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RESEARCH ARTICLE

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Circadian vulnerability of cisplatin-induced ototoxicity in the cochlea

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Abstract

The chemotherapeutic agent cisplatin is renowned for its ototoxic effects. While hair cells in the cochlea are established targets of cisplatin, less is known regarding the afferent synapse, which is an essential component in the faithful temporal transmission of sound. The glutamate aspartate transporter (GLAST) shields the auditory synapse from excessive glutamate release, and its loss of function increases the vulnerability to noise, salicylate, and aminoglycosides. Until now, the involvement of GLAST in cisplatin-mediated ototoxicity remains unknown. Here, we test in mice lacking GLAST the effects of a low-dose cisplatin known not to cause any detectable change in hearing thresholds. When administered at nighttime, a mild hearing loss in GLAST KO mice was found but not at daytime, revealing a potential circadian regulation of the vulnerability to cisplatin-mediated ototoxicity. We show that the auditory synapse of GLAST KO mice is more vulnerable to cisplatin administration during the active phase (nighttime) when compared to WT mice and treatment during the inactive phase (daytime). This effect was not related to the abundance of platinum compounds in the cochlea, rather cisplatin had a dose-dependent impact on cochlear clock rhythms only after treatment at nighttime suggesting that cisplatin can modulate the molecular clock. Our findings suggest that the current protocols of cisplatin administration in humans during daytime may cause a yet undetectable

Abbreviations: ABR, auditory brainstem response; CDDP, cis-diamminedichroroplatinum, cisplatin; CtBP2, C-terminal binding 2 protein; DPOAE, distortion product of otoacoustic emissions; EDTA, ethylenediaminetetraacetic acid; GLAST, glutamate aspartate transporter; ICP-MS, induced-coupled mass spectrometry; GluA2, glutamate receptor subunit A2; HET, heterozygous; IHC, inner hair cell; IPC, inner phalangeal cell; KO, knockout; OC, organ of Corti; OHC, outer hair cell; PBS, phosphate-buffered saline; PER2::LUC, Period2 Luciferase; PFA, paraformaldehyde; SCN, suprachiasmatic nucleus; SGN, spiral ganglion neuron; SV, Stria vascularis; tdTom, Rosa26-tdTomato; WT, Wild-type; ZT, zeitgeber time.

[Corrections added on August 26, 2020, after first online publication: Affiliation '1' has been added for Christopher R. Cederroth.]

damage to the auditory synapse, more so in already damaged ears, and severely impact auditory sensitivity in cancer survivors.

KEYWORDS

afferent synapse, circadian, cisplatin ototoxicity, hearing loss, GLAST, PER2, synaptic pairing

1 | INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II, CDDP) is a highly effective chemotherapeutic agent commonly used in the treatment of solid tumors and several malignant tumors, such as those of the head and the neck, in adults and children.[1,2] Cisplatin is known to have numerous debilitating side effects such as permanent hearing loss, neurotoxicity, and nephrotoxicity.[2,3] The impact of cisplatin on auditory function has been observed near half a century ago,[4] and yet, is not receiving enough attention in current medical practice.[5,6] The prevalence of hearing loss following cisplatin treatment is dependent upon cumulative dose,[5] and has been reported as being between 4% and 90% depending on factors such as age and gender of the patient, a combination of other ototoxic agent(s) or irradiation used, exposure to concomitant noise, duration of the treatment, and administration methods.[7] A study on North Americans showed that 18% have profound hearing loss, and 40% develop tinnitus after increasing cumulative cisplatin doses.[5] Children generally show greater vulnerability to cisplatin-mediated hearing loss than adults.[8-10] As hearing loss has recently been ranked fourth leading cause of years lived with disability,[11] while also being an established risk factor for dementia,[12] understanding the mechanisms underlying cisplatin-mediated ototoxicity is of major health relevance and of great need for the prevention and treatment of hearing loss.

In humans and experimental animals, cisplatin-induced hearing loss usually impairs the high frequency regions of the cochlea and extends to lower frequencies with prolonged treatment. A variety of procedures have been used to identify the key structures causing hearing loss by cisplatin and found that marginal cells of the stria vascularis (SV), spiral ligament, the organ of Corti (OC), (primarily outer hair cells [OHC] and subsequently inner hair cells [IHC]), supporting cells and the spiral ganglion neurons (SGN) are particularly susceptible and prone to cisplatin retainment for a long period of time.[3,13,14] Cisplatin targets tumor proliferative cells through DNA damage.[1] Since cochlear cells do not proliferate, it is more reasonable that ototoxicity is caused by mitochondrial damage.[15] A dominant pathway for cisplatin ototoxicity is NOX3 signaling, leading to the production of reactive oxygen species (ROS), which in turns induce inflammatory reactions promoting apoptotic and necrotic cell death.[15,16]

While the understanding of the actions of cisplatin on hair cell death has increased in the last decade, very little is known on the impact of cisplatin on the IHC/afferent neuron synapse, which dysfunction perturbs the propagation of auditory information along the auditory nerve. Indeed, recent work has suggested that the most vulnerable elements in the auditory periphery are actually the cochlear nerve terminals rather than the hair cells or the stria vascularis.[17,18] Even after noise exposures causing only transient threshold elevation, there can be significant loss of synaptic connections between the IHCs and the peripheral terminals of cochlear nerve fibers, despite no loss of hair cells or persistent threshold elevation. Although threshold shift is a sensitive metric of hair cell damage, diffuse loss of IHC synapses, or their cochlear nerve fibers has to exceed 80%-90% before behavioral thresholds rise significantly and can reach 40%-50% without elevating thresholds of compound neural potentials such as the auditory brainstem responses (ABR).[17] This type of auditory neuropathy results from damage or loss of presynaptic ribbons, a structure in hair cells responsible for the release of neurotransmitters, such as glutamate at sub-millisecond temporal precision and maintenance of signal transmission.[19] This glutamatergic synapse is shielded by the glutamate aspartate transporter (GLAST), which has been found in supporting inner phalangeal cells (IPCs) cells surrounding the IHCs, fibrocytes in the spiral ligament and the spiral limbus, and satellite cells of the SGNs.[20] Similar to the central nervous system, GLAST limits the accumulation of extracellular glutamate, thereby preventing tonic activation of receptors and subsequent excitotoxicity.[21] Consistently, mice lacking GLAST show greater hearing threshold shifts and synaptic damage than do wild-type (WT) mice after noise exposure,[22] as well as greater vulnerability to aminoglycoside and salicylate ototoxicity.[23,24] As guinea pigs and rats are more susceptible than mice to cisplatin-mediated ototoxicity,[25] GLAST may potentially be involved in the protection of the synapse subjected to cisplatin treatment. Furthermore, since humans do not display GLAST expression in IPCs,[26] GLAST KO mice may more closely reflect the vulnerability of the human synapse to auditory insults, and thus, facilitate the translation of preclinical findings to humans.

Another aspect poorly investigated, is the consideration of circadian aspects in the ability to mitigate cisplatin ototoxicity. The cochlea is indeed a highly circadian organ, with circadian rhythms of the protein PER2 as ample as what is found in the liver, and expressing elements of the core clock machinery

such as *Per1*, *Per2*, *Bmal-1*, and *Rev-erbα* mainly in hair cells and SGNs.[27] At the cellular level, oscillatory patterns propagate from the apex toward middle turns and cellular synchronicity within the cochlea requires the function of voltage-gated potassium channel and extracellular calcium.[28] Two consequences from this auditory clock have been evidenced: first, the cochlea is more vulnerable at nighttime to noise damage,[29] a process that requires circulating glucocorticoids [30]; second, the action of drugs (eg, dihydroxyflavone, a selective TrkB agonist, and dexamethasone) to protect from noise-damage depending on the time-of-the-day, the TrkB agonist is effective when administered at nighttime, and dexamethasone at daytime.[29,30] Thus, drugs may have a different impact depending on the time-of-the-day when administered. Whether the ototoxic effects of cisplatin on the auditory system depend on the time-of-the-day is poorly known. Here, we evaluate the impact of GLAST loss of function after day or night cisplatin administration using a protocol of mild ototoxicity.

2 | METHODS

2.1 | Experimental animals

All experimental procedures on animals were performed in accordance with the guidelines and the regulations of Karolinska Institutet and approved by the regional ethics committee (Stockholm's Norra Djurförsöksetiska Nämnd, N156/14 and N140/15).

GLAST KO mice[31] and their WT littermates were obtained from heterozygous (HET) crosses or from KO males crossed with HET females. Similar to what we previously reported,[24] we observed a non-mendelian distribution in the progeny of crosses between heterozygous KO mice (n = 200): 29% wild-type animals, 54% heterozygous knockout animals, and 15% homozygous knockout animals. Two percent of the pups had an unclear genotype. Males and females aged between 2 and 3 months were pooled for this project, since separated genders did not present any significant differences and used for audiological, morphological, and molecular measurements. GLAST-CreER^{T2} transgenic mice[32] were crossed to the Rosa26-tdTomato (tdTom) Cre-reporter line (obtained from the Jackson Laboratory, B6.Cg-Gt(Rosa)26Sor$^{tm14(CAG-tdTomato)Hze}$/J, JAX stock: 007914)[33] to generate GLAST-CreER^{T2};R26R-tdTom mice, in which CreER^{T2} is hemizygous and Rosa26-tdTom is either heterozygous or homozygous. All animals were ≥8 weeks old at the onset of experiments. Genetic recombination was induced by a daily intraperitoneal injection of 2 mg of tamoxifen (Sigma, 20 mg/mL in 1:9 ethanol: corn oil) for five consecutive days. PER2::LUC (mPer2Luc) mice[34] were used in order to track PER2 bioluminescence from cochlear explants in the presence or absence of cisplatin treatment. All animals were in a C57BL/6J genetic background. Animals had ad libitum access to food (Lactamin R34) and water. Temperature in the animal facility was maintained between 19 and 21°C and lights were on at 6:00 AM and off at 6:00 PM Work in darkness was performed under red light.

2.2 | In vivo and in vitro drug treatment

For in vivo experiments, GLAST WT and KO male and female mice were used. Forty-nine mice (24 WT and 25 KO) underwent auditory measurements prior to the treatment (baseline measurements) and at the end of the treatment (24 hours and 2 weeks post). Cisplatin treated mice (479306, Sigma-Aldrich) received an intraperitoneal (i.p.) injection once-daily in a dose of 4 mg/kg (30 mg/kg, 200 mL/30 g body weight) (cisplatin diluted in 0.9% NaCl) for four consecutive days, either at daytime, at 09.00 AM (Zeitgeber time 3, ZT3) or at nighttime at 09.00 PM (ZT15). Zeitgeber time is used for indicating the phase of an entrained circadian cycle in which ZT 0 is the time when lights are on and ZT 12 when the lights are turned off. Twelve mice (6 WT and 6 KO) were used as control animals since they were treated with saline (0.9% NaCl) for four consecutive days either at day or nighttime. GLAST WT and KO male mice (14 WT and 14 KO) were used for measuring the platinum compounds in the OC/SGN, SV, and plasma 1 hour after one single i.p. cisplatin injection (4 mg/kg) either administered at daytime (08:00 AM) or at nighttime (08:00 PM). For in vitro experiments, cisplatin dissolved in phosphate-buffered saline (PBS) was used in different concentrations of 40, 80, 160, 400, 800, and 1600 nM. Final concentrations of cisplatin were made from a stock of 2 mM.

2.3 | Auditory measurements

Stimuli were generated using BioSigRP software which was connected to Tucker-Davis Technologies System III hardware. For distortion product otoacoustic emissions (DPOAE) recordings, two independent signals were broadcast from a single multifunction processor (RX6) to two separate programable attenuators (PA5). Each attenuator connected to a dedicated speaker driver (ED1) that played the stimulus over a dedicated electrostatic speaker (EC1). Sound from the two speakers merged in a custom made, ear-canal probe with a soft plastic tip for closed field stimulation. For ABR measurements, the signal went from a processor to an attenuator. It was then fed to an amplifier (SA1) and output through an open field speaker (MF1).

Stimulus intensity was calibrated using a ¼-inch microphone and a conditioning preamplifier (4939 A 011 and 2690 A 0S1, Brüel and Kjær) connected to an RX6

processor. For DPOAE stimuli the microphone was coupled as closed field to the ear-canal probe and mimicked the location of the eardrum. For ABR stimuli the microphone's position approximated the location of an experimental animal's ear and had its membrane facing the open field speaker. Speakers were calibrated before the beginning of each experiment using a frequency sweep (4-32 kHz). The output was corrected to produce a flat spectrum at 100 dB SPL (open field speaker, ABR) and 70 and 80 dB SPL (closed field speakers, DPOAE).

Mice were anesthetized with a mixture of ketamine (ketaminol 50 mg/mL, Intervet, 511485) and xylazine (Rompun 20 mg/mL, Bayer, KP0A43D) (100 and 10 mg/kg body weight, 200 μL/30 g, respectively) and placed in a custom-made acoustic enclosure with sound absorbing material on the walls and ceiling ($60 \times 60 \times 100$ cm^3). Body temperature was maintained at 36.5°C using a heating pad (HB 101/2 Panlab, Harvard Apparatus). Temperature was under control during each experiment by placing a thermometer under the mouse's body. We first recorded DPOAEs. In short, the acoustic coupler was inserted into the ear canal. A microphone (EK 23103, Knowles) inserted in the acoustic coupler with a preamplifier (ER-10B+, Etymotic Research) and connected to a processor (200 kHz sample rate) measured sound intensity in the ear canal. Each speaker played one of two primary tones (f_1 and f_2) and swept in 5 dB steps from 80 to 10 dB SPL (for f_2). To avoid distortion of no physiological origin, stimulus levels were kept ≤80 dB SPL. The $2f_1 - f_2$ distortion product was measured with f_2 = 8, 12, 16, 24 kHz, f_2/f_1 = 1.25, and stimulus levels $L_1 = L_2 + 10$ dB SPL. DPOAE thresholds were defined as the lowest level of f_1 required to produce a DPOAE ≥ −5 dB SPL.

Subsequently, we measured ABRs. Stainless-steel subdermal needle electrodes were placed one at the head vertex (positive), one under the right ear pinna (negative), and the third one above the right leg (ground). ABRs were evoked by tone bursts (0.5 ms rise/fall time, 5 ms duration) of 8, 12, 16, and 24 kHz, presented 33.3 times per second. Signals were collected via a low-impedance head stage (RA4LI) connected to a preamplifier (RA4PA) and digitally sampled with a processor (200 kHz sample rate). Responses to 1000 bursts were bandpass filtered at 0.3-3 kHz using BioSigRP and averaged at each intensity to determine the threshold. For each frequency, sound intensity was decreased from 90 to 5 dB SPL in 5 dB steps, until threshold was reached and confirmed with 2 or 3 replicate measurements. Threshold was defined as the lowest sound intensity with visually identifiable and reproducible waves. Wave 1 amplitude was determined as the difference between the first wave peak and its subsequent trough.

ABR and DPOAE measurements were performed 5-7 days prior to cisplatin or saline treatment (baseline), 24 hours and 2 weeks after the last injection.

2.4 | Immunohistochemistry and quantification of synaptic receptors

For immunostaining, 2 weeks after the last administration of cisplatin or saline (control), mice underwent transcardiac perfusion with 4% of paraformaldehyde in PBS and cochleae were decalcified in 0.12 M of ethylenediaminetetraacetic acid (EDTA) for 2-3 days. For the demonstration and quantification of presynaptic ribbons, postsynaptic glutamatergic AMPA-receptors (GluA2) and their coupling, cochlear surface preparations were micro-dissected and stained for (a) C-terminal binding protein 2 (mouse (IgG1) anti-CtBP2, 612044 from BD-Biosciences, used at 1:200), to quantify presynaptic ribbons; (b) Glutamate receptor subunit A2 (mouse (IgG2a) anti-GluA2, MAB397 from Millipore, used at 1:1000), to quantify postsynaptic receptors and (c) rabbit anti-Myosin VIIa, (25-6790 from Proteus Biosciences, used at 1:200) to delineate the hair cell bodies. Primary antibody incubations were performed overnight at 37°C, followed by 2 hours incubation at 37°C with secondary antibodies coupled to Alexa fluor dyes (IgG1 goat anti-mouse AF568 at 1:500, IgG2a goat anti-mouse AF488 at 1:500 and IgG donkey anti-rabbit AF647 at 1:200), correspondingly. The cochlear pieces were mounted in Vectashield with DAPI, cover-slipped and sealed with nail polish. Cochlear frequency mapping was then performed using a custom plug-in to ImageJ from NIH (Measure_Line.class from the Liberman laboratory at the Eaton-Peabody Laboratory). This gave the total length of the cochlea and their respective frequency points and was used as a guide obtaining confocal images for discrete frequency regions (6, 8, 12, 16, 24, 32, 48, and 64 kHz) along the length of the cochlea. Confocal z-stacks (20-40), with dimensions 1024 × 1024 and 16-bit image, along discrete regions of the basilar membrane were made with a 63× oil immersion objective (NA1.40) on a Zeiss LSM 880 confocal microscope. A z-stack of 642 nm and interval 1 μm was used to capture all synaptic structures of at least 10 hair cells. Image stacks were analyzed using Imaris software (x64 9.2.0, Bitplane AG, Zurich, Switzerland) for the number of ribbons or postsynaptic receptors as well as for their synaptic pairing. Number of ribbons per IHC was confirmed in ImageJ software. For assessing the total number of ribbons or postsynaptic GluA2 puncta per IHC, surface and masking properties were identified. Then, the region of interest (approximately 10 hair cells) was tagged for counting by using the "spots" function. After adjusting for the thresholds, puncta with pixel intensities greater than 0.6 μm on an 8-bit scale (0-255) were counted. For determining the synaptic pairing, "distance transformation" and "spot co-localization" was used. The threshold/distance between the spots was set to 0.7-1 μm. The volume of each puncta was determined by using different spot sizes

function. Then, the same procedure as for the number of puncta was followed in order to specify the size. The group where a sample belonged to, was blinded to the analyst.

2.5 | Detection of GLAST expression in the cochlea

To visualize the expression of GLAST in cochlear tissue, GLAST-dtTom mice were intracardially perfused with PBS followed by 4% of paraformaldehyde dissolved in PBS prior to removing the cochlea. The cochleae were decalcified as described above, soaked in 20% of sucrose in PBS for 24 hours in 4°C for cryoprotection. After embedding in 20% of gelatin, the cochlear specimens were quickly deep frozen and stored in −70°C. Serial mid-mediolar sections with thickness of 14 µm were cut on a cryostat (HM 500 M, Zeiss) at −20°C. Prior to the staining procedure, the sections were air dried for 2 hours and rinsed in PBS at 37°C for 20 minutes in order to remove the gelatin. The sections were incubated with 5% of normal goat serum in 0.3% of Triton-X in PBS for 30 minutes at room temperature followed by overnight incubation with calbindin antibody (1:400, ab11426, Abcam). Following the rinsing in PBS, the sections were incubated with goat anti-rabbit secondary antibody (Alexa 488, A11008, Thermo fisher) for 2 hours. The sections were rinsed with PBS and incubated with Hoechst 33258 at 2 µg/mL (H3569, Thermo fisher) for 20 minutes. Following subsequent wash in PBS, the slides were mounted with prolong anti-fade mounting medium (Thermo fisher). The slides were analyzed under a Zeiss LSM 880 confocal microscope at different magnifications and photographed.

2.6 | Induced-coupled plasma Mass spectrometry

Platinum levels were measured 1 hour after one single injection of 4 mg/kg cisplatin, administered either at daytime (08.00 AM) or at nighttime (08.00 PM) from the OC including SGNs, SV and blood from GLAST WT and KO male mice (N = 28). Mice were then euthanized by isoflurane inhalation, followed by decapitation. In order to collect plasma samples, blood was obtained from the trunk after decapitation. The cochlear compartments were snapped frozen in dry ice and kept at −80°C until induced-coupled plasma Mass spectrometry (ICP-MS) measurements were made. The blood was centrifuged in order to separate plasma which was then kept at −80°C until the platinum analysis. The procedure of Breglio et al,[14] was used for the analysis and quantification of platinum compounds. The data were presented as the concentration of platinum compounds normalized to protein concentration of each tissue.

2.7 | Organotypic cultures and PER2::LUC oscillations

Adult cochleae were dissected from PER2::LUC male and female knock-in mice (N = 46) and cultured organotypically on a membrane (Millipore, PICMORG50) as previously described.[29] Cochleae, dissected free of bone and SV, were kept in culture for a minimum of 6 days whose morphology has been previously characterized.[28] Isolated cochleae from PER2::LUC mice (8-12 weeks of age) were used. The bioluminescence emission from the cochlea was measured for 1 minutes every 10th minute with Lumicycle, a microplate luminometer equipped with photomultiplier tubes (Actimetrics, Wilmette, IL). Parameters of PER2::LUC rhythmicity (amplitude, phase, and period) were quantified. Amplitude and phase were analyzed using Origin software 8.1 SR1 (Microcal Software, Northampton, MA, USA) and period was identified by using the Lumicycle Analysis program from where we extracted the raw values. Data from each recording trace was first normalized by subtracting the 24 hours baseline drift from the raw data. The amplitude was calculated as the difference between the highest (peak) and the lowest (trough) photon count within one cycle. The calculation was performed from trough-to-peak and from peak-to-trough, thus, giving two values (half-cycles) within one cycle. For each amplitude data point, three half-cycles (1.5 cycles) were used for amplitude analyses. The very first peak after culture start was not used. Phase was determined as maximum (peak) luminescence between 24 and 48 hours (peak between day 1 and 2) of recording. The average peak time of each treatment group was calculated and statistically compared with the respective sham group. The period of one complete cycle was defined as the time between two consecutive peaks (ie, the highest photon count within one cycle) and five consecutive peaks were used for averaging periods. Ex vivo cultures were treated with cisplatin or PBS (control) either at the trough (day 3 of culture) or at the peak (day 3.5) of the oscillation. These two time points were chosen since they correspond to the peak and trough levels of cochlear PER2 protein expression, slightly delayed from peak (ZT12) and trough (ZT0) Per2 mRNA levels. The drug was washed out 3 days later, when fresh culture medium was added. This was not performed immediate after cisplatin treatment to avoid confounding resetting effects upon medium change, known to synchronize oscillations in culture.

2.8 | Statistical analysis

One, two or three-way ANOVA with Sidak's or Tukey's post hoc were used for statistical analysis (Prism version 8.0, GraphPad software). Difference, which was below 0.05, considered significant and depicted with an asterisk (*). All data

points are shown as mean values ± SEM. Reported n is the number of animals/samples analyzed.

3 | RESULTS

3.1 | Expression of GLAST in the cochlea

We first used dtTom (red) reporter mice under the control of the GLAST promoter to determine where GLAST is expressed in the cochlea. Expression was found in the supporting cells (inner border and inner and outer phalangeal cells) surrounding the inner hair cells, the satellite cells surrounding the spiral ganglion neurons, the fibrocytes of the spiral ligament and in the fibrocytes of the spiral limbus (Figure S1). Staining was not observed in the stria vascularis, outer hair cells or the interdental cells of the spiral limbus. The Purkinje cells of the cerebellum were used as a positive control and high expression was found in these cells (data not shown).

3.2 | Nighttime administration of cisplatin causes hearing loss in GLAST KO mice

To investigate the circadian impact of cisplatin on auditory function, we applied a 4 mg/kg/day dose of the drug for four consecutive days during either daytime (ZT3) or nighttime (ZT15). Baseline values for ABR thresholds did not differ between GLAST KO and WT mice (with Sidak's post hoc analysis, $P = .051$-$.44$ for all frequencies tested, Figure S2A).

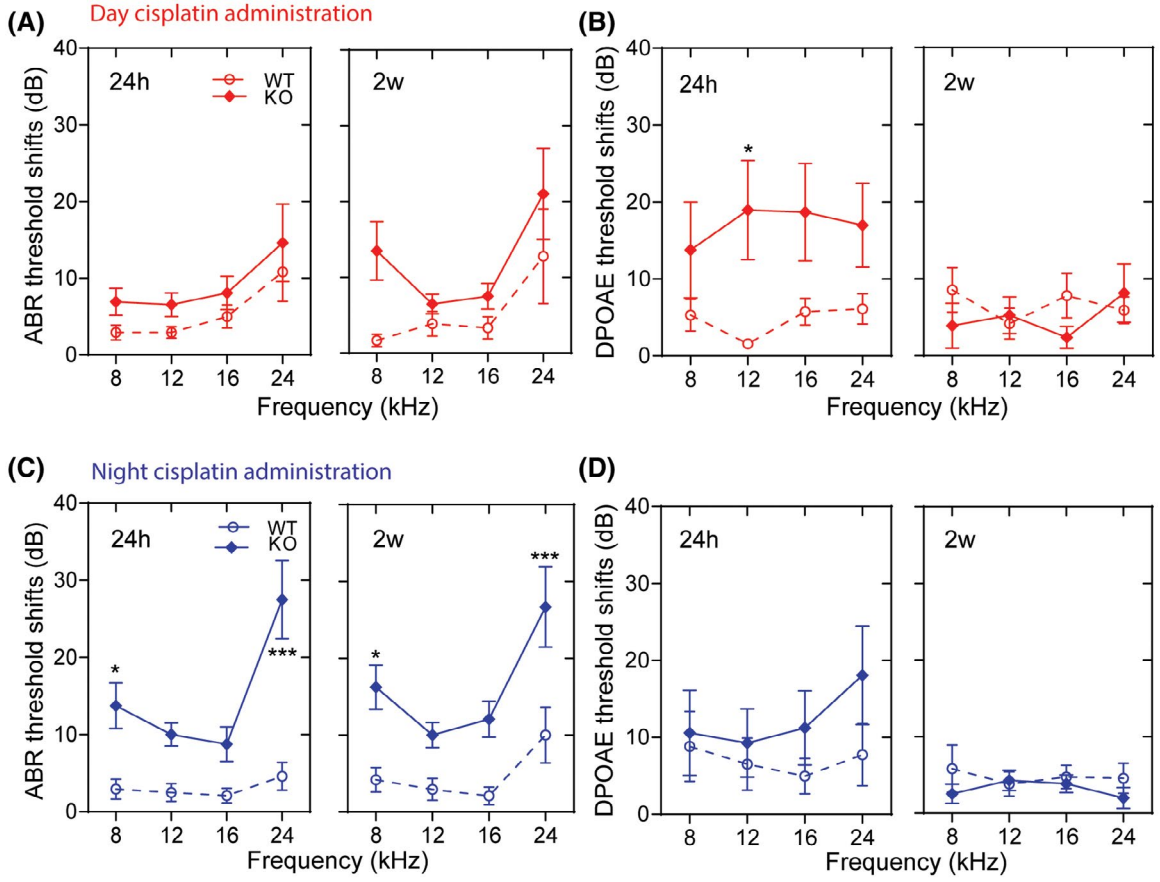

FIGURE 1 Cisplatin administration at nighttime affects hearing threshold shifts. A, ABR threshold shifts from GLAST WT (red open circles) and GLAST KO (red filled diamonds) mice exposed to 4 mg/kg cisplatin for 4 days, at daytime (09.00 AM), were measured 24 hours (left panel) and 2 weeks (right panel) after the last injection. Results are mean values ± SEM; n = 10-13. $*P < .05$; $**P < .01$; $***P < .001$; two-way ANOVA with Sidak's post hoc analysis. B, DPOAE threshold shifts from GLAST WT and KO mice 24 hours (left panel) and 2 weeks (right panel) after day cisplatin administration. Results are mean values ± SEM; n = 10-13. $*P < .05$; $**P < .01$; $***P < .001$; two-way ANOVA with Sidak's post hoc analysis. C, ABR threshold shifts from WT (blue open circles) and KO (blue filled diamonds) mice exposed to 4 mg/kg cisplatin for 4 days, at nighttime (09.00 PM), measured 24 hours post (left panel) and 2 weeks post (right panel). Results are mean values ± SEM; n = 10-12. $*P < .05$; $**P < .01$; $***P < .001$; two-way ANOVA with Sidak's post hoc analysis. Stars depicted above KO at 8 and 24 kHz showed statistical significance compared to WT. D, DPOAE threshold shifts from WT and KO mice 24 hours post (left panel) and 2 weeks post (right panel) after the last night injection. Results are mean values ± SEM; n = 10-12. $*P < .05$; $**P < .01$; $***P < .001$; two-way ANOVA with Sidak's post hoc analysis

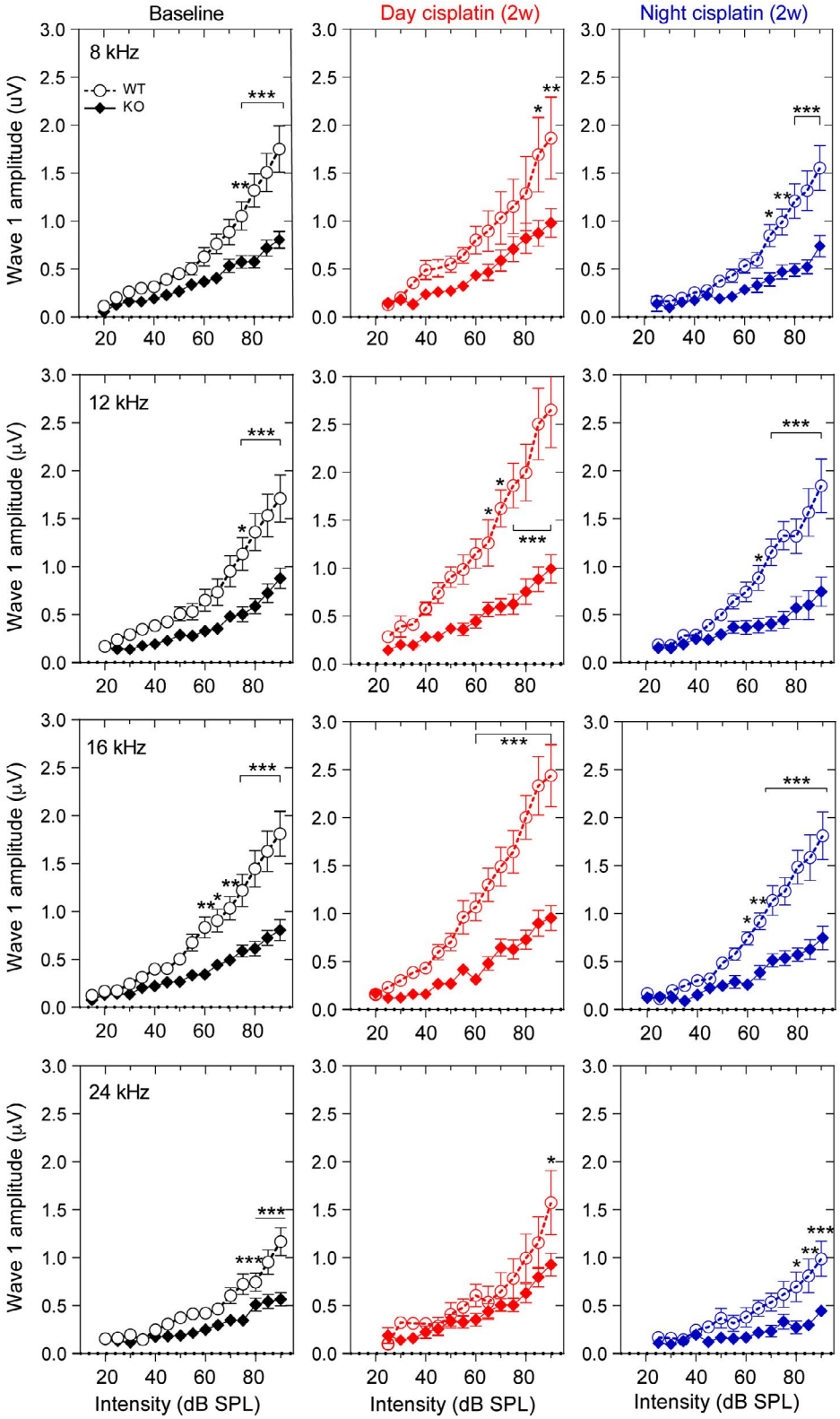

FIGURE 2 Effects of cisplatin on ABR Wave 1 amplitude of GLAST WT and KO mice. Quantification of ABR Wave 1 amplitude of GLAST WT (open circles) and GLAST KO (filled diamonds) mice at baseline levels (left panel), 2 weeks after day cisplatin administration (middle panel), and 2 weeks after night cisplatin administration (right panel) at 8, 12, 16, and 24 kHz. Results are mean values ± SEM; n = 10-13. **P* < .05; ***P* < .01; ****P* < .001; two-way ANOVA with Sidak's post hoc analysis. Stars above KO line at specific dB levels depict statistical significance compared to WT mice

Similarly, DPOAE thresholds did not differ between the two genotypes at baseline level (with Sidak's post hoc analysis, $P = .93$-$.99$ for all frequencies tested, Figure S2B). We next assessed ABR threshold shifts 24 hours and 2 weeks post day or night cisplatin administration from GLAST WT against KO mice. While the ANOVA revealed an effect of genotype 2 weeks post day treatment ($F_{(1, 68)} = 7.026$, $P = .01$), no differences were found in Sidak's post hoc analyses (2 weeks post day treatment, WT vs KO: $P = .086$-$.9976$ for all frequencies tested; Figure 1A). We assessed DPOAE threshold shifts 24 hours and 2 weeks post day or night cisplatin administration from WT and KO mice and we found a transient effect of genotype 24 hours post day treatment (two-way ANOVA, 24 hours post day treatment: Genotype Factor $F_{(1, 92)} = 14.17$, $P = .0003$; Frequency Factor $F_{(3, 92)} = 0.1349$, $P = .94$; with statistical difference between WT and KO at 12 kHz after Sidak's post hoc analysis, $P = .03$), but not 2 weeks after (two-way ANOVA, 2 weeks post day treatment: Genotype Factor: $F_{(1, 92)} = 0.817$, $P = .368$; Frequency Factor: $F_{(3, 92)} = 0.323$, $P = .8$; Figure 1B), suggesting that DPOAEs from WT mice show a transient decrease after day cisplatin treatment, which recovers when assessed 2 weeks post treatment. In contrast, greater ABR threshold shifts were found in KO mice compared to WT 2 weeks after night administration (two-way ANOVA, 2 weeks post day: Genotype Factor: $F_{(1, 88)} = 33.28$, $P < .0001$; Frequency Factor: $F_{(3, 88)} = 7.557$, $P = .0001$; Figure 1C), in spite of unaltered DPOAE threshold shifts (two-way ANOVA, Genotype Factor: $F_{(1, 88)} = 1.58$, $P = .212$; Frequency Factor: $F_{(3, 88)} = 0.1331$, $P = .94$; Figure 1D). When comparing day vs night treatment, we could not detect any difference in ABR threshold shifts. Table S1 provides the summary statistics from a three-way ANOVA evaluating the threshold shifts measured 2 weeks post day or night noise trauma in GLAST WT or KO mice. While an effect on time and frequency was detected (three-way ANOVA, Frequency Factor: $F_{(3, 156)} = 11.97$, $P < .0001$; Genotype Factor: $F_{(1, 156)} = 32.87$, $P < .0001$), no effect of time of treatment was revealed (three-way ANOVA, Time of treatment Factor: $F_{(1, 156)} = 1.22$, $P = .27$). These findings suggest that nighttime administration of cisplatin in GLAST KO mice causes mild hearing loss.

3.3 | GLAST KO mice display lower ABR Wave 1 amplitude at baseline, which is not further worsened by cisplatin administration

We previously reported that GLAST KO mice display near 50% reduction in the amplitude of the ABR Wave 1 in the absence of any noise or ototoxic drug exposure.[35] We thus quantified the ABR Wave 1 amplitude at baseline and 2 weeks after the last day or night cisplatin administration between WT and KO mice. Consistent with our former findings, GLAST KO mice showed reduced ABR Wave 1 amplitudes when compared to WT mice (two-way ANOVA, $P < .0001$ for all frequencies tested, Figure 2). When we compared the Wave 1 amplitude at the highest stimulus (90 dB Sound Pressure Level-SPL) of the respective WT or KO mice across baseline, 2 weeks post day or night treatment (Figure S3), no changes were found 24 hours or 2 weeks post day or night cisplatin treatment when compared to their respective baseline at any frequency ($P > .17$), with the exception of the 24 kHz in the KO mice which were slightly elevated 2 weeks after day treatment when compared to baseline ($P = .04$, Figure S3).

3.4 | Presynaptic ribbons and synapses are more vulnerable to cisplatin at nighttime in the absence of GLAST

Cochlear synaptopathy is inferred from reduced ABR Wave 1 amplitude in the absence of changes in hearing thresholds.[36] We thus assessed the abundance of presynaptic ribbons and paired synapses in saline treated GLAST WT and KO mice and confirmed that the number of ribbons remained unaffected in KO mice (two-way ANOVA, Genotype factor: $F_{(1, 147)} = 0.097$, $P = .755$; Frequency Factor: $F_{(7, 147)} = 23.30$, $P < .0001$, Figure 3A,B), whereas there was a decrease in the proportion of paired synapse (two-way ANOVA, Genotype factor: $F_{(1, 44)} = 34.75$, $P < .0001$; Frequency Factor: $F_{(7, 44)} = 6.335$, $P < .0001$, Figure 3A,C), most evident at 12 and 16 kHz ($P = .0006$ and $.003$, respectively), in line with the reduced ABR Wave 1 amplitude (Figure 2).

Consistent with a greater impact on hearing thresholds after night cisplatin administration, a greater loss of presynaptic ribbons and synaptic pairing was found in GLAST KO mice when compared to WT (Figure 3). While loss of presynaptic ribbons was evident in GLAST KO mice at 12 and 24 kHz 2 weeks after night cisplatin treatment (two-way ANOVA, Genotype factor: $F_{(1, 141)} = 27.50$, $P < .0001$; Frequency Factor: $F_{(7, 141)} = 10.79$, $P < .0001$), such differences were not found in day treated animals, where instead an increase in ribbons could be found in the KO treated during daytime (two-way ANOVA, Genotype factor: $F_{(17, 149)} = 10.34$, $P = .0016$; Frequency Factor: $F_{(7, 149)} = 8.519$, $P < .0001$; with Sidak's post hoc analysis, $P = .17$-$.99$ across all frequencies; Figure 3B), consistent with the greater increase in Wave 1 amplitude shown in Figure S3. Notably, there was a significant reduction of synaptic pairing (coupled synapses) in GLAST WT mice, at 16 kHz both after day and night cisplatin administration when compared to WT saline mice (two-way ANOVA with Sidak's post hoc test, day treatment: $P = .04$, night treatment: $P = .01$, Figure 3C). On

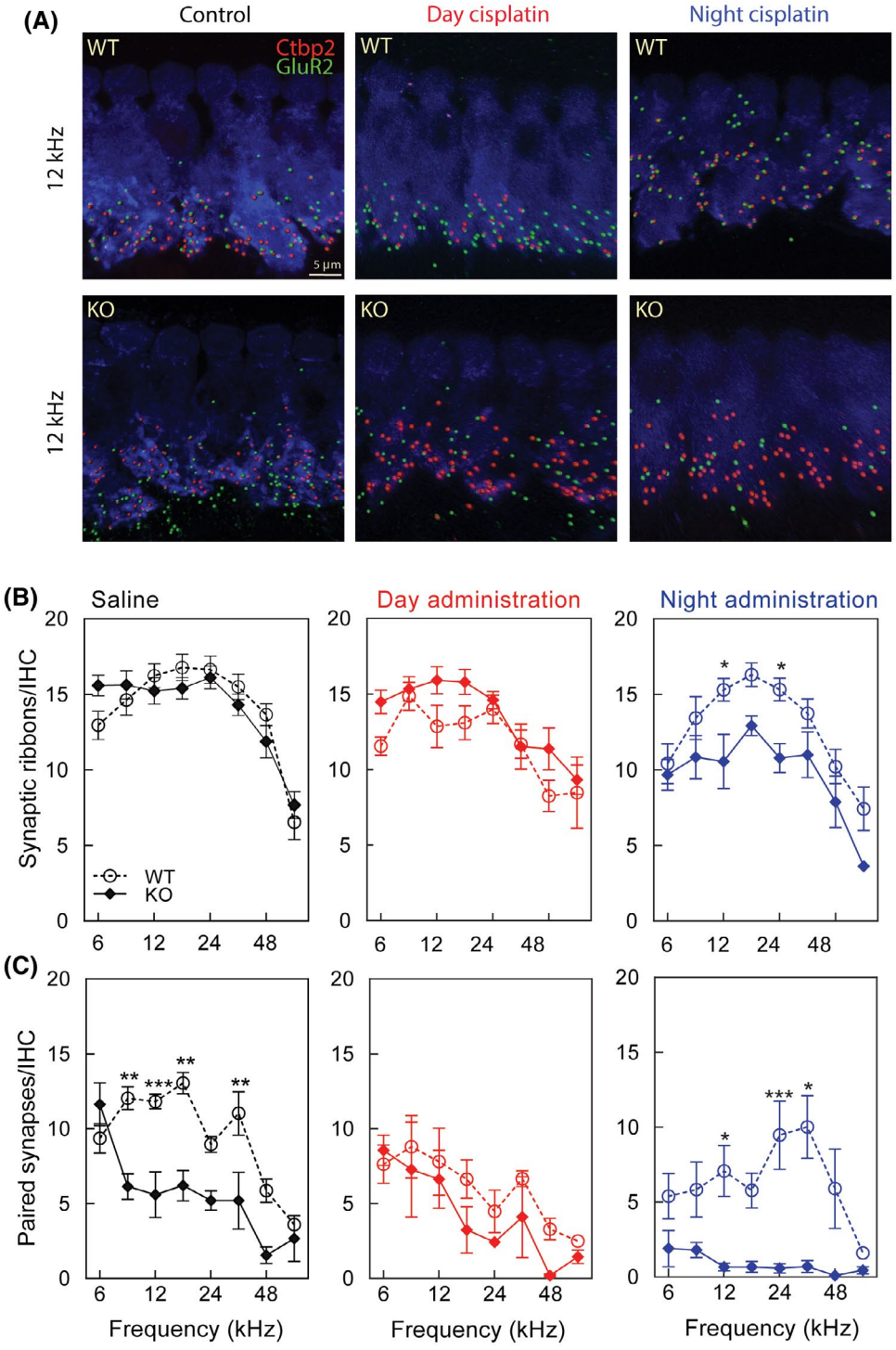

FIGURE 3 Cisplatin treatment at nighttime influences synaptic ribbons and abolishes synaptic coupling. A, 3D Imaris illustration from confocal imaging of presynaptic ribbons (red dots), postsynaptic GluA2 (green dots), and their synaptic pairing in 5 IHCs labeled with Myosin-VIIA (blue) at 12 kHz, in WT (upper) and KO (below) mice, from control (left panel), day treated group (middle panel), and night treated group (right panel). B, Quantification of the amount of presynaptic ribbons in each IHC, along the whole cochlea of WT and KO mice, 2 weeks after saline (control) injections (left panel), day cisplatin administration (middle panel), and night cisplatin administration (right panel). Results are mean values ± SEM; n = 4-13. *$P < .05$; **$P < .01$; ***$P < .001$; two-way ANOVA with Sidak's post hoc analysis. C, Quantification of number of paired synapses per IHC across the length of the cochlea of WT and KO mice, in saline, day and night treated group. Results are mean values ± SEM; n = 1-6. *$P < .05$; **$P < .01$; ***$P < .001$; two-way ANOVA with Sidak's post hoc analysis

the contrary, the impact of night cisplatin administration was more evident on synaptic coupling of GLAST KO, with a near complete loss at 12, 24, and 32 kHz compared to WT (two-way ANOVA with Sidak's post hoc test, $P = .04$, $P = .0002$, and $P = .01$, respectively; Figure 3C), something that was not depicted in day treated animals (two-way ANOVA with Sidak's post hoc test, $P > .94$ on all frequencies, Figure 3C).

3.5 | Circadian effects on synaptic vulnerability to cisplatin

To assess the circadian impact on ribbon counts and synaptic pairing, we performed a three-way ANOVA comparing the impact of the genotype, the time of treatment, and the frequency along which ribbon counts or synapse pairing was evaluated (Table S1). While ribbon counts were impacted in terms of frequency and time of treatment (three-way ANOVA, Frequency Factor: $F_{(7, 290)} = 17.03$, $P < .0001$; Time of Treatment Factor: $F_{(1, 290)} = 13.92$, $P = .0002$), genotype had a strong tendency, but without reaching significance (three-way ANOVA, Genotype Factor: $F_{(1, 290)} = 3.136$, $P = .08$). In contrast, synapse pairing differed along frequencies, between genotypes and by time of treatment (three-way ANOVA, Frequency Factor: $F_{(7, 91)} = 2.276$, $P = .04$; Genotype Factor: $F_{(1, 91)} = 25.61$, $P < .0001$; Time of Treatment Factor: $F_{(1, 91)} = 4.280$, $P = .04$). Overall, the impact of cisplatin was greater on synapse pairing rather than on the synaptic ribbon alone, whereby in absence of glutamate buffering capacity (GLAST KO), administration during the active phase caused a dramatic reduction of coupled IHC/afferent neuron synapses. These findings suggest that GLAST may confer a protective role against circadian-dependent cisplatin injuries on the auditory synapse.

3.6 | Cisplatin bioavailability in the cochlea is not influenced by the time-of-the-day

The greater impact of cisplatin on hearing thresholds and synaptic loss after night administration may be due to greater bioavailability when compared to day administration. We performed a pharmacokinetic analysis of cisplatin bioavailability in the SV and the combination of OC with SGN, 1 hour after day or night administration using ICP-MS. Consistent with greater renal and liver clearance during the active phase, measurements in the blood showed lower abundance of platinum compounds after night administration when compared to day administration, and the absence of GLAST function did not impact the bioavailability of circulating platinum (two-way ANOVA, Genotype factor: $F_{(1, 24)} = 0.258$, $P = .615$; Time-of-the-day Factor: $F_{(1, 24)} = 31.75$, $P < .0001$; Figure 4A). Interestingly, in cochlear compartments (eg, OC/SGN and SV), platinum compounds were equally available after day or night treatment, irrespectively of GLAST function (two-way ANOVA, OC/SGN: Genotype factor: $F_{(1, 24)} = 0.523$, $P = .476$; Time-of-the-day Factor: $F_{(1, 24)} = 0.057$, $P = .812$; SV: Genotype factor: $F_{(1, 23)} = 0.001$, $P = .97$; Time-of-the-day Factor: $F_{(1, 23)} = 0.19$, $P = .66$; Figure 4B,C). These findings suggest that the damage in the cochlea after night cisplatin administration is not due to greater levels of cisplatin being present in the cochlear tissues.

3.7 | Effects of cisplatin on cochlea PER2::LUC rhythms ex vivo

The differential impact of cisplatin on the cochlea after day or night administration, prompted independent of

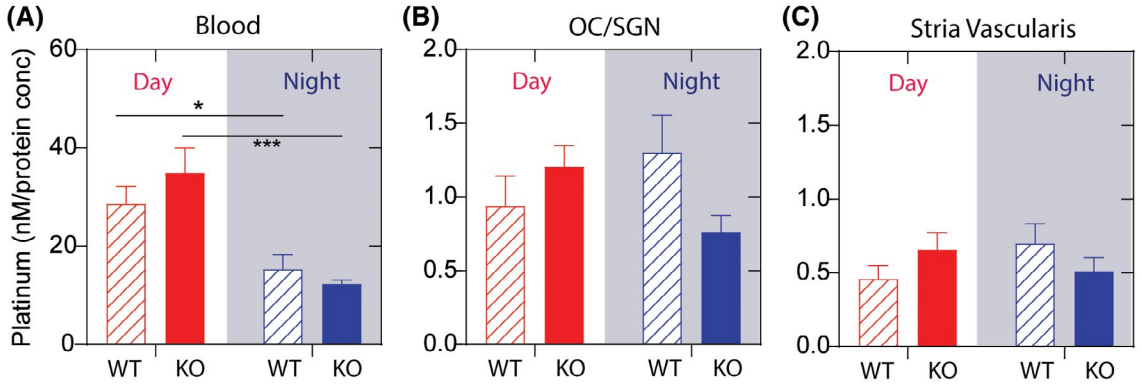

FIGURE 4 Differential platinum accumulation in the blood and the cochlear compartments. A, Platinum concentration in the plasma of GLAST WT (patterned bar) and GLAST KO (filled bar) mice, collected 1 hour after single cisplatin injection at daytime (08.00 AM) and at nighttime (08.00 PM). Platinum concentrations are normalized to protein concentration. Results are mean values ± SEM; n = 6-8. *$P < .05$; **$P < .01$; ***$P < .001$; two-way ANOVA with Sidak's post hoc analysis. Platinum concentrations in microdissected cochlear compartments as (B) OC/SGN and (C) stria vascularis from WT and KO mice. Results are mean values ± SEM; n = 6-8. *$P < .05$; ***$P < .001$; two-way ANOVA with Sidak's post hoc analysis

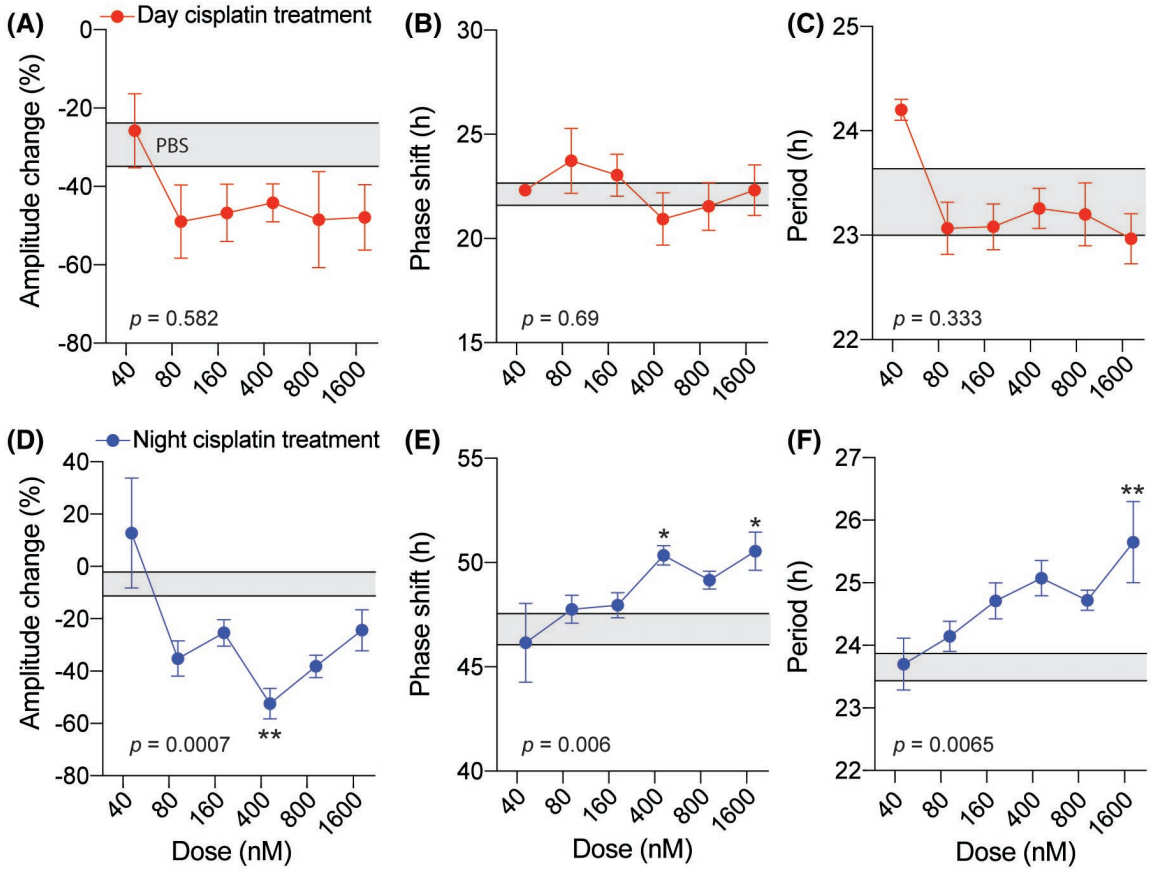

FIGURE 5 Differential effects of cisplatin on PER2::LUC circadian rhythms in the cochlea ex vivo. A, Cisplatin dose response in amplitude expressed as percentage change, phase shift (hour) and period (hour) of PER2::LUC ex vivo cochleae treated at the trough of the oscillations (daytime). PBS was used as a control. Cisplatin administration was followed by washing out period. Results are mean values ± SEM; n = 3-9. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; one-way ANOVA with Sidak's post hoc analysis. B, Cisplatin dose curve in amplitude (%), phase shift (hour) and period (hour) of PER2::LUC ex vivo cultures treated at the peak of the oscillations (nighttime). PBS was used as control. Cisplatin administration was followed by adding fresh medium. Results are mean values ± SEM; n = 3-9. $^{*}P < .05$; $^{**}P < .01$; one-way ANOVA with Sidak's post hoc analysis

bioavailability us to evaluate whether cisplatin could directly modulate cochlear clock rhythms. More specifically, we tested whether cisplatin affects the period of the oscillations (the interval between two reference points), the phase (reflecting a change in the peak time of synchronous cell oscillations) and the amplitude (reflecting either a change in PER2 expression or altered synchrony). Cochlear explants from PER2::LUC reporter mice, cultured for a period of 6-9 days, were exposed for a duration of 3 days to six different doses of cisplatin (40, 80, 160, 400, 800, and 1600 nM), applied either at the trough of the oscillations at day 3 of the culture (day administration) or at the peak of the oscillations, at day 3.5 of the culture (night administration) in the absence of presynchronization. Day cisplatin treatment did not show any effect on PER2 rhythms on the amplitude (one-way ANOVA, $F_{(6, 27)} = 0.794$, $P = .582$), phase (one-way ANOVA, $F_{(6, 30)} = 0.65$, $P = .69$), and period (one-way ANOVA, $F_{(6, 24)} = 1.214$, $P = .333$, Figure 5A-C). In contrast, night cisplatin administration caused a decrease in the amplitude (one-way ANOVA, $F_{(6, 29)} = 5.505$, $P = .0007$) and delay in the phase (one-way ANOVA, $F_{(6, 31)} = 3.769$, $P = .006$), and the period (one-way ANOVA, $F_{(6, 29)} = 3.793$, $P = .0065$) (Figure 5D-F). These findings illustrate that cisplatin directly impacts on clock function in the cochlea when administered at nighttime, but not at daytime, and provides evidence of a direct relationship between cisplatin and clock rhythms.

4 | DISCUSSION

The present findings demonstrate a contribution of glutamate homeostasis in the circadian vulnerability to cisplatin-mediated ototoxicity in mice, and more importantly, the afferent synapse, which appears more sensitive in glutamate transporter deficient mice when cisplatin is administered during the active phase (nighttime). Assuming this circadian ototoxic vulnerability would hold true in humans, who are diurnal unlike mice, this would suggest a greater risk for developing hearing loss and synaptopathy when treated during daytime. As the human inner phalangeal cells, which are the

supporting cells surrounding the auditory afferent synapse, lack EAAT1,[26] the homolog of rodent GLAST, the human synapse may thus display some of the vulnerability features reported here in the GLAST KO mice treated with cisplatin. Since ototoxicity is a major side effect from cancer treatments using cisplatin,[15] our study suggests that chronopharmacological approaches to cancer treatment may prove beneficial to decrease side effects, as exemplified with clinical trials using chronomodulated infusion of oxaliplatin, an analogue of cisplatin.[37,38] Given the leading contribution of hearing loss in years lived with disability,[39] considering the time-of-the-day in cancer treatments using cisplatin may not only increase the likelihood of success, but also decrease side effects that have a non-negligible impact on surviving patient's life quality.

The increased vulnerability of the mouse auditory synapse to cisplatin administration at nighttime may stem from a preexisting cochlear synaptopathy, as defined by a near 50% lower ABR Wave 1 amplitude and reduced paired synapses in normal hearing GLAST deficient mice in the absence of any ototoxic insult. On the contrary, cochleae with normal glutamate buffering abilities are less vulnerable and not equally subjected to circadian influences. In humans, there is evidence that patients with existing hearing deficits are more vulnerable to cisplatin ototoxicity.[13,40] Thus, auditory screens prior to cancer treatment could identify individuals at risk for greater auditory damage. This would be even more relevant in children, who show more severe ototoxicity compared to adults.[41] However, as severe neuropathy cannot be captured by conventional audiometry,[42] new methods to diagnose damages at the auditory synapse are required.

Indeed, the decline in paired synapses in GLAST KO mice after night cisplatin administration did not correlate with changes in Wave 1 amplitude, suggesting that there is not always a direct relationship between synaptic status and Wave 1 amplitude, as previously suggested.[36,43] Indeed, there have been debates on how reliable Wave 1 amplitude is as an indirect measure of synaptopathy in rodents and humans.[44] In animals, the lack of clear direct relationships between synapse status and Wave 1 amplitude may be due to the type of trauma, filter settings, processor, and type of anesthetics used during ABR measures. Alternative methods to assess synaptopathy are under way, such as ABR latency,[45] the envelope following response (EFR),[44,46] and the middle ear muscle reflex.[47] GLAST KO mice may prove useful as a model to test these methods and develop new technologies.

The greater vulnerability to cisplatin-mediated ototoxicity at nighttime was not related with drug bioavailability in cochlear compartments. Platinum compounds were found lower in the blood during the active phase probably due to greater liver detoxification and renal clearance during nighttime. However, the equally abundant platinum compounds in the cochlea after day or night cisplatin administration, irrespective of GLAST function, suggest a greater uptake at nighttime. Differences in drug uptake in the cochlea depending on the time-of-the-day have never been investigated, but the recent findings that the blood-brain barrier is under circadian control[48] suggest that the blood labyrinth barrier may also subjected to such regulations.

Our findings, however, contrast those previously reported in Fischer 344 rats, where cisplatin treatment (12 mg/kg) within six groups treated at different time points around the clock caused greater hearing damage and OHC loss at higher frequencies at daytime.[49] This greater vulnerability to ototoxicity in rats correlates with greater lethality when cisplatin is administered during their inactive phase (daytime) compared to their active phase.[50] In contrast, antibiotic-mediated ototoxicity, influenced by circadian rhythms, appeared to be more damaging when administered at nighttime in Sprague-Dawley rats, which is consistent with our findings.[51,52] Moreover, PER1/2 KO mice showed higher toxicity, increased tumor volume and reduction of body weight, 2 weeks after 10 mg/kg of cisplatin administered at nighttime, with an enhancement in immune response probably due to a disrupted clock.[53] As different circadian responses to auditory insults appear to occur depending on the species and strains, the circadian impact of cisplatin-mediated toxicity in humans remains to be established.

There are several limitations to be acquainted in the present study. First, we did not examine the endocochlear potential after the day and night cisplatin treatment. Indeed, the SV, which is the generator of the endocochlear potential that drives current through the hair cells, cumulates platinum compounds more than the OC and the SGNs[14]—something we could not observe here since OC and SGNs were sampled together. Second, GLAST protein is not only expressed in IHC/synapse, but also in satellite cells surrounding SGNs, and in spiral limbus fibrocytes.[54] Thus, the vulnerability of GLAST KO mice to cisplatin at nighttime may not be restricted to the synapse. Third, while we observe that cisplatin affects PER2 oscillations only when administered at the peak expression (nighttime), the molecular mechanisms underlying the regulation of clock rhythms by cisplatin remain unknown. Since it is well established that cisplatin impacts the mitochondria,[3] and that mitochondrial function is an essential element of clock regulation,[55] cisplatin may impact clock function via altered mitochondrial metabolism at a specific times-of-the-day. Reversely, it could also be that the circadian expression of mitochondrial genes in the cochlea such as *Sod2* and *Lrp2*, which peak at nighttime in the cochlea,[30] could render the cochlea more vulnerable at nighttime. Indeed, should the function of the hearing organ be already subjected to high metabolic demand during the active phase, there would not be enough capacity for triggering appropriate damage response mechanisms. Since these two genes are strongly associated with ototoxicity in humans,[56] future studies will need to address both genetic and circadian

impacts on cisplatin-mediated ototoxicity. While the current study does not directly address the mechanism(s) underlying the circadian vulnerability of the cochlea to cisplatin, our previous data evidencing the role of the suprachiasmatic nucleus (SCN) and adrenal-derived glucocorticoids in the increased vulnerability to noise trauma at nighttime[30] offers a framework for the design of a chronopharmacological treatment with repeated cisplatin administration.[14] The clock in the cochlea is under the control of the SCN, which electrolytic lesion suppresses the rhythmic expression of core clock genes and protects from night noise trauma.[30] Downstream of the SCN, are the adrenal glands that use glucocorticoids as a powerful synchronizer of peripheral clock rhythms.[30] Their surgical removal also protects from night noise exposure, however, only affects the rhythmicity of near 22.5% of circadian genes in the cochlea, some of which are related to inflammation.[30] Since our previous data shows that SCN lesions or adrenalectomy are protective, a direct test would require surgical procedures in GLAST KO mice. Since these show near 50% mortality at birth, a conditional (cochlea or support cell specific) GLAST KO will be necessary to determine the contribution of clock elements in the protection to cisplatin-mediated ototoxicity.

Cisplatin ototoxicity poses a serious problem in cancer survivor's daily life due to hearing impairment.[57] Major efforts have been made to find treatment strategies to reduce the side effects of cisplatin and other chemotherapeutic agents,[58] however, the different molecular and cellular mechanisms involved in cisplatin-induced hearing loss illustrate the complexity of this condition, making it difficult for an appropriate otoprotective agent to be found. One promising but underappreciated approach is the assessment of chronopharmacological approaches and how these could potentially reduce the side effects of anticancer drugs, while improving their efficacy,[59] as it has been exemplified by oxaliplatin, a cisplatin derivative, widely used for the treatment of colorectal cancer in humans.[37,38] Given the impact of time-of-the-day and cochlear function, treating cancer patients at the right time-of-the-day may prove useful not only for improving survival rates, but also for long-term life quality.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

E. Tserga, H. Sarlus, C.R. Cederroth, E. Björn, and B. Canlon performed experiments. E. Tserga, C.R. Cederroth, E. Björn, and B. Canlon analyzed the data and performed statistical analysis; C.R. Cederroth, B. Canlon, and R. Moreno-Paublete conceived experiments; C.R. Cederroth and E. Guimaraes were responsible for the genetic models; E. Tserga, C.R. Cederroth, and B. Canlon wrote the manuscript. All authors contributed to its editing.

REFERENCES

1. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov*. 2005;4(4):307-320.
2. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol*. 2014;740:364-378.
3. Sheth S, Mukherjea D, Rybak LP, Ramkumar V. Mechanisms of cisplatin-induced ototoxicity and otoprotection. *Front Cell Neurosci*. 2017;11:338.
4. Lippman AJ, Helson C, Helson L, Krakoff IH. Clinical trials of cis-diamminedichloroplatinum (NSC-119875). *Cancer Chemother Rep*. 1973;57(2):191-200.
5. Frisina RD, Wheeler HE, Fossa SD, et al. Comprehensive audiometric analysis of hearing impairment and tinnitus after cisplatin-based chemotherapy in survivors of adult-onset cancer. *J Clin Oncol*. 2016;34(23):2712-2720.
6. Greenzang KA. Hearing loss. *J Clin Oncol*. 2018;36(1):94-95.
7. Landier W. Ototoxicity and cancer therapy. *Cancer*. 2016;122(11):1647-1658.
8. Knight KR, Kraemer DF, Neuwelt EA. Ototoxicity in children receiving platinum chemotherapy: underestimating a commonly occurring toxicity that may influence academic and social development. *J Clin Oncol*. 2005;23(34):8588-8596.
9. Kushner BH, Budnick A, Kramer K, Modak S, Cheung NK. Ototoxicity from high-dose use of platinum compounds in patients with neuroblastoma. *Cancer*. 2006;107(2):417-422.
10. Gurney JG, Tersak JM, Ness KK, et al. Hearing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the Children's Oncology Group. *Pediatrics*. 2007;120(5):e1229-e1236.
11. Wilson BS, Tucci DL, Merson MH, O'Donoghue GM. Global hearing health care: new findings and perspectives. *Lancet*. 2017;390(10111):2503-2515.
12. Thomson RS, Auduong P, Miller AT, Gurgel RK. Hearing loss as a risk factor for dementia: a systematic review. *Laryngoscope Investig Otolaryngol*. 2017;2(2):69-79.
13. Chirtes F, Albu S. Prevention and restoration of hearing loss associated with the use of cisplatin. *Biomed Res Int*. 2014;2014:925485.

14. Breglio AM, Rusheen AE, Shide ED, et al. Cisplatin is retained in the cochlea indefinitely following chemotherapy. *Nat Commun*. 2017;8(1):1654.
15. Rybak LP, Ramkumar V. Ototoxicity. *Kidney Int*. 2007;72(8):931-935.
16. Rybak LP, Mukherjea D, Jajoo S, Ramkumar V. Cisplatin ototoxicity and protection: clinical and experimental studies. *Tohoku J Exp Med*. 2009;219(3):177-186.
17. Liberman LD, Suzuki J, Liberman MC. Dynamics of cochlear synaptopathy after acoustic overexposure. *J Assoc Res Otolaryngol*. 2015;16(2):205-219.
18. Liberman MC, Epstein MJ, Cleveland SS, Wang H, Maison SF. Toward a differential diagnosis of hidden hearing loss in humans. *PLoS One*. 2016;11(9):e0162726.
19. Moser T, Starr A. Auditory neuropathy–neural and synaptic mechanisms. *Nat Rev Neurol*. 2016;12(3):135-149.
20. Jin ZH, Kikuchi T, Tanaka K, Kobayashi T. Expression of glutamate transporter GLAST in the developing mouse cochlea. *Tohoku J Exp Med*. 2003;200(3):137-144.
21. Glowatzki E, Cheng N, Hiel H, et al. The glutamate-aspartate transporter GLAST mediates glutamate uptake at inner hair cell afferent synapses in the mammalian cochlea. *J Neurosci*. 2006;26(29):7659-7664.
22. Hakuba N, Koga K, Gyo K, Usami SI, Tanaka K. Exacerbation of noise-induced hearing loss in mice lacking the glutamate transporter GLAST. *J Neurosci*. 2000;20(23):8750-8753.
23. Shimizu Y, Hakuba N, Hyodo J, Taniguchi M, Gyo K. Kanamycin ototoxicity in glutamate transporter knockout mice. *Neurosci Lett*. 2005;380(3):243-246.
24. Yu H, Vikhe Patil K, Han C, et al. GLAST deficiency in mice exacerbates gap detection deficits in a model of salicylate-induced tinnitus. *Front Behav Neurosci*. 2016;10:158.
25. Poirrier AL, Van den Ackerveken P, Kim TS, et al. Ototoxic drugs: difference in sensitivity between mice and guinea pigs. *Toxicol Lett*. 2010;193(1):41-49.
26. Ahmed S, Vorasubin N, Lopez IA, Hosokawa S, Ishiyama G, Ishiyama A. The expression of glutamate aspartate transporter (GLAST) within the human cochlea and its distribution in various patient populations. *Brain Res*. 2013;1529:134-142.
27. Basinou V, Park JS, Cederroth CR, Canlon B. Circadian regulation of auditory function. *Hear Res*. 2017;347:47-55.
28. Park JS, Cederroth CR, Basinou V, et al. Differential phase arrangement of cellular clocks along the tonotopic axis of the mouse cochlea ex vivo. *Curr Biol*. 2017;27(17):2623-2629.
29. Meltser I, Cederroth CR, Basinou V, Savelyev S, Lundkvist GS, Canlon B. TrkB-mediated protection against circadian sensitivity to noise trauma in the murine cochlea. *Curr Biol*. 2014;24(6):658-663.
30. Cederroth CR, Park JS, Basinou V, et al. Circadian regulation of cochlear sensitivity to noise by circulating glucocorticoids. *Curr Biol*. 2019;29(15):2477-2487.e6.
31. Watase K, Hashimoto K, Kano M, et al. Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. *Eur J Neurosci*. 1998;10(3):976-988.
32. Slezak M, Goritz C, Niemiec A, et al. Transgenic mice for conditional gene manipulation in astroglial cells. *Glia*. 2007;55(15):1565-1576.
33. Madisen L, Zwingman TA, Sunkin SM, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci*. 2010;13(1):133-140.
34. Yoo SH, Yamazaki S, Lowrey PL, et al. PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*. 2004;101(15):5339-5346.
35. Tserga E, Damberg P, Canlon B, Cederroth CR. Auditory synaptopathy in mice lacking the glutamate transporter GLAST and its impact on brain activity. *Prog Brain Res*. 2020. https://doi.org/10.1016/bs.pbr.2020.04.004
36. Fernandez KA, Guo D, Micucci S, De Gruttola V, Liberman MC, Kujawa SG. Noise-induced cochlear synaptopathy with and without sensory cell loss. *Neuroscience*. 2020;427:43-57.
37. Levi F, Zidani R, Misset JL. Randomised multicentre trial of chronotherapy with oxaliplatin, fluorouracil, and folinic acid in metastatic colorectal cancer. International Organization for Cancer Chronotherapy. *Lancet*. 1997;350(9079):681-686.
38. Levi F. Circadian chronotherapy for human cancers. *Lancet Oncol*. 2001;2(5):307-315.
39. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet*. 2017;390(10100):1211-1259.
40. Pearson SE, Taylor J, Patel P, Baguley DM. Cancer survivors treated with platinum-based chemotherapy affected by ototoxicity and the impact on quality of life: a narrative synthesis systematic review. *Int J Audiol*. 2019;58(11):685-695.
41. Yancey A, Harris MS, Egbelakin A, Gilbert J, Pisoni DB, Renbarger J. Risk factors for cisplatin-associated ototoxicity in pediatric oncology patients. *Pediatr Blood Cancer*. 2012;59(1):144-148.
42. White-Schwoch T, Anderson S, Kraus N. Long-term follow-up of a patient with auditory neuropathy and normal hearing thresholds. *JAMA Otolaryngol Head Neck Surg*. 2020;146(5):499.
43. Kujawa SG, Liberman MC. Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. *J Neurosci*. 2009;29(45):14077-14085.
44. Hickox AE, Larsen E, Heinz MG, Shinobu L, Whitton JP. Translational issues in cochlear synaptopathy. *Hear Res*. 2017;349:164-171.
45. Mehraei G, Hickox AE, Bharadwaj HM, et al. Auditory brainstem response latency in noise as a marker of cochlear synaptopathy. *J Neurosci*. 2016;36(13):3755-3764.
46. Shaheen LA, Valero MD, Liberman MC. Towards a diagnosis of cochlear neuropathy with envelope following responses. *J Assoc Res Otolaryngol*. 2015;16(6):727-745.
47. Valero MD, Hancock KE, Maison SF, Liberman MC. Effects of cochlear synaptopathy on middle-ear muscle reflexes in unanesthetized mice. *Hear Res*. 2018;363:109-118.
48. Zhang SL, Yue Z, Arnold DM, Artiushin G, Sehgal A. A circadian clock in the blood-brain barrier regulates xenobiotic efflux. *Cell*. 2018;173(1):130-139.e10.
49. Bielefeld EC, Markle A, DeBacker JR, Harrison RT. Chronotolerance for cisplatin ototoxicity in the rat. *Hear Res*. 2018;370:16-21.
50. Hrushesky WJ, Levi FA, Halberg F, Kennedy BJ. Circadian stage dependence of cis-diamminedichloroplatinum lethal toxicity in rats. *Cancer Res*. 1982;42(3):945-949.
51. Yonovitz A, Fisch JE. Circadian rhythm dependent kanamycin-induced hearing loss in rodents assessed by auditory brainstem responses. *Acta Otolaryngol*. 1991;111(6):1006-1012.
52. Soulban G, Smolensky MH, Yonovitz A. Gentamicin-induced chronotoxicity: use of body temperature as a circadian marker rhythm. *Chronobiol Int*. 1990;7(5–6):393-402.
53. Dakup PP, Porter KI, Little AA, et al. The circadian clock regulates cisplatin-induced toxicity and tumor regression in

melanoma mouse and human models. *Oncotarget*. 2018;9(18):14524-14538.

54. Furness DN, Lawton DM, Mahendrasingam S, Hodierne L, Jagger DJ. Quantitative analysis of the expression of the glutamate-aspartate transporter and identification of functional glutamate uptake reveal a role for cochlear fibrocytes in glutamate homeostasis. *Neuroscience*. 2009;162(4):1307-1321.
55. Sardon Puig L, Valera-Alberni M, Canto C, Pillon NJ. Circadian Rhythms And Mitochondria: Connecting The Dots. *Front Genet*. 2018;9:452.
56. Tserga E, Nandwani T, Edvall NK, et al. The genetic vulnerability to cisplatin ototoxicity: a systematic review. *Sci Rep*. 2019;9(1):3455.
57. Paken J, Govender CD, Pillay M, Sewram V. Cisplatin-associated ototoxicity: a review for the health professional. *J Toxicol*. 2016;2016:1809394.
58. Rybak LP, Whitworth CA. Ototoxicity: therapeutic opportunities. *Drug Discov Today*. 2005;10(19):1313-1321.
59. Cederroth CR, Albrecht U, Bass J, et al. Medicine in the fourth dimension. *Cell Metab*. 2019;30(2):238-250.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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