Differential effects of noise exposure between substrains of CBA mice

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ABSTRACT

Noise trauma involves a plethora of mechanisms including reactive oxygen species, apoptosis, tissue damage, and inflammation. Recently, circadian mechanisms were also found to contribute to the vulnerability to noise trauma in mice, with greater damage occurring during their active phase (nighttime), when compared to similar noise exposures during their inactive phase (daytime). These effects seem to be regulated by mechanisms involving Bdnf responses to noise trauma and circulating levels of corticosterone (CORT). However, recent studies using different noise paradigms show contradicting results and it remains unclear how universal these findings are. Here we show that these findings differ even between substrains of mice and are restricted to a narrow window of noise intensity. We found that CBA/Sca mice exposed to 103 dB SPL display differential day/night noise sensitivity as measured by auditory brainstem responses (ABRs), but not at 100 (where full recovery is observed in day or night exposed mice) or 105 dB SPL (where permanent damage is found in both groups). In contrast, neither CBA/Caj or CBA/JRj displayed such differences in day/night noise sensitivity, whatever noise intensity used. These effects appeared to be independent from outer hair cell function, as distortion product otoacoustic emissions appeared equally affected by day or night noise exposure, in all strains and in all noise conditions. Minor differences in ribbon counts or synaptic pairing were found in CBA/Sca mice, which were inconsistent with ABR wave I amplitude changes. Interestingly, CORT levels peaked in CBA/Sca mice at the onset of darkness at zeitgeber time 12 reaching levels of 43.8 ng/ml, while in the CBA/Caj and the CBA/JRj, levels were 11.9 and 15.6 ng/ml respectively and peaking 4 h earlier (zeitgeber time 8). These findings were consistent with higher period of daily rhythm in CBA/Sca mice when measured in complete darkness using running wheels (23.7 h), than in CBA/Caj (23.45 h) or CBA/JRj (23.13 h). In conclusion, our study suggests that the differential vulnerability to noise trauma between inactive and active phase is not universal and is as sensitive as strain differences that might be governed by the circadian amplitude of the circulating CORT profiles.

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1. Introduction

While the fundamental knowledge around the molecular regulation of the clock gene feed-forward loop is rather well established, less is known about the physiological processes under the control of these molecular switches. The cochlea and the inferior colliculus show persistent oscillations of their molecular clocks, ex vivo (Meltsers et al., 2014; Park et al., 2016), and expression of core clock genes (Bmal1, Per1, Per2, and Rev Erb-a) follows a circadian pattern up to six-fold change in amplitude (Meltsers et al., 2014). The clock in the cochlea is under the control of the suprachiasmatic nucleus (SCN) (Cederroth et al., 2019), two hypothalamic nuclei orchestrating organ rhythms via paracrine signaling. Locally, cochlear rhythms rely at least in part on Bmal1 since its depletion in the cochlea leads to a progressive decline in PER2::LUC oscillations when placed in culture, in absence of any circadian and systemic cues (Cederroth et al., 2019). In the cochlea, it has been shown that CBA/Sca mice exposed to moderate noise levels at daytime (inactive phase), recover from their hearing loss, while those exposed at nighttime (active phase), show permanent threshold shift, however in absence of outer hair cell (OHC) loss (Meltsers et al., 2014). However, this differential sensitivity to

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noise does not seem to apply to impulse noise in C57BL/6 J mice (Harrison and Bielefeld, 2018). Rats exposed to intermittent noise for a duration of 12 h also do not display evidence of such differences of sensitivity depending on when the noise is applied (Sheppard et al., 2019). Plausibly, differences in the protocols in noise exposure, exposure duration, the strain background, and the species involved may influence the outcome. Regarding the latter, differences in rats and mice have been reported in the literature with the acoustic startle showing circadian variations in both species but with greater responses in rats during their active phase (Frankland and Ralph, 1995), and in mice during their resting phase (Cederroth et al., 2019). Similarly, cisplatin treatment causes greater damage in rats when administered during their inactive phase (Bielefeld et al., 2018), while in mice, greater vulnerability is seen after administration during the active phase (Tserga et al., 2019).

Circadian influences not only affect the vulnerability of the cochlea to noise trauma, but also affect the cochlea’s response to drug treatments. Administration of the TrkB agonist dihydroxyflavone (DHF) to mice is effective when administered during the active phase, but not during the inactive phase (Meltser et al., 2014). Reversely, the synthetic glucocorticoid receptor agonist dexamethasone (DEX) appears more effective after being administered during the inactive phase, but less so after administration during the active phase (Cederroth et al., 2019). Indeed, recent evidence points towards a crucial role of circulating glucocorticoids (GCs) in regulating the circadian vulnerability to noise in CBA/Sca mice. Removal of the adrenal glands abolishes the differential day/night sensitivity in these mice, albeit maintaining mildly perturbed core clock rhythms (Cederroth et al., 2019).

As we were investigating the impact of chronopharmacology in the prevention of noise-induced hearing loss, the information of our animal provider Scanbur about the imminent termination of the CBA/Ca/Sca colony, which was used in our former publications (Cederroth et al., 2019; Meltser et al., 2014; Park et al., 2016, 2017), arrived to us too late. This led us to explore two additional providers to recapitulate our findings (CBA/Ca from Jackson and CBA/JR from Janvier). In parallel, we searched throughout Scandinavian academic entities and identified a batch of cryopreserved CBA/Ca/Sca embryos from 2008 in another facility of the Karolinska Institute and re-derived these animals. As we moved from a conventional animal facility to a new specific pathogen free (SPF) facility, the aim of the study was to replicate our former findings in CBA/Ca/Sca mice on the effects of day or night exposure on hearing loss and verify whether these effects were also seen in CBA/Ca and CBA/JRj mice.

Our primary outcome measure for animal hearing loss was auditory brainstem response (ABR), as it was consistently found to be different in CBA/Ca/Sca mice exposed either during day- or nighttime (Cederroth et al., 2019; Meltser et al., 2014). Additionally, a range of secondary outcome measures covering different domains of animal auditory ability and circadian aspects were also included: ABR wave 1 amplitude, distortion product otoacoustic emissions (DPOAE), synaptic ribbon counts, synaptic pairing counts, circadian circulating CORT levels, circulating CORT response after day or night noise exposure, and locomotor period. Locomotor activity was also recorded because it is controlled by an endogenous circadian clock (Cederroth et al., 2019; Saini et al., 2013; Yoo et al., 2004). This measure can be easily recorded using running-wheels in rodents, reflecting their activity status. Their locomotor activity rhythm provides a reliable marker of their circadian system under constant environmental conditions (e.g., light/dark cycles or LD), as well as their endogenous rhythm assessed in constant darkness (in absence of entrainment or in constant darkness DD), which reveals their endogenous period (Tau).

2. Materials and methods

2.1. Experimental design and animals

We acquired CBA mice from three different breeders: CBA/Caj (The Jackson Laboratory, ME USA), CBA/JRj (Janvier Labs, France), CBA/Ca/Sca (originally from Scanbur, Sweden, but cryopreserved in 2008 at the Karolinska Institutet Huddinge) hereafter named CBA/Sca. CBA/Caj mice were sent as cryopreserved embryos, rederived and bred at the Karolinska Institutet Comparative Medicine Biomedical facility (KMB). In contrast, CBA/JRj breeding pairs were sent to KMB directly. CBA/Sca were rederived and bred at KMB. Experiments were conducted on male mice aged between 2 and 5 months old and sequentially performed on CBA/JRj, CBA/Caj, and finally CBA/Sca mice. Females were excluded as these were not part of the primary aims of the study. All animals were housed in the presence of their littermates, in individually ventilated cages with ad libitum access to CRM (P) food pellets (SDS # 801.722) and water. We note that this food also differed from our past reports (Cederroth et al., 2019; Meltser et al., 2014; Park et al., 2016, 2017). Nesting material was dust-free soft and springy paper called Sizzlenest (Datesand, #51A09), cardboard play tunnels were used as enrichment (Datesand, # CS3B01). Background noise levels inside the cages during normal animal husbandry routines were up to 60 dB(A) SPL. All animals were ear marked and tail handled. Temperature in the animal facility was maintained between 19 °C and 21 °C and lights were on at 6:00 a.m. and off at 6:00 p.m. For internal validity (Voelkl et al., 2020), auditory brainstem responses (primary outcome) were performed by three different researchers. All procedures followed the regulations of Karolinska Institutet and were approved by the regional ethics committee of Stockholm (Stockholm’s Norra Djurförsöksätska nämnd, N156–14, N140–15).

2.2. Noise exposure

Individual mice were transferred to wire mesh cages (10 × 10 × 10 cm). Four cages were placed on a single rotating round platform (40 cm diameter, 2 rotations per minute) inside a 225 × 120 × 100 cm³ sound attenuating chamber. Inside the chamber downward-facing speakers (JBL 075) were fitted in the four upper corners. A PC running WaveLab Studio 6 software played broadband noise (6–12 kHz; 100, 103, 105 dB SPL). The noise signal was delivered to the speakers through an amplifier (Sampson Servo 200). To ensure animals were exposed without any interruptions, sound level was monitored throughout the experiment using a microphone (SwanTek SV17) connected to a sound level meter (A-weighted SwanTek 979) and SwanPC++ software. Animals were randomly assigned either to noise or sham (no noise) group. Noise levels above the rotating platform at a mouse’s ear level varied less than 3 dB(A) SPL. We note that this noise exposure setup and calibration differed from our previous work as we found reduced variability in the outcome (personal observations). The noise exposure was for one hour, either starting at ZT3 (zeitgeber time) or ZT12. Sham mice were used as negative controls and were placed inside the mesh cages following a similar procedure as the noise exposed, however without any noise exposure.

2.3. Auditory measurements

Signal generation and acquisition was done using Tucker-Davis Technologies System II hardware and software. Stimuli were generated using BioSigRZ software running on a PC connected to a signal processor (R2B). For DPOAEs, two independently driven MFI speakers merged in a custom made, ear-canal probe with a soft
plastic tip for closed field stimulation. For ABRs, the stimulus was output through an open field speaker (MF1). A Brüel and Kær ¼-inch microphone and a conditioning pre-amplifier (4939 A 011 and 2690 A 051) was used to calibrate the stimulus level. For the DPOAE stimuli the microphone was coupled closed field to the ear canal probe and mimicked the location of the eardrum. For ABR stimulus 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 one at a time using a frequency sweep (4–32 kHz). The output was corrected to produce a flat spectrum at 90 dB SPL (open field speaker, ABR) and 80 dB SPL (closed field speakers, DPOAE). Mice were anesthetized with a mixture of ketamine (ketaminol 50 mg/ml, Intervet, 511,485) and xylazine (Rompun 20 mg/ml, Bayer, KPOA43D) (100 and 10 mg/kg body weight, respectively) and placed in a custom-made acoustic enclosure with sound absorbing material on the walls and ceiling (60 × 60 × 100 cm³). Body temperature was maintained at 36.5 °C using a heating pad (Homeothermic Monitoring System 55–7020, Harvard Apparatus). We first recorded DPOAEs. In short, the acoustic coupler was inserted into the ear canal. A microphone (EK 23,103, Knowles) was inserted in an acoustic coupler connected to a pre-amplifier (ER-10B+, Etymotic Research) and a processor (200 kHz sample rate) to measure sound level in the ear canal. Each speaker played one of two primary tones (f1 and f2) and swept in 5 dB steps from 80 to 10 dB SPL (for f2). The 2f1 − f2 distortion product was measured with f2 = 8, 12, 16, 24, 32 kHz, f2/f1 = 1.25, and stimulus levels L1 = L2 = 10 dB SPL. Subsequently, we measured ABRs. Stainless-steel subdermal needle electrodes were placed at the head vertex (positive), under the right ear pinna (negative) and above the right leg (ground). ABRs were evoked by tone bursts (0.5 ms rise/fall time, 5 ms duration) of 8, 12, 16, 24 and 32 kHz presented 21 times per second. Signals were collected via a low-impedance head stage (RA4LI) connected to a pre-amplifier (RA4PA) and digitally sampled (200 kHz sample rate). Responses to 200–3000 bursts were bandpass filtered at 0.3–3 kHz using BioSigRZ and averaged at each level. For each frequency, sound level decreased from 90 dB SPL in 5 dB steps. DPOAEs and ABRs were recorded 3–21 days before noise exposure (baseline), within 24 h after exposure (24 h), and 14 days after exposure (2 wk). A summary of the number of animals used per strain and across groups for the primary outcome is provided in Supplementary Table 1.

2.4. Immunohistochemistry and synaptic coupling quantification

Immunohistochemistry was performed on cochleae 2 weeks after day (ZT3) or night (ZT15) noise exposure. Four percent paraformaldehyde (PFA) in PBS was used to perform a transcardiac perfusion. The cochleae were decalified in 0.12 M ethylene-diaminetetraacetic acid (EDTA) for 3–4 days and then micro-dissected and stained for (a) C-terminal binding protein 2 (mouse IgG1 anti-CtBP2, 612,044 from BD-Biosciences, used at 1:200) for the demonstration and quantification of the presynaptic ribbons; (b) Glutamate receptor subunit A2 (mouse IgG2A anti-GluA2, MAB397 from Millipore, used at 1:800), in order to quantify the glutamatergic postsynaptic receptors and (c) anti-myosin VI A (rabbit IgG, 25–6790 from Proteus Biosciences, used at 1:200) for delineation of the hair cell bodies. Cochlear samples were incubated with primary antibodies overnight at 37 °C, followed by 2 h incubation at 37 °C with secondary antibodies coupled to Alexa flour dyes (a) goat anti-mouse IgG1 AF568 (red) at 1:500, (b) goat anti-mouse IgG2A AF488 (green) at 1:500 and (c) donkey anti-rabbit IgG AF647 (far red) at 1:200, correspondingly to primary antibodies. Then cochlear pieces were mounted in Vectashield antifade mounting medium with DAPI (H-1200, Vector Laboratories), cover-slipped and sealed with nail polish. Next, cochlear frequency mapping was performed using a custom ImageJ software plug-in, provided by the NIH (Measure_ Line.class from Liberman research group at the Eaton-Peabody laboratory). This mapping provides with the total length of each mouse cochlea and some respective frequency points, which were used as guide for obtaining high quality images in the confocal microscope for discrete frequency regions (6, 8, 12, 16, 24, 32, 48 and 64 kHz) across the whole cochlear length. Z-stacks (20–40), with dimensions 1024 × 1024 and 16-bit image, were taken with a 63x oil immersion objective (NA 1.40) on a Zeiss LSM 880 confocal microscope. A Z-stack of 642 nm and interval 1 μm was used to capture all pre- and postsynaptic structures of at least 10 hair cells. Images were collectively analyzed and were blinded to the noise condition.

For assessing the total number of ribbons and postsynaptic GluA2 puncta per inner hair cell (IHC), surface and masking structures were created. Image stacks were analyzed using Imaris software (x64 9.2.0, Bitplane AG, Zurich, Switzerland) for the number of ribbons, postsynaptic receptors and their synaptic pairing. Then, the region of interest (approximately 10 IHCs) was tagged for counting by using the “spots” function. After adjusting for the thresholds, puncta with pixel intensities 0.6–0.8 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. We note that GluR2 staining is notoriously difficult and therefore some of the images had to be excluded for quantification of paired synapses. Therefore, synaptic pairing may appear underpowered at some frequencies (e.g. n = 1–2). In some instances, low number of images (n = 1–2) are in the 64 kHz region, which a poor dissection may have affected the staining quality.

2.5. Correlation between wave 1 amplitude and synaptic status

We compared ribbon and synapse counts to the ABR wave 1 amplitude at 70 dB SPL. We chose the amplitude at this sound level because it strikes a balance between evoking a good to reasonable response at baseline and after noise exposure, and over-stimulation of the inner ear. High sound levels recruit larger areas of the cochlear partition, which makes comparison to a specific frequency stimulation less precise. Overstimulation can also lead to saturation of the ABR, decreasing the strength of a potential correlation between ribbon and synapse counts.

2.6. Corticosterone EIA ELISA

The CORT from plasma was assessed by ELISA. While the blood collection in our former study was performed in free running animals (maintained in constant darkness (DD) for three days), here animals were maintained in a normal day/night schedule (LD), consistent with previous studies showing the maintenance in the phase and amplitude of circulating or fecal CORT circadian rhythms when shifted into DD (Cederoth et al., 2019; Husse et al., 2014). Plasma collection has been performed for each substrate during two different conditions; every 4th hour around the clock (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20) at baseline levels and immediately after day (ZT3) or night (ZT15) noise exposure at 103 dB. For the CORT levels after noise exposure, 2 groups of sham animals were included for each substrate, sham animals in their home cage and sham animals treated with the same restrained conditions as the ones in the noise group. Mice were decapitated under isoflurane anesthesia and blood from the trunk was collected in heparin coated tubes and was placed on ice. The collected blood samples were centrifuged at 1400 rpm, 4 °C, for 10 min and the supernatant plasma was transferred in clean tubes and placed on ice. Then, samples were treated with Steroid Displacement Reagent
(SDR, 10 μl plasma + 10 μl SDR) and kept at 4 °C till further analysis. The plasma CORT levels were measured with the CORT EIA kit according to the manufacturer's instructions (ADI-900–097, Enzo Life Sciences).

2.7. Actograms

To record the rhythm of locomotor activity, adult mice (at least 8-weeks old) were individually housed in activity wheel-equipped cages (#ENV-044, Med-Associates, St Albans City, VT) under LD 12:12 for at least 24 days, after which they were shifted to complete darkness or dark/dark cycle (DD). Locomotor activity was recorded using Wheel Manager software (#SOF-860, Med-Associates, St Albans City, VT) and analyzed using ClockLab software (Actimetrics, Wilmette, IL). Fluorescent lights (300–600 lux inside the cage) were used for behavior experiments. Wheel-running activity was recorded and analyzed as previously published (Vitaterna et al., 2006). The circadian period (Tau) was extracted from the constant darkness (DD) phase of the wheel running activity records by linear regression analysis of activity onset, optimized to account for actograms with low activity levels and slightly abnormal activity onsets.

2.8. Statistical analysis

Statistical analyses for ABR and DPOAE data were done using Python (SciPy, statsmodels) and R (nlme, lsmeans). DPOAE thresholds were defined as the lowest level of f1 required to produce an emission ≥ −5 dB SPL (8, 12 kHz), ≥ 0 dB SPL (16, 24 kHz), ≥ 5 dB SPL (32 kHz). If no level of f1 generated an emission meeting this criterion, threshold was assumed to be 85 dB SPL. For different ABR experiments, one of a group of three experienced individuals (C.P.C.V, J.M.F., R.M.P) visually identified thresholds as the lowest sound level with identifiable synchronous waves. Separating each mouse strain and experimental condition, we used the non-parametric Kruskal-Wallis H-test (SciPy, kruskal) to find differences in thresholds between baseline, ZT3 and ZT15 within a frequency group. If a significant difference was found (alpha = 0.05) we did a post hoc analysis using the Mann-Whitney U rank test (SciPy, mannuheney) to identify which groups showed significant differences (alpha = 0.05). The wave 1 amplitude is defined as the difference between the first peak and first trough of the ABR. The same individual that identified the threshold visually identified these points. The aggregated wave 1 data were preprocessed with outlier detection. For each time point (baseline, 24 h, or 2 w) and frequency (8, 12, 16, 24, or 32 kHz) we performed a robust linear regression on the relation between stimulus level and log (wave 1 amplitude). Next, we normalized these data to their mean and standard deviation. Separated for each mouse strain, we pooled all normalized data and fit a skewed normal distribution to these data. Any data point that fell outside this distribution’s 95% confidence interval was labeled as outlier and removed. We fitted linear mixed models to the wave 1 amplitude data as previously described (Schrode et al., 2018; Patel, 2020). The use of these models is motivated by their ability to deal with repeated measures, and missing data points caused by our outlier removal. In brief, the models were fit to the log-transformed wave 1 amplitude to linearize their relation to stimulus level. Model variables were stimu-

Fig. 1. ABR thresholds as a function of stimulus frequency two weeks after day (ZT3) or night (ZT15) noise trauma. Panel rows correspond to different CBA substrains (CBA/CaJ, CBA/JR) and CBA/Sca; panel columns correspond to different noise levels (100, 103 and 105 dB). Following the colors, gray line indicates baseline thresholds, red indicates thresholds after day noise exposure and blue line indicates thresholds after night noise exposure, dots mark individual data points and lines indicate their means. Typically, ten data points in one panel represent a single mouse: five frequencies (8, 12, 16, 24 and 32 kHz) tested as baseline and the same tested two weeks later after noise exposure at either ZT3 or ZT15. Asterisks (ZT3 vs ZT15), 4-pointed crosses (baseline vs ZT15), and 3-pointed crosses (baseline vs ZT3) indicate significant differences (p<0.05) using post hoc Mann-Whitney U rank test; Number of mice/strain; CBA/CaJ: N = 8–11; CBA/JR: N = 7–14; CBA/Sca: N = 3–11 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).
lus level and experimental condition (baseline, ZT3, ZT15), and included their main effect and interaction term. Stimulus level was used as weighting term. We confirmed that model residuals were homoscedastic by visual inspection of quantile-quantile plots. Post hoc comparison of the linear mixed models using multivariate adjustment revealed significance of differences. For the statistical analysis of synaptic ribbons and synaptic pairing, two-way ANOVA with Sidak’s multiple comparisons test was performed in GraphPad software (Prism version, 9.0.1). For the levels of the plasma CORT around the clock in the 3 different mouse substrains, two-way ANOVA with Sidak’s multiple comparisons test was performed and for the CORT values after day or night noise/sham exposure for each substrate, two-way ANOVA with multiple comparisons was used in GraphPad. Statistical differences which were below 0.05, were considered significant and depicted with an asterisk (*). All data points were shown as mean values ± SEM or mean values ± SD.

3. Results

3.1. ABR and DPOAE

All substrains showed similar basal thresholds before noise exposure, however these substrains showed differences in noise-induced ABR threshold changes (three-way ANOVA, 100 dB SPL noise exposure: Strain Factor: $F(2, 134) = 8.722$, $p = 0.0002$; 103 dB SPL noise exposure: Strain Factor: $F(2, 226) = 10.345$, $p < 0.0001$; CBA/Caj Strain Factor: $F(2, 177) = 83.7$, $p < 0.0001$; Fig. 1). The ABR thresholds of the CBA/Caj strain gradually increased from baseline with elevating noise levels. The ABR thresholds for the CBA/JRj substrain increased between 100 dB SPL and 103 dB SPL but no further increases were noted after the 105 dB SPL exposure. A similar pattern was found for the CBA/Sca substrain between 100 and 103 dB SPL but the change in threshold was smaller compared to the CBA/JRj substrain (Fig. 1). Differences in day or night noise-induced ABR thresholds were not found for the CBA/Caj or the CBA/JRj substrains (Fig. 1a–f). In contrast, the CBA/Sca mice exposed at a noise level of 103 dB SPL showed a differential day night response where thresholds of the ZT15 group were significantly greater than the thresholds of the ZT3 group at higher frequencies (Fig. 1h; post hoc Mann-Whitney U test, $p = 0.0317$ (16 kHz), $p = 0.0086$ (24 kHz), $p = 0.0102$ (32 kHz)). No differences between day and night noise exposures were found at 100 and 105 dB SPL (Fig. 1g,l).

Fig. 2 shows an example of wave 1 data for a 24 kHz stimulus. For most frequencies, wave 1 amplitudes were significantly reduced after noise exposure. A large range of ABR stimulus levels generated a significant difference between the ZT3 and ZT15 groups for all substrains at a noise level of 103 dB SPL, as well as for CBA/Caj at a 105 dB SPL noise level and CBA/Sca at a 100 dB SPL noise level. In these strain-noise combinations wave 1 amplitude was further reduced after exposure at ZT15 than after exposure at ZT3. Supplementary Figs. 1–3 reveals many more instances where ZT3 and ZT15 differed significantly across the three substrains at other noise exposure levels. Average waveforms for all strains and all conditions are illustrated in Supplementary Fig.
4. The most common difference is a lowered wave 1 amplitude after exposure at ZT15 compared to ZT3. However, CBA/JRj mice exposed at 100 dB SPL sometimes showed the inverse effect where exposure at ZT3 lowered amplitudes significantly more. Comparing all combinations of noise exposure levels and ABR frequencies for the three substrains, CBA/Sca showed a significant difference in 13 out of 15 combinations. The other two substrains showed significant differences in 9 out of 15 combinations only. The DPOAE thresholds shown in Fig. 3 reveal different sensitivities to OHC damage for the three substrains, but a circadian effect is absent. In general, after noise exposure DPOAE thresholds were increased with increasing noise level, but the increase varied for the three substrains. Threshold increase was largest for CBA/JRj, with a significant difference between nearly all baseline and noise-exposed groups. The CBA/Caj substrain showed an intermediate increase, with significant differences for all frequencies after noise exposure at 103 and 105 dB SPL. At 100 dB SPL only 12 and 16 kHz groups showed significant differences between baseline and the noise-exposed groups. Finally, threshold increase was the smallest for CBA/Sca mice where nearly all frequencies showed significant changes after noise exposure at 103 and 105 dB SPL, but typically no change in threshold after exposure at 100 dB SPL. We note that the loss of outer hair cells (OHC) is minimal for all strains at any noise intensity (Supplementary Table 2), with no loss of OHCs observed in the CBA/Caj even at 105 dB SPL noise exposure with near 40 dB permanent threshold shifts.

With higher sensitivity to OHC damage more DPOAE thresholds exceed what can be measured, “saturating” at 85 dB SPL (Fig. 3b−f). This saturation results in an underestimation of the mean DPOAE thresholds. However, the Kruskall–Wallis H-test and Mann-Whitney U test are nonparametric and saturation does not affect statistical significance. It is important to note that even at baseline we report DPOAE thresholds exceeding 70 dB SPL. These high thresholds are likely caused by mispositioning of the ear canal probe and not the actual threshold. This is the case for 16 out of 68 (24%) CBA/Caj mice, 14 out of 61 (23%) CBA/JRj mice, and 2 out of 55 (<1%) CBA/Sca mice. Where present, these spurious data points effectively increase the estimated threshold. We included these data because a similar ratio of data points could be affected after noise exposure. However, after noise exposure it is impossible to distinguish between a spurious data point or an actual threshold increase.

3.2. Synaptic ribbons and pairing

Synaptic-ribbon counts and synaptic coupling were determined two weeks after the ZT3 and ZT15 noise exposures (Fig. 4). Representative images of the stainings are shown in Supplementary Fig. 5. When comparing the 100 dB SPL noise trauma to the other exposure levels, it was found that there was a decrease in the number of ribbons per IHC for the CBA/Caj substrain after both the 103 dB day and night exposure (two-way ANOVA, p < 0.0357; Fig. 4a–c). No day-night differences were found at any noise exposure level in the CBA/Caj substrain. The CBA/JRj strain showed a decrease in ribbon number when comparing the 103 and 105 dB day and the night exposures (two-way ANOVA, p < 0.018; Fig. 4e, f). There was also a decrease when comparing the 100 dB day or night exposure to the 105 dB night exposure (two-way ANOVA, p < 0.018; Fig. 4d, f). The only day-night difference that was found for this substrain was detected after the 103 dB exposure at 6 kHz (two-way ANOVA, p < 0.0459; Fig. 4e). The CBA/Sca substrain did not show any graded decrease in ribbon counts after the 103 or
105 dB day exposure compared to the 100 dB day exposure. The CBA/Sca substrain however displayed a reduced number of ribbons per IHC after the 100 dB and 103 dB night noise exposure compared to the 100 and 103 dB day exposure. These reductions in ribbon number were found at 16 and 12 kHz, respectively (two-way ANOVA, p < 0.05 and p < 0.008; Fig. 4a, b). The synaptic pairing was also evaluated for the different substrains after noise exposure (Fig. 5). The CBA/CaJ strain showed a decrease in synaptic pairing between the 100 dB and the 103 dB day exposures (two-way ANOVA, p < 0.0001; Fig. 5a, b) but an increase after the 105 dB day exposure compared to the 103 dB (two-way ANOVA, p < 0.0002; Fig. 5b, c). No day-night differences were found after any exposure level or at any particular frequency. The CBA/JRj substrain showed a decrease in pairing after the 103 dB and the 105 dB day and night exposure compare the 100 dB day and night exposure (two-way ANOVA, p < 0.018; Fig. 5d-f). A significant day-night difference was found after the 105 dB exposure at 8 kHz frequency (two-way ANOVA, p < 0.02; Fig. 5f). The CBA/Sca substrain showed a decrease in pairing between the 100 dB and 105 dB day exposures (two-way ANOVA, p < 0.0038; Fig. 5g, i). A day-night difference was detected after the 103 dB exposure (two-way ANOVA, p < 0.0004; Fig. 5h). Fig. 5h shows that at the 12 kHz region the CBA/Sca mice showed a lower number of intact synapses per IHC in the group exposed to 103 dB SPL noise at day compared to night. Interestingly, we found little concordance between ABR wave 1 amplitude and synapse status after noise exposure (Figs. 6 and 7).

3.3. Corticosterone measures and locomotor activity

Our previous study suggested that CORT can be used as a marker for detecting differential day night differences to noise trauma (Cederroth et al., 2019). The circadian profile of CORT samples obtained from the three different substrains illustrates different peaks of expression (two-way ANOVA, Time Factor: F(5, 169) = 14.07, p < 0.0001; Substrain Factor: F(2, 169) = 15.08, p < 0.0001; Fig. 8a). The CBA/Sca strain showed a later peak compared to the CBA/JRj and CBA/CaJ strains by 4 h. These findings were consistent with a greater period in CBA/Sca mice than the other strains when maintained in DD conditions [CBA/Sca Tau = 23.7; CBA/CaJ Tau = 23.13; CBA/JRj Tau of 23.45, Fig. 8b, c]. The CORT concentration at ZT16 also varied among the substrains with the CBA/Sca showing the highest average concentration (43.8 ng/ml ± SEM 21.49) compared to the CBA/CaJ (11.89 ng/ml ± SEM 8.83) and CBA/JRj (15.58 ng/ml ± SEM 11.09) (p < 0.0001 with Sidak’s post-hoc testing).

In order to determine how these differences in CORT expression affect the response to a noise trauma we have performed an extensive study comparing CORT plasma values in (1) the home cage environment during daytime; (2) after 1 h in the exposure cage placed in the noise chamber (without noise exposure) during daytime; (3) after 1 h day noise exposure; (4) the home cage environment during nighttime; (5) after 1 h in the exposure cage placed in the noise chamber (without noise exposure); (6) after 1 h night noise exposure. The different strains reacted dif-
Fig. 5. Quantification of synaptic coupling of auditory nerve fibers to ribbon synapses per HIC for different cochlear frequency regions two weeks after noise trauma at 100, 103 and 105 dB, for each substrain. Layout, markers and coloring follow those of Fig. 4. Stars indicate significant differences between noise exposures at ZT3 and ZT15 for each substrain (* * *: p < 0.005); two-way ANOVA with Sidak’s post hoc analysis. Results are mean values ± SEM; Number of cochlea/strain; CBA/CaJ: N = 4–6; CBA/JRj: N = 3–6; CBA/Sca: N = 3–6. In some cochlear samples, at the higher frequency (64 kHz region), there is a small number of images (n = 1–2) due to either poor dissection or inadequate quality of GluR2 staining.

Served in CBA/Sca mice (Cederroth et al., 2019; Meltser et al., 2014), the principle of nighttime vulnerability was less clear when the experiments were broadened to assess other strains and noise exposure levels. Thus, the differential vulnerability to noise trauma between the inactive and active phases is unlikely to be universal. Subtle differences, at the level of genetic substrains, potentially governed by the circadian amplitude of the circulating CORT profiles, could be grounds to such discrepancies.

In this respect, the present results suggest a predictive value in circadian CORT profiles with regards to the outcome from noise trauma, whether occurring during the active or inactive phase. Mice that have an ample CORT profile, such as CBA/Sca mice, displayed greater noise vulnerability at nighttime when compared to daytime. Such differences in day/night noise sensitivity were not observed in CBA/CaJ or CBA/JRj. In contrast, CORT responses to noise trauma did not appear to correlate with the differential day/night noise sensitivity. Our study thus proposes that important variations in substrate CORT profiles around the clock may underlie the differential vulnerability to day/night noise trauma. CBA/CaJ and CBA/JRj substrains showed an increase in CORT after night noise trauma, which could be an explanation for being protected against night noise trauma and allowing them to recover from the overstimulation. In contrast, CBA/Sca mice did not demonstrate a night noise induced CORT response, which may be an underlying cause for their lack of recovery after night noise trauma. The lack of CORT response in the CBA/Sca mice after night noise trauma was likely due to the already high levels of CORT at nighttime suggesting that glucocorticoid receptors in the cochlea are maximally occupied and thus not allowing a potential pro-

4. Discussion

Our study reveals that while the specific results previously reported were reproduced i.e. a day/night noise difference is ob-
tective response to occur. Noteworthy, CBA/Sca generally displayed lower threshold shifts than the other two strains, which could be potentially due to a tonic protective effect of CORT, a finding that remains to be assessed. While the present findings illustrate the importance of hormonal differences between different mouse substrains in basal and in challenged situations, these experiments are only descriptive. Future studies will need to manipulate corticosterone levels at nighttime in CBA/Caj or CBA/JRj to verify whether the phenotype of CBA/Sca can be recapitulated. Reversely, blocking glucocorticoid receptors at nighttime in CBA/Sca may also abolish the differential day/nocturnal noise sensitivity in this strain.

Circadian rhythms can be measured by behavioral differences between activity and rest. Voluntary activity on running wheels is one common method to reliably determine the circadian rhythm of a mouse. Rodents are nocturnal and, when kept in constant darkness (free-running conditions), the circadian clock will dominate the behavior of the animal without influences of external light conditions. It should be noted that the period of these different mouse strains is maintained under constant darkness conditions and is shorter than 24 h. Under constant darkness conditions, the overall running activity has been shown to differ between different strains such that C57BL/6 J mice demonstrate higher activity than 129S1/SvlmJ (Hossain et al., 2004). Running wheel activity had also illustrated strain differences in BALB/c, C57BL/6 J, DBA and 129/J mice. The DBA mice had a short circadian period of 23.31 h compared with 23.73 h in C57BL/6 mice, 22.9 h in BALB/c mice and 23.93 h in 129/J mice (Possidente and Stephan, 1988; Schwartz and Zimmerman, 1990). In the current study, differences in the locomotor activity between the substrains were also found. The period of CBA/Sca mice was highest (23.7 h), while being 15 min shorter in CBA/JRj and 34 min shorter in CBA/Caj. These findings indicate that the different substrains will have a different amount of time needed to synchronize their biological clocks and adjust their physiology to the light entrainment. As a result of these differences the animals will have different times of the day where they will have peak sensitivities to different insults. The locomotor activity of each mouse strain illustrates individual chronotypes with their preferred time of activity and rest, which is an important factor to consider when designing experiments.

Whereas the CBA/Sca displayed differential day/nocturnal sensitivity to noise, this was only applicable to the 103 dB SPL exposure, not the lower level of 100 dB SPL, nor the 105 dB SPL. This suggests a fine tuning of the circadian effects regulating this greater vulnerability seen during the active phase. When the levels of exposure were too high, no differences were observed between day and night noise exposures, likely since daytime exposure caused a major shift in hearing. Similarly, when levels were too low, they were insufficient in revealing a greater sensitivity during the active phase. In contrast to our former studies, we here included DPOAE measures, which impact after noise exposure did...
not differ between day- or nighttime, whatever the substrain assessed, suggesting that OHC function was not involved in the differential day/night sensitivity to noise. Wave 1 amplitude instead showed differences between day- or nighttime noise exposures, with CBA/Sca being the most affected. However, the genuine impact of noise on wave 1 amplitude was difficult to assess when hearing thresholds were affected, which was the case in all noise exposures. Often used as a correlate of noise impacting wave 1 amplitude, ribbon or paired synapses counts showed few differences between day and night noise exposures in CBA/Sca mice only. CBA/JRj and CBA/CaJ also displayed differences in ABR wave 1 amplitudes in 103 and 105 dB SPL noise exposures when comparing day and night exposures, however no differences were seen histologically at the synaptic level. Indeed, previous studies have suggested ABR Wave 1 amplitude as a correlate for auditory sinciphalopathy in mouse or humans exposed to noise (Bramhall et al., 2019; Hickox et al., 2017), however, the poor and scattered correlation between ABR Wave 1 amplitude and synaptic status across all hearing frequencies, suggests that Wave 1 amplitude cannot be used as a surrogate marker of synaptic damage. Consistent with this hypothesis, we previously found that, in absence of noise exposure, GLAST KO mice displayed reduced ABR Wave 1 amplitude and halved synaptic pairing while displaying normal hearing thresholds and normal synaptic ribbon abundance (Tserga et al., 2021). However, when treated with cisplatin at nighttime, GLAST KO mice displayed an almost complete loss of pairing and mildly reduced ribbon numbers despite unaffected ABR Wave 1 amplitudes (Tserga et al., 2020).

The CBA/Sca that were used in our two former publications displayed a differential day/night noise sensitivity that correlated with large amplitudes of CORT, up to 90 ng/ml at CT12 (Cederroth et al., 2019; Meltser et al., 2014). In the present study, the differential day/night sensitivity to noise was still observed but to a less prominent degree than in our previous reports (44 ng/ml). We believe that one explanation for this difference is due to the origins and housing conditions of these CBA/Sca strains. In the first studies, animals were obtained from Scanbur and housed in a low barrier conventional facility. The Scanbur colony was terminated in 2017. We then identified a batch of cryopreserved CBA/Sca embryos, which was used to rederive and house the colony in a new specific pathogen free (SPF) facility that uses individual ventilated cages. Indeed, rederivation into SPF facilities has been shown to impact on the phenotype (either attenuating or creating new ones) in various studies. For instance, TLR5 mutants develop spontaneous colitis in conventional facilities, but not longer when animals were rederived in SPF conditions where they instead develop metabolic syndrome, possibly through altered microbiota (Vijay-Kumar et al., 2010, 2007). The NOD mouse strain develops diabetes depending on the microbial environment in the animal housing facility (Kriegel et al., 2011; Wen et al., 2008). Exposing SPF mice to
Fig. 8. (a) Corticosterone values from CBA/CaJ, CBA/JRj, and CBA/Sca maintained in LD, whose blood was collected every 4th hour around the clock. (b) Average Tau value for each CBA substrain when maintained 3 weeks in constant darkness (DD). Mean values for each individual are shown. (c) Average double-plot actograms showing locomotor activity for each strain in light/dark (LD) cycles and in dark/dark (DD) cycles. On the horizontal axis, each line represents a day. Dark horizontal bars represent the active phase and the white horizontal bar the inactive phase. The red line indicates the fit with which the Tau (b) was quantified. Stars with brackets connecting two groups indicate significant differences between these groups (*: p<0.05; **: p<0.01; ***: p<0.005); two-way ANOVA with Sidak’s post hoc analysis. Results are mean values ± SEM; Number of mice/strain; CBA/Sca: N = 8–12, CBA/JRj: N = 6–12, CBA/CaJ: N = 6–10 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 9. Corticosterone values after different interventions at ZT3 (day) and ZT15 (night). Each of ZT3 and ZT15 has three different interventions: handling only (open circles and open diamonds), sham noise exposure (open red circles for ZT3 and open blue diamonds for ZT15), and actual noise exposure at 103 dB SPL (filled red circles for ZT3 and filled blue diamonds for ZT15). Each panel shows data for a single substrain (CBA/CaJ, CBA/JRj and CBA/Sca), split and clustered above the corresponding intervention. Markers show raw data, and their means with standard deviations are indicated by horizontal lines with whiskers. Stars with brackets connecting two groups indicate significant differences between these groups (*: p<0.05; **: p<0.01; ***: p<0.005); two-way ANOVA with Sidak’s post-hoc analysis. Results are mean values ± SD; Number of mice/strain; CBA/CaJ: N = 7–10; CBA/JRj: N = 6–12; CBA/Sca: N = 8–12 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Specific pathogens can impact on graft rejections, resistance to infection, and adaptive immune responses among many other traits (Barthold, 2002; Masopust et al., 2017). Thus, it is likely that the attenuated phenotype stems from the rederivation of the CBA/Sca strain into SPF conditions.

In conclusion, while our past research was not replicated to its full extent, the present study suggests that subtle differences in housing milieu take part in the difficulty in replicating auditory research findings between and within laboratories. Rederivation and sanitary conditions (standard vs SPF) may contribute to such hurdles. For instance, we have previously observed that a single administration of 4 mg/kg cisplatin to GLAST KO mice caused limited auditory damage when administered during daytime at the Karolinska Institutet (Tserga et al., 2020), whereas the same experiment caused a 20–40 dB threshold shift when performed at the Rockefeller University (Cederroth, unpublished observations). Similarly, the embryonic lethality we previously reported in 50% of GLAST KO mice when bred at the Karolinska Institutet (Tserga et al., 2020; Yu et al., 2016), was never observed when bred at the Rockefeller University (Cederroth, unpublished...
observations). In addition to rederivation and sanitary conditions, subtle differences in the genetic background as those evidenced here at the strain level can impact on the outcome. Our findings suggest that the subtle interplay between corticosterone circadian profiles, the genetic background, and the housing conditions influences the vulnerability to noise trauma at different times of the day.

Supplementary figures and tables

CRediT authorship contribution statement

Corstiaen P.C. Versteegh: Visualization, Data curation, Formal analysis, Conceptualization, Writing – original draft, Writing – review & editing. Evangelia Tserga: Visualization, Data curation, Formal analysis, Conceptualization, Writing – original draft, Writing – review & editing. Jacopo M. Fontana: Visualization, Data curation, Formal analysis, Conceptualization. Rocio Moreno-Paublete: Visualization, Data curation, Formal analysis, Conceptualization. Heela Sarsus: Visualization, Data curation, Formal analysis, Conceptualization. Georgios-Alkis Zisidas: Visualization, Data curation, Formal analysis, Conceptualization. Christopher R. Cederroth: Visualization, Data curation, Formal analysis, Conceptualization, Writing – original draft, Writing – review & editing. Barbara Canlon: Visualization, Data curation, Formal analysis, Conceptualization, Writing – original draft, Writing – review & editing.

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Data availability

Raw data and Python code are available as Supplementary material.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.heares.2021.108395.

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