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AMP A receptor inhibition by synaptically released zinc

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The vast amount of fast excitatory neurotransmission in the mammalian central nervous system is mediated by AMPA-subtype glutamate receptors (AMPA receptors). As a result, AMPA-mediated synaptic transmission is implicated in nearly all aspects of brain development, function, and plasticity. Despite the central role of AMPA receptors in neurobiology, the fine-tuning of synaptic AMPA responses by endogenous modulators remains poorly understood. Here we provide evidence that endogenous zinc, released by single presynaptic action potentials, inhibits synaptic AMPA currents in the dorsal cochlear nucleus (DCN) and hippocampus. Exposure to low sound reduces presynaptic zinc levels in the DCN and abolishes zinc inhibition, implicating zinc experience-dependent AMPA synaptic plasticity. Our results establish zinc as an activity-dependent, endogenous modulator of AMPA receptors that tunes fast excitatory neurotransmission and plasticity in glutamatergic synapses.

T he development, function, and experience-dependent plasticity of the mammalian brain depend on the refined neuronal interactions that occur in synapses. In the majority of excitatory synapses, the release of the neurotransmitter glutamate from presynaptic neurons opens transmembrane ion channels in postsynaptic neurons, the ionotropic glutamate receptors, thereby generating the flow of excitatory signaling in the brain. As a result, these receptors play a fundamental role in normal function and development of the brain, and they are also involved in many brain disorders (1).

The ionotropic glutamate receptor family consists of AMPA, kainate, and NMDA receptors (NMDARs). Although kainate receptor-mediated postsynaptic responses occur in a few central synapses (2), AMPA receptors (AMPARs) and NMDARs are localized in the postsynaptic density of the vast majority of glutamatergic synapses in the brain, mediating most of excitatory neurotransmission (1). NMDAR function is regulated by a wide spectrum of endogenous allosteric neuromodulators that fine-tune synaptic responses (3–5); however, much less is known about endogenous AMPAR neuromodulators [(1, 5), but see refs. 6 and 7]. Recent structural studies revealed that the amino terminal domain (ATD) and ligand-binding domain (LBD) are tightly packed in NMDARs but not AMPARs (8–10). These structural differences explain some of the functional differences in allosteric modulation between AMPARs and NMDARs, such as why the ATD of NMDARs, unlike that of AMPARs, modulates function and contains numerous binding sites for allosteric regulators. Nonetheless, given the importance of fine-tuning both synaptic AMPAR and NMDAR responses for brain function, it is puzzling that there is not much evidence for endogenous, extrasynaptic AMPAR modulation. The discovery and establishment of endogenous AMPAR modulators is crucial both for understanding ionotropic glutamate receptor signaling and for developing therapeutic agents for the treatment of AMPAR-related disorders, such as depression, cognitive dysfunctions associated with Alzheimer’s disease, and schizophrenia (1, 11).

Free, or readily chelatable, zinc is an endogenous modulator of synaptic and extrasynaptic NMDARs (12–15). Free zinc is stored in glutamatergic vesicles in many excitatory synapses in the cerebral cortex, limbic, and brainstem nuclei (16). In some brain areas, such as in the hippocampus, 50% of boutons synaptic onto CA1 neurons and all mossy fibers synaptic onto CA3 neurons contain synaptic free zinc (17). Whereas earlier studies demonstrated that exogenous zinc inhibits AMPARs (18–21), more recent work suggests that endogenously released synaptic zinc does not modulate AMPARs in central synapses (14, 22). This conclusion was derived from the inability to efficiently chelate and quantify synaptic zinc with the zinc-selective chelators and probes used (15), in apparent support of the hypothesis that zinc acts at low levels of released zinc during synaptic stimulation (14).

Recent work in our laboratories used new chemical tools that allowed us to intercept and visualize mobile zinc efficiently (15). These studies revealed modulation of extrasynaptic NMDARs by zinc and led us to reinvestigate whether synaptically released zinc might be an endogenous modulator of AMPARs as well. In the present study, we applied these same tools in electrophysiological, laser-based glutamate uncaging and in imaging experiments using wild type and genetically modified mice that lack synaptic zinc.

Results

ZnT3-Dependent Synaptic Zinc Inhibits AMPAR EPSCs in Dorsal Cochlear Nucleus Synapses. First, we explored the effect of synaptic zinc on AMPAR-mediated excitatory post synaptic currents (EPSCs) in the dorsal cochlear nucleus (DCN), a zinc-rich auditory brainstem nucleus. The DCN is a cerebellum-like structure (23), where glutamatergic parallel fibers (PFs) are zinc-rich (24) and innervate interneurons and principal neurons, cartwheel cells (CWCs), and fusiform cells (FCs), respectively. Bath application of 100 µM ZnX1, an extracellular fast, high-affinity zinc chelator (13, 15), potentiated cartwheel cell AMPAR EPSCs evoked by a single PF stimulus (PF EPSCs) (Fig. 1 A–C). This finding suggested, for the first time to our knowledge in a mammalian synapse, that endogenous zinc inhibits AMPAR EPSCs.

To determine whether the effects of synaptic zinc on PF EPSCs were mediated by presynaptic mechanisms, we used paired-pulse ratio (PPR) and coefficient of variation (CV) analysis, two

Significance

Ionotropic glutamate AMPA receptors (AMPARs) play a fundamental role in normal function and plasticity of the brain, and they are also involved in many brain disorders. Despite the central role of AMPARs in neurobiology, the modulation of synaptic AMPA responses by endogenous modulators remains not well understood. Here, in three synapses found in two different brain areas, we provide the first evidence, to our knowledge, that endogenous zinc is coreleased with glutamate and modulates the strength of synaptic AMPA responses. Because in many neocortical areas more than 50% of excitatory presynaptic terminals contain zinc within their glutamatergic vesicles, our findings establish zinc as a general neuromodulator that allows for fine-tuning and plasticity of glutamatergic fast synaptic transmission in the brain.


Conflict of interest statement: The authors declare no conflict of interest.

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Synaptic ZnT3-dependent zinc inhibits AMPAR EPSCs in DCN parallel fiber synapses via a postsynaptic mechanism. (A) Schematic of the experimental setup for electrophysiological experiments in cartwheel cells. In this figure, AMPAR EPSCs were recorded from cartwheel cells and evoked by parallel fiber stimulation (PF EPSCs). (B) Representative PF EPSCs before and after ZX1 application. (C) Time course of PF EPSC amplitude before and after ZX1 application (PF EPSC amplitude, 15–20 min after ZX1 application: 140.22 ± 7.66% of baseline, n = 7, P < 0.01). (D) Representative PF EPSCs in response to two stimuli 20 ms apart: before and after ZX1 application. (E) Summary graph of paired-pulse ratio (n = 7, P = 0.36 for control vs. ZX1). (F) Summary graph of normalized 1/CV (n = 7, P = 0.01 for second pulse vs. first pulse; n = 7, P = 0.95 for control first pulse vs. ZX1 first pulse). (G) Representative PF EPSCs in control, after tricine, and after tricine and ZX1 application. (H) Time course of PF AMPAR EPSC amplitude before, after tricine, and after tricine and ZX1 application (PF AMPAR amplitude: 15–20 min after tricine application: 100.07 ± 3.35% of baseline, n = 5, P = 0.97; 15–20 min before tricine and after ZX1 application: 146.51 ± 7.20% of baseline, n = 5, P < 0.01). (I) Representative PF EPSCs from ZnT3WT and ZnT3KO mice before and after ZX1 application. (J) Time course of PF AMPAR EPSC amplitude from ZnT3WT and ZnT3KO mice before and after ZX1 application (PF AMPAR amplitude, 15–20 min after ZX1 application: 100.07 ± 3.35% of baseline, n = 5, P < 0.01; ZnT3KO: 94.69 ± 6.85% of baseline, n = 5, P = 0.16; ZnT3WT vs. ZnT3KO: P < 0.01). Values represent mean ± SEM. For details of statistical tests and detailed values shown in main figures, see SI Materials and Methods.
of ZnT3-dependent vesicular zinc mediates the inhibition of PF EPSCs.

Evoked Action Potential Driven Release of Zinc from Presynaptic Terminals Mediates AMPAR EPSC Inhibition. ZX1 potentiation of PF EPSCs is consistent with the hypothesis that chelation of stimulus-driven, synaptically released zinc removes AMPAR inhibition by the metal ion. Alternatively, there might be a tonic level of ZnT3-dependent zinc, arising from spontaneous release of zinc from presynaptic vesicles, which inhibits AMPARs but is independent of synaptic stimulation. Low nanomolar tonic zinc levels in DCN brain slices inhibit extrasynaptic NMDARs and potentiate glycine receptors (15, 29). To determine whether tonic zinc modulates AMPAR currents, we used glutamate uncaging to activate AMPARs and bypass synaptic stimulation. When we uncaged glutamate onto the dendritic arbor of CWCs in the molecular layer (Fig. 2A), we evoked pharmacologically isolated AMPAR currents that were not potentiated by the addition of ZX1. This finding indicates that tonic zinc does not modulate AMPARs and is consistent with the nanomolar zinc affinity of NMDARs containing NR2A subunits (30) and glycine receptors containing the α1 subunit (31), compared with the lower zinc affinity of AMPARs (18, 19). Moreover, ZX1 application did not affect the amplitude, frequency, or kinetics of spontaneous EPSCs (sEPSCs; Fig. 2D–F), which are elicited by random, nonevoked firing of presynaptic granule cells, indicating that inhibition of PF EPSCs by zinc requires evoked, action potential-driven release of zinc from presynaptic vesicles.

Zinc Inhibition of AMPAR EPSCs Is Input Specific in DCN Synapses. The DCN is a laminar structure with layers that contain glutamatergic PF zinc-rich terminals synapsing onto different neurons, as well as layers that harbor zinc-lacking glutamatergic terminals. In particular, fusiform cells receive zinc-rich PF input at their apical dendrites in the molecular layer, and zinc-lacking auditory nerve (AN) input at their basal dendrites in the deep layer (Fig. 3E) (32). Moreover, fusiform cells express AMPARs containing GluA2-3 subunits in their apical dendrites, whereas cartwheel cells express GluA1-3 subunits (33). To determine whether zinc modulation of PF EPSCs is input-specific in fusiform cells and whether zinc modulates PF EPSCs in another synapse with different AMPAR composition, we took advantage of this anatomical and functional segregation of synaptic inputs in the DCN. First, we demonstrated that the DCN molecular layer is zinc-rich by using a cell-permeable, acetylated zinc fluorescent sensor diacetylated Zinpyr-1 (DA-ZP1) (34). Consistent with previous anatomical studies (32), our imaging experiments revealed a zinc-specific fluorescent signal that is ZnT3 dependent and specific to the molecular layer of the DCN (Fig. 3A). Next, we used an extracellular fluorescent sensor, ZP1-6COOH, to determine whether we could observe input-specific zinc release. We performed two-pathway imaging experiments in the same slice, which showed that PF stimulation in the molecular layer generated a fluorescent response, indicating zinc release, whereas stimulation of AN fibers in the deep layer did not generate any zinc fluorescent signal (Fig. 3B–D). To test for input-specific inhibition of PF EPSCs by zinc, we used two-pathway electrophysiological experiments to record PF EPSCs and AN EPSCs from the same fusiform cell (Fig. 3E and F). Note that PF EPSCs, but not AN EPSCs, showed paired-pulse facilitation, further confirming our ability to stimulate two independent, anatomically and functionally distinct inputs (Fig. 3F and Fig. S3A). Application of ZX1 potentiated only PF EPSCs without affecting PPR, but left AN EPSCs unaffected (Fig. 3G and H and Fig. S34). These results show that zinc-mediated modulation of AMPAR EPSCs in fusiform cells is input-specific, occurring only at glutamatergic synapses that contain zinc, and is mediated by postsynaptic mechanisms. Moreover, these results indicate that zinc modulates synapses with AMPARs containing GluA1-3 subunits.

Zinc Inhibition of AMPAR EPSCs in Hippocampal Synapses. To determine whether synaptic zinc-mediated inhibition of AMPAR EPSCs is a general modulatory mechanism of AMPAR neurotransmission across different zinc-containing synapses, we explored the effect of zinc on AMPAR EPSCs in the hippocampus. We stimulated zinc-rich Schaffer collateral fibers (SCs) (14) (Fig. 3J), and we recorded from hippocampal CA1 neurons, which express AMPARs containing GluA1-3 subunits (35). ZX1 potentiated CA1 SC EPSCs in ZnT3KO mice, but left CA1 SC EPSCs unaffected in ZnT3KO mice (Fig. 3J and K). Similar to the DCN, ZX1 did not affect PPR and CV of SC EPSCs in ZnT3WT mice (Fig. S3 B and C), indicating that synaptic zinc modulates CA1 SC EPSCs via postsynaptic mechanisms. Finally, PPR, CV, and kinetic properties of CA1 SC EPSCs were not different between ZnT3WT and ZnT3KO mice, suggesting that the lack of effect of ZX1 in SC EPSCs in ZnT3KO mice was due to the lack of synaptic zinc and not to differences in baseline synaptic transmission between ZnT3WT and ZnT3KO mice (Fig. S3 B–D and Table S1). Moreover, we investigated the effects of ZX1 on the presynaptic fiber volley and the size of the accompanied field EPSP (fEPSP) recorded in the stratum radiatum and evoked
Plasticity of AMPAR EPSCs by Sound-Evoked Reduction of Presynaptic Zinc Levels in DCN Synapses. The results depicted in Figs. 1–3 show that synaptic zinc is an endogenous neuromodulator that controls the strength of baseline synaptic responses in zinc-containing synapses. Potential plasticity of zinc levels would give zinc a dynamic role in shaping excitatory synaptic transmission in an activity-dependent manner and would add to the complexity of synaptic plasticity in mammalian synapses. In the neocortex, levels of synaptic zinc are rapidly and dynamically regulated. In particular, in the barrel cortex, increased sensory stimulation leads to decreased levels of synaptic zinc, whereas decreased sensory stimulation leads to increased synaptic zinc (36). These anatomical studies have established the experience-dependent modulation of synaptic zinc levels; however, the effect of this modulation on synaptic strength remains unknown. We examined the effect of auditory experience on zinc-mediated effects on PF EPSCs in fusiform neurons, which receive direct AN input in their basal dendrites (Fig. 3E). We examined mice that were exposed to sustained loud sound (see SI Materials and Methods for details), which caused hearing loss, as evidenced by increased threshold of auditory brainstem responses (ABRs) in noise-exposed mice (Fig. S4 A–D). We also studied sham-exposed mice, which underwent the same procedure but were not exposed to sound. As expected, ZX1 enhanced PF EPSCs in fusiform neurons from sham-exposed mice, but, strikingly, did not affect PF EPSCs in fusiform neurons from noise-exposed mice (Fig. 4A and B). Moreover, PPR, CV, and kinetic properties of PF EPSCs were not different between sham- and noise-exposed mice, suggesting that the lack of an effect of ZX1 on PF EPSCs in noise-exposed mice was not a result of changes in glutamatergic synaptic transmission (Fig. S4 E and F and Table S1). Quantal analysis on stimulus-evoked AN EPSCs from sham- and noise-exposed mice showed that CV was increased in noise-exposed mice but PPR was unaltered (Fig. S4 G and H). These results indicate decreased quantal content (n Pr) without changes in Pr in sound-exposed mice; such changes are consistent with a reduced number of release sites. Moreover, these results are consistent with reduced ABR thresholds (Fig. S4 A–D) and with previous studies showing damage of AN terminals even after milder acoustic trauma (37, 38).

We hypothesized that the lack of a ZX1 effect on PF EPSCs may be due to a decrease in zinc inhibition in PF EPSCs. Consistent with this hypothesis, we found that DCN slices from noise-exposed mice showed a significant decrease in synaptic zinc levels, by afferent stimulation also in the stratum radiatum. ZX1 increased synaptic strength, measured as increases in the slope of the EPSP, without affecting the amplitude of presynaptic fiber volley (Fig. S3 E and F). Because the amplitude of the fiber volley is proportional to the number of presynaptic fibers activated by the stimulus and thus serves as an estimate of the strength of an afferent input, we conclude that ZX1 increases synaptic strength but does not affect afferent input (Fig. S3 E and F). Next, we measured the effect of ZX1 on the spiking output of DCN granule cells, the cells from which zinc-rich parallel fibers originate. ZX1 did not affect either action potential threshold or the current-firing frequency (f-I) function in these neurons (Fig. S3 G and H), further suggesting that ZX1 does not affect the spiking output of presynaptic neurons. Taken together, our results suggest that zinc inhibition of AMPAR EPSCs is a general postsynaptic modulatory mechanism in zinc-containing synapses that express GluN1-3.

Fig. 3. Zinc-mediated inhibition of AMPAR EPSCs is input specific in DCN synapses and occurs in hippocampal synapses. (A, Left) Brightfield image of a DCN slice showing the molecular and deep layer of the DCN where parallel fiber (PF) and auditory nerve (AN) inputs reside, respectively. (Center) DA-ZP1, a cell-permeable fluorescent zinc sensor reveals zinc-mediated fluorescence in the molecular but not deep layer of a DCN slice from a WT mouse. (Right) Absence of DA-ZP1 fluorescence in a DCN slice from a ZnT3KO mouse. (B) Illustration of two-pathway imaging experiments with stimulating electrodes placed in the molecular and deep layer of the DCN. (C) In response to a 100-Hz, 1-s stimulation in the molecular layer, ZP1-6COOH, a membrane-impermeable fluorescent zinc sensor reveals evoked zinc signals in the molecular but not in the deep layer of the DCN. No fluorescence is evoked by identical electrical stimulation in the deep layer. (D) Representative ZP1-6COOH fluorescent responses in response to a 100-Hz, 1-s electrical stimulation. (E) Schematic of the experimental setup for two-pathway electrophysiological experiments in fusiform cells. (F) Representative traces from two-pathway experiment showing, in response to paired-pulse stimulation, PF EPSCs, and AN EPSCs recorded from the same fusiform cell. (G) Representative PF and AN EPSCs recorded from the same fusiform cell as shown in F, before and after ZX1 application. (H) Time course of PF and AN EPSC amplitude before and after ZX1 application (AMPAR EPSC amplitude, 10–15 min after ZX1 application: AMPAR EPSC: 151.09 ± 7.05% of baseline, n = 3, P < 0.01; AN EPSC: 100.01 ± 1.66% of baseline, n = 3, P = 0.79; PF EPSC vs. AN EPSC: P < 0.01). (I) Schematic of the experimental setup for experiments in the hippocampus, including stimulation of Schaffer collaterals (SC) and recording from CA1 neurons (J) Representative SC CA1 EPSCs from ZnT3WT and ZnT3KO mice before and after ZX1 application. (K) Time course of ZnT3WT and ZnT3KO CA1 EPSC amplitude before and after ZX1 application (AMPA EPSC amplitude, 15–20 min after ZX1 application: ZnT3WT: 146.71 ± 5.66% of baseline; n = 5, P < 0.01; ZnT3KO: 92.23 ± 9.20% of baseline; n = 5, P = 0.18; ZnT3WT vs. ZnT3KO: P < 0.01).
as evidenced by reduced DA-ZP1 fluorescence in these mice (Fig. 4 C and D). Next, we compared evoked zinc release between sham- and noise-exposed mice. To quantify evoked zinc levels in DCN slices, we incubated the slices in ACSF containing the ratiometric zinc sensor LZN (2 μM), measured zinc-mediated fluorescence in response to PF electrical stimulation (Fig. 4E), and used the equation shown in SI Materials and Methods to convert fluorescence ratios to extracellular zinc levels, which were subsequently normalized to sham-exposed levels (15). We found that evoked zinc release was significantly reduced in noise-exposed mice (Fig. 4 E and F). This result suggests that sound-dependent reduction in vesicular zinc levels and vesicular zinc release abolished the inhibitory effect of zinc on PF AMPAR EPSCs. Because noise exposure caused reduction of AN inputs, we suggest that the sound-dependent removal of the inhibitory effect of zinc enhances PF EPSCs and is consistent with a compensatory, presynaptic homeostatic response that restores the overall excitatory strength in fusiform cells. This finding indicates that experience-dependent changes of presynaptic zinc levels caused AMPAR plasticity even in the absence of changes in glutamatergic transmission.

**Discussion**

Our results show that endogenous, synaptically released zinc modulates AMPAR EPSCs in two different brain areas. Although earlier studies had suggested that exogenous zinc modulates AMPARs, this modulation has been considered physiologically irrelevant, because recent work failed to reveal any effect of endogenous zinc on AMPAR EPSCs in hippocampal and in DCN synapses (14, 22). However, these studies either used tricine or compared AMPAR EPSCs between WT and ZnT3KO mice. The use of tricine is problematic, because, unlike ZX1, tricine cannot efficiently prevent zinc from binding high-affinity zinc-binding sites and therefore is not an appropriate chelator for studying the role of zinc in synapses (15). Consistent with these results, ZX1, but not tricine, revealed the effect of endogenous zinc on AMPAR EPSCs (Fig. 1 G and H).

Finally, the lack of difference in the size of AMPAR EPSCs, evoked by trains of synaptic stimuli, between WT and ZnT3KO in hippocampal synapses, has been interpreted as evidence for the lack of effect of endogenous zinc on MF AMPA EPSCs (14). However, this result does not exclude compensatory, non–zinc-mediated mechanisms that maintain AMPAR EPSCs unchanged in ZnT3KO mice. Together, our results establish vesicular zinc as an endogenous AMPAR modulator that adjusts fast excitatory synaptic transmission in the brain.

Fig. 4. Plasticity of AMPAR EPSCs by sound-evoked reduction of presynaptic zinc levels in DCN parallel fiber synapses. (A) Representative PF EPSCs from sham- and noise-exposed mice before and after ZX1 application. (B) Time course of PF EPSC amplitude from sham- and noise-exposed mice before and after ZX1 application (PF EPSC amplitude: sham-exposed: 145.55 ± 6.87% of baseline, n = 5, P < 0.01; noise-exposed: 96.98 ± 4.85% of baseline, n = 5, P = 0.83; sham- vs. noise-exposed: P < 0.01). (C) Representative zinc-mediated fluorescence signals in sham- and noise-exposed mice at increasing concentrations of DA-ZP1. (D) Summary graph of fluorescence intensity at different concentrations of DA-ZP1 (fluorescence intensity in arbitrary units: sham- vs. noise-exposed, n = 5, P = 0.02 for 0.25 μM; P = 0.15 for 0.5 μM; P < 0.01 for 0.75 μM; P = 0.03 for 1 μM). (E, Left) Representative evoked, zinc-mediated fluorescent signals in sham- and noise-exposed mice in response to increasing number of pulses at 100 Hz. (Right) Time course of representative ratiometric fluorescent signals. (F) Summary graph of normalized extracellular zinc concentrations. Concentrations from noise-exposed mice are normalized to sham-exposed average concentrations.

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How could stimulation by noise exposure, which targets AN inputs, lead to changes in vesicular zinc in PF inputs? Recent results have revised the DCX circuitry and support the notion that, through electrical coupling with fusiform cells, stellate cells, a class of interneurons in the molecular layer, sense ongoing auditory activity, thus providing a link between AN and PF activity (43, 44). Based on these findings, auditory signals are able to rapidly recruit or suppress stellate cells and control the efficacy of PF activity. Auditory-evoked changes in PF activity through this pathway may provide the trigger for plasticity in presynaptic zinc levels. Moreover, other studies indicate that coincident synaptic activation of PF and AN inputs leads to induction of spike-timing dependent synaptic plasticity (STDP) of parallel fiber inputs (45), by analogy with the climbing fiber and parallel fiber inputs in the cerebellum. According to this scheme, auditory and noise-exposed increases in fusiform cell spiking could also provide the trigger to induce plastic changes in PF inputs. Alternatively, because granule cells receive auditory nerve input from higher auditory centers such as auditory cortex, the changes in auditory stimulation might cause changes in vesicular zinc via this pathway (46).

Previous studies have established activity-dependent AMPAR synaptic plasticity via changes in pre- and postsynaptic glutamatergic neurotransmission (47). Such AMPAR plasticity is involved in memory, learning, and development of the CNS and is crucial for the proper functioning and adaptability of the mammalian brain. The sound-dependent plasticity of presynaptic zinc levels and zinc-mediated inhibition of AMPARs (Fig. 4) adds zinc as a key player in the complexity of AMPAR synaptic plasticity in the mammalian brain.

Materials and Methods
All animal procedures were approved by Institutional Animal Care and Use Committees of the University of Pittsburgh. Methods for preparing brain slices, electrophysiological recordings, noise exposure, and imaging of acoustic brainstem responses, and fluorescence imaging are provided in SI Materials and Methods. Data analysis, statistical tests, and detailed values presented in main figures are also provided in SI Materials and Methods.

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