MATERIALS AND METHODS

Animals

Forty-eight male C57BL/6J mice weighing 18–21 g were randomly divided into two groups: control group and 72hSD group. The mice were conditioned for 5 days before the experiment, and the animals were housed in the Experimental Animal Center of the Military Medical Sciences Academy and maintained a 12/12 h light/dark cycle at controlled temperature (23 ± 2 °C) and humidity (50 ± 5%), and the background noise was controlled to be less than 45 dB sound pressure level (SPL). The mice had free access to food and filtered water. All animal experimental procedures are in accordance with the standards of the Military Medical Sciences Academy Ethics Committee (Animal Ethics Number: IACUC of AMMS-04-2023-033).

SD Procedure

According to the protocol of SANG et al.[1], the CPW paradigm was applied to the 72hSD group for SD. The mice in the 72hSD group were placed in a drum (20 cm in height and 25 cm in diameter) containing an 8 mm shallow water layer for 72 h for SD, maintained at a water temperature of 23 ± 1 °C, and filled with water and food at a height of 3 cm from the bottom of the barrel. When the mice fell asleep, their muscles were awake when the loss of muscle tone caused their heads to touch the water. The water in the bucket was changed every 12 h to maintain a clean environment for the sleep-deprived mice. The mice of control group are placed in rearing cages filled with dry litter.

Auditory Brainstem Responses (ABR)

The hearing threshold was evaluated using ABR tests at various time points after SD (D0: immediately after SD; D3, D7 represent 3 or 7 days after SD, respectively) (Supplementary Figure S1A). Mice were anesthetized with sodium pentobarbital (dose of 0.1 mL/10 g) in 1% induction, and the body temperature was maintained using a heating pad. Three stainless steel needle electrodes were inserted subcutaneously into the test auricle (reference electrode), median position of the cranial parietal (recording electrode), and skin of the hind paw (grounded electrode).

The hearing threshold and ABR wave I amplitude of brain waves (click) and pure tones (tone) at each frequency were measured using an auditory electrophysiology workstation TDT RZ6 (Tucker-Davis Technologies, Alachua, USA) in a standard soundproof room. A loudspeaker (placed 10 cm away from the detection ear) was placed in an open-field configuration to deliver acoustic stimuli. The tone detection frequency was set at 4, 8, 16, and 32 kHz, the number of superimpositions was 1024, and the stimulus intensity started at 90 dB, decreasing every 10 dB, decreasing in 5 dB steps near the hearing threshold until the eigenwave II disappeared, and the sound intensity corresponding to this wave was recorded as the animal's auditory response threshold.

Immunofluorescence Staining

The removed cochlea was fixed in 4% paraformaldehyde (PFA) for 24 h, followed by decalcification in 10% ethylenediaminetetraacetic acid (EDTA) for 3 days at 4 °C. The cochlear basement membrane was isolated under a stereomicroscope (Olympus SZX7, Japan), evenly divided into three segments (apical, middle, and basal), cochlear tissue was infiltrated with dilution (1% Triton X-100, 0.1 mol/L PBS solution) for 30 min, incubated with blocking solution (5% BSA, 0.5% Triton X-100, and 0.1 mol/L PBS solution) for 1 h, and with the hair cell marker rabbit anti-Myosin-VIIa (1:1,000, 25-6790) or the synaptic ribbons marker rabbit anti-CtBP2 (1:500, BS2287) was incubated overnight at 4 °C. The samples were then incubated with goat anti-rabbit 549 antibody (1:500, BS10023) for 1 h in the dark. The basement membrane was placed on a slide, sealed with an anti-quenching sealer containing DAPI, and the staining results were observed under a fluorescence microscope (Olympus BX51, Japan) or a Zeiss microscope (Zeiss Mica, Germany).

Proteomics Analysis

The analysis was performed using data independent acquisition (DIA) quantitative proteomics technology using 10 mg of protein from cochlear tissue as the starting material. Samples were trypsinized into peptides, desalted on a C18 column, vacuum-lyophilized, and the digested peptides were dried and reconstituted with 0.1% formic acid. The peptides were separated using UHPLC (Thermo Scientific,

USA) and analyzed using an Orbitrap Astral mass spectrometer (Thermo Scientific, USA) for DIA mass spectrometry. All mass spectrometry data were merged using the software, and the database search of DIA mass spectrometry data and protein DIA quantification were completed. Differential expression analysis was performed on the identified proteins using the DIA-NN system for further analysis (FC > 1.2 or FC < 1/1.2, $P < 0.05$). Differentially expressed proteins (DEPs) were compared to the Metascape database, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) identification, functional annotation, and pathway enrichment were performed.

Enzyme-Linked Immunosorbent Assay (ELISA)

A corticosterone (CORT) assay (ELISA) kit (Mlbio, China) was used to detect the stress marker CORT. The inflammatory markers IL-1β, IL-6, and IL-17A were detected using a mouse IL-1β, IL-6, and IL-17A ELISA kit (Jonlnbio, China). The ELISA protocol was performed according to the manufacturer's instructions.

Western Blot Analysis

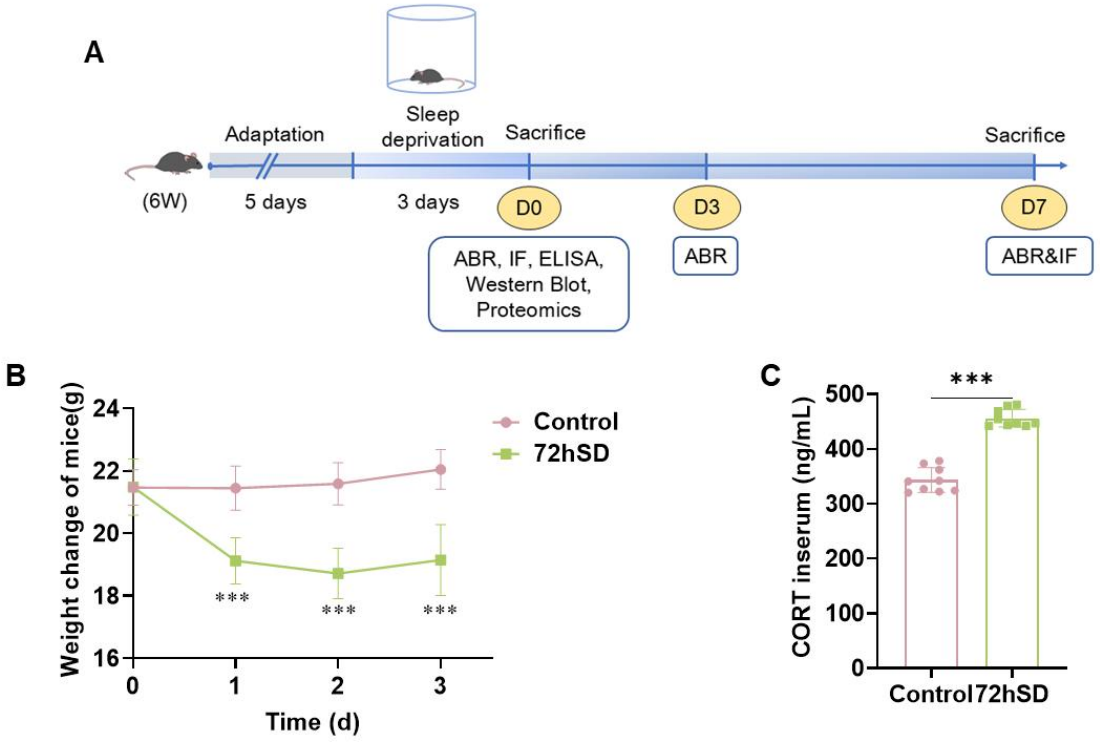
The anesthetized mouse (sodium 1% pentobarbital) was decapitated, and the cochlea was quickly removed from the skull and placed in a Petri dish on ice. Six cochleae (from three mice) were combined into one sample, and the entire cochlea, including the cochlea, basement membrane, and lateral wall, was dissected using a stereomicroscope (Olympus SZX7, Japan). The tissue was immediately transferred to a pre-prepared RIPA lysis buffer (Solarbio, China) with 1% protease inhibitor homogenate, centrifuged at 12,000 rpm for 15 min at 4 °C, and stored at -80 °C for subsequent western blot analysis. The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF membrane. 5% skim milk or BSA prepared with TBST buffer was used to block non-specific binding sites. Then, the membrane was incubated overnight at 4 °C with the primary antibodies, including β-actin (1:3,000, 20536-1-AP), HO-1 (1:1,000, 10701-1-AP), 4-HNE (1:1,000, HNE11-S-12-C), NLRP3 (1:1,000, A21906), GSDMD (1:1,000, ab219800), Caspase-1 (1:1,000, A16792), ASC (1:1,000, ab309497), IL-1β (1:500, sc-12742), TLR4 (1:1,000, sc-293072), NF-κB p65 (1:1,000, sc-8008), IκBα (1:1,000, A24909), MyD88

(1:1,000, sc-74532), respectively. After three rinses in TBST buffer for 10 min, goat anti-rabbit (1:20,000, BS20241-Y) or goat anti-mouse (1:20,000, BS20242-Y) secondary antibodies conjugated to horseradish peroxidase (HRP) were incubated for 1 h, depending on the type of primary antibody. Finally, the membranes were rinsed three times with TBST for 10 min and treated with an enhanced chemiluminescent reagent (ECL; Invigentech, USA). Each analysis was performed in triplicate.

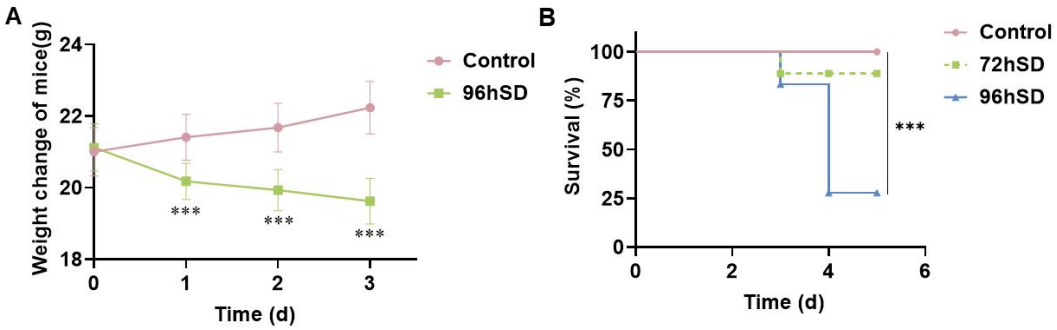
Analysis and Statistics

Statistical analyses were performed using SPSS 27.0 software (SPSS Inc., USA) and GraphPad Prism software version 8.0 (GraphPad Software, USA). Student's t-test was used to compare the differences in western blot analyses between the control group and 72hSD group. One-way analysis of variance (ANOVA) and LSD post hoc tests were applied to analyze the changes in ABR data and synapse counting. $P < 0.05$ was considered a statistically significant difference. All data are presented as the mean ± standard deviation.

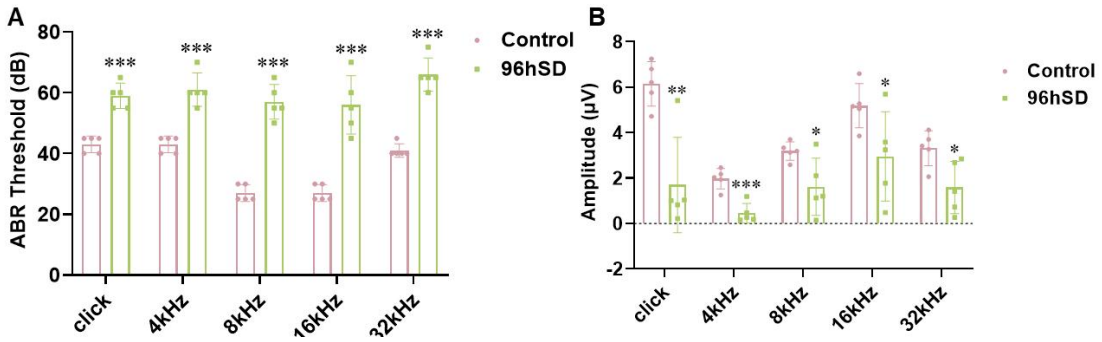
1. SANG D, LIN K, YANG Y, et al. Prolonged sleep deprivation induces a cytokine-storm-like syndrome in mammals. Cell, 2023; 186, 5500-16.



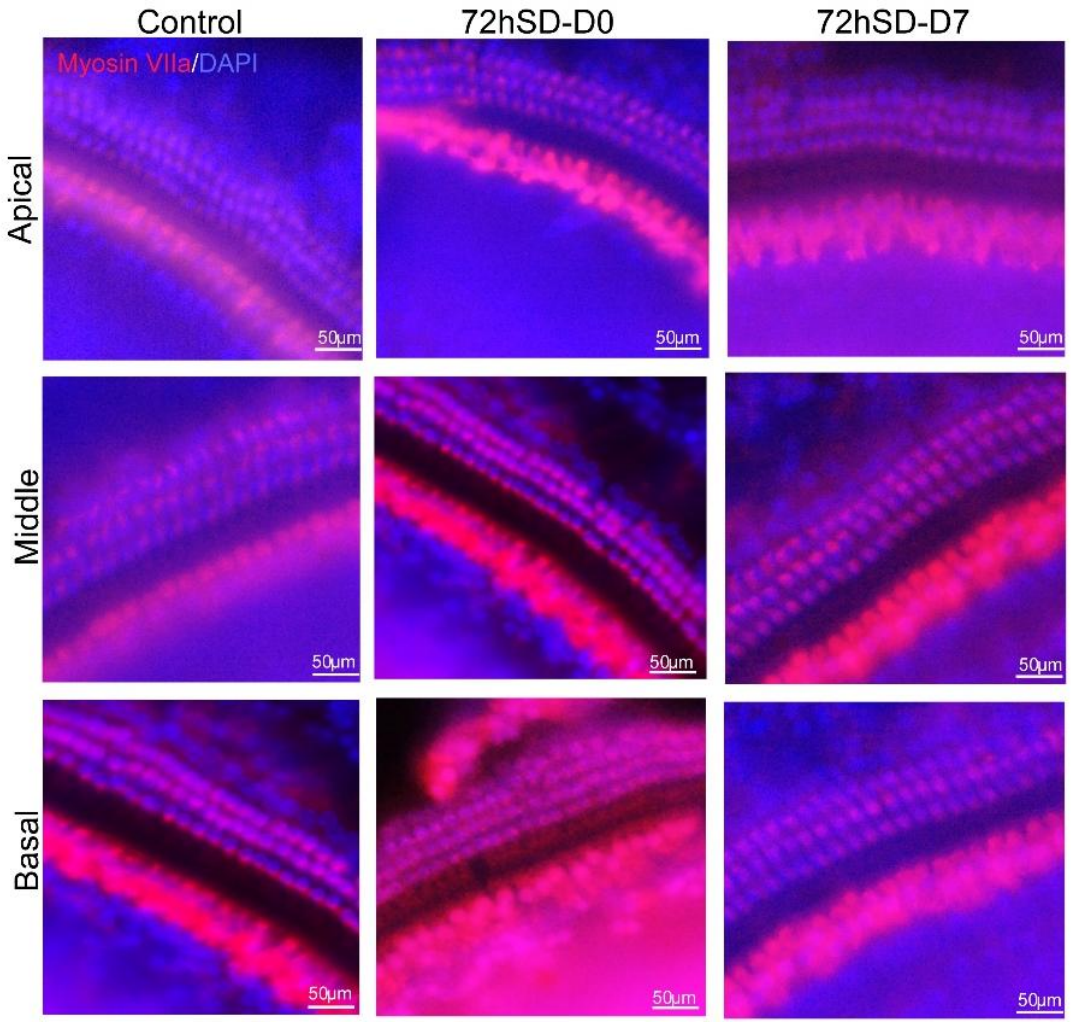
Supplementary Figure S1. Effects of 72 h SD on body weight and CORT. (A) Schematic timeline; (B) Change in body weight of 72 h SD (*n* = 13); (C) Levels of corticosterone in plasma (*n* = 9). Where, ***: *P* < 0.001 compared with the control group. ABR, auditory brainstem responses; IF, immunofluorescence; ELISA, enzyme-linked immunosorbent assay; CORT, corticosterone; SD, sleep deprivation.



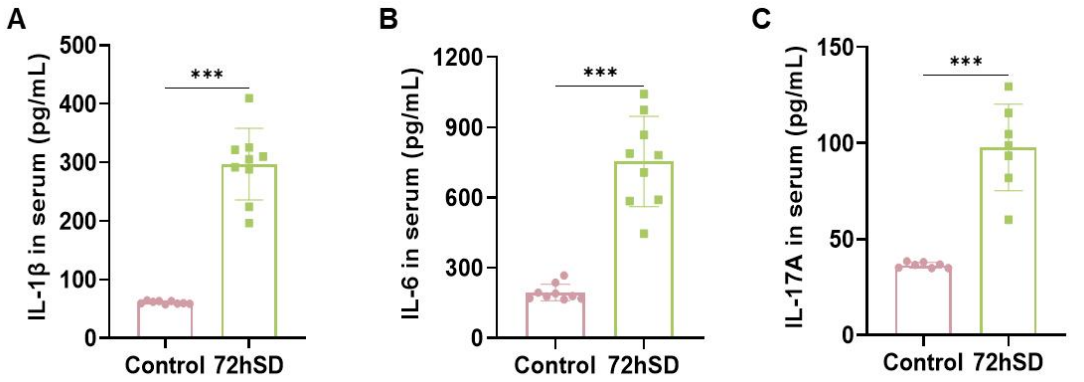
Supplementary Figure S2. Effect of SD on body weight and survival in mice. (A) Changes in body weight of mice after 96 h of SD. (B) Survival analysis of the three groups of mice. (*n* = 18). Where, ***: *P* < 0.001 compared with the control group. SD, sleep deprivation.



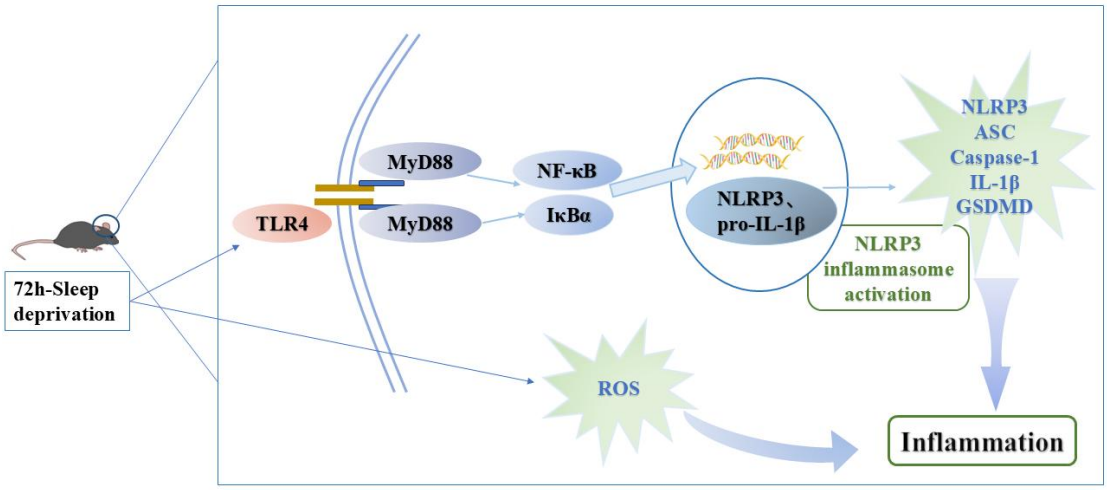
Supplementary Figure S3. Effect of 96 h SD on hearing in mice. (A, B) Changes in ABR hearing threshold and I wave amplitude at D0 after 96 h SD under the stimulus of click and pure tone (4, 8, 16, and 32 kHz). (n = 5). Where, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ compared to the control group. ABR, auditory brainstem responses; SD, sleep deprivation.



Supplementary Figure S4. Representative images of HC density across the cochlea (red, anti-Myosin VIIa; blue, DAPI).



Supplementary Figure S5. Effect of 72h SD on inflammation. (A–C) Serum levels of inflammatory markers IL-1β (A), IL-6 (B), and IL-17A (C) after SD. (*n* = 7–9). Where, ***: *P* < 0.001 compared with the control group. IL-1β, interleukin-1β; IL-6, interleukin-6; IL-17A, interleukin-17A; SD, sleep deprivation.



Supplementary Figure S6. Schematic representation of the activation of TLR4/NF-κB/NLRP3 signaling pathway by SD to induce inflammation. SD promoted the activation of NF-κB via TLR4/MyD88, and the activated NF-κB further activated NLRP3 and other factors after entering the nucleus, promoting the production of the NLRP3 inflammasome. This causes cochlear inflammation. ASC, apoptosis-associated speck-like protein containing CARD; Caspase-1, cysteine aspartate-specific protease1; GSDMD, gasdermin-D; IκBα, nuclear factor kappa B inhibitor alpha; IL-1β, interleukin-1β; MyD88, Myeloid differentiation protein 88; NF-κB, nuclear factor kappa-B; NLRP3, NOD-like receptor thermal protein domian associated protein 3; ROS, reactive oxygen species; SD, sleep deprivation; TLR4, toll-like receptors 4.