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1 **Transcriptome Variant Analysis of Noise Susceptibility in C57BL/6J Mice**

2

3 **Authors:** Siyue Wang[1a], Jing Cai[1a], Ligang Kong[a], Lei Xu[a], Zhaoming Fan[a], Haibo Wang[*a]

4

5 **Affiliation:** [a]Department of Otolaryngology-Head and Neck Surgery, Shandong Provincial

6 ENT Hospital, Shandong University, No. 4, Duanxing West Road, Jinan, Shandong 250022,

7 China

8

9 [*]**Corresponding author:** Haibo Wang,

10 Department of Otolaryngology-Head and Neck Surgery,

11 Shandong Provincial ENT Hospital, Shandong University,

12 No. 4, Duanxing West Road, Jinan,

13 Shandong 250022, China

14 E-mail: whboto11@163.com

15

16 [1]These authors contributed equally to this work.

17

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19

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23 **Abstract**

24 ***Background:*** Susceptibility to noise varies dramatically between mice of the same genetic
25 background; however, the underlying molecular mechanism remains unknown.

26 ***Methods:*** C57BL/6J (B6) mice of the same sex, age, and strain were exposed to noise of the
27 same intensity and duration, and the auditory brainstem response (ABR) threshold was
28 determined 48 h later. Some mice had significant hearing loss, while some did not; the ABR
29 threshold measured in these two groups of mice was significantly different. The cochlea of
30 the two groups of mice was dissected, and RNA sequencing and analysis were performed.
31 Differentially expressed genes (DEGs) between the two groups were selected, Kyoto
32 Encyclopedia of Genes and Genomes pathway analysis was performed, and protein–protein
33 interaction network maps were listed.

34 ***Results:*** This study showed that noise exposure of the same intensity and duration caused
35 different degrees of hearing loss in C57BL/6J (B6) mice. This was the result of the
36 up-regulation or down-regulation of many genes, such as Nop2, Bysl, Rrp9, Spsb1, Fbxl20,
37 and Fbxo31. Changes in the transcriptome of these genes may affect cochlear susceptibility to
38 noise.

39 ***Conclusion:*** The DEGs identified in this experiment may provide more insight into protocols
40 for gene therapy in the clinical practice of hearing loss.

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45

46 ## 1. Introduction

47 Noise-induced hearing loss is a form of sensorineural deafness that results from
48 prolonged exposure to a noisy environment and a combination of factors. At present, the
49 recognized mechanisms are mechanical damage to the cochlea, metabolic damage, immune
50 and inflammatory damage, and genetics (Ding et al. 2019). Current studies have found that
51 the genes involved in noise-induced hearing loss are associated with oxidative stress, DNA
52 repair, gap junctions, apoptosis, K^+ recycling, and heat shock proteins (Ding et al. 2019; Mao
53 and plasticity 2021; Sliwinska-Kowalska and research 2013). It is well known that
54 susceptibility to noise varies significantly among individuals, and not everyone experiences
55 the same hearing loss after the same noise exposure (Sliwinska-Kowalska and research
56 2013).

57 Prior to this, some animal studies have demonstrated that NOX3, FOXO3, NRF2, CX26,
58 CRFR2, BHMT, A1AR, and MYH14 (Beaulac et al. 2021; Fu et al. 2016; Graham et al. 2010;
59 Honkura et al. 2016; Lavinsky et al. 2015; Partearroyo et al. 2019; Vlajkovic et al. 2017;
60 Zhou et al. 2016) knockout mice are more sensitive to noise than wild-type mice. These
61 studies in knockout mice have shown that genetic defects in mice, which disturb specific
62 paths and structures within the cochlea, make mice more sensitive to noise (Le et al. 2017).
63 There are also many other studies of genetic mouse noise susceptibility (Fairfield et al. 2005;
64 Holme and JARO 2004; Kozel et al. 2002; Ohlemiller et al. 1999; Ohlemiller et al. 2000;
65 Schick et al. 2004; Tabuchi et al. 2005; Yan et al. 2013), and it has been demonstrated that
66 noise susceptibility varies for different strains of mice. For example, B6 and 129 mice

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67 showed differences in gene expression after noise exposure; HSP70, HSP40, GADD45b, and
68 P21Cip1 were significantly induced and up-regulated at the protein level in 129 mice, and
69 their up-regulation may have a protective effect on hearing in these mice (Gratton et al. 2011).
70 Inbred C57BL/6J (B6) mice are more likely to acquire noise-induced hearing loss than inbred
71 CBA/Cal (CB) mice (oto-laryngologica 1992; Shone et al. 1991) because the AHL gene is
72 reported to influence susceptibility to noise-induced hearing loss (Davis et al. 2001; Erway et
73 al. 1996; Harding et al. 2005).

74 Many studies have shown that people working in environments with similar noise levels
75 often show varying degrees of hearing loss (Henderson et al. 1993), especially in
76 occupational noise exposure. The noise susceptibility of this population is more pronounced,
77 with approximately 33% showing noise-induced hearing impairment and 16% showing
78 substantial hearing impairment (Themann and America 2019). In China, the prevalence of
79 occupational noise-induced hearing loss is 21.3% (Zhou et al. 2020); therefore, not all people
80 exposed to noise suffer from noise-induced hearing loss. In addition, many studies have
81 employed single nucleotide polymorphism screening methods in noise-exposed populations
82 (Liu et al. 2021; Miao et al. 2019; Zhang et al. 2019b; Zhang et al. 2019c), demonstrating that
83 genes play an important role in noise susceptibility.

84 We have also found that the same batch of mice showed different degrees of hearing loss
85 under the same noise exposure. Some mice show severe deafness immediately after noise
86 exposure, while some do not. However, there is no study on the transcriptome of mice of the
87 same strain with different susceptibilities to noise. Therefore, we investigated the
88 transcriptome variation of inbred C57BL/6J (B6) mice with different noise susceptibility.

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89 RNASeq is the first sequencing-based method to detect the entire transcriptome in a
90 high-throughput and quantitative manner, and it can accurately quantify the expression levels
91 of genes (Marioni et al. 2008; Wang et al. 2009b). In recent years, the advent of RNASeq
92 technology has allowed us to discover new genes and transcriptomes for a wide range of
93 diseases, contributing to the discovery of disease-causing factors.

94 Therefore, we established a noise-induced hearing loss mouse model. After noise
95 exposure, the degree of hearing loss was determined by auditory brainstem response (ABR)
96 measurement, and some mice were selected as the noise-resistant group (R_NE) and some as
97 the noise-sensitive group (S_NE). The mouse cochleae were collected, and using RNASeq
98 technology, differentially expressed genes (DEGs) related to noise susceptibility were
99 selected. The functions of these differential genes were summarized by Kyoto Encyclopedia
100 of Genes and Genomes (KEGG) pathway analysis in order to determine their roles in the
101 etiopathology of noise-induced hearing loss for future studies.

102

103 2. Materials and Methods

104 *2.1. Mice*

105 Fifty inbred C57BL/6J (B6) male 8-week-old normal hearing mice (all purchased from
106 Pengyue Company, Jinan, China) were selected and maintained in a quiet environment in the
107 specific pathogen-free animal room of the Shandong Institute of Otolaryngology. All mice
108 lived in a room with constant temperature (approximately 22–25°C) and were given adequate
109 food and water. One week later, 40 mice were randomly selected as the experimental group
110 and exposed to noise to establish the noise-induced hearing loss model, and 10 mice were

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111 used as the control group without noise exposure (Control). According to the hearing results
112 after noise exposure, we further subdivided the experimental group: those with a hearing
113 threshold range above one standard deviation of the mean hearing threshold of the
114 experimental group comprised the S_NE, and those with a hearing threshold range below one
115 standard deviation of the mean hearing threshold of the experimental group comprised the
116 R_NE.

117 All animal experiments were approved by the Ethics Committee of the Shandong
118 Provincial ENT Hospital, Shandong University, and the experiments complied with the
119 relevant ethical regulations for animal testing and research. All efforts were made to
120 minimize the number of animals used and to prevent their suffering.

121

122 ***2.2. Noise exposure***

123 Mice were anesthetized intraperitoneally with a mixture of ketamine hydrochloride (100
124 mg/kg) and xylazine (4 mg/kg) and then exposed to 100 dB sound pressure level (SPL) white
125 noise for 2 h. The noise stimuli were synthesized by a noise generator (SF-06, Random Noise
126 Generator, RION, USA) and amplified by an amplifier (CDi 1000 Power Amplifier, Crown,
127 USA). The bottom of the cage and the center of the speaker were placed on the same
128 horizontal line, and the distance between the two was determined using a noise meter. The
129 noise meter radio was placed in the center of the bottom of the cage, and the noise measured
130 in the center was ensured to be 100 dB each time.

131

132 ***2.3. ABR measurement***

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133 All mice were tested for ABR thresholds prior to the experiment, and the experimental
134 group underwent re-testing for ABR 48 h after noise exposure. The anesthesia employed was
135 the same as above, and the anesthetized mice were placed in a sound-proof chamber to
136 measure ABR responses under sound stimuli at 4, 8, 12, 16, 24, and 32 kHz, with 1024
137 repetitions of stimulation per recording (Tucker-Davis Technology, USA). The left ear of the
138 mouse was oriented towards the speaker (MF1; TDT) at a distance of approximately 5 cm,
139 and the recording electrode was inserted into the subcutaneous tissue of the middle of the two
140 ears, the reference electrode was fixed at the ipsilateral ear, and the ground electrode was
141 placed at the back. The sound level was decreased by 5 dB from 90 dB until no hearing curve
142 appeared. We ensured that each frequency was judged by the same person and that reliable
143 results were obtained. It is also essential to duplicate this operation for low SPLs close to the
144 threshold to guarantee the stability of the signal. After ABR audiometry, the mice were laid
145 on a warming mat to maintain body temperature and ensure awakening.

146

147 ***2.4. Sample collection and preparation***

148 ABR data were recorded for comparison, and the cochleae of mice in the R_NE, the
149 S_NE, and the Control were collected. First, the mice were anesthetized with the same drug
150 dose and methods, and cardiac perfusion was performed using normal saline. The left and
151 right cochleae were removed and rapidly placed in RNA later (Invitrogen, AM7021)
152 overnight at 4°C and transferred to -20°C for long-term storage. Total RNA was extracted and
153 tested for quality. Polymerase chain reaction amplification was performed to complete the
154 entire library preparation work.

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155

156 ### *2.5. RNA sequencing*

157 Qualified libraries were sequenced with an Illumina NovaSeq6000 sequencer with a
158 sequencing strategy of PE150 to obtain high-quality sequences (Clean Reads).

159

160 ### *2.6. DEG analysis*

161 DESeq2 (1.20.0, method = 'per-condition') was used for gene differential expression
162 analysis. Differential gene screening primarily means the fold difference (fold change value)
163 and q value (padj value, corrected P value) are related metrics. The criteria for differential
164 gene screening in this experiment were more than 1.5-fold difference and $q < 0.05$.

165

166 ### *2.7. Protein–protein interaction network maps*

167 Using the STRING protein interaction database, combined with the results of DEG
168 analysis and the interaction pairs included in the database, the DEG sets can be directly
169 mapped to the protein–protein interaction (PPI) network of this species.

170

171 ### *2.8. KEGG pathway analysis*

172 KEGG is a record base for the systematized analysis of genome functions that link
173 genomic and higher-order functional information. Pathway analysis was performed by
174 applying a hypergeometric test to each pathway in KEGG to identify pathways that were
175 evidently enriched in DEGs.

176

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177 ## 3. Results

178 ### *3.1. Detection of ABR thresholds in mice before and after noise exposure*

179 To compare the differences in hearing of mice before and after noise exposure, we
180 measured the ABR threshold of all mice before noise exposure, followed by noise exposure
181 immediately after in the experimental group, and then measured the ABR threshold of noise
182 in mice 48 h later; the data were then compared (Figure 1). According to the hearing results
183 after noise exposure, we screened out the R_NE and the S_NE. The susceptibility of
184 C57BL/6J (B6) mice to noise showed individual differences under the same noise exposure.

185

186 ### *3.2. Gene expression in mouse cochleae with different susceptibility to noise*

187 C57BL/6J (B6) mice showed different hearing loss under the same noise exposure. To
188 compare the differences in gene expression among the three groups (the R_NE, the S_NE,
189 and the Control), we performed principal component analysis of RNASeq data, estimated the
190 PC1 and PC2 values of each sample, and plotted the results (Figure 2). The gene expression
191 analysis of the three groups cluster together and converge into three parts, indicating that
192 there is a significant difference in gene expression between the R_NE, the S_NE, and the
193 Control.

194 Differential analysis was performed between the three groups; the differential genes
195 were plotted on a Wayne diagram (Figure 3). In total, there were 802 differential genes, 559
196 up-regulated and 243 down-regulated, in the S_NE compared to the Control. Further, there
197 were 2646 differential genes, 1576 up-regulated and 1070 down-regulated, in the R_NE
198 compared to the Control. These three groups shared 529 common differential genes. These

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199 529 differential genes were all noise-induced variants and were significant within the R_SE
200 and the S_NE.

201 Next, we focused on the differential genes between the R_NE and the S_NE. A total of
202 695 differential genes were obtained from these two groups by sequence analysis, with 366
203 genes up-regulated and 329 genes down-regulated in the R_NE compared to the S_NE (Table
204 S1). The 695 genes were plotted in a heat map showing the significant differences in Figure
205 4.

206

207 ### *3.3. KEGG pathway analysis of DEGs*

208 The KEGG analysis of the 529 common differential genes in the three groups showed
209 that the top 20 enriched pathways (Figure 5) suggested that these genes play an important
210 role in focal adhesion, cytoskeleton, hormone synthesis, HIF-1, cellular matrix, viral infection,
211 and Rap1. In addition, these pathways are significant in noise resistance and noise sensitivity,
212 and it can be concluded that noise exposure leads to mutations in these 529 genes at the
213 transcriptional level and is associated with the above pathways.

214 The KEGG analysis of 695 differential genes in the R_NE and the S_NE showed that
215 these genes were significantly enriched in ribosome synthesis, apoptosis, NF-κB signaling
216 pathway, nucleic acid metabolism, and insulin resistance pathways in eukaryotes. We believe
217 that these pathways are significant in noise susceptibility and play a crucial part in the
218 individual differences in hearing loss in mice after the same noise exposure.

219

220 ### *3.4. PPI network of DEG protein products in the R_NE and S_NE*

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221 The 695 DEGs between the R_NE and the S_NEwere constructed into a PPI network
222 (Figure 7), indicating that the interactions of the proteins encoded by these genes are also
223 closely complex, and the regulation of these genes may be controlled by interactions with
224 other members. Analysis was performed using the MCODE plugin of Cytoscape, and the top
225 two most significant subnetworks were selected from the resulting subnetworks (Figure 8).
226 Blue represents that the expression level of this gene was down-regulated in the R_NE, and
227 red represents that the expression level of this gene was up-regulated in the S_NE. The
228 obtained genes in the subnetwork are ranked according to the magnitude of the P value; the
229 smaller the P value, the more meaningful the gene in the R_NE. Therefore, we focused on the
230 top three down-regulated genes, Nop2, Bysl, and Rrp9, and the top three up-regulated genes,
231 Spsb1, Fbxl20, and Fbxo31.
232

233 ## 4. Discussion

234 ### *4.1. Related DEGs for noise susceptibility*

235 In this experiment, mice with the same genetic background showed different hearing loss
236 due to noise exposure. Their genes were analyzed for variation arising at the transcriptional
237 level. We found that 695 genes were differentially expressed in the R_NE and the S_NE and
238 constructed a PPI network map (Figure 7) to screen the top two subnetworks (Figure 8) that
239 were related to noise susceptibility in mice. Nop2, Bysl, and Rrp9 were the down-regulated
240 genes in the R_NE, with P values in the top three in the subnetwork. Nop2 had the smallest P
241 value among the selected subnetworks, illustrating that down-regulation of this gene was
242 significantly associated with noise susceptibility. It has been demonstrated that the degree of

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243 methylation of NOP2 is associated with the dedifferentiation potential of postmitotic
244 supporting cells into otic stem cells. NOP2 may therefore play a role in regulating the
245 stemness of the organ of Corti (Waldhaus et al. 2012). We have reason to believe that NOP2
246 can affect cochlear susceptibility to noise; however, this needs to be confirmed in further
247 studies. In addition, Bysl and Rrp9 are not currently being studied in the field of otology.
248 However, some scholars have found that in liver cancer, loss of Bysl induces apoptosis (Wang
249 et al. 2009a). Therefore, we have inferred from our experimental results that down-regulation
250 of Bysl increases noise-induced apoptosis, resulting in cochlear sensitivity to noise; however,
251 this requires further verification. Rrp9 is less well studied; it is a U3 snoRNA-binding protein
252 consisting of a WD-repeat domain and an n-terminus region (Zhang et al. 2013). Rrp9 is
253 important in the processing of pre-rRNA (Du et al. 2021), but we are currently unable to
254 determine the effect of this gene on noise susceptibility.

255 Spsb1, Fbxl20, and Fbxo31 were up-regulated genes in the R_NE, with P values in the
256 top three in the subnetwork; hence, they are also significantly correlated with noise
257 susceptibility. Several studies have shown that up-regulation of Spsb1, Fbxl20, and Fbxo31
258 inhibits apoptosis in cancer (Feng et al. 2014; Kim et al. 2019; Liu et al. 2018; Manne et al.
259 2021; Qin and discovery 2014); hence, it is speculated that high expression of these three
260 genes may inhibit noise-induced apoptosis and thus resist noise. We aim to continue to
261 investigate whether up- or down-regulation of these genes protects or impairs hearing in
262 noisy environments in subsequent experiments.

263

264 ***4.2. Pathways involved in susceptibility to noise***

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265 We further investigated the function of these differential genes. The common differential
266 genes of the Control, the R_NE, and the S_NE were caused by noise exposure, and their
267 pathways were associated with noise-induced hearing loss. In the present study, the pathways
268 closely related to noise-induced hearing loss and of interest to us were the focal adhesion,
269 regulation of actin cytoskeleton, and HIF-1 signaling pathways. The involvement of DEGs
270 associated with noise-induced hearing loss in focal adhesion was mentioned in an article on
271 proteomics (Miao et al. 2021). In another study, it was shown that noise can increase the
272 expression of focal adhesion kinase, and in noise-exposed Corti organs, FAK p-Tyr577 can be
273 detected in the outer hair cell stereocilia in noise-damaged areas (Jamesdaniel et al. 2011). A
274 similar article also mentions focal adhesion, which highlights that nonerythroid spectrin alpha
275 II plays a key role in the morphology and auditory function of hair cell stereocilia by
276 modulating focal adhesion signaling (Yao et al. 2022). Stereocilia are actin-based protrusions
277 on auditory and vestibular sensory cells that are necessary for hearing and balance. They are
278 regulated by myosin motors, actin cross-linkers, and capping proteins (McGrath et al. 2017).
279 Studies on the relationship between regulation of the actin cytoskeleton and noise-induced
280 hearing loss are also ongoing. Previous reports have clearly described that F-actin cleavage
281 occurs in the hair cells of guinea pigs and cochleae of dragon cats after noise exposure (Hu et
282 al. 2002; Raphael and neurology 1992). Thus, it can be inferred that the signaling pathway of
283 regulation of actin cytoskeleton should maintain the morphology of hair cells, and that noise
284 will imbalance the pathway and lead to the degeneration of hair cells. If there are genes that
285 can protect this pathway from noise-induced destruction, the hair cells can be protected,
286 thereby protecting hearing.

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287 In humans, hypoxia is an influential causative element of inner ear disease, and the role
288 of HIF-1 in the regulation of oxygen homeostasis in the inner ear, such as regulation, energy
289 supply, cell proliferation, or death is of interest. Insufficient blood supply after noise exposure
290 leads to a decrease in the oxygen partial pressure, leaving the cochlea in a hypoxic state
291 (pathology 2009). Hypoxic environmental preconditioning prevents noise-induced hearing
292 loss in CBA/J and CBA/CAJ mice by upregulating HIF-1α in the organ of Corti (Gagnon et
293 al. 2007). Another study proposed the use of cobalt chloride treatment, which up-regulates
294 HIF-1α and protects hearing in noise-exposed mice (Chung et al. 2011). We suggest that the
295 HIF-1 signaling pathway, which activates the transcription of diverse genes that enable cells
296 to survive under hypoxic conditions, plays a crucial role in triggering protective metabolic
297 changes in response to hypoxia (pathology 2009), resists noise, maintains cochlear
298 homeostasis, and thus protects hearing.

299 The differential genes selected in the R_NE and the S_NE were due to different
300 susceptibility of the mouse cochlea to noise; therefore, the enrichment pathways were
301 associated with noise susceptibility. Among them, apoptosis and the NF-κB signaling
302 pathway deserve our attention. Numerous studies have now demonstrated that cochlear hair
303 cells can undergo apoptosis under noise exposure. Many drugs or methods have also been
304 found to inhibit hair cell apoptosis to protect hearing. Therefore, we suggest that noise
305 initiates the apoptosis pathway in hair cells, making the cochlea increasingly sensitive to
306 noise.

307 While the NF-κB signaling pathway may be a defense pathway, it has been demonstrated
308 that in the auditory system, the NF-κB signaling pathway can be activated by noise (Zhang et

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309 al. 2019a) and can prevent noise-induced hearing loss (Tahera et al. 2006). Investigations on
310 the use of photobiomodulation (PBM) for noise-induced hearing loss found that PBM can
311 activate NF-κB to protect the cochlea from oxidative stress and apoptosis (Tamura et al.
312 2016). In other studies, mice lacking the p50 subunit of NF-κB were found to have a higher
313 sensitivity to noise exposure (Lang et al. 2006). Therefore, there is no doubt that the NF-κB
314 signaling pathway can resist noise damage.

315 In this experiment, we found that ABR can be used to determine the presence of hearing
316 loss. Otoacoustic emission can also be performed to detect cochlear amplification function
317 and hair cell function integrity. In addition, there may have been asymmetric hearing loss, as
318 we only tested the hearing threshold of one ear. Further experiments could be performed in
319 the future to address these limitations.

320

321 **5. Conclusions**

322 This study revealed that mice with the same genetic background show different
323 susceptibility to noise, and transcriptome specific changes were apparent after noise exposure.
324 DEGs were found through variant analysis. Bioinformatic analysis revealed the functional
325 implication of these genes. Changes in the transcriptome of these genes may affect cochlear
326 susceptibility to noise. In the next step, the experimental results of this study should be
327 verified using knockout mice. Noise-induced hearing loss is an irreversible disease; hence, its
328 prevention is particularly important. Workers exposed to the same noise over the same
329 occupational years experience varying degrees of noise-induced hearing loss. We can use
330 gene therapy to prevent work-related injuries in these workers. As the society continues to

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331 evolve, people are becoming more susceptible to noise, which in turn damages their hearing.
332 This study can help with future genetic screening, predict individual susceptibility to noise,
333 and prevent noise-induced hearing loss using gene therapy.
334

335 **Data availability**

336 The authors affirm that all data necessary for confirming the conclusions of the article are
337 present within the article, figures, and tables. Supplementary material has been uploaded to
338 https://gsajournals.figshare.com/.
339

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345

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350 **Conflict of Interest**

351 The authors declare no relevant conflict of interests.
352

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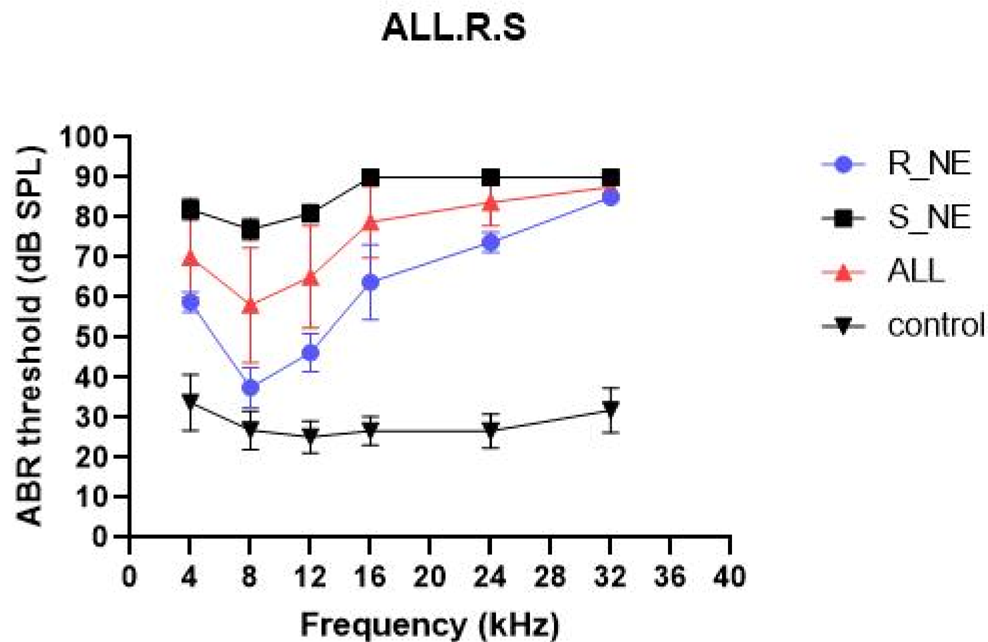
485

486 **Figure Captions**



487

488 Figure 1. Determination of auditory brainstem response threshold in the three groups of mice

489 The R_NE is the noise-resistant group, the S_NE is the noise-sensitive group, the NE is the

490 experimental group, and the control is the control group without noise.

491

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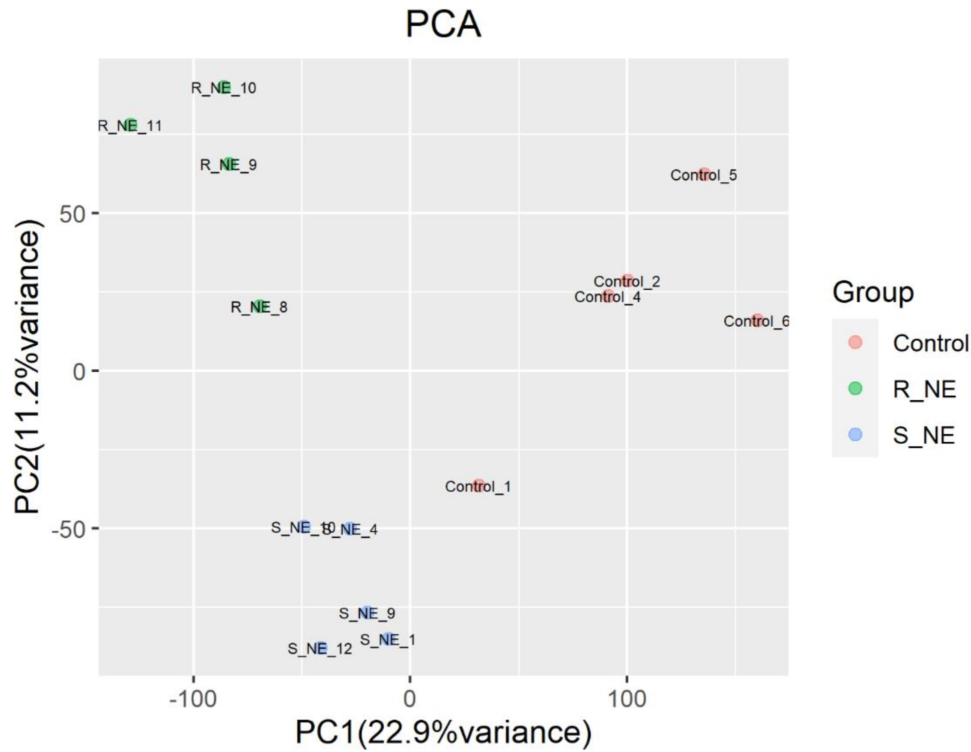

492

493 Figure 2. Principal component analysis

494 Red dots are the Control, green dots are the R_NE, and blue dots are the S_NE. The three

495 groups of samples are clustered into three parts, which indicates that there are significant

496 differences in gene expression in the cochlear samples.

497

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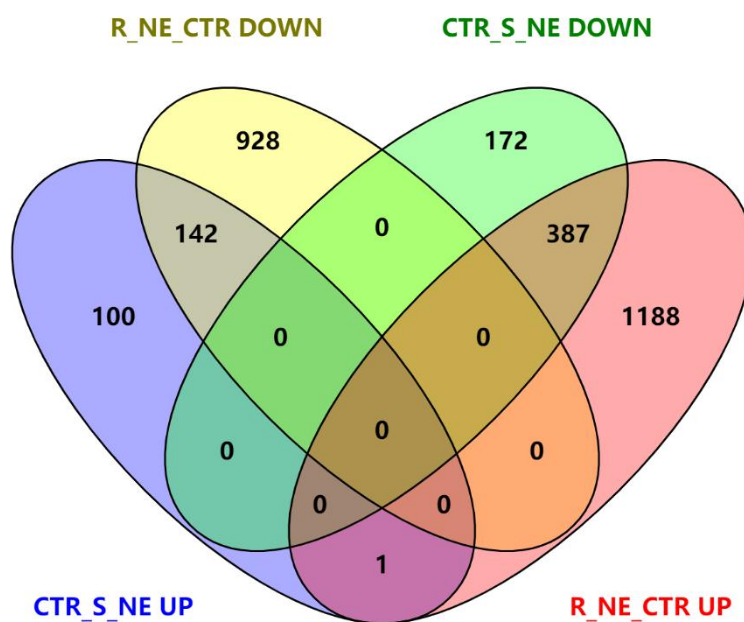

498

499 Figure 3. Wayne diagram

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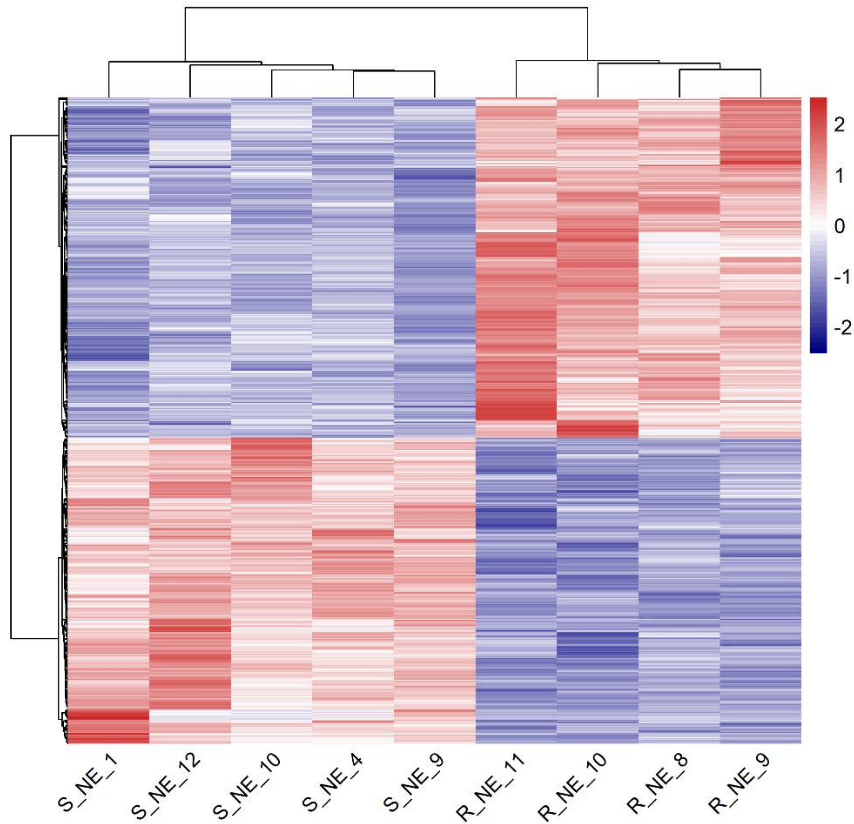

501

502 Figure 4. Heat map of differential genes in the R_NE and the S_NE. The red color represents

503 high correlation, and the blue color represents low correlation. It can be seen in the figure that

504 there are significant differences in differential genes between the R_NE and the S_NE.

505

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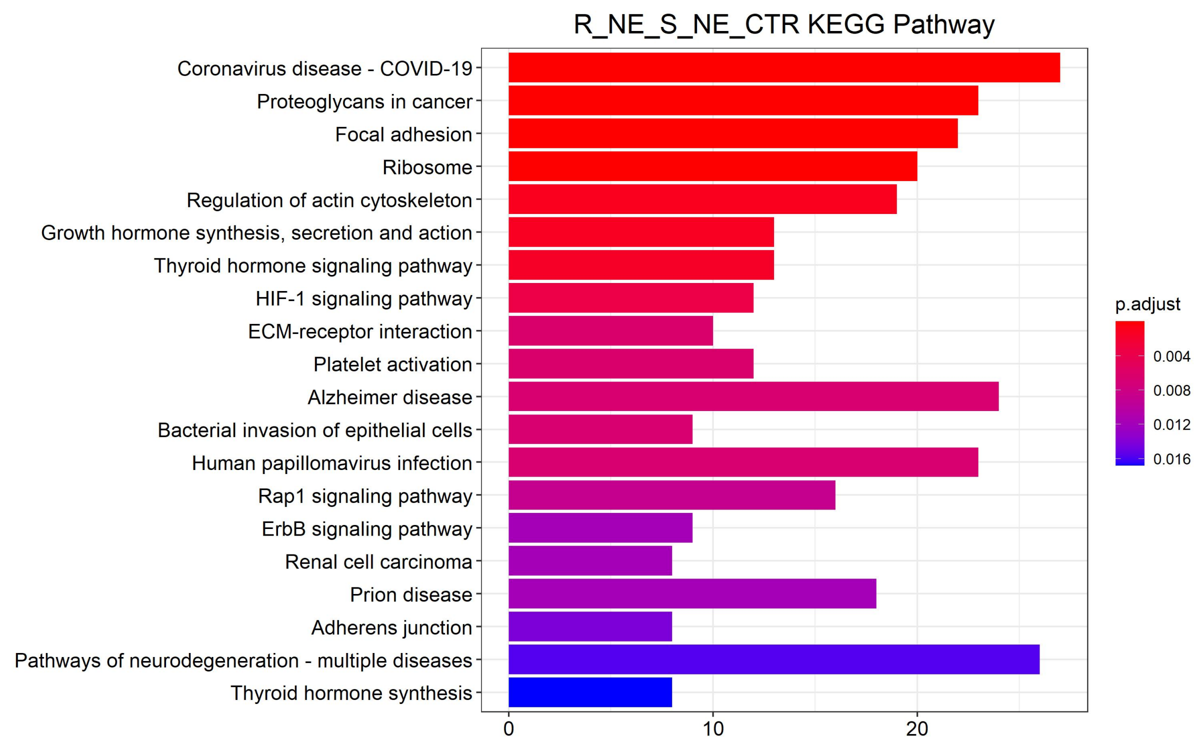
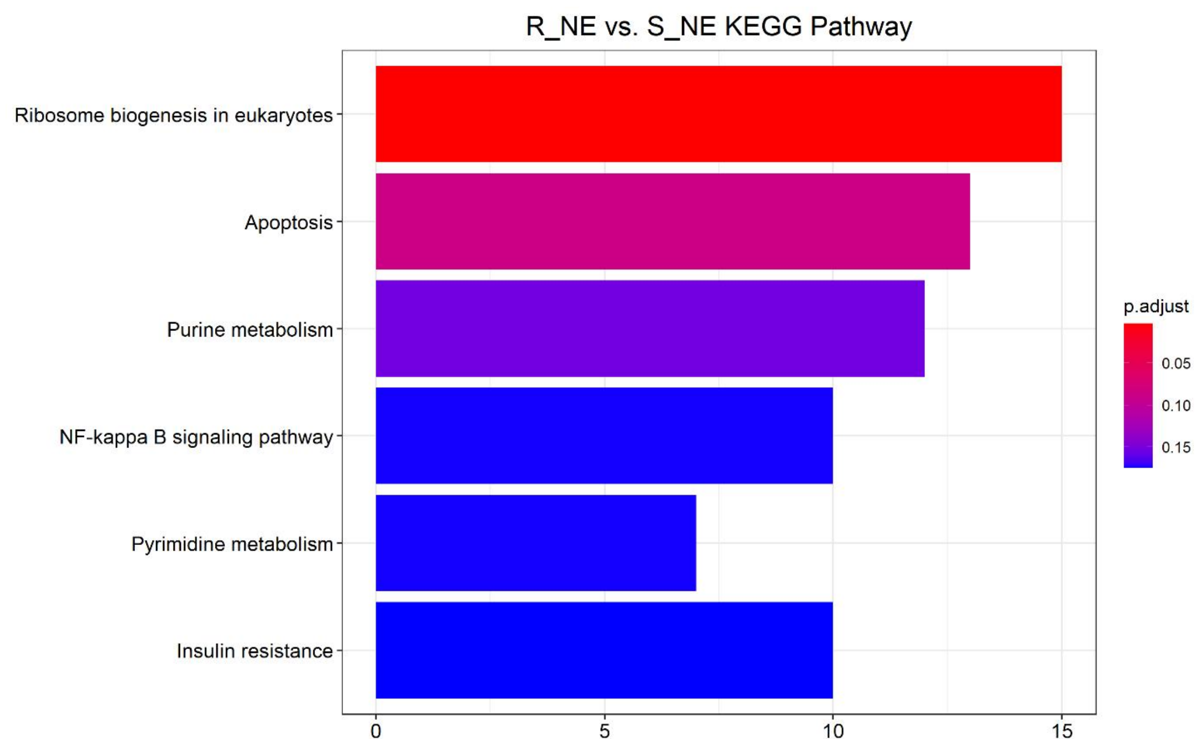


506

507 Figure 5. Enrichment analysis of common differential genes in the Control, the R_NE, and

508 the S_NE

509



510

511 Figure 6. Enrichment analysis of differential genes in the R_NE and the S_NE

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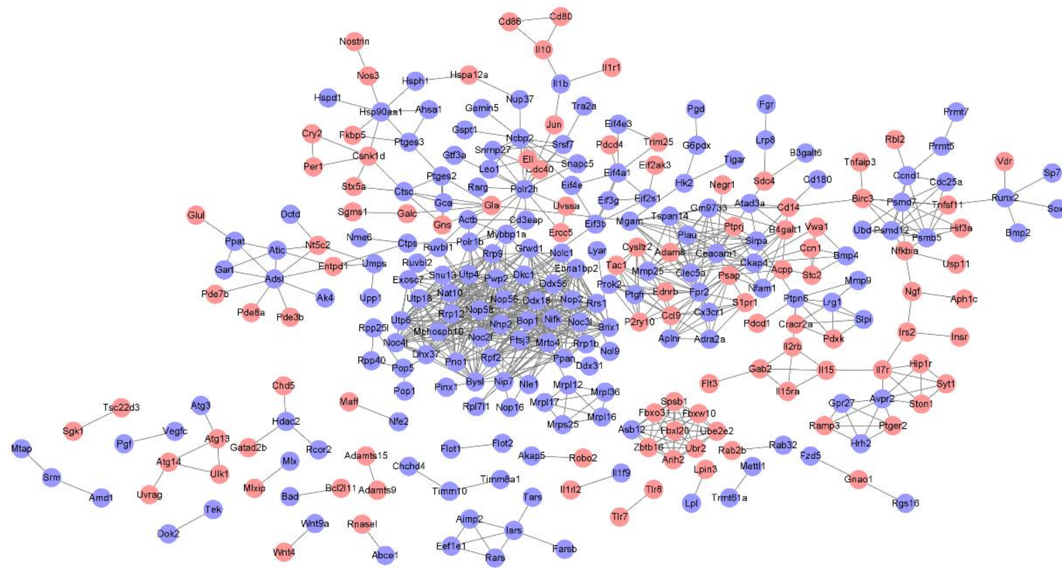


512

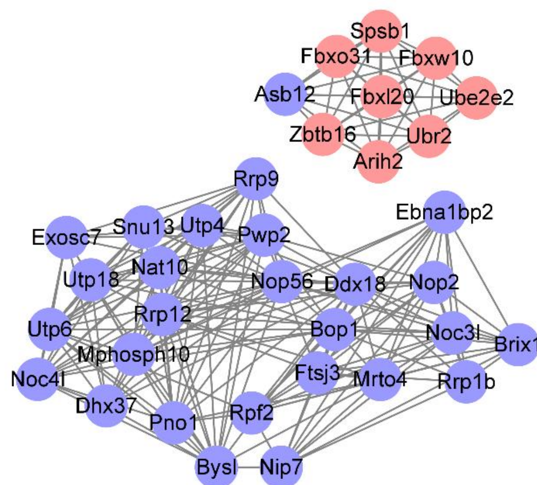
513 Figure 7. Protein-protein interaction network maps of differential genes in the R_NE and the

514 S_NE

515 Blue represents genes down-regulated in the R_NE, and red represents genes up-regulated in

516 the S_NE.

517



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519 Figure 8. Protein-protein interaction network maps

520 The analysis was performed using the MCODE plugin of Cytoscape, and the top two
521 subnetworks were selected from the resulting subnetworks (upper graph). Blue represents
522 genes down-regulated in noise resistance, and red represents genes up-regulated in noise
523 resistance.