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1 **Time course of activity-dependent changes in auditory nerve synapses reveals multiple underlying**
2 **cellular mechanisms**

3

4 Abbreviated title: Time course of synaptic changes at endbulbs

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26

27 **Abstract**

28 Abnormal levels of acoustic activity can result in hearing problems such as tinnitus and language
 29 processing disorders, but the underlying cellular and synaptic changes triggered by abnormal activity are
 30 not well understood. To address this issue, we studied the time course of activity-dependent changes
 31 that occur at auditory nerve synapses in mice of both sexes after noise exposure and conductive hearing
 32 loss. We found that EPSC amplitude and synaptic depression decreased within two days of noise
 33 exposure, through a decrease in the probability of vesicle release (P_r). This was followed by a gradual
 34 increase in EPSC amplitude, through a larger pool of releasable vesicles (N). Occlusion of the ear canal
 35 led to a rapid decrease in EPSC amplitude, through a decrease in N , which was followed by an increase in
 36 EPSC amplitude and synaptic depression through an increase in P_r . After returning to normal sound
 37 levels, synaptic depression recovered to control levels within 1 to 2 d. However, repeated exposure to
 38 noise for as little as 8 hr/d caused synaptic changes after 7 d, suggesting recovery did not fully offset
 39 changes. Thus, there appear to be three activity-dependent mechanisms in auditory nerve synapses:
 40 bidirectional changes in P_r in 1 to 2 d, slower bidirectional changes in N through synaptic growth or
 41 retraction, and rapid down-regulation of N with low activity. The dynamic changes indicate that multiple
 42 mechanisms are present to fine-tune synaptic fidelity across different acoustic conditions in a simple
 43 relay.

44

45 **Significance**

46 Hearing Impairments can arise from exposure to noise or conductive hearing loss. This appears to result
 47 from changes in the brain, but the mechanisms are not well understood. We study this issue by

48 studying the synapses made by auditory nerve fibers called endbulbs of Held. These synapses undergo
 49 bidirectional changes in size and release probability of neurotransmitters in response to increased or
 50 decreased activity. Here we made a close examination of how quickly these synaptic characteristics
 51 change, which indicates there are at least three cellular mechanisms underlying changes. Furthermore,
 52 repeated exposure to brief periods of noise can produce cumulative effects. These changes could
 53 significantly affect hearing, especially since they occur at the start of the central auditory pathway.

54 55 **Introduction**

56 Acoustic experience can influence the transmission of information throughout the auditory
 57 pathway (Rubio, 2020; Walmsley et al., 2006). Increased activity, such as resulting from exposure to loud
 58 noise, can lead to tinnitus (Shore and Wu, 2019). Decreased activity, such as occurs during otitis media,
 59 can result in language processing disorders (Holm and Kunze, 1969; Whitton and Polley, 2011). These
 60 hearing deficits are caused by changes in the central auditory pathway (Sanes and Bao, 2009). It is
 61 important to identify the cellular processes involved in activity-dependent synaptic changes to develop
 62 prevention and treatment strategies. It is also important to understand the time course of the effects,
 63 so that therapeutic interventions can be made before hearing is permanently affected.

64 Synaptic changes can come about through changes in the size of the readily releasable pool of
 65 vesicles (N), the probability of vesicle release (P_r), or the quantal size (Q). There is extensive evidence in
 66 multiple systems that N , P_r , and Q can change in response to activity *in vitro* (Castillo, 2012; Davis, 2006;
 67 Luscher et al., 2000; Turrigiano, 2012). These synaptic properties have also been shown to be modulated
 68 *in vivo* (Chen et al., 2011; Hengen et al., 2013; Keck et al., 2013; Knott et al., 2006). In the auditory
 69 system, noise exposure and conductive hearing loss can affect N , P_r , or Q (Grande et al., 2014; Mendoza
 70 Schulz et al., 2014; Mowery et al., 2019), but how these different synaptic properties change and how

71 quickly changes take place is not well understood. By studying the time course, it may provide
 72 information about how many cellular mechanisms are involved.

73 A useful synapse to study the effects of acoustic experience is the endbulb of Held, which is
 74 formed by auditory nerve fibers onto bushy cells (BCs) in the cochlear nucleus (Ryugo and Fekete, 1982;
 75 Ryugo and Sento, 1991; Spirou et al., 2005). BCs relay precise timing information to the superior olive
 76 (Grothe et al., 2010; Joris et al., 1998; Oertel, 1999), so changes in endbulb function may interfere with
 77 sound localization. Endbulbs are sensitive to levels of sound-driven activity, as exposure to non-
 78 traumatic levels of noise for a week results in a decrease in P_r and an increase in N (Ngodup et al., 2015).
 79 The opposite effects, increased P_r and decreased N , occur in genetic models of deafness (Mendoza
 80 Schulz et al., 2014; Oleskevich et al., 2004), or one week after a bilateral occlusion of the ear canal
 81 (Zhuang et al., 2017). Monaural deprivation leads to larger postsynaptic densities, increased AMPA
 82 receptor expression, and reduced VGLUT1 expression (Clarkson et al., 2016), and the overall effect on Q
 83 is unclear. Changes in P_r appear to result from changes in presynaptic calcium influx (Zhuang et al.,
 84 2020). A better understanding of the time-course of changes in synaptic properties could provide
 85 insight into the number and diversity of activity-dependent mechanisms at endbulbs.

86 We addressed this issue by assessing N , P_r , and Q at multiple time points following noise
 87 exposure and ear occlusion. We found bidirectional changes in P_r in response to increased or decreased
 88 activity with similar time-courses, probably reflecting a single process. By contrast, ear occlusion
 89 seemed to result in a rapid down-regulation in N , whereas noise exposure led to a slow increase in N ,
 90 suggesting they are initially mediated by different cellular mechanisms. We also found that these
 91 mechanisms could be triggered by brief, repeated noise exposure, suggesting they could have significant
 92 impact on hearing.

93

94 **Materials and Methods**

95 All experiments were performed with the approval of the University at Buffalo's Institutional
 96 Animal Care and Use Committee. Electrophysiology experiments used CBA/CaJ mice (Jackson Labs, stock
 97 #654). Immunohistochemistry experiments were performed on mice expressing YFP in a subset of
 98 auditory nerve fibers (see *Endbulb structure*, below). All experiments utilized mice of either sex ranging
 99 in age from postnatal day 20 (P20) to P31.

100

101 *Noise exposure or ear occlusion*

102 Mice were primarily housed in facilities with ambient sound levels measuring 21 to 38 dB SPL
 103 (average of 27 dB) over a frequency range 1 to 20 kHz as measured by a Larson-Davis 824 sound meter
 104 at 1/3 octave intervals. For noise exposure, mice were housed in a separate room, and a speaker (Fostex
 105 FT28D) was placed above the cage, and driven by a noise generator (Aco-Pacific 3025). Noise intensities
 106 in the cage measured 76 to 85 dB SPL (average of 81 dB SPL). Noise exposure was started at P21.

107 For mice undergoing occlusion, at P21 both ear canals were either surgically ligated or plugged
 108 with a silicone elastomer (Kwik-Cast, WPI). Mice undergoing ligation were anesthetized with 200 mg/kg
 109 ketamine plus 10 mg/kg xylazine. The skin was depilated with Nair, and treated with betadine. An
 110 incision was made ventral to the pinna to expose the ear canal, which was ligated with surgical silk
 111 (Harvard Apparatus). The incision was closed using suturing and Vetbond. Recovering mice were given
 112 carprofen (5 mg/kg) the following day, and were housed in normal acoustic conditions. Before ear-
 113 plugged mice were used for brain slice electrophysiology or immunohistochemistry, the efficacy of ear
 114 plugs was confirmed using auditory brainstem responses (ABRs). Mice with a < 15 dB shift in ABR
 115 threshold were excluded from further study.

116

117 *Auditory brainstem responses*

118 Mice were anesthetized with 200 mg/kg ketamine plus 10 mg/kg xylazine and placed on a 37°C
 119 heating pad (Gaymar) in a sound booth (Med Associates) lined with Sonex sound-attenuating foam
 120 (Acoustical Solutions). ABRs were recorded with a vertex electrode, an electrode inserted behind the
 121 pinna ipsilateral to the stimulated ear, and a ground electrode inserted contralateral to the stimulated
 122 ear. Clicks (100 μ s) were presented through a speaker (MF1, Tucker-Davis Technologies) placed 10 cm
 123 from the stimulated ear canal driven by a TDT ABR rig powered by a WS4 computer with an R26
 124 processor. ABR threshold was obtained by reducing the stimulus intensity from 90 to 20 dB SPL in steps
 125 of 10 dB, with 5 dB steps near threshold. ABR recordings were made and analyzed using BioSig software
 126 (Tucker-Davis Technologies Inc.).

127
 128 *Electrophysiology*

129 To prepare brain slices for electrophysiology, mice were anesthetized with 200 mg/kg ketamine
 130 plus 10 mg/kg xylazine, then perfused transcardially with ice-cold sucrose solution (in mM: 76 NaCl, 75
 131 sucrose, 25 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂). Then the brain was removed,
 132 and sagittal slices were cut using a Campden Integraslice 7550 MM or a Leica VT1200 (142 μ m), and
 133 incubated in standard recording solution (in mM: 125 NaCl, 26 NaHCO₃, 20 glucose, 2.5 KCl, 1.25
 134 NaH₂PO₄, 1 MgCl₂, 1.5 CaCl₂, 4 Na L-lactate, 2 Na-pyruvate, 0.4 Na L-ascorbate, bubbled with 95% O₂-5%
 135 CO₂) at 34°C for 20 minutes. Afterwards, slices were kept at room temperature until recordings. During
 136 recordings, 1 μ M strychnine was added to block spontaneous glycinergic IPSCs. Whole-cell voltage-
 137 clamp recordings were made from BCs in the anteroventral cochlear nucleus (AVCN) using borosilicate
 138 patch pipettes of resistance 1.3-2.3 M Ω . Pipettes were filled with internal solution containing (in mM):
 139 35 CsF, 100 CsCl, 10 EGTA, 10 HEPES, and 1 QX-314, pH 7.3, 300 mOsm. BCs were patched under an
 140 Olympus BX51WI microscope with a Multiclamp 700B (Molecular Devices) controlled by an ITC-18

141 interface (Instrutech), driven by custom-written software (mafPC) running in Igor (WaveMetrics). The
 142 bath was perfused at 3–4 ml/min using a pump (403U/VM2; Watson-Marlow), with standard recording
 143 solution running through an inline heater to maintain the temperature at 34°C (SH-27B with TC-324B
 144 controller; Warner Instruments). BCs were held at –70 mV with access resistance 5 to 15 MΩ
 145 compensated to 70%. Cells were identified as BCs by having EPSCs with fast decay kinetics ($\tau < 0.2$ ms)
 146 and half-widths (< 0.5 ms). Single auditory nerve fibers were stimulated using a glass microelectrode
 147 placed 30 to 50 μ m away from the soma with currents of 4 to 20 μ A through a stimulus isolator (WPI,
 148 A360). Single or paired pulses were applied every 8 s. For mean-variance analysis (Fig. 3), the
 149 concentration of CaCl_2 : MgCl_2 was modified to (in mM): 1 : 2.5, 1.5 : 1, 3 : 0.1, or 4.5 : 0.05.

151 *Endbulb structure*

152 Endbulb structure was assessed in transgenic mice expressing YFP in auditory nerve fibers.
 153 These mice were generated by crossing mice expressing Cre off a promotor for advillin (AVIL-Cre) (Zhou
 154 et al., 2010), with mice derived from Ai32 mice from Jackson Laboratories (strain 024109) that have a
 155 floxed construct of channelrhodopsin-EYFP. Both strains were individually back-crossed into the
 156 CBA/CaJ line for over 10 generations before crossing for experiments. In these mice (AVIL-YFP),
 157 expression of EYFP is in a subset of auditory nerve fibers, despite widespread expression of advillin in
 158 auditory nerve fibers (Hasegawa et al., 2007; Zhang-Hooks et al., 2016). AVIL-YFP mice were
 159 anesthetized with 200 mg/kg ketamine and 10 mg/kg xylazine, then perfused transcardially with 0.9%
 160 saline followed by 4% buffered paraformaldehyde. Brains were removed and postfixed (room
 161 temperature, 1 hr) before cryoprotecting in 20% sucrose (4°C). Sagittal sections were cut frozen at 25 to
 162 50 μ m thickness on a sliding microtome (American Optical) and washed in phosphate-buffered saline
 163 (0.9% NaCl, 0.2 M phosphate; PBS, pH = 7.4). Slices were permeabilized with 0.5% Triton X-100 in PBS
 164 (PBST) for 10 min, washed in PBS three times, blocked in 1% normal goat serum in PBST for 1 hr at room

165 temperature, and incubated overnight at 4°C in primary antibody solution containing anti-VGluT1
 166 (polyclonal guinea pig, 1:500, Synaptic Systems, #135304), and anti-GFP (polyclonal rabbit, 1:1000,
 167 Invitrogen, A-6455) in PBST. Specificity of the anti-VGluT1 antibody was validated by Synaptic Systems
 168 using western blot in VGluT1-knockout animals (see Synaptic Systems website). Specificity of the anti-
 169 GFP antibody was verified by the absence of auditory nerve labelling in animals that lacked the
 170 transgene in auditory nerve fibers (data not shown). The sections were then washed three times in PBS
 171 and incubated for 2 hr with secondary antibodies Alexa 488 goat anti-rabbit (1:250, Invitrogen, A-11008)
 172 and Alexa 594 goat anti-guinea pig (1:250, Invitrogen, A-11076).

173 Immunostained sections were imaged on a Leica TCS SP8 confocal microscope system installed
 174 on a Leica DMI8 inverted stand. Stacks of images were obtained at 0.3 μm interval, and imported into
 175 Wavemetrics Igor. Structures were traced with a custom-written program based on Reconstruct (Fiala,
 176 2005). Tracing was done blind to the acoustic experience of the animal.

177

178 *Experimental design and statistical analysis*

179 Average results are reported throughout as mean $\pm$ standard error of the mean (SEM) and the
 180 number of cells per group is indicated in the text. Because of the variability in endbulb properties, 15 to
 181 20 cells were needed for most measurements. Before statistical comparison, normality was evaluated
 182 for each dataset using Shapiro-Wilks tests. Most experiments assessed synaptic features at multiple
 183 time points. We first used ANOVA (parametric) or Kruskal-Wallis tests (non-parametric) to test for
 184 significant changes across all groups, and if significant, we tested the significance of individual time
 185 points with post-hoc tests. We did not detect changes with age in control animals, so control data were
 186 pooled for greater statistical power. For post-hoc tests comparing multiple experimental groups to a
 187 single control, we used the Tamhane-Dunnett (parametric) or Anderson-Darling many-to-one (non-
 188 parametric) tests as implemented in R (adManyOneTest and tamhaneDunnettTest in the PMCMRplus

189 package). When experimental groups were compared to each other, we used the Anderson-Darling all-
 190 pairs test (adAllPairsTest). Post-hoc tests used Holm's correction method for multiple comparisons. The
 191 significance level was set at $\alpha = 0.05$. Exact p values are shown in the corresponding text.

192

193 **Results**

194 *Synaptic changes after noise exposure and occlusion*

195 We wanted to determine how quickly noise-exposure or ear-occlusion induced changes in
 196 synaptic function. EPSCs were recorded from BCs in whole-cell voltage-clamp following electrical
 197 stimulation of single presynaptic auditory nerve synapses. To ensure the stimulation of a single synaptic
 198 input, we verified that EPSCs were all-or-none near threshold. We first evaluated the stability of synaptic
 199 properties between P20 and P31 (Fig. 1B). At these ages, mice are not yet sexually mature, but the
 200 properties of endbulbs appear largely stable (Pliss et al., 2009). We confirmed EPSC₁ stability first by
 201 performing linear regression which yielded a slope of 10 ± 202 pA/d (mean $\pm$ standard error of the
 202 mean, 146 cells, 39 mice), which was not significantly different from zero ($p > 0.5$, t test), indicating no
 203 change in EPSC₁ over these ages (Fig. 1B). Second, we assigned EPSCs to 5 age groups (P20–21: 7.7 ± 1.6
 204 nA, 22 cells, 5 mice; P22: 6.7 ± 1.5 nA, 16 cells, 6 mice; P23: 9.4 ± 1.9 nA, 10 cells, 3 mice; P24–26: $9.0 \pm$
 205 1.3 nA, 45 cells, 13 mice; P27–31: 7.4 ± 0.9 nA, 53 cells, 12 mice). We detected no significant difference
 206 among these groups (Kruskal-Wallis, $p = 0.8$). Therefore, we pooled control EPSC₁ data for subsequent
 207 comparisons (8.0 ± 0.6 nA; 146 cells).

208 We then manipulated auditory nerve activity by exposing mice to non-traumatic, broadband
 209 noise, or by bilaterally ligating the ear canal beginning at P21, and tracked changes in EPSC₁ amplitude.
 210 Following noise exposure, EPSC₁ appeared to decrease between 0.75 to 4 d compared to control (0.25 d:
 211 7.8 ± 1.7 nA, 16 cells, 7 mice; 0.5 d: 7.1 ± 0.8 nA, 45 cells, 6 mice; 0.75 d: 4.7 ± 0.7 nA, 13 cells, 3 mice; 1
 212 d: 6.0 ± 0.6 nA, 42 cells, 6 mice; 2 d: 4.7 ± 0.6 nA, 19 cells, 4 mice; 3–5 d: 5.5 ± 1.1 nA, 23 cells, 9 mice; 6–

213 10 d: 9.4 ± 1.1 nA, 37 cells, 15 mice) (Fig. 1C, red symbols). EPSC₁ amplitude also appeared to decrease
 214 below control between 2–5 d following ear ligation (1 d: 6.8 ± 0.8 nA, 36 cells, 7 mice; 2 d: 5.9 ± 0.8 nA,
 215 23 cells, 9 mice; 3–5 d: 4.3 ± 0.6 nA, 30 cells, 8 mice; 6–10 d: 8.8 ± 1.1 nA, 35 cells, 9 mice) (Fig. 1C, blue
 216 circles).

217 We compared the noise-exposed and ear-ligated groups against control and found there was a
 218 significant difference (Kruskal-Wallis, $p < 0.001$). Post-hoc comparisons indicated only one condition
 219 that differed significantly from control, at 3–5 d following ligation ($p = 0.01$ Anderson-Darling many-to-
 220 one test). We were concerned that the highly conservative corrections for multiple comparisons
 221 coupled with the large variance in EPSC₁ amplitude might obscure real changes following noise exposure
 222 or ear ligation. We had found previously that N increases and P_r decreases following noise exposure,
 223 while N decreases and P_r increases following ear ligation, with no detectable changes in Q (Ngodup et
 224 al., 2015; Zhuang et al., 2017). We were interested to determine if N and P_r might change with different
 225 time courses, so we analyzed the noise-exposure and ear-ligated data using regression with two linear
 226 segments:

$$EPSC(t) = \begin{cases} A + B_1 t, & t < t_0 \\ A + B_2(t - t_0) + B_1 t_0, & t \geq t_0 \end{cases}$$

227 where A is the amplitude of EPSC₁ before the manipulation (i.e. 8.0 nA), B_1 and B_2 are the rates with
 228 which two processes affect EPSC₁ amplitude, with each process dominating before or after time t_0 . For
 229 EPSCs after noise exposure, the fit yielded $t_0 = 1.5 \pm 0.5$ d, $B_1 = -2.3$ nA/d and $B_2 = 0.5 \pm 0.1$ nA/d, with
 230 both B_1 and B_2 highly significantly different from zero ($p < 0.001$, t test, 195 cells, red line in Fig. 1C). This
 231 indicates that EPSC₁ amplitude decreases rapidly immediately after noise exposure, and returns to
 232 control levels more slowly after several days. For ear ligation, the fit yielded $t_0 = 3.8 \pm 0.7$ d, $B_1 = -1.1 \pm$
 233 0.4 nA/d and $B_2 = 1.4 \pm 0.4$ nA/d, with both B_1 and B_2 highly significantly different from zero ($p < 0.01$, t
 234 test, 124 cells, blue line in Fig. 1C). This indicates that EPSC amplitude also decreases following ear
 235 ligation, but more slowly, and then returns to control levels beginning around 4 days.

236 To understand the cellular mechanisms underlying these processes, we wanted to specifically
 237 characterize changes in N , P_r , and Q . We assessed P_r by eliciting pairs of EPSCs with an interval of 3 ms,
 238 and calculating the ratio of the EPSC amplitudes (paired-pulse ratio, $PPR = EPSC_2/EPSC_1$) (Fig. 1A). PPR is
 239 sensitive to changes in P_r , where an increase in P_r is reflected as a lower PPR, and a decrease in P_r is
 240 reflected as a higher PPR. We first examined the stability of PPR in control mice. Linear regression of
 241 PPR from P20 to P31 had a slope of -0.0026 ± 0.0044 /d, which was not significantly different from zero
 242 ($p = 0.56$, 146 cells, 39 mice), indicating no change in PPR over these ages (Fig. 1D). Furthermore, there
 243 were no significant changes in average PPR across the age groups (P20–21: 0.56 ± 0.04 , 22 cells, 5 mice;
 244 P22: 0.53 ± 0.04 , 16 cells, 6 mice; P23: 0.58 ± 0.04 , 10 cells, 3 mice; P24–26: 0.48 ± 0.02 , 45 cells, 13
 245 mice; P27–31: 0.54 ± 0.02 , 53 cells, 12 mice; ANOVA, $p = 0.13$) (Fig. 1D). Therefore, control PPR was
 246 pooled across all age groups in subsequent analyses (0.53 ± 0.01 , 146 cells).

247 Next, we recorded PPR from mice exposed to noise or with ear ligation for up to 13 d (Fig. 1A, E,
 248 red and blue markers). We fit each set of data to an exponential function: $f(t) = y_{start} + (y_{end} - y_{start})(1 -$
 249 $e^{-t/\tau})$, where y_{start} is the PPR before manipulation (i.e. 0.53), y_{end} is the ending PPR, and τ is the time
 250 constant. Following noise exposure, the PPR increased with $\tau = 1.07 \pm 0.58$ d and $y_{end} = 0.68 \pm 0.03$ (160
 251 cells, Fig. 1E, red line), and following ear ligation, PPR decreased with $\tau = 1.97 \pm 1.12$ d and $y_{end} = 0.40 \pm$
 252 0.03 (124 cells, Fig. 1E, blue line). Analysis with ANOVA indicated that PPR differed significantly
 253 following noise exposure and ear ligation ($p < 0.001$). Post-hoc analysis identified the specific days
 254 showing significant changes. Following noise exposure, PPR increased significantly beginning at 1 d and
 255 reached a plateau thereafter (1 d: 0.65 ± 0.03 , 33 cells, 7 mice; 2 d: 0.63 ± 0.03 , 19 cells, 3 mice; 3 to 5 d:
 256 0.68 ± 0.05 , 23 cells, 8 mice; 6 to 13 d: 0.70 ± 0.05 , 22 cells, 9 mice; $p < 0.05$ each group, Tamhane-
 257 Dunnett's test). By contrast, following ear ligation, PPR decreased significantly after 2 d and reached a
 258 plateau thereafter (2 d: 0.45 ± 0.02 , 23 cells, 4 mice; 3–5 d: 0.38 ± 0.02 , 30 cells, 7 mice; 6–10 d: $0.42 \pm$
 259 0.02 , 35 cells, 5 mice; $p < 0.03$ each group, Tamhane-Dunnett test).

260 Thus, noise exposure leads to a decrease in P_r within 1 d, consistent with the initial decrease in
 261 EPSC₁. Because P_r remains low, it does not appear likely to explain the subsequent return of EPSC₁ to
 262 control levels, indicating that other synaptic changes must be taking place, such as increases in Q or N .
 263 Even more strikingly, ear ligation leads to an increase in P_r within 2 d, but EPSC₁ decreases at that time,
 264 suggesting the increase in P_r is more than offset by other synaptic changes, such as decreases in Q or N .
 265 The increase in P_r could contribute to the slower increase in EPSC₁ observed 4 d after ligation.

266

267 *Quantal size and frequency*

268 We next assessed changes in quantal size (Q) by recording spontaneous mEPSC amplitude and
 269 frequency (Fig. 2A). Previous studies had found no significant changes in mEPSC amplitude or frequency
 270 after one week of noise exposure or ear ligation (Ngodup et al., 2015; Zhuang et al., 2017), and we
 271 wanted to evaluate whether changes take place on a faster time scale that could contribute to the
 272 changes in EPSC₁ we observed. We first examined mEPSC frequency and amplitude between P20 to P30
 273 in control endbulbs, and both appeared stable over this age range. Linear regression of mEPSC
 274 amplitude had slope of -1.2 ± 1.6 pA/d, and mEPSC frequency had slope of -0.09 ± 0.28 s⁻¹/d, neither of
 275 which differed significantly from zero (64 cells, 13 mice, both $p > 0.4$, t test, Fig. 2B, C). In addition, we
 276 grouped mEPSC amplitudes and frequencies according to age ranges P20–22, P24–26, and P28–30, and
 277 no significant differences were detected between the groups (P20–22: amplitude 134 ± 7 pA, frequency
 278 6.1 ± 1.7 s⁻¹, 19 cells, 4 mice; P24–26: 125 ± 8 pA, 7.1 ± 1.5 s⁻¹, 27 cells, 5 mice; P28–30: 132 ± 9 pA, $5.3 \pm$
 279 0.8 s⁻¹, 18 cells, 4 mice; Kruskal-Wallis test, $p = 0.58$ for both mEPSC amplitude and frequency) (Fig. 2B,
 280 C). Therefore, we pooled control measurements across all ages (amplitude 130 ± 5 pA; frequency $6.3 \pm$
 281 0.8 s⁻¹).

282 We next examined mEPSCs in bushy cells of mice following noise exposure or ear ligation. There
 283 were no significant changes in mEPSC amplitude at any time point following the onset of noise or after

284 ear ligation (red and blue markers, Fig. 2D, both $p > 0.15$, Kruskal-Wallis test). Thus, the effects of noise
 285 exposure or ear ligation on Q are minor, and seem unlikely to contribute to the changes in PPR or EPSC₁
 286 observed in Fig. 1.

287 We also investigated changes in mEPSC frequency, which may be influenced by changes in N and
 288 P_r (Burrone et al., 2002; Prange and Murphy, 1999) which might change with different time courses.
 289 There were no significant changes in mEPSC frequency after noise exposure for any of the age groups (p
 290 $= 0.53$, Kruskal-Wallis). We also fit mEPSC frequency data using segmented regression (see equation
 291 above), constraining A to the average rate of controls (6.3 s^{-1}). For noise exposure, the fit yielded $t_0 =$
 292 $0.49 \pm 0.77 \text{ d}$, and $B_1 = -3.0 \pm 4.6 \text{ s}^{-1}/\text{d}$, and $B_2 = 0.05 \pm 0.15 \text{ s}^{-1}/\text{d}$. Neither slope was significantly
 293 different from zero ($p > 0.5$ both slopes, t test, 141 cells, 16 mice, red line, Fig. 2E), indicating negligible
 294 effects of noise exposure on mEPSC frequency.

295 By contrast, mEPSC frequency changed significantly following ear-ligation ($p = 0.02$, Kruskal-
 296 Wallis). The mEPSC frequency appeared to decrease between 1 and 4 d following ligation, and post-hoc
 297 analysis revealed a significant decrease at 2 d (1 d: $4.3 \pm 0.4 \text{ s}^{-1}$, 42 cells, 7 mice, $p = 0.79$; 2 d: $3.4 \pm 0.4 \text{ s}^{-1}$,
 298 35 cells, 9 mice, $p = 0.04$; 4 d: $3.3 \pm 0.4 \text{ s}^{-1}$, 31 cells, 11 mice, $p = 0.06$; 8 d: $5.6 \pm 1.1 \text{ s}^{-1}$, 33 cells, 10
 299 mice, $p = 0.79$; Anderson-Darling many-to-one test) (Fig. 2E). Segmented regression yielded $t_0 = 1.7 \pm$
 300 0.5 d , $B_1 = -2.0 \pm 0.6 \text{ s}^{-1}/\text{d}$, and $B_2 = 0.4 \pm 0.2 \text{ s}^{-1}/\text{d}$, and both slopes differed significantly from zero (both
 301 $p < 0.01$, blue line, Fig. 2E). This suggests that after ligation, one process drives a rapid decrease in
 302 mEPSC frequency, followed by a slower process by which mEPSC frequency increases beginning around
 303 2 d. P_r increases slowly during this period (i.e. PPR decreases, Fig. 1E), so the initial decrease in mEPSC
 304 frequency most likely reflects a rapid decrease in the number of functional release sites, consistent with
 305 the decrease in EPSC₁ observed shortly after ear ligation.

306

307 *Mean-variance analysis*

308 To explicitly quantify the effects of sound exposure on N , P_r , and Q , we used mean-variance
 309 analysis. The relationship between the mean (μ) and variance (σ^2) of EPSC₁ amplitude can be described
 310 by the equation $\sigma^2 = Q\mu - (\mu^2/N)$, when the extracellular calcium concentration (Ca_e) is varied to
 311 influence P_r (Foster and Regehr, 2004; Meyer et al., 2001; Silver, 2003). Recording durations permitted
 312 three Ca_e during each experiment, either 1, 1.5, and 3 mM or 1.5, 3, and 4 mM. Assessing N and P_r in the
 313 context of individual vesicles would require application of a low-affinity antagonist, to prevent receptor
 314 saturation and desensitization (Xu-Friedman and Regehr, 2004). However, in preliminary experiments
 315 with 2 mM kynurenate, we could not raise P_r high enough to constrain the fit. Therefore, we did not use
 316 a low-affinity antagonist in the experiments presented here, so the values of N , P_r , and Q do not simply
 317 reflect vesicle properties. Figure 3A shows an example recording of a BC from a control animal. Fitting to
 318 the equation above yielded $N = 63.6$, and $Q = 185$ pA. Using the values of Q and N , we calculated release
 319 probability ($P_r = \text{EPSC}_1/QN$). For the example in Figure 3A, P_r in 1.5 Ca_e was 0.22. On average, control
 320 synapses had N of 73 ± 9 , Q of 182 ± 18 pA, and P_r of 0.48 ± 0.03 in 1.5 Ca_e (33 cells, 13 mice).

321 We next examined the effects of noise exposure (Fig. 3B–D) and ear ligation (Fig. 3E–G) on N , P_r ,
 322 and Q . Comparing all groups simultaneously showed there was a significant change in N ($p < 0.001$,
 323 Kruskal-Wallis test). Post-hoc analysis indicated there was no significant change in N after noise
 324 exposure for 1 d (75 ± 12 , 13 cells, 3 mice, $p = 0.68$, Anderson-Darling many-to-one test; Fig. 3H), but
 325 there was a significant increase at 7 d (153 ± 28 , 16 cells, 7 mice, $p = 0.02$, Anderson-Darling many-to-
 326 one test; Fig. 3H). The effects at 7 d are similar to findings in a previous study (Ngodup et al., 2015).
 327 Thus, noise exposure leads an increase in N between day 1 and 7, which may contribute to the increase
 328 in EPSC₁ over this period (Fig. 1C).

329 We next considered the effects of ear ligation after 2 and 7 d with post-hoc analyses. We saw a
 330 significant decrease in N at both 2 and 7 d (2 d: 29 ± 7 , 16 cells, 4 mice, $p = 0.003$; 7 d: 43 ± 5 , 20 cells, 7

331 mice, $p = 0.04$; Anderson-Darling many-to-one test, Fig. 3H). The results at 7 d are similar to the
 332 findings of a previous study (Zhuang et al., 2017). Thus, N decreased by 2 d after ear ligation, which
 333 likely contributed to the initial decrease in EPSC₁ (Fig. 1C) and mEPSC frequency (Fig. 2E).

334 No significant changes were detected in either P_r or Q following noise exposure or ear ligation (p
 335 > 0.50 , Kruskal-Wallis test). The lack of change in Q is consistent with previous studies (Ngodup et al.,
 336 2015; Zhuang et al., 2017) and the present measurements of mEPSC amplitude (Fig. 2D). However, the
 337 lack of change in P_r is unexpected, especially considering the robust changes in PPR in Fig. 1E. One
 338 possible reason for this difference is that the value of P_r in mean-variance analysis is derived from EPSC₁
 339 at a single Ca_e , so quantifying P_r may be subject to more error than Q and N , that are produced by fitting
 340 over multiple data points. Overall, it seems most likely that after noise exposure, P_r decreases followed
 341 by a slower increase in N , whereas after ear ligation N initially decreases followed by an increase in P_r ,
 342 with neither manipulation causing a change in Q .

343

344 *Changes in endbulb structure*

345 The changes in N following noise exposure or ear ligation likely result from changes in the
 346 number of release sites. We wanted to see if there were corresponding anatomical changes in endbulb
 347 structure. Our strategy was to label endbulbs using an antibody against VGluT1 (Lauer et al., 2013),
 348 which reveals puncta surrounding BC somata (Fig. 4A, yellow). VGluT1 labels synaptic vesicles in
 349 endbulbs, so we considered VGluT1-positive puncta as likely functional synaptic regions of the endbulb.
 350 BCs in mice receive 2 to 5 endbulbs (Cao and Oertel, 2010; Chanda and Xu-Friedman, 2010b; Nicol and
 351 Walmsley, 2002; Wang and Manis, 2008). To aid reconstruction of individual endbulbs, we used a strain
 352 of mice expressing YFP in a subset of auditory nerve fibers (AVIL-YFP), such that one or two endbulbs
 353 were labelled per BC (Fig. 4A, cyan). To ensure that the expression of YFP did not affect the size of the
 354 synapse, we compared the areas of YFP-positive and -negative fibers, and found that they were not

355 significantly different (YFP-positive: $47.47 \pm 7.09 \mu\text{m}^3$, 23 endbulbs; YFP-negative: $39.78 \pm 5.60 \mu\text{m}^3$, 22
 356 endbulbs; 3 mice; $p = 0.40$, t-test). The expression of YFP did not appear to correlate with calretinin
 357 immunolabelling (data not shown), so it is probably unrelated to auditory nerve fiber subtype. We used
 358 the YFP labeling to aid in identifying puncta from the same endbulb. VGluT1-positive structures were
 359 traced and linked between optical sections (Fig. 4B). The areas of traces were used to calculate the
 360 volumes of synaptic regions of each endbulb.

361 We performed reconstructions of endbulbs from age-matched control, noise-exposed, and ear-
 362 ligated mice. Tracing was done blind to the manipulation. A Kruskal-Wallis test found that there was a
 363 significant difference between the groups ($p < 0.001$). Post-hoc analysis revealed that there was no
 364 significant change in VGluT1⁺ regions of endbulbs after 3 d of noise exposure compared to P24 controls
 365 (control: $38.4 \pm 5.2 \mu\text{m}^3$, 32 endbulbs, 3 mice; noise-exposed: $40.9 \pm 4.2 \mu\text{m}^3$, 48 endbulbs, 3 mice; $p >$
 366 0.50 , Anderson-Darling all-pairs test) (Fig. 4C). However, VGluT1⁺ regions were significantly larger in mice
 367 exposed to noise for 7 d compared to P28 controls (control: $43.0 \pm 4.4 \mu\text{m}^3$, 50 endbulbs, 3 mice; noise-
 368 exposed: $63.5 \pm 8.1 \mu\text{m}^3$, 54 endbulbs, 3 mice; $p = 0.02$, Anderson-Darling all-pairs test, Fig. 4C). For ear
 369 ligation, VGluT1⁺ regions of endbulbs were not significantly different after 2 d ($35.5 \pm 4.9 \mu\text{m}^3$, 24
 370 endbulbs, 3 mice, $p > 0.50$, Anderson-Darling all-pairs test), but were significantly smaller after 7 d of ear
 371 ligation ($21.5 \pm 1.7 \mu\text{m}^3$, 59 endbulbs, 3 mice, $p < 0.001$, Anderson-Darling all-pairs test, Fig. 4C). These
 372 data suggest that elevated or decreased acoustic activity causes morphological changes in the extent of
 373 the vesicle pool in the endbulb, not immediately, but after several days.

374 The slow increase in the extent of the vesicle pool following noise exposure could correspond to
 375 the gradual increase in EPSC₁ amplitude (Fig. 1C), and the increase in N in mean-variance analysis (Fig.
 376 3H). However, the slow decrease in vesicle pool area following ear ligation does not directly match the
 377 faster time course of the decrease in EPSC₁ (Fig. 1C), mEPSC frequency (Fig. 2E), or N in mean-variance

analysis (Fig. 3H). Thus ear ligation appears to trigger another mechanism that drives a decrease in N , distinct from large-scale changes in morphology.

Occluding with ear plugs

We wanted to study the rate of recovery of P_r after returning to normal sound conditions. We were concerned that repeated surgery and anesthesia associated with ligating and un-ligating the ear might obscure changes in P_r . Therefore, we evaluated the effectiveness of occlusion by plugging the ear canals with a fast-setting silicone, which could be applied and removed without anesthesia or surgery. We compared the effectiveness of plugging vs. ligating by comparing ABRs to click stimuli. A Kruskal-Wallis test showed that there was a significant difference in ABR threshold between groups ($p < 0.001$). Subsequent post-hoc tests indicated that ABR thresholds increased significantly from 35.5 ± 1.1 dB SPL (11 ears, 6 mice) to 57.5 ± 2.5 dB SPL after ear-plugging (8 ears, 4 mice, $p < 0.001$, Anderson-Darling all-pairs test), and to 69.5 ± 2.9 dB SPL after ear-ligating (21 ears, 11 mice, $p < 0.001$, Anderson-Darling all-pairs test) (Fig. 5A, B). Furthermore, while ear plugs were effective, they were significantly less effective at increasing ABR threshold than ligating ($p < 0.001$, Anderson-Darling all-pairs test).

We verified whether ear-plugging could induce synaptic changes, by quantifying changes in PPR. Ear-ligation and ear-plugging had broadly similar effects on PPR, and they were not significantly different at any of the comparable time points (ear-plugging 1 d: 0.53 ± 0.02 , 15 cells, 3 mice; 2 d: 0.43 ± 0.03 , 13 cells, 3 mice; 4 d: 0.41 ± 0.03 , 21 cells, 3 mice; 8 d: 0.42 ± 0.04 , 8 cells, 3 mice; all $p > 0.50$, t-tests comparing corresponding ear-ligation time points; Fig. 5C). Thus, the effects of ear-plugging and ligation seem similar, and may be useful for studying recovery from occlusion.

400 *Recovery of P_r*

401 We explored the recovery of PPR following a return to normal sound conditions after 3 or 10 d
 402 of noise exposure. A Kruskal-Wallis test indicated significant differences in PPR between the different
 403 exposure groups ($p = 0.001$). We then conducted post-hoc comparisons using the Anderson-Darling
 404 many-to-one test to identify specific differences. For 3 d of noise exposure, PPR remained significantly
 405 elevated by 6 hr in quiet conditions (0.76 ± 0.03 , 9 cells, 3 mice, $p < 0.001$), and was indistinguishable
 406 from control levels beginning at 12 hr (12 h: 0.57 ± 0.04 , 7 cells, 2 mice; 24 hr: 0.54 ± 0.06 , 12 cells, 3
 407 mice; 36 hr: 0.57 ± 0.05 , 15 cells, 4 mice; $p > 0.50$ each test; Fig. 6A, red circles). For mice exposed to
 408 noise for 10 d, a Kruskal-Wallis test also indicated significant differences between groups ($p = 0.001$).
 409 Post-hoc analysis using Anderson-Darling many-to-one tests showed that PPR remained significantly
 410 elevated after 12 hr in quiet conditions (0.78 ± 0.04 , 19 cells, 2 mice, $p < 0.001$), slightly elevated above
 411 control over the following 24 hr (1 d: 0.64 ± 0.06 , 14 cells, 2 mice, $p = 0.049$), and was indistinguishable
 412 from control after 1.5 d (0.61 ± 0.04 , 16 cells, 4 mice; $p = 0.13$; Fig. 6A, purple circles). This suggests that
 413 P_r recovers quickly, with the rate depending on the duration of noise exposure.

414 We also studied the recovery of PPR after ear-plugging for 3 d. The Kruskal-Wallis test indicated
 415 that there were significant differences between the treatment groups ($p = 0.009$). Post-hoc analysis
 416 using the Anderson-Darling many-to-one test indicated that PPR was significantly more depressed than
 417 control 6 hr and 12 hr after unplugging (6 hr: 0.40 ± 0.04 , 10 cells, 2 mice, $p = 0.04$; 12 hr: 0.36 ± 0.07 , 8
 418 cells, 2 mice, $p = 0.04$), and was indistinguishable from control by 1 d (0.50 ± 0.03 , 9 cells, 2 mice, $p =$
 419 0.45 ; Fig. 6B). Thus, P_r appears to recover on a similar time scale following occlusion or noise exposure.

420

421 *Repeated exposure*

422 We examined how periods of recovery between repeated bouts of noise exposure might affect
 423 auditory nerve synapses by exposing mice to noise for a number of hours per day for 7 d before slices

were cut to assess synaptic properties (Fig. 7A). The 0 hr/d condition (i.e. no noise exposure) had relatively depressed PPR after 7 d (0.53 ± 0.02 , 19 cells, 5 mice), very similar to the control value used throughout, while 24 hr/d is the same as constant noise for 7 d or more in Fig. 1. A Kruskal-Wallis test indicated significant differences between groups ($p < 0.001$). Subsequent post-hoc analysis using the Anderson-Darling many-to-one test indicated a significant increase in PPR after 7 d of noise exposure for 8 hr/d (0.65 ± 0.03 , 19 cells, 2 mice, $p = 0.02$), and 12 hr/d (0.71 ± 0.03 , 27 cells, 3 mice, $p < 0.001$) compared to 0 hr/d (Fig. 7B), but no increase in PPR after 4 hr/d (0.47 ± 0.04 , 15 cells, 2 mice). By contrast, EPSC₁ showed no significant changes for any duration of noise exposure (4 hr/d: 4.9 ± 0.9 nA; 8 hr/d: 7.1 ± 0.8 nA; 12 hr/d: 7.4 ± 0.9 nA; $p = 0.23$, Kruskal-Wallis). These results suggest that the effects of even brief noise exposure can accumulate, resulting in changes in P_r within a few days.

Discussion

We tracked how quickly changes in acoustic environment lead to physiological and anatomical changes in auditory nerve synapses, which revealed multiple activity-dependent processes (Fig. 8). PPR changes after 1 or 2 d of noise exposure or occlusion, suggesting activity levels act on a single mechanism to bidirectionally regulate release probability (P_r). Occlusion causes a decrease in EPSC amplitude and mEPSC frequency, most likely through a decrease in N , with changes in endbulb structure occurring after several days. This suggests that the rapid mechanism acts by disabling release sites, followed by slower structural remodeling. Noise exposure leads to a slow increase in N , with physiological and anatomical features changing together, presumably because adding release sites is more complex.

Recovery from noise or occlusion appeared to be very fast. That might suggest that noise exposure followed by equal time for recovery would produce little effect on synaptic function. However, repeated exposure to noise for as little as 8 hr/d induced changes in PPR. This may have

448 implications for people that are exposed daily to loud environments, despite having time for recovery.
 449 Furthermore, it suggests that the cellular mechanisms involved in detecting the activity levels retain
 450 some residual activation from one bout of noise exposure to the next.

451

452 *Changes in N and P_r , but not Q*

453 This study shows that for manipulations of both noise exposure and occlusion, endbulbs
 454 respond by changing the presynaptic properties of N and P_r . PPR results show most clearly that P_r is
 455 bidirectionally sensitive to activity, with changes becoming evident in 1 or 2 d. It is notable that the
 456 changes take longer to develop than presynaptic forms of long-term potentiation and depression (LTP
 457 and LTD) *in vitro* (Malenka and Bear, 2004), which appear otherwise similar. It is possible the cellular
 458 mechanisms are actually the same, but that acoustically-driven activity is less effective at triggering
 459 changes. Protocols for inducing LTP/LTD *in vitro* are very refined, and may be more powerful at
 460 recruiting induction mechanisms than acoustic stimuli *in vivo*. This may also explain the somewhat
 461 faster rate of change in P_r with noise exposure compared to occlusion (1.1 vs. 2.0 d), because the
 462 increase in activity with noise is likely to be substantial, while the drop-in activity with occlusion is likely
 463 to be smaller.

464 It is more difficult to evaluate changes in N than in P_r , so we used multiple lines of evidence. For
 465 noise-exposure, both mean-variance analysis and VGluT1 immunohistochemistry suggested that noise
 466 does not drive an increase in N within 1 d, but does after 7 d. Noise-driven changes in EPSC₁ amplitude
 467 are more complex. Shortly after the start of noise exposure, EPSC₁ amplitude decreases, most likely
 468 because of a decrease in P_r . Then, after ~1.5 days, EPSC₁ amplitude begins to increase, most likely from
 469 an increase in N (because Q does not change measurably).

470 The changes in N with occlusion are more complex. Mean-variance analysis and mEPSC
 471 frequency suggest N decreases within 2 to 4 days after occlusion, but structural changes in VGluT1

immunohistochemistry are not visible until 7 d. Remarkably, the overall effect is an initial decrease in EPSC₁, despite P_r increasing. Taken together, these results suggest that occlusion triggers a rapid down-regulation of N , with slower increase in P_r , followed by structural rearrangements that maintain a low N .

It is striking that the changes in N and P_r compensate almost perfectly for each other, yielding the same average EPSC₁ after 7 d. During noise exposure, first P_r decreases and then N increases, whereas after occlusion, N appears to decrease first followed by increasing P_r . This is surprising because EPSC₁ *in vitro* represents release from a fully rested and recovered synapse, which auditory nerve synapses are unlikely to be *in vivo*, especially in the presence of noise. Even so, recruiting additional release sites would also increase the amplitude of chronically-depressed EPSCs, thereby preserving the fidelity of the synapse. In addition, EPSC amplitude would also be expected to affect the timing of postsynaptic spikes, because postsynaptic latency and jitter are sensitive to EPSP amplitude (Chanda and Xu-Friedman, 2010a; Xu-Friedman and Regehr, 2004; Yang and Xu-Friedman, 2009). This is important, because the timing of BC spikes is used for sound localization (Oertel, 1999), so shifts in EPSC amplitude resulting from changes in activity levels during noise exposure or occlusion could disrupt interaural comparisons of sound level or timing. Adjusting N and P_r together may minimize such disruption.

Our methods of noise exposure and occlusion did not perturb quantal size (Q). This is striking, because changes in activity do affect Q in other systems both *in vitro* (Turrigiano et al., 1998; Wierenga et al., 2006) and *in vivo* (Hengen et al., 2013; Torrado Pacheco et al., 2021; Wu et al., 2021). Furthermore, changes in Q have been observed in the auditory cortex following occlusion *in vivo* (Mowery et al., 2019). Endbulbs from knockouts of the synaptic protein Bassoon have deeper depression and show increased Q (Mendoza Schulz et al., 2014). In addition, monaural occlusion leads to lower VGLUT1 density on endbulb vesicles and more AMPA receptors at BC postsynaptic densities (Clarkson et al., 2016), which could affect Q . An increase in Q would be consistent with the homeostatic effect of synaptic scaling. It is not clear why noise exposure or binaural occlusion do not also cause a

496 change in Q . Insight into this issue may arise with better understanding of the induction mechanisms
 497 driving changes in Q at endbulbs.

498

499 *Cellular mechanisms of changes in P_r and N*

500 Our results suggest that during noise exposure, P_r decreases before N increases, whereas during
 501 occlusion, N decreases before P_r increases. The changes in P_r appear to occur at roughly the same time
 502 following a change in acoustic conditions, suggesting increases and decreases share a common activity-
 503 dependent mechanism. Imaging experiments suggest that changes in P_r at the endbulb and calyx of
 504 Held result from modulation of presynaptic calcium influx (Fekete et al., 2019; Zhuang et al., 2020). The
 505 time course for changes in P_r was more than a day, which does not put strong constraints on the
 506 possible cellular mechanisms. Calcium influx could be modulated through phosphorylation of channels
 507 (Catterall and Few, 2008), which can occur much faster than one day. It is possible that *in vivo*, the
 508 signaling pathway is weakly activated, so phosphorylation could occur more slowly. Alternatively, the
 509 cellular mechanism may be intrinsically slow, such as requiring gene expression. Indeed, occlusion leads
 510 to a shift in calcium channel subtypes driving release at endbulbs (Zhuang et al., 2020), which could
 511 require changes in gene expression, although it is possible that different subtypes are already available
 512 in a reserve pool of channels.

513 Our results suggest two different mechanisms regulating N , one following occlusion that rapidly
 514 down-regulates release sites, and one following noise exposure that slowly upregulates release sites.
 515 The slow increase appears to parallel structural changes following noise exposure. It seems reasonable
 516 that structural changes and addition of new release sites could take a significant amount of time, for
 517 example if vesicles and proteins associated with neurotransmitter release must be generated *de novo*
 518 (Ataman et al., 2008). By contrast, the rapid decrease in N following occlusion could use fast
 519 mechanisms such as removing receptors from the postsynaptic density, as occurs in postsynaptic forms

of LTD (Luscher et al., 2000), producing a rapid decrease in EPSC₁ amplitude. It would be interesting to determine if the same release sites that are rapidly disabled after occlusion are then gradually removed by structural changes, or if there are larger reworkings of the synapse taking place.

Implications for hearing

Our findings represent a major change in our understanding of the function of auditory nerve synapses. Until recently, endbulbs were thought to be relatively stable synapses, simply relaying precise temporal information about sounds from the cochlea to the superior olive for sound localization. Now we understand that there are multiple processes regulating N and P_r based on activity. We have shown that these forms of long-term synaptic plasticity can be triggered through realistic exposure. Exposure to noise for 8 hr/d is sufficient to cause changes in synaptic function, despite even longer periods of recovery. Because endbulbs are a major conduit for auditory activity into the brain, changes in these synapses may have a significant effect on hearing.

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653

654 **Figure Legends**

655

656 **Figure 1.** Speed of activity-dependent changes in EPSC₁ amplitude and PPR at auditory nerve synapses.

657 **A**, Representative traces showing EPSC₁ and PPR for 3 ms interval in control, after noise exposure (12 hr,
658 18 hr, 1 d, 2 d, 7 d), and after ear ligation (1 d, 2 d, and 7 d). Manipulations were started at P21. **B**, EPSC₁
659 amplitude for control endbulbs over the ages studied. Small dots are EPSC₁ amplitude from 146 cells
660 from 39 mice, and open circles are averages for the age or exposure ranges demarcated by different
661 shading. There were no significant differences between groups ($p = 0.8$, Kruskal-Wallis test). Line is
662 regression of individual data. **C**, Effects of noise exposure (195 cells, 50 mice, red) and ear ligation (124
663 cells, 33 mice, blue) on EPSC₁ amplitude. Lines are fits to individual data using segmented regression
664 (see Results). A Kruskal-Wallis test indicated significant differences between exposure groups ($p >$
665 0.001), and asterisks mark points that were significantly different from control ($p < 0.05$, Anderson-
666 Darling many-to-one post-hoc test). **D**, PPR for control endbulbs over the ages studied. There were no
667 significant differences between age groups ($p = 0.13$, ANOVA). Line is regression of individual data. **E**,
668 Effects of noise exposure (red) or ear ligation (blue) on PPR. Lines are fits of individual data to the
669 exponential decay function (see Results). ANOVA indicated significant differences between groups ($p <$
670 0.001), and asterisks mark points that differed significantly from control ($p < 0.05$, Tamhane-Dunnett's
671 test). Markers throughout are mean $\pm$ SEM.

672

673 **Figure 2.** Changes in quantal size and frequency after noise exposure or ear ligation. **A**, Representative

674 traces of spontaneous synaptic activity for auditory nerve synapses from control (left, black), noise-
675 exposed (center, red), and ear-ligated (right, blue) mice. **B**, **C**, Amplitude (**B**) and frequency (**C**) of
676 mEPSCs in control mice. Small markers are individual experiments, and open circles are averages for age
677 groups demarcated by shading. Kruskal-Wallis tests showed no significant differences between groups in

678 mEPSC amplitude or frequency (both $p > 0.5$). Lines are regressions to individual data. **D, E**, Amplitude
 679 (**D**) and frequency (**E**) of mEPSCs after noise exposure (red) or ear ligation (blue). Small dots are
 680 individual experiments, and open circles are averages for the exposure durations demarcated by
 681 shading. Lines in **E** are fits to individual data using segmented regression (see Results). There were no
 682 significant effects of noise exposure or ear ligation on mEPSC amplitude (both $p > 0.15$, Kruskal-Wallis),
 683 nor mEPSC frequency after noise exposure ($p = 0.53$), but mEPSC frequency decreased significantly
 684 following ear ligation ($p = 0.02$), and asterisks mark points that differed significantly from control ($p <$
 685 0.05 , Anderson-Darling many-to-one test). Black line and grey shaded area represent pooled average $\pm$
 686 SEM from control cells.

687
 688 **Figure 3.** Activity-dependent changes assessed using mean-variance analysis. **A**, Representative
 689 experiment recording from a BC of a control animal. Markers are EPSC₁ amplitudes measured over the
 690 course of an experiment in 3 different external calcium concentrations (Ca_e ; 1, 1.5, or 3 mM). **B, C**,
 691 Representative experiments with BCs from animals noise-exposed for 1 d (**B**) or 7 d (**C**). **D**, Mean and
 692 variance from control experiment in **A** (closed black circles), noise-exposed for 1 d in **B** (open red
 693 circles), and noise-exposed for 7 d in **C** (closed red circles). Lines are fits to the mean-variance
 694 relationship (see Results) to estimate N , P_r , and Q . **E, F**, Representative recordings made from BCs in
 695 animals ear-ligated for 2 (**E**) or 7 d (**F**). **G**, Mean and variance from control experiment in **A** (closed black
 696 circles), ear-ligated 2 d in **E** (open blue circles), and ear-ligated 7 d in **F** (closed blue circles). Lines are fits
 697 to the mean-variance relationship (see Results). **H–J**. Histograms for N (**H**), Q (**I**), and P_r (**J**) for control,
 698 noise-exposed 1 or 7 d, and ear-ligated 2 or 7 d. Dots are individual data. Bars are mean $\pm$ SEM.
 699 Asterisks (*) denote significant difference from control ($p < 0.05$, Kruskal-Wallis followed by Anderson-
 700 Darling many-to-one test).

701

702 **Figure 4.** Structural changes in endbulbs after noise exposure and ear ligation. **A**, Optical section from
 703 the cochlear nucleus of an AVIL-YFP mouse immunostained for VGlut1. YFP labelling is shown in cyan,
 704 and VGlut1 in yellow. **B**, Traces of four different endbulbs (red, magenta, blue, black) around the BC in
 705 **A**. Optical sections were sampled every 0.3 μm , with every third section shown here. The tracings at
 706 the left of the third row (asterisk) correspond to the images in **A**. **C**, Synaptic volumes from P24 animals
 707 in control conditions, noise-exposed for 3 d, ear-ligated for 3 d, P27 control animals, noise-exposed for 7
 708 d, and ear-ligated for 7 d. Dots are individual synaptic volumes, and bars are averages $\pm$ SEM. Asterisks
 709 (*) denote significant difference from age-matched controls ($p < 0.05$, Kruskal-Wallis followed by
 710 Anderson-Darling all-pairs test).

711
 712 **Figure 5.** Efficacy of ear-plugging vs. ear-ligation. **A**. Representative ABRs from control, plugged, and
 713 ligated ears. Arrowheads mark threshold. **B**. ABR threshold for control, plugged, and ligated ears. **C**, PPR
 714 recorded from BCs in ear-plugged animals. Small triangles are PPRs from individual cells, and open red
 715 triangles are averages. Average PPRs for ear-ligated cells are duplicated from Fig. 1 for comparison
 716 (open blue circles). The black line and dark grey bar indicate control mean $\pm$ SEM. PPR after ear-plugging
 717 did not differ significantly from ear-ligating at any time point ($p > 0.5$, each comparison).

718
 719 **Figure 6.** Recovery of PPR after noise exposure and ear-plugging. **A**. PPR after 0, 0.25, 0.5, 1, or 1.5 d
 720 recovery in quiet conditions, following noise exposure for 3 d (red) or 10 d (purple). Dots are individual
 721 data, and open circles are averages. **B**. PPR after 3 d of ear-plugging. Black line and grey bar in **A** and **B**
 722 represent the control mean $\pm$ SEM. Asterisks (*) denote points significantly different from control ($p <$
 723 0.05, Kruskal-Wallis followed by Anderson-Darling many-to-one test).

724

725 **Figure 7.** Effects of periodic noise exposure. **A**, Treatment paradigm. Grey bars represent times that the
726 noise was on each day for a week. On day 7, slices were prepared for electrophysiology immediately
727 after ending noise exposure. **B**, **C**, PPR (**B**) and EPSC₁ amplitude (**C**) following 7 d of noise exposure for 0,
728 4, 8, 12, 24 hr/d (15 to 27 cells per point). Small dots indicate individual data, and open circles are
729 averages. Asterisks (*) denote PPRs significantly greater than 0 hr/d ($p < 0.05$, Kruskal-Wallis followed
730 by Anderson-Darling many-to-one test). EPSC₁ did not change significantly ($p = 0.23$, Kruskal-Wallis).

731

732 **Figure 8.** Graphical summary of synaptic changes. Diagram shows an auditory nerve fiber (blue)
733 contacting a bushy cell (black outline). Release sites are indicated as orange dots. Noise exposure leads
734 to decreased P_r (yellow), followed by structural changes and addition of release sites. Conductive
735 hearing loss (CHL) induces rapid inactivation of release sites (grey), followed by an increase in P_r (red),
736 and then by structural changes

Figure 1

Figure 2

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