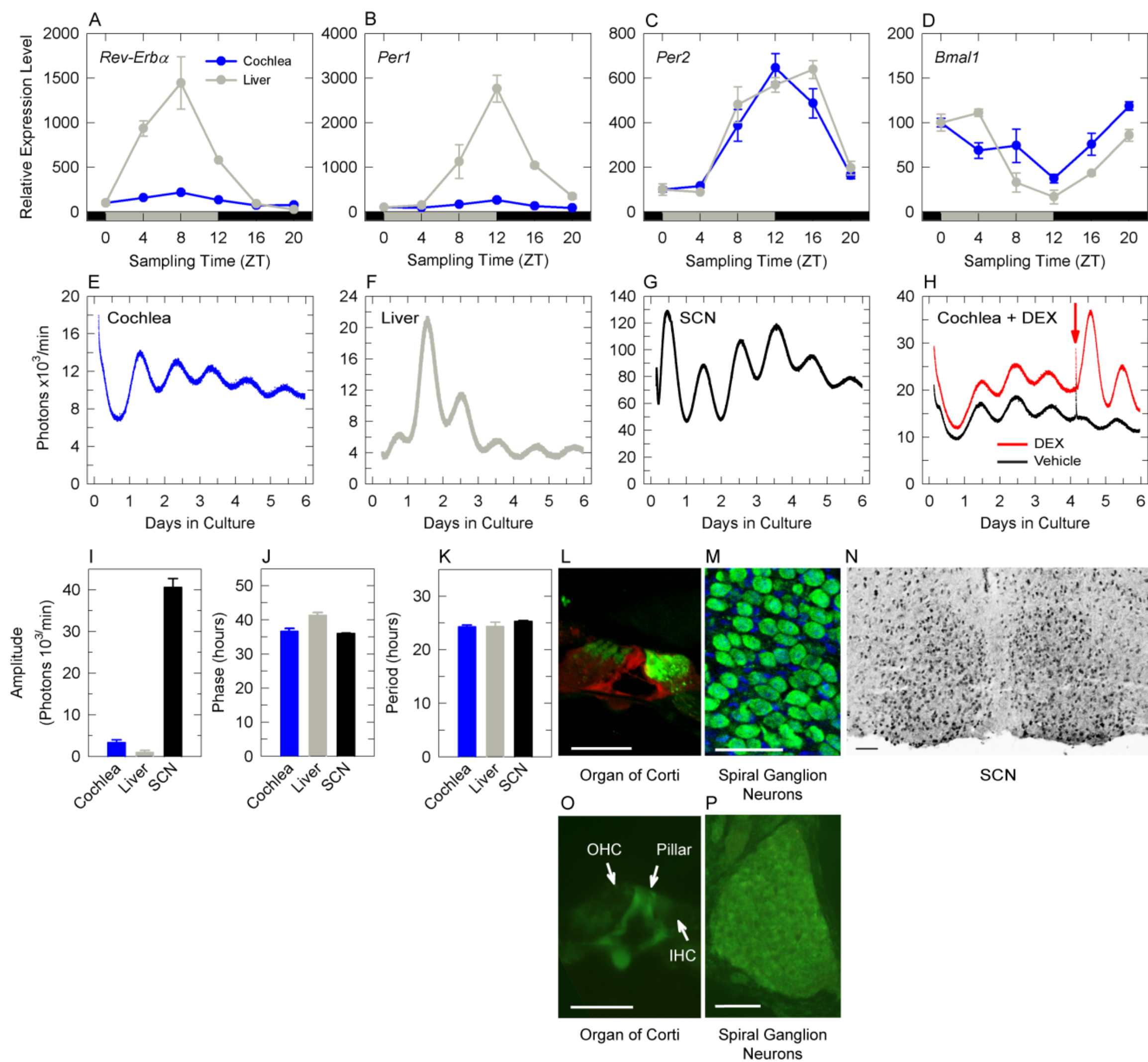


**Supplementary Figure 1. Circadian rhythmicity in the adult cochlea.** Temporal expression of *Reverb-α* **(a)**, *Per1* **(b)**, *Per2* **(c)**, and *Bmal1* **(d)** mRNAs in the cochlea (blue circles) and in the liver (gray circles). *Per1* mRNA levels oscillated with an amplitude of about 30-fold in the liver, whereas the amplitude in the cochlea was only about two-fold. *Per2* transcripts in the cochlea and liver displayed similar circadian accumulation (6 fold amplitude). Transcript levels of *Reverb-α* rose more than 10-fold in the liver but with an amplitude of two-fold in the cochlea. The well-known anti-phase circadian regulation of *Bmal1* was found in both the liver and the cochlea. Results

are mean values ± SEM (n = 3-4) and the Zeitgeber times (ZT) at which the animals were sacrificed are indicated. Without medium exchange or other pharmacological manipulations, isolated cochleae demonstrated robust self-sustained molecular rhythmicity for at least 6 days as in the liver and the SCN. Representative bioluminescence records of circadian PER2::LUC expression in cultured adult cochleae **(e)**, liver **(f)** and SCN **(g)** explants. Treatment with dexamethasone, a synchronizing agent, restored rhythmicity when applied 4 days after damping **(h)**, reflecting that cochlear cells became asynchronous with time rather than dying off. Quantification of the rhythmic components are shown by the average amplitude **(i)**, phase **(j)** and period **(k)** for each organs. The average PER2::LUC rhythm amplitude in photon/min was 3365 ± 648 in adult cochleae, 916 ± 551 in the liver and 40605 ± 2129 in the SCN **(i)**. The phase of the oscillations was analyzed by measuring the time of peak expression between 24 and 48 h in culture. PER2::LUC in the cochlea was maximally expressed around Zeitgeber Time (ZT) 11-12 approximately at the same time as in the SCN (cochlea at 36.7 ± 0.9; liver at 41.3 ± 0.5; SCN at 36 ± 0.2) **(j)**. The period of the PER2::LUC rhythm was 24 ± 0.2 h in the cochlea, 24.4 ± 0.5 in the liver, and 25.4 ± 0.1 h in the adult SCN **(k)**. Results are mean values ± SEM, n = 4-9. Immunostaining of PER2 in a cochlea of intact adult CBA/CaJ mouse shows the localization of the protein in inner and outer hair and supporting cells of the organ of Corti **(l)** and in the spiral ganglion neurons **(m)** of the cochlea. PER2 immunostaining of a unilateral SCN (coronal section) is shown as positive control **(n)**. As a negative control, $mPer2^{Brdm1}$ mutant cochleas (having a frame deletion that produces an unstable PER2 protein) were used and immunoreactivity was not expressed in the hair cells or spiral ganglion neurons (data not shown). Scale bar: 50 µm.

**Supplementary Table 1.** Sybr Green Primers

| | | |
|---|---|---|
| mPERIOD1 | *mPer1* | CATTCCGCCTAACCCCATATG |
| | | CGGGGAGCTTCATAACCAGAG |
| mCLOCK | *mClock* | AGAGATGACAGTAGTATTTTTGATGGATTG |
| | | TCTCTACGTTTCTTTTCTGATTTGTTTCT |
| mBMAL1 | *mBmal1* | CTCAGCTGCCTCGTTGCAATCGGG |
| | | GTACTCCATAGATTTCACCCGTATTTC |
| mREVERB-A | *mReverb-*$\alpha$ | ATG CCC ATG ACA AGT TAG GC |
| | | GGG CTA CCT GAT GCA TGA TT |
| mTUBB | *mTubb* | GCAGTGCGGCAACCAGAT |
| | | AGTGGGATCAATGCCATGCT |
| mGAPDH | *mGapdh* | TCCATGACAACTTTGGCATTG |
| | | CAGTCTTCTGGGTGGCAGTGA |
| mTRF1R | *mTrf1R* | GGAATCCCAGCAGTTTCTTTTTG |
| | | CAATGCCTCATAGGTATCCAATCTAG |
| mTUBA2 | *mTubA2* | AGGAGCTGGCAAGCATGTG |
| | | CGGTGCGAACTTCATCGAT |
| mHPRT | *mHPRT* | GCTCGAGATGTCATGAAGGAGAT |
| | | AAAGAACTTATAGCCCCCCTTGA |
| mCYCLO B | *mCycloB* | ATG TGG TTT TCG GCA AAG TT |
| | | TGA CAT CCT TCA GTG GCT TG |

## EXPERIMENTAL PROCEDURES

### *Ethics Statement and animal handling*

All experimental procedures on animals were performed in accordance with the guidelines and regulations set forth by Karolinska Institutet and “Stockholm’s Norra Djurförsöksetiska Nämnd”. Recordings of circadian oscillations of the PER2 protein were performed using tissues obtained from knock-in PERIOD2::LUCIFERASE (PER2::LUC) transgenic mice with a C57BL/6 background [1], generously provided by Prof. J. Takahashi. CBA/J and PER2::LUC male mice aged between 2 and 4 months mice were used for audiological, morphological and molecular experiments. Animals had free access water and to food (Lactamin R34, Lantmännen). Food pellets contained 43 mg/kg daidzin, 60 mg/kg genistin, 10 mg/kg glycitin, 2 mg/kg daidzein, 1.6 mg/kg genistein (Lantmännen report). Temperature was maintained between 19° and 21°C. Because lights were on at 6 a.m. and off by 6 p.m., we set 6 a.m. as the Zeitgeber time ZT 0. Handling at ZT 14-16 (darkness) was performed in red light.

### *Organotypic cultures*

Adult cochleae, suprachiasmatic nucleus (SCN) and livers were dissected from PER2::LUC mice and cultured organotypically on a membrane (Millipore, PICMORG50). The isolated tissues were cultured as described [2, 3]. Cochleae, dissected free of bone and stria vascularis, were kept in culture for minimum 6 days. Real time luciferase reporter technology is described [1-3]. PER2::LUC rhythms (amplitude, phase, and period) were analyzed using Origin software 8.1 SR1 (Microcal Software, Northampton, MA, USA). Data from each recording trace was first de-trended by subtraction of the 24 h baseline drift from the raw data. The period of one complete cycle was defined as the time between two consecutive peaks (i.e. the highest photon count within one cycle) and consecutive peaks were used for averaging periods. The amplitude was calculated as the difference between highest (peak) and lowest (trough) photon count within one cycle. The

calculation was performed from trough-to-peak and from the peak-to-trough, thus giving two values (half-cycles) within one cycle. In each recording, three half-cycles were used for amplitude analyses. The very first peak after culture start was not used. The phase relationships were analyzed by comparing the time of the peak (maximum bioluminescence) between 24 and 48 h after culture start in each tissue. Phase shifts were calculated by comparing the time of the peak right before treatment with the time of the peak right after the treatment.

***Drug treatment***

Mice were given a 4 ml/kg intraperitoneal injection of DHF (5 mg/kg) dissolved in the vehicle (18 % DMSO in PBS solution) 2 h prior to noise trauma. For in vitro treatments, two protocols were used for the treatment of cochlear explants:

1) DHF day-night experiments (Figure 3D-F): Cochlear explants were exposed to DMSO vehicle or DHF 60 µM either at ZT 3 or ZT12. Dexamethasone 21 phosphate disodium salt (Sigma Aldrich, D1159) was applied on day 4. 2) DHF/ANA12 experiments (Figure 3G-I): Cochlear explants were pre-exposed on day 0 and day 2 with DMSO vehicle or ANA12 (Sigma Aldrich, SML0209) at ZT 4. On day 3 the explants were co-treated with DHF (Tocris, 3826) and vehicle or ANA12 at ZT 4.

***Acoustic trauma and auditory brainstem response (ABR)***

To generate hearing loss, awake and unanesthetized animals were treated with free field broadband noise at 6 - 12 kHz at intensity of 100 dB SPL for 1 h, similar to previously described noise trauma paradigm [4]. Auditory sensitivity was assessed with ABR thresholds for the frequency of 6, 12,16 and 24 kHz as described previously [5]. Post-trauma measurements where performed either 24 h after the day or night exposures. However, the final measurements made at 2 weeks post-trauma were made only during the day because i) we found no differences in basal ABR levels between day and night and ii) after 2 weeks post-trauma, the ABR levels are considered stable.

***Acoustic startle response (ASR)***

CBA male mice were tested for the ASR at sleep (ZT 3-6) and awake (ZT 14-16) phases. Same animals were used for the experiment with 7 days interval between day and night sessions. The ASR paradigm was previously described [6]. The startle magnitude (Vmax, V - the mean maximal startle response to the “pulse-alone” trials) and startle latency ($T_{max}$, msec - the mean latency to the maximal response to the “pulse-alone” trials) were calculated.

***Immunocytochemistry and quantification of synaptic ribbons***

For immunostaining of PER2, wild type or *mPer2*$^{Brdm1}$ mutants (having a frame deletion that produces an unstable PER2 protein) [7] underwent transcardiac perfusion with 4% paraformaldehyde and cochleae were decalcified in EDTA 2% for 4 days and cryosectioned (14µm). Sections were immunostained with a rabbit-antibody to PER2 (PER21-A, Alpha Diagnostic, Texas USA; 1:100). For the quantification of synaptic ribbons, surface preparations from fixed cochleae, decalcified for twelve hours in EDTA 2%, were stained for C-terminal binding protein 2 (mouse anti-CtBP2, 612044 from BD-Biosciences, used at 1:200) and secondary FITC-conjugated goat anti-rabbit and TRIC-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch; Pennsylvania USA; 1:100). Cochlear lengths were obtained for each sample and a cochlear frequency map computed to precisely localize inner hair cells (IHCs) throughout the length of the cochlea [8]. Confocal z-stacks from every 0.3 mm distance along the basilar membrane were made with a z-step-size of 642 nm. Averages were then made for every mm region. Image stacks were then analyzed using Image J software. Synaptic ribbons were counted in 5-10 adjacent inner hair cells and divided the number of cells counted in order to obtain the number of ribbons per cell. Quantification was performed using an automated particle counting after

converting the image to grayscale and thresholding the image. This technique was manually validated before collecting the data.

***Quantitative Real Time-PCR***

SybrGreen qRT-PCR assays were performed as previously described [9, 10] with minor modifications. CBA male mice were put into darkness for 72 hours prior their exposure to either silence or sound (100 dB, 6-12 kHz narrow band white noise, for 1 hour). To allow comparisons, the cochleas (controls, day and night noise exposure) were collected at fixed ZT time points. Animals were decapitated under isofluorane anesthesia and their cochleae and liver collected. Total RNAs were extracted from whole cochleae using the Direct-zol™ RNA MiniPrep kit from ZymoResearch (Nordic Biolabs AB, Sweden) according to the manufacturer's protocol, and immediately followed by DNAse I treatment (Invitrogen). RNA integrity was assessed using RNA 6000 nanochips with an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA) and quantity was evaluated with Nanodrop. RT-PCR assays were performed with 0.5 μg total RNA from 3-4 adult cochleae, or livers, collected every 4 h around the clock. Total RNAs were reverse transcribed with the Superscript II RT-kit from Invitrogen (Life Sciences, Sweden) according to manufacturer's instructions, and one-twentieth cDNA dilution was used as template for each PCR. cDNA was PCR amplified in a 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR master mix (Applied Biosystems). Raw threshold-cycle (Ct) values were obtained from Sequence Detection Systems 2.0 software (Applied Biosystems). Relative quantities (RQs) were calculated with the formula $RQ = E - Ct$, using efficiencies calculated for each run with the Data Analysis for Real-Time PCR (DART-PCR) algorithm, as described [11]. A mean quantity was calculated from triplicate PCR for each sample, and this quantity was normalized with the geometric mean of two to four most stable genes out of six reference genes (tubulin β, Tubb; glyceraldehyde-3-phosphate dehydrogenase, G3pdh; transferring

receptor 1, Trf1R; Tubulin α2, Tuba2; hypoxanthine phosphoribosyltransferase, HPRT; and Cyclophilin B) selected using the geNorm algorithm as described [12]. Normalized quantities were averaged for three technical replicates for each data point and represented as the mean ± SD. The highest normalized relative quantity was arbitrarily designated as a value of 1.0. Fold changes were calculated from the quotient of means of these normalized quantities and reported as ± SEM. The primers used for quantitative RT-PCR (qRT-PCR) are listed in **Supplemental Table 1**.

For the evaluation of *Bdnf* mRNA transcript levels, we performed Taqman qRT-PCR assays (Applied Biosystems, CA, USA). Mice were decapitated under isofluorane anesthesia; cochleae were extracted from the temporal bone and further dissected in RNAlater RNA stabilization reagent (Qiagen). The outer bony shell of the cochlea and the vascular tissue (stria vascularis) were removed, and cochleae were left in RNA stabilization reagent for 12-24 h at room temperature. *Bdnf* (Mm01334042_m1) gene expression was quantified as described above using *Cyclophilin E* (Mm00450929_m1) and *Hprt* (Mm00446968_m1) as the most stable normalization genes.

***Statistics***

Data are presented as a mean ± SEM. Statistical analysis of PER2::LUC rhythms and the qRT-PCR was performed with GraphPad Prism 5.04 (GraphPad Software Inc., CA, USA). Analysis of other experiments was performed using SigmaStat v 3.5. The tests used for each experiment are described in the figure legends.

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