of calcium-related genes was generated in STRING (accessed June 2026), visualized in Cytoscape (version 3.10), and ranked by degree and betweenness using NetworkAnalyzer. Calcium-related genes were grouped into influx, efflux, and regulatory modules for time-resolved analysis at 0, 12, and 24 h after cisplatin withdrawal. Candidate findings were evaluated in independent qPCR, immunoblotting, loss-of-function, and pharmacological experiments with five biological replicates per group. Raw sequencing data are available in the Genome Sequence Archive under accession CRA050592 (BioProject PRJCA075109).

2.11. Western blotting

Samples were lysed in RIPA buffer with protease and phosphatase inhibitors (Roche), and protein was quantified by BCA (Thermo Fisher Scientific). For cochlear Western blotting, five independently prepared pooled samples were analyzed per group; each pool comprised 20 cochleae from 10 animals. Equal protein (20 μg) was separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA), and blocked in 5% non-fat milk. Primary antibodies were diluted in Western blot primary-antibody dilution buffer and incubated overnight at 4 °C: GNA14 (Proteintech, 13350-1-AP, 1:200), HSPA5/GRP78 (Proteintech, 11587-1-AP, 1:2000), ATF4 (Proteintech, 10835-1-AP, 1:500), CHOP (Abcam, ab11419, 5

μg/mL), cleaved caspase-3 (Proteintech, 25128-1-AP, 1:500), caspase-3 (Invitrogen, 700182, 0.1 μg/mL), GAPDH (MedChemExpress, YA6008, 1:10000), phospho-p38 (Cell Signaling Technology, 9211, 1:1000), p38 (MedChemExpress, YA252, 1:500), phospho-JNK (Cell Signaling Technology, 9251, 1:1000), JNK (Proteintech, 81629-1-RR, 1:20000), phospho-ERK1/2 (Invitrogen, 14-9109-82, 1 μg/mL), and ERK1/2 (Proteintech, 11257-1-AP, 1:2000). HRP-conjugated goat anti-rabbit IgG (H+L) (Invitrogen, 31460, 1:10000) or goat anti-mouse IgG (H+L) (Invitrogen, 31430, 1:5000) was used as appropriate for the primary antibody. Bands were detected by ECL (Millipore) on a ChemiDoc system (Bio-Rad). Manufacturer-provided product information was used to document application validation and specificity boundaries. For GNA14 antibody 13350-1-AP, Proteintech reports reactivity with human and mouse samples and application testing in WB, IHC, and IF/ICC, including mouse-lung WB and HeLa/HepG2 IF. For cleaved-caspase-3 antibody 25128-1-AP, Proteintech states that the antibody recognizes cleaved caspase-3 fragments and does not recognize full-length caspase-3. These are manufacturer validation statements, not independent validation in cochlear tissue. No independent antibody-specificity validation was performed in this study, and application images alone were not interpreted as knockout-based specificity evidence.

2.12. Quantitative real-time PCR

RNA was reverse-transcribed with the PrimeScript RT kit (Takara, Dalian, China), and qPCR was run on a CFX96 Touch system (Bio-Rad) with SYBR Green (Takara). Relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method, with GAPDH as the reference for cochlear and HEI-OC1 samples. Gna14 was also measured in cisplatin-treated 6-10B and CNE-2 cells, with β-actin as the reference. Primers are listed in Online Resource 1, Table S1. For cochlear qPCR, five independent pooled samples were analyzed per group, each containing 20 cochleae from 10 animals. RNA and protein were obtained from separately collected pools.

2.13. siRNA transfection

siRNAs were delivered with siRNA-mate plus (GenePharma, Shanghai, China). siRNA against Gna14 and a non-targeting control (GenePharma) were mixed with reagent, incubated 20 min, and added to HEI-OC1 cells or explants at 30 nM. Knockdown was confirmed at 48 h by qPCR and Western blot. Sequences are listed in Online Resource 1, Table S2.

2.14. Molecular docking

The Gna14 structure was taken from the AlphaFold Protein Structure Database and the p-CA structure from PubChem. Docking used AutoDock Vina, with structures prepared in AutoDockTools and the search grid centered on the predicted pocket. The lowest-energy pose was visualized in PyMOL.

2.15. Cellular thermal shift assay

Cellular target engagement was assessed by live-cell cellular thermal shift assay (CETSA) in HEI-OC1 cells. Cells were incubated with p-CA (30 μ M) or an equal volume of DMSO at 37 °C for 30 min. Treated cells were then aliquoted and heated from 50 to 90 °C in 5 °C increments for 3 min, followed by cooling, lysis, and centrifugation to remove aggregated protein. The soluble fraction was immunoblotted for Gna14, and band intensities were normalized to the lowest temperature to generate melting curves. Eight independent experimental batches were analyzed, with matched DMSO and p-CA conditions within each batch. Curve-level area under the curve (AUC) and apparent melting temperature (T_m) values were compared using a two-sided exact Wilcoxon signed-rank test.

2.16. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD), with individual biological-replicate values shown whenever applicable. The independent biological unit, rather than individual cells, microscopic fields, technical wells, ears, or TEM images, was used for inference. Fields or cells were first averaged within each well or specimen, bilateral auditory measurements were averaged within each mouse, and TEM fields were averaged within each mouse. The primary explant sample size was estimated prospectively from a pilot middle-turn hair-cell-density experiment (three explants per group; means 24.5 and 29.0 cells/100 μ m; pooled SD 1.87; standardized effect size 2.41). A two-sided two-sample calculation with α = 0.05 and 80% power required four explants per group; five were used to allow for experimental loss. Other assays used the prespecified number of independently prepared biological samples feasible for the assay, and no retrospective power calculation was used to reinterpret nonsignificant findings. Distributional shape was inspected and Shapiro–Wilk and Brown–Forsythe diagnostics were recorded where calculable; because these diagnostics have limited power at n = 5–8, test selection also considered the experimental design and endpoint scale. Prespecified contrasts between independent continuous outcomes were analyzed using two-sided Welch t tests, which do not assume equal variances. Matched continuous measurements were analyzed using two-sided paired t tests. For replicate-level cell-count or density contrasts in which an arm was constant, including a group with no detected positive cells, all observed zeros were retained and an exact two-sided permutation test of the mean difference was used instead of treating zeros as missing or forcing a zero variance estimate. P values were adjusted using the Holm procedure only within the prespecified family that addressed the same biological question. The prespecified contrasts were Control versus Cisplatin and Cisplatin versus the relevant genetic or pharmacological intervention; dose-response panels additionally compared Cisplatin with each p-CA dose. Time-course experiments used independent explants at each time point and were analyzed using prespecified contrasts within time point, with Holm correction across the displayed time points for the same contrast; no repeated-measures or group-by-time interaction was inferred. Outcomes measured across cochlear turns or frequencies were summarized at the mouse level and analyzed within each displayed stratum, with Holm correction across the corresponding prespecified strata; no frequency-by-treatment or turn-by-treatment interaction was claimed. CETSA curve-level AUC

and apparent T_m were compared between eight matched DMSO and p-CA batches using two-sided exact Wilcoxon signed-rank tests. RNA-sequencing differential expression was analyzed separately using DESeq2 with false-discovery-rate control. Exact adjusted P values for the principal prespecified comparisons are reported in the Results and figure legends. Complete prespecified test results, group-level summaries, and assumption diagnostics are reported in Supplementary Table S3; this table does not include individual raw observations. Statistical tests were two-sided and adjusted $P < 0.05$ was considered significant. Analyses were performed using GraphPad Prism 9 and independently checked from the source data. In vitro cochlear imaging and immunofluorescence generally used five independent explants per group; cochlear Western blotting and qPCR used five independently prepared pooled samples per assay (20 cochleae from 10 animals per pool, with separate pools for protein and RNA); TEM morphometry used five independent mice per group; HEI-OC1 MitoSOX and TMRM imaging used five independent experiments per group; and in vivo assays used eight mice per group. In Fig. 6I, 10 cells were quantified in each of five independent experiments and the experiment-level mean was analyzed (biological $n = 5$). Group allocation was randomized by drawing lots. Experiments and outcome assessment were performed blinded to group allocation; group identities were revealed for statistical analysis. Microscopic fields were selected randomly. No samples were excluded.

3. Results

3.1. Cisplatin induces a compensated-to-decompensated calcium transition before cochlear injury

We assessed calcium signals in independent cochlear explants at defined time points during cisplatin exposure and recovery. Explants were exposed to 30 μM cisplatin for 24 h, followed by drug withdrawal, and Fluo-4 AM measurements extended to 72 h from the start of exposure (Fig. 1A).

The calcium signal was biphasic rather than steadily progressive (Fig. 1B,C). Early measurements showed fluctuations, followed by a pronounced and sustained elevation at later sampling points. We refer to these early and late patterns as the compensated and decompensated phases, respectively. Elevated fluorescence persisted after cisplatin withdrawal, indicating sustained calcium dysregulation under these experimental conditions (Danese et al. 2021; Lu et al. 2019).

We then compared calcium and mitochondrial superoxide signals in the same explants at each sampling point (Fig. 1D,E). Both signals remained lower in controls. In cisplatin-treated explants, Fluo-4 AM and MitoSOX fluorescence increased at the 24-h sampling point and remained elevated at 36 and 48 h, linking sustained calcium dysregulation with mitochondrial oxidative stress.

Hair-cell loss appeared later. Myosin7a-positive cell counts were preserved during the compensated phase and then declined, with the greatest loss at 72 h and in the basal turn (Fig. 1F). The later fall in Fluo-4 signal may partly reflect loss of probe signal as cells died. Together, these observations associate sustained calcium dysregulation with mitochondrial oxidative stress and subsequent cochlear injury.

3.2. Time-resolved transcriptomics identifies Gna14 at the calcium-transition boundary

We next used time-resolved RNA sequencing to identify molecular changes associated with the early calcium trajectory. We first examined enriched pathways, then calcium-related functional modules, and finally individual candidate genes.

The calcium signaling pathway was among the enriched KEGG terms (Fig. 2A). Calcium-related differentially expressed genes were grouped into influx, efflux, and regulatory modules (Fig. 2B,C). The influx module increased from 0 to 24 h. The efflux and regulatory modules changed less over the same period. These post-withdrawal changes were associated with the sustained calcium dysregulation observed in Fig. 1.

Clustering within the influx module identified genes with progressive induction (Fig. 2D,E). We also generated a STRING protein–protein interaction network and ranked nodes by centrality in Cytoscape (Fig. 2F) (Szklarczyk et al. 2023; Shannon et al. 2003). Gna14 increased over time and occupied a high-centrality position (degree = 10; betweenness = 0.15). Gene Ontology analysis showed enrichment of sarcolemmal, synaptic-membrane, receptor-complex, and calcium/calmodulin-dependent compartments (Fig. 2G).

These analyses prioritized Gna14 near the transition to sustained calcium dysregulation. We therefore tested whether reducing Gna14 altered the injury phenotype.

3.3. Gna14 contributes to cisplatin-induced hair-cell injury and engages Gq–IP₃R signaling

We used Gna14 loss-of-function together with pharmacological modulation of Gq–IP₃R signaling.

Gna14 immunoreactivity was low in control explants and increased in parvalbumin-positive hair cells after cisplatin exposure (Fig. 3A). FR900359, an inhibitor of the Gq family, reduced the proportion of Gna14-high hair cells (Fig. 3A) (Schrage et al. 2015). FR900359 is not selective for Gna14, so this result supports involvement of Gq signaling rather than Gna14-specific pharmacology. Western blotting and qPCR also showed increased Gna14 protein and transcript abundance after cisplatin exposure (Fig. 3B and Online Resource 1, Fig. S2A).

Gna14 silencing reduced the cisplatin-induced Gna14 signal and preserved Myosin7a-positive hair cells. Non-targeting control siRNA did not provide the same protection (Fig. 3B,C,E and Online Resource 1, Fig. S2B). The key Holm-adjusted P values were 0.0317 for the exact-permutation Gna14-positive-cell comparison and 2.67×10^{-5} for the Welch-test hair-cell rescue comparison (Cisplatin versus siGna14+Cisplatin). These results support a functional contribution of Gna14 to cisplatin-induced hair-cell injury.

We next activated the broader Gq pathway with carbachol (Fig. 3D,F). Carbachol alone increased Fluo-4 fluorescence and reduced Myosin7a-positive cell counts relative to Control. However, adding carbachol to cisplatin did not significantly change either outcome relative to Cisplatin alone. In contrast, 2-APB reduced the calcium increase and preserved hair cells (Fig. 3D,F). These data implicate Gq-IP₃R signaling in the injury phenotype. They do not show that selective Gna14 activation alone is sufficient.

Taken together, Gna14 induction, the protective effect of Gna14 silencing, and Gq-pathway modulation connect Gna14-associated signaling with the transition to sustained calcium dysregulation.

3.4. The Gna14-associated calcium transition is accompanied by ER-mitochondria remodeling and apoptosis

We next asked whether the decompensated calcium state was accompanied by changes at ER-mitochondria contacts and by mitochondrial injury.

Metascape analysis highlighted membrane-contact and organelle compartments (Fig. 4A) (Zhou et al. 2019). Gna14 expression correlated with several genes involved in ER-mitochondria calcium transfer, including Itpr1, Itpr2, Vdac1, Mcu, and Plcb4 (Fig. 4B) (De Stefani et al. 2011; Giorgi et al. 2018). Gq-PLCβ signaling converges on IP₃ receptors, so these associations are compatible with a link to contact-site calcium transfer. They do not establish causality (Szabadkai et al. 2006).

Transmission electron microscopy showed greater ER-mitochondria apposition after cisplatin treatment (Fig. 4C). MERC coverage and the proportion of mitochondria in contact with the ER were increased (Fig. 4D,E). Cisplatin-treated mitochondria also had a lower aspect ratio and reduced cristae density (Fig. 4F,G), consistent with mitochondrial structural injury.

Cisplatin also increased cleaved caspase-3 and TUNEL labeling in hair cells (Fig. 4H,I). Gna14 silencing reduced both signals. For Control versus Cisplatin and Cisplatin versus Cis+siGna14, respectively, the Holm-adjusted P values were 0.000585 and 0.000585 for cleaved caspase-3 and 0.0159 and 0.0159 for TUNEL labeling. These findings support a contribution of Gna14 to the apoptotic response to cisplatin. However, MERC architecture was not measured after selective Gna14 manipulation, so the present data do not establish a direct causal link between Gna14 and MERC remodeling.

3.5. p-CA promotes Gna14 thermal stabilization and attenuates the decompensated calcium transition

Docking predicted a possible p-CA-binding pocket in Gna14 (Fig. 5A) (Jumper et al. 2021; Trott and Olson 2010). Across eight paired experimental batches, live-cell CETSA showed higher curve-level AUC and apparent T_m after p-CA treatment than after DMSO (both exact Wilcoxon P = 0.0078; Fig. 5B),

supporting cellular target engagement (Martinez Molina et al. 2013). CETSA does not define binding affinity or the binding site. In five independent experiments, p-CA also reduced the cisplatin-associated increase in Gna14 protein abundance (Holm-adjusted $P = 1.70 \times 10^{-5}$ for Cisplatin versus Cis+p-CA; Fig. 5C). The corresponding transcript data are shown in Online Resource 1, Fig. S2A.

p-CA attenuated the cisplatin-induced calcium increase at 4 and 6 h and consistently from 24 to 72 h, whereas the 2- and 12-h comparisons were not significant (Fig. 5D,E). Fluo-4 fluorescence rose markedly after 24 h in cisplatin-treated explants, whereas this late increase was reduced by p-CA. These data indicate that p-CA attenuated the transition to sustained calcium dysregulation.

Carbachol restored the calcium increase despite continued p-CA exposure (Holm-adjusted $P = 5.89 \times 10^{-5}$ for Cis+p-CA+Carbachol versus Cis+p-CA; Fig. 5F). Thus, broader Gq-pathway activation could overcome the calcium-lowering effect of p-CA. Together with the Gna14 loss-of-function and CETSA results, this finding supports involvement of Gna14-associated Gq signaling. The molecular selectivity and binding mode of p-CA remain to be established.

3.6. Pharmacological interception of the calcium transition is accompanied by reduced ER–mitochondria remodeling and organelle stress

p-CA reduced the cisplatin-associated increase in ER–mitochondria contacts (Fig. 6A–C). The proportion of mitochondria in contact with the ER and MERC coverage were lower with p-CA than with cisplatin alone. Mitochondrial aspect ratio and cristae density were also improved (Fig. 6D,E,H), consistent with partial preservation of mitochondrial ultrastructure.

Cisplatin increased the ER-stress proteins HSPA5, ATF4, and CHOP, whereas p-CA reduced these changes (Fig. 6F). For Control versus Cisplatin and Cisplatin versus Cis+p-CA, respectively, the Holm-adjusted P values calculated from the normalized relative-expression values displayed in the graphs were 0.000245 and 0.000163 for HSPA5, 0.000110 and 3.63×10^{-5} for ATF4, and 1.77×10^{-5} and 3.43×10^{-6} for CHOP. By qPCR, Chop, Atf4, and Hspa5 all showed significant changes in the corresponding contrasts (adjusted $P \leq 0.000305$; Fig. 6G). Both analyses were based on five independent experiments. JC-1 staining showed an increased green-to-red fluorescence ratio after cisplatin exposure, consistent with mitochondrial membrane depolarization. This increase was attenuated by p-CA (Fig. 6I). MitoSOX and TMRM measurements in HEI-OC1 cells are shown separately in Online Resource 1, Fig. S3. MAPK activation was also lower after p-CA treatment (Online Resource 1, Fig. S4) (Peng et al. 2018).

These results show that attenuation of sustained calcium dysregulation by p-CA was accompanied by less ER–mitochondria remodeling, lower ER-stress signaling, and better preservation of mitochondrial function.

3.7. p-CA dose-dependently protects hair cells and spiral ganglion neurons in vitro

Cisplatin caused marked loss of Myosin7a-positive hair cells along the tonotopic axis. p-CA preserved hair cells in a dose-dependent manner in the apical, middle, and basal turns (Fig. 7A,D–F). Higher doses were needed to achieve a similar degree of protection in the more vulnerable basal turn.

p-CA also protected the neural component of the cochlea. Cisplatin disrupted the neurite network and reduced spiral ganglion neuron integrity. p-CA preserved SGN innervation and Tuj1-positive neurites (Fig. 7B,C). Fiber density, fiber length, and SGN soma counts also moved toward control values (Fig. 7G–I). Similar dose-dependent protection was observed in HEI-OC1 cells (Online Resource 1, Fig. S1) (Kalinec et al. 2003).

3.8. Systemic p-CA preserves hearing and cochlear structure in vivo

p-CA was then tested in the chronic mouse model (Fig. 8A). Cisplatin increased auditory brainstem response thresholds across the tested frequencies. p-CA attenuated this threshold elevation, with a numerically larger effect at 5 mg/kg than at 2 mg/kg (Fig. 8B). All eight cisplatin-treated mice had no measurable response at the 90-dB ceiling at 32 kHz, whereas measurable responses were retained in both p-CA groups. For Cisplatin versus Cis+p-CA (5 mg/kg), Holm-adjusted $P = 0.00280$ at each displayed stimulus. For the 2-mg/kg group, the 4-kHz comparison was not significant (adjusted $P = 0.154$), whereas the remaining displayed comparisons had adjusted $P = 0.00280$.

The functional effect was accompanied by structural protection. p-CA reduced hair-cell loss (Fig. 8C,D), preserved spiral ganglion peripheral fibers (Fig. 8E,F), and improved ribbon-synapse integrity. It increased paired CtBP2/Homer1 puncta and reduced orphaned CtBP2-positive ribbons across coding frequencies (Fig. 8G,H).

3.9. p-CA does not reduce cisplatin cytotoxicity in the tested tumor cell lines

Cisplatin produced small but statistically detectable changes in Gna14 transcript abundance in 6-10B and CNE-2 cells (Holm-adjusted $P = 0.00216$ and 0.00977 , respectively), but these changes were far smaller than the increase observed in cochlear hair cells (adjusted $P = 3.78 \times 10^{-6}$; Online Resource 1, Fig. S5C) (Hao et al. 2024). p-CA also did not reduce the loss of viability caused by cisplatin in either line (Online Resource 1, Fig. S5A,B). These findings are limited to the two tested in vitro tumor models and require confirmation in other tumor settings (Benkafadar et al. 2017).

4. Discussion

This study reveals a time-dependent reorganization of calcium homeostasis and ER–mitochondria interactions during cisplatin ototoxicity. Early fluctuations in calcium fluorescence were followed by sustained dysregulation that persisted after cisplatin withdrawal. This transition was associated with mitochondrial oxidative stress and cochlear injury and was accompanied by increased ER–mitochondria apposition, greater MERC coverage, and mitochondrial structural injury. Time-resolved transcriptomics further identified Gna14 near the transition boundary. Gna14 silencing protected hair cells, while pharmacological modulation linked the phenotype to Gq–IP₃R signaling. Together, these findings place the calcium transition and ER–mitochondria remodeling within the same temporal framework of cisplatin-induced cochlear injury and identify Gna14-associated signaling as a molecular component of this process.

The temporal pattern of the calcium response is important because Ca²⁺ is required for normal hair-cell physiology, including mechanotransduction, synaptic transmission, adaptation, and mitochondrial metabolism (Berridge et al. 2003; Chan and Rouse 2016; Fettiplace and Nam 2019). An increase in intracellular Ca²⁺ therefore does not necessarily indicate irreversible injury. Previous work has implicated altered calcium handling in cisplatin ototoxicity, while oxidative stress and mitochondrial dysfunction are established features of later cochlear damage (Lu et al. 2019; Ramkumar et al. 2021; Wang et al. 2023). Our results add a temporal dimension to these observations. The early phase of relatively preserved Ca²⁺ homeostasis may reflect residual buffering capacity. Sustained elevation emerged later and was accompanied by increased mitochondrial superoxide and cochlear injury. The persistence of this elevation after cisplatin withdrawal further suggests that removal of the initiating toxicant may not immediately restore homeostasis once the decompensated state has developed. Thus, failure of calcium control may represent an intermediate stage between the initial cisplatin insult and overt cochlear injury.

A second major finding is the remodeling of the ER–mitochondria interface during this transition. MERCs are now recognized as dynamic signaling platforms that coordinate local Ca²⁺ transfer, mitochondrial metabolism, proteostasis, lipid exchange, and stress responses rather than as static structural contacts (Csordás et al. 2018; Larrañaga-SanMiguel et al. 2025; Silva-Pavez et al. 2026). Recent work has emphasized that changes in MERC architecture can distinguish adaptive from maladaptive cellular states. This concept is increasingly relevant in neurobiology. MAM remodeling has been linked to ER stress, mitochondrial calcium overload, and neuronal injury in several recent studies, including work published in Cellular and Molecular Neurobiology (Mohan and Talwar 2026; Li et al. 2026) and other neurological injury models (Xue et al. 2026; Zhang et al. 2026). These studies support the view that altered ER–mitochondria coupling can participate directly in the transition from cellular adaptation to organelle dysfunction.

In the present study, cisplatin increased ER–mitochondria apposition and MERC coverage at the stage when sustained Ca²⁺ dysregulation and mitochondrial injury emerged. These ultrastructural changes

were accompanied by a lower mitochondrial aspect ratio, reduced cristae density, activation of ER-stress signaling, loss of mitochondrial membrane potential, and hair-cell apoptosis. p-CA attenuated both the sustained calcium phenotype and the MERC abnormalities while preserving mitochondrial structure and function. These findings extend previous descriptions of mitochondrial injury in cisplatin ototoxicity by showing that the spatial relationship between the ER and mitochondria is also remodeled during cochlear injury. The parallel changes in Ca^{2+} homeostasis, MERC architecture, ER stress, and mitochondrial function suggest that inter-organelle calcium handling is an important feature of the transition from compensated stress to hair-cell injury.

This interpretation is also consistent with the established role of the IP_3R –GRP75–VDAC1 axis at ER–mitochondria contact sites. IP_3 receptors release Ca^{2+} from the ER and, together with GRP75 and VDAC1, create a local route for efficient Ca^{2+} transfer toward mitochondria (Szabadkai et al. 2006; Rizzuto et al. 2012; Giorgi et al. 2018). Recent neurological studies have further connected enhanced MAM coupling and IP_3R -associated Ca^{2+} transfer with mitochondrial calcium overload and neuronal apoptosis (Xue et al. 2026; Zhang et al. 2026). In our transcriptomic data, Gna14 expression correlated with several genes involved in ER–mitochondrial calcium handling, including *Itpr1*, *Itpr2*, *Vdac1*, *Mcu*, and *Plcb4*. Together with the Gq– IP_3R pharmacological experiments, these observations place Gna14-associated calcium signaling and MERC remodeling within a common injury framework.

Gna14 provides a plausible molecular link to this framework. GNA14 belongs to the Gq family of heterotrimeric G-protein α subunits and can couple receptor signaling to $\text{PLC}\beta$ -dependent IP_3 production and ER Ca^{2+} release (Hubbard and Hepler 2006; Zou et al. 2019). In our model, Gna14 was upregulated in association with sustained calcium dysregulation, occupied a central position in the calcium-related interaction network, and showed a protective loss-of-function phenotype. Gna14 silencing reduced hair-cell injury and apoptosis. Broader pathway manipulation produced results in the same direction: carbachol increased calcium accumulation, whereas inhibition of downstream IP_3R -associated calcium release reduced the phenotype. These findings support a functional contribution of Gna14-associated Gq– IP_3R signaling to the calcium transition. Gna14 is unlikely to act alone, however, because the transcriptomic response also involved other calcium influx, efflux, and regulatory pathways.

p-CA provided a pharmacological test of whether this injury transition could be attenuated. Previous work had already reported biochemical and histopathological protection by p-CA against cisplatin ototoxicity in rats (Ekinci Akdemir et al. 2018), and p-CA has also been studied more broadly for antioxidant and cytoprotective effects (Pei et al. 2016; Chen et al. 2024). The present study extends those observations by linking p-CA treatment to the temporal calcium transition, MERC remodeling, and Gna14-associated signaling. p-CA reduced the late Ca^{2+} increase, attenuated MERC remodeling and ER-stress signaling, preserved mitochondrial structure and membrane potential, and protected cochlear hair cells and neural elements. These cellular effects were accompanied by preservation of auditory thresholds in the chronic cisplatin model. CETSA further showed p-CA-dependent thermal stabilization of Gna14 in living cells, and broader Gq activation with carbachol overcame the calcium-lowering effect

of p-CA. Taken together, these findings connect the pharmacological phenotype of p-CA with Gna14-associated calcium signaling and downstream organelle stress.

The target-engagement data should be interpreted at the level supported by the experiments. CETSA demonstrates a p-CA-dependent change in Gna14 thermal stability in cells, while docking provides a structural hypothesis for a possible interaction. These approaches do not determine binding affinity or molecular selectivity. Additional biophysical studies and comparison with other Gq-family members will be useful for defining the interaction more precisely. Similarly, although Gna14-associated calcium signaling and MERC remodeling converge within the same injury process, the direction of this relationship remains to be resolved. Direct measurement of MERC architecture and ER-to-mitochondrial Ca^{2+} transfer after selective Gna14 manipulation would be an important next step.

For an otoprotective adjunct, preservation of cisplatin cytotoxicity is also important. In 6-10B and CNE-2 nasopharyngeal carcinoma cells, p-CA did not reduce cisplatin-induced loss of viability, and the cisplatin-associated Gna14 transcript changes were much smaller than those observed in the cochlear model (Hao et al. 2024). These findings provide preliminary evidence that cochlear protection can be separated from tumor-cell protection under the conditions tested. Other experimental approaches have likewise shown that otoprotection can coexist with cisplatin-mediated tumor control (Benkafadar et al. 2017; Zhao et al. 2026). Nevertheless, the present tumor experiments were limited to two cell lines, and confirmation in additional tumor types and tumor-bearing animal models will be required before drawing broader oncological conclusions.

Several limitations remain. The compensated-to-decompensated transition was defined primarily in an ex vivo cochlear model and should be validated in adult and in vivo settings. The phase labels describe the observed fluorescence pattern in this experimental system rather than a universal biological cutoff. Direct Gna14 gain-of-function was not examined, and the available pharmacological modulators were not Gna14-selective. MERC structure was not measured after selective Gna14 manipulation, preventing assignment of a direct causal sequence from Gna14 to MERC remodeling. Finally, the tumor-sparing analysis was limited to two nasopharyngeal carcinoma cell lines. Despite these limitations, the combined temporal, genetic, pharmacological, ultrastructural, and in vivo evidence supports a model in which sustained calcium dysregulation and ER-mitochondria remodeling mark a critical stage of cisplatin ototoxicity, with Gna14-associated Gq-IP₃R signaling contributing to this process.

5. Conclusion

Cisplatin ototoxicity showed a transition from compensated calcium handling to sustained dysregulation. Gna14-associated Gq-IP₃R signaling contributed to this transition, and p-CA attenuated the calcium phenotype and downstream organelle stress. In the chronic mouse model, p-CA also preserved hearing and cochlear structure. The results support a time-resolved view of cisplatin ototoxicity and identify sustained calcium dysregulation as a potential pharmacological target.

Abbreviations

p-CA: p-coumaric acid; MERC: mitochondria–ER contact; MAM: mitochondria-associated ER membrane; IP₃R: inositol 1,4,5-trisphosphate receptor; VDAC1: voltage-dependent anion channel 1; MCU: mitochondrial calcium uniporter; PLCβ: phospholipase C beta; UPR: unfolded protein response; CC3: cleaved caspase-3; ROS: reactive oxygen species; $\Delta\Psi_m$: mitochondrial membrane potential; ABR: auditory brainstem response; SGN: spiral ganglion neuron; IHC: inner hair cell; CETSA: cellular thermal shift assay; DEG: differentially expressed gene; PPI: protein–protein interaction; TEM: transmission electron microscopy; 2-APB: 2-aminoethoxydiphenyl borate.

Declarations

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 82571307) and the Shanghai Municipal Science and Technology Commission (Grant No. 25ZR1401049).

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

Shun-Xun Lai: Investigation, Formal analysis, Visualization, Writing – original draft. Hong-Bo Yu: Investigation, Methodology. Yong-Kang Sun: Investigation, Methodology. Qi-Ling Tong: Investigation, Methodology. Ying-Zi He: Conceptualization, Supervision, Writing – original draft, Writing – review and editing. Hui-Qian Yu: Conceptualization, Supervision, Writing – original draft, Writing – review and editing. All authors read and approved the final manuscript.

Ethics Approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Fudan University (approval no. IACUC-DWZX-2023-031) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Data Availability

The RNA-sequencing data have been released in the Genome Sequence Archive at the National Genomics Data Center under accession CRA050592 (BioProject PRJCA075109). Other datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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