Rab3-interacting molecules 2α and 2β promote the abundance of voltage-gated $\text{Ca}_V1.3$ $\text{Ca}^{2+}$ channels at hair cell active zones

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Ca$^{2+}$ influx triggers the fusion of synaptic vesicles at the presynaptic active zone (AZ). Here we demonstrate a role of Ras-related in brain 3 (Rab3)–interacting molecules 2α and β (RIM2α and RIM2β) in clustering voltage-gated $\text{Ca}_V1.3$ Ca$^{2+}$ channels at the AZs of sensory inner hair cells (IHCs). We show that IHCs of hearing mice express mainly RIM2α but also RIM2β and RIM3γ, which all localize to the AZs, as shown by immunofluorescence microscopy. Immunohistochemistry, patch-clamp, fluctuation analysis, and confocal Ca$^{2+}$ imaging demonstrate that AZs of RIM2α-deficient IHCs cluster fewer synaptic $\text{Ca}_V1.3$ Ca$^{2+}$ channels, resulting in reduced synaptic Ca$^{2+}$ flux. Using superresolution microscopy, we found that Ca$^{2+}$ vesicles remained clustered in stripes underneath anchored ribbons. Electron tomography of high-pressure frozen synapses revealed a reduced fraction of membrane-tethered vesicles, whereas the total number of membrane-proximal vesicles was unaltered. Membrane capacitance measurements revealed a reduction of exocytosis largely in proportion with the Ca$^{2+}$ current, whereas the apparent Ca$^{2+}$ dependence of exocytosis was unchanged. Hair cell-specific deletion of all RIM2 isoforms caused a stronger reduction of Ca$^{2+}$ influx and exocytosis and significantly impaired the encoding of sound onset in the postsynaptic spiral ganglion neurons. Auditory brainstem responses indicated a mild hearing impairment on hair cell-specific deletion of all RIM2 isoforms or global inactivation of RIM2α. We conclude that RIM2α and RIM2β promote a large complement of synaptic Ca$^{2+}$ channels at IHC AZs and are required for normal hearing.

Significance

Sound encoding relies on Ca$^{2+}$-regulated transmitter release from inner hair cells (IHCs). Here we demonstrate a role of Ras-related in brain 3 (Rab3)–interacting molecule 2 (RIM2) in Ca$^{2+}$ channel-clustering and vesicle-tethering at the active zones of IHCs. Active zones of RIM2α-deficient IHCs cluster fewer synaptic voltage-gated $\text{Ca}_V1.3$ Ca$^{2+}$ channels, resulting in reduced synaptic Ca$^{2+}$ influx. Exocytosis was diminished in RIM2α-deficient IHCs, likely contributing to the mild hearing impairment of RIM2α knockout mice. Hair cell-specific disruption of all RIM2 isoforms caused a stronger decrease of Ca$^{2+}$ current and exocytosis in IHCs and impaired the encoding of sound onset in spiral ganglion neurons. We conclude that RIM2α and RIM2β promote synaptic clustering of Ca$^{2+}$ channels at IHC active zones and are required for normal hearing.

Additional signficance

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A role of RIM1 and RIM2 in clustering Ca\(^{2+}\) channels at the AZ was demonstrated by analysis of RIM1/2-deficient presynaptic terminals of cultured hippocampal neurons (14), auditory neurons in slices (18), and Drosophila neuromuscular junction (19). Because α-RIMS also bind the vesicle-associated protein Ras-related in brain 3 (Rab3) via the N-terminal zinc finger domain (20), they are also good candidates for molecular coupling of Ca\(^{2+}\) channels and vesicles (18, 21, 22). Finally, a role of RIMS in priming of vesicles for fusion is the subject of intense research (18, 21–27). RIMS likely contribute to priming via disinhibiting Munc13 (26) and regulating vesicle tethering (27). Here, we studied the expression and function of RIM in IHCs. We combined molecular, morphologic, and physiologic approaches for the analysis of RIM2α knockout mice [RIM2α SKO (28); see Methods] and of hair cell-specific RIM1/2 knockout mice (RIM1/2 cDKO). We demonstrate that RIM2α and RIM2γ are present at IHC AZs of hearing mice, positively regulate the number of synaptic Cav1.3 Ca\(^{2+}\) channels, and are required for normal hearing.

**Results**

RIM2α, RIM2β, and RIM3γ Are Present at IHC AZs of Hearing Mice. The mature IHC ribbon synapse shows an unconventional molecular composition (reviewed in refs. 29–31). Recently, the expression of RIM2α was shown to colocalize with synaptic ribbons was demonstrated for immature mouse IHCs. However, IHCs of hearing mice were reported to lack RIM2 protein as well as RIM1–RIM4 mRNA (13). Here we revisited the expression of RIMs in IHCs of hearing mice, using RT-PCR and immunohistochemistry, using knockout tissue as control. We used the previously published conditional RIM1/2 DKO (14) and crossed it to a new transgenic mouse line expressing Cre-recombinase under the promoter of Vglut3 [Vglut3-Cre, generated as described in SI Appendix, Fig. S1A, active in IHCs (31, 32); a detailed characterization of the Vglut3-Cre line will be published as a separate study] for hair cell-specific deletion of all RIM1 and RIM2 isoforms (named RIM1/2 cDKO). Using a floxed-GFP reporter, Cre recombination was observed in >99% of IHCs and in some outer hair cells, but not in neurons or supporting cells within the cochlea (SI Appendix, Fig. S1B). Different from a previous report (13), we detected mRNA for RIM1–RIM4 in organs of Corti of wild-type mice after the onset of hearing [SI Appendix, Fig. S2A and SI Appendix, Experimental Procedures, postnatal day (p) 14–16, not differentiating the individual RIM1/2 isoforms]. Nested RT-PCR revealed RIM2 and RIM3γ mRNA in single IHCs (p14–p16; SI Appendix, Fig. S2B). Nested RT-PCR for specific RIM2-isoforms detected RIM2α mRNA in most IHCs (SI Appendix, Fig. S2C). mRNAs for RIM2β and RIM2γ were rarely detectable in single IHCs but were always found at the organ of Corti level (SI Appendix, Fig. S2C).

Next, we studied the presence and localization of RIM proteins by labeling IHCs of wild-type, RIM2α SKO, and RIM1/2 cDKO mice with antibodies to RIM1, RIM2, and RIM3γ. In wild-type IHCs, we found spot-like RIM2 (Fig. 1A) and RIM3γ (SI Appendix, Fig. S3A) immunofluorescence that colocalized with C-terminal binding protein 2 (CtBP2)/RIBEYE–marked ribbon-occupied AZ of IHCs, whereas no obvious RIM1 immunofluorescence was detected (SI Appendix, Fig. S3B). In addition, we found RIM2 immunofluorescence separated from ribbon (Fig. 1A and C–F). This most likely reflects RIM2 expression at synapsin-expressing conventional efferent synapses (blue in Fig. 1A, synapsin is absent from IHCs) (34) of lateral olivocochlear neurons with spiral ganglion neurons (SGNs; connectivity is illustrated in Fig. 1B) underneath the IHCs (Fig. 1D). Using two-color stimulated emission depletion (STED) microscopy, we observed a stripe-like shape of the RIM2 immunofluorescence, which, according to synapse orientation, appeared to mark the base of the synaptic ribbon (Fig. 1C), indicating that RIM2 is part of the Bassoon-positive presynaptic density of the AZ, which occupies the space between the ribbon and membrane (4). RIM2 immunofluorescence,
although weaker, remained at the ribbon-occupied AZs of RIM2α SKO IHCs (Fig. 1E) but was absent from AZs of IHCs of RIM1/2 cDKO mice lacking all RIM2 isoforms (Fig. 1F). This indicates the additional presence of RIM2β at IHC AZs, as the RIM2 antibody does not recognize RIM2γ.

**Reduced Ca\(^{2+}\) Channel Immunofluorescence at AZs of RIM2c-Deficient IHCs.** First, we studied the role of RIM2α, the likely most abundant RIM2 isoform in IHCs, in regulating presynaptic Ca\(^{2+}\) influx. We approached the overall organization of RIM2α SKO IHC synapses using immunohistochemistry for Ca\(^{2+}\), and CtBP2/RIBEYE, marking the presynaptic Ca\(^{2+}\) channel cluster and ribbons as well as GluA2/3, indicating the postsynaptic AMPA receptor cluster. Ribbon-occupied synapses, identified as CtBP2/RIBEYE immunofluorescent spots juxtaposed to GluA2/3 immunofluorescence (Fig. 2A), were found in normal number in RIM2α SKO IHCs (Fig. 2B). Ca\(^{2+}\) channels remained clustered at each ribbon-type IHC AZ (Fig. 2C and D), but the Ca\(^{2+}\) channel immunofluorescence intensity was reduced in RIM2α SKO IHCs. Using semiquantitative immunohistochemistry with identical and parallel tissue processing, imaging, and analysis of immunofluorescence, we found a reduction by 48% (Fig. 2D and E; P < 0.001, 417 RIM2α SKO AZs in 56 IHCs in three organs of Corti from two mice; 390 RIM2α control (con) AZs in 50 IHCs in four organs of Corti from three mice). A similar result was obtained in two further sets of experiments. Using two-color STED microscopy, we found stripe-like Ca\(^{2+}\) channel clusters at ribbon-occupied RIM2α SKO AZs, which, on inspection, in single XY sections, seemed to be qualitatively comparable in size and shape to those of RIM2α con IHCs (Fig. 2C). We did not detect significant differences in immunofluorescence intensity for RIBEYE (Fig. 2D; P = 0.27, same AZs analyzed as for Ca\(^{2+}\) earlier) and GluA2/3 (P = 0.26; n = 650 synapses in 72 IHCs in four organs of Corti from RIM2α SKO mice; n = 673 synapses in 78 IHCs and four organs of Corti for RIM2α con).

**Disruption of RIM2α and RIM2β Reduces Synaptic Ca\(^{2+}\) Influx in IHCs.** To study the effect of RIM2 disruption on presynaptic Ca\(^{2+}\) influx, we performed perforated patch-clamp recordings of Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) and confocal imaging of presynaptic Ca\(^{2+}\) signals in IHCs of RIM2α SKO. We found a mild reduction of the whole-cell I\(_{\text{Ca}}\) amplitude on deletion of RIM2α (16.8%; Fig. 3A and B). The deletion of all RIM2 isoforms in IHCs of RIM1/2 cDKO mice decreased I\(_{\text{Ca}}\) by 51.4% (Fig. 3A and SI Appendix, Fig. S5C; P = 0.01 relative to Cre-negative littermate controls: RIM1/2 con). I\(_{\text{Ca}}\) amplitudes were not significantly different between IHCs of RIM1/2 cDKO (lacking all RIM1 and RIM2 isoforms, eight IHCs in five organs of Corti) and RIM2 cSKO (lacking all RIM2 isoforms (see Methods) seven IHCs in four organs of Corti; P = 0.5; SI Appendix, Fig. S5C), supporting the notion that IHC AZs operate without RIM1. Next, we performed a detailed analysis of the biophysical properties of the Ca\(^{2+}\) channels in RIM2α SKO IHCs. Voltage dependence (V\(_{0.5}\) = −14.8 ± 2.7 mV for nine RIM2α SKO IHCs vs. −12.4 ± 3.5 mV for eight RIM2α con IHCs; P = 0.58) and kinetics of activation of I\(_{\text{Na}}\) as well as I\(_{\text{Ca}}\) inactivation were unaltered (SI Appendix, Fig. S4A and B). The reduced synaptic Ca\(^{2+}\) influx in RIM2α SKO IHCs suggested a diminished number of synaptic Ca\(^{2+}\) channels. To test this hypothesis and probe for potential changes in single-channel current or open probability, we performed a nonstationary fluctuation analysis as described (Fig. 3B) (35). The estimated number of Ca\(^{2+}\) channels in RIM2α SKO IHCs (eight IHCs) amounted to 1,102 ± 65, which was 17.8% less than in RIM2α con IHCs (1,341 ± 73; seven IHCs), and hence, the reduced number of Ca\(^{2+}\) channels accounts for the reduction of the whole-cell Ca\(^{2+}\) current. Neither the single channel current nor the open probability changed significantly (SI Appendix, Table S3).

![Fig. 2. Disruption of RIM2α reduces the synaptic Ca\(^{2+}\) immunofluorescence. (A) Representative projections of confocal images showing GluA2/3 (green) and RIBEYE (magenta) across the basal region of rows of RIM2α con (Top) and RIM2α SKO (Bottom) IHCs. Orderly juxtaposed presynaptic ribbons and postsynaptic AMPA receptor clusters indicate intact ribbon-anchorage. (Scale bar: 10 \(\mu\)m.) (B) Average number of ribbon-occupied IHC synapses: comparable number of synapses in both genotypes. (C) Two-color STED image of immunolabeled ribbons (CtBP2/RIBEYE, magenta) and Ca\(^{2+}\) channel clusters (green) in RIM2α con and RIM2α SKO IHCs. Ca\(^{2+}\) channel clusters retain the stripe-like morphology. The images are maximum-intensity projections after smoothing with a Gaussian (σ = 1 pixel) in ImageJ. (Scale bar: 1 \(\mu\)m.) (D) Organs of Corti from RIM2α con (Left) and RIM2α SKO (Right) mice immunolabeled for Ca\(^{2+}\) (green) and CtBP2/RIBEYE (magenta). Clusters of Ca\(^{2+}\) were concentrated in the basal poles near synaptic ribbons in IHCs of both genotypes: each synaptic ribbon was accompanied by a cluster of Ca\(^{2+}\). (Scale bars: 10 \(\mu\)m.) (E) Quantification of synaptic Ca\(^{2+}\) immunofluorescence revealed a reduction in intensity for AZs of RIM2α SKO IHCs (Left), no change in CtBP2/RIBEYE immunofluorescence (Right).](image-url)
Because the whole-cell recordings sum \( I_{Ca} \) of all AZs and extrasynaptic membranes, we sought to further analyze \( Ca^{2+} \) influx at the single AZ, using confocal \( Ca^{2+} \) imaging at fluoscently tagged AZs (2, 36, 37). IHCs were depolarized for 20 ms to \( -7 \) mV to fully activate \( Ca^{2+} \) channels, and \( Fluo-5N \) fluorescence was studied using line scans across the center of “Ca^{2+} microdomains” (Fig. 3 C and D), identified as hotspots of \( Fluo-5N \) fluorescence change during a preceding 254-ms depolarization. Ca^{2+} microdomains of RIM2α SKO (40 AZs in 13 IHCs in seven organs of Corti) and RIM2α con (35 AZs in 15 IHCs in seven organs of Corti) showed comparable spatial extents (Fig. 3C and SI Appendix, Fig. S5B) and kinetics (Fig. 3D). However, we found a 36% reduction of the maximal fluorescence change (Fig. 3D and SI Appendix, Fig. S5A; \( P < 0.002 \)), which is less than the decrease of synaptic \( Ca^{2+} \) immunofluorescence (48%) but exceeds the loss of the whole-cell \( Ca^{2+} \) current (18%). The fluorescence change varied greatly among the AZs in IHCs of RIM2α SKO mice, as previously described for wild-type IHCs (36). The coefficients of variation were comparable in both genotypes (0.65 for RIM2α con and 0.66 for RIM2α SKO IHCs), again contrasting the synaptic phenotype of Bassoon-deficient IHCs that showed reduced \( Ca^{2+} \) signaling heterogeneity. In summary, RIM2α and RIM2β promote a large complement of synaptic Ca^{1.3} \( Ca^{2+} \) channels. This function of RIM2 seems to involve the immobilization of the channels at the AZ, at least in addition to promoting channel trafficking to the plasma membrane, as the reduction of synaptic \( Ca^{2+} \) channels exceeded that of the whole-cell \( Ca^{2+} \) current.

Disruption of RIM2α Reduces the Fraction of Membrane-Tethered Synaptic Vesicles. Next, we studied the ultrastructure of the AZ, using electron microscopy and tomography. To obtain a close to native structural preservation, we used high-pressure freezing in combination with freeze-substitution. Tomographic reconstructions revealed that the AZ ultrastructure was generally maintained in RIM2α SKO IHCs showing normally anchored, sized [average ribbon height: 258.4 ± 24.6 nm for RIM2α con (n = 10 tomograms) vs. 270.2 ± 18.0 nm for RIM2α SKO (n = 10 tomograms)] and shaped synaptic ribbons with an unaltered complement of ribbon-associated synaptic vesicles (29.0 ± 3.9 for RIM2α SKO vs. 28.2 ± 3.5 for RIM2α con per tomogram, typically containing approximately half of the ribbon). The total number of membrane-proximal vesicles within 50 nm from the AZ membrane (Fig. 4C) was unaltered in RIM2α SKO compared with controls (8.0 ± 1.0 for RIM2α SKO vs. 6.9 ± 0.7 for RIM2α con; Fig. 4 D and F). Performing electron tomography allowed the identification of tethers to the vesicles as well as the determination of the tether length in 3D (SI Appendix, Fig. S6 A and B). Interestingly, the fraction of membrane-tethered vesicles (number of tethered vesicles/total number of membrane-proximal vesicles) was significantly reduced at RIM2α SKO AZs (0.24 ± 0.03 for RIM2α SKO vs. 0.41 ± 0.02 for RIM2α con; \( P < 0.002 \); Fig. 4 D and E), suggesting RIM2α plays a role in tethering vesicles to the AZ membrane of IHCs. The tether length, however, was unchanged (average tether length, 22.7 ± 1.6 nm for RIM2α con vs. 22.9 ± 1.5 nm for RIM2α SKO; SI Appendix, Fig. S6C).

Disruption of RIM2α and RIM2β Reduces IHC Exocytosis. How does the disruption of RIM2 affect hair cell exocytosis? The reduction of \( Ca^{2+} \) channels and the impaired vesicle tethering are likely to diminish exocytosis. Moreover, as RIM2α is a candidate for linking vesicles and Ca^{2+} channels, a potential disruption of \( Ca^{2+} \) influx-exocytosis coupling might result. We tested these hypotheses by measuring exocytic membrane capacitance changes (\( \Delta C_{mem} \)) in RIM2α SKO and RIM1/2 cDKO IHCs in response to depolarizations of various durations to the maximum \( Ca^{2+} \) current potential in perforated-patch recordings (Fig. 5 A and B). On the basis of previous work (35, 38-40), we interpret the fast and saturating component (0–20 ms depolarization duration) as exocytosis of a standing readily releasable pool (RRP) (41), and the slower component (>20 ms depolarization duration) as sustained exocytosis, primarily reflecting vesicle supply to the RRP and subsequent fusion (Fig. 5B). The exocytic \( \Delta C_{mem} \), in response to 20-ms depolarization, was reduced by 36% in RIM2α SKO IHCs (14 IHCs, \( P = 0.015 \) relative to RIM2α con) and by 52% in RIM1/2 cDKO (15 IHCs, \( P = 0.003 \) relative to RIM1/2 con and \( P < 0.001 \) relative to RIM2α SKO). The rate of sustained exocytosis was diminished, as well [from 0.76 ± 0.14 fF/ms in RIM2α con IHCs to 0.38 ± 0.07 fF/ms in RIM2α SKO IHCs (\( P = 0.02 \) relative to RIM2α con) and 0.09 ± 0.02 fF/ms in RIM1/2 cDKO IHCs (\( P < 0.001 \) relative to RIM1/2 con) (nine IHCs, 0.5 ± 0.08 fF/ms; \( P < 0.001 \) relative to RIM2α SKO)]. Exocytosis was not significantly different between IHCs of RIM1/2 cDKO and RIM2α cSKO (\( P = 0.5 \); SI Appendix, Fig. S5D), supporting the notion that hair cell AZ operates without RIM1. Relating the exocytic \( \Delta C_{mem} \) to the...
changes of $[\text{Ca}^{2+}]_{\text{m}}$. (Fig. 5D, Upper) and reduction of open $\text{Ca}^{2+}$ channels by slow perfusion of the dihydropyridine channel antagonist isradipine (10 μM; Fig. 5D, Lower). We obtained $m$ by fitting the relationship of $\Delta C_{\text{m}}$ to $Q_{\text{Ca}}$ for each cell with a power function: $\Delta C_{\text{m}} = A(Q_{\text{Ca}})^m$ (43), and for the case of the single $\text{Ca}^{2+}$ channel current change, we restricted fitting to the $Q_{\text{Ca}}$ range that did not show obvious saturation of $\Delta C_{\text{m}}$ (4) (Fig. 5D). The estimates of $m$ were statistically indistinguishable between RIM2a SKO and RIM2a con IHCs for both manipulations [isradipine, $1.58 \pm 0.12$ vs. $1.51 \pm 0.11$ ($P = 0.62$); $\Delta [\text{Ca}^{2+}]_{\text{m}}$, $2.63 \pm 0.09$ vs. $2.59 \pm 0.14$ ($P = 0.8$); $n = 6$ IHCs for both genotypes]. We conclude that the RIM2a disruption impairs RRP size and replenishment, but leaves Ca$^{2+}$ influx-exocytosis coupling largely intact.

**RIM2 Disruption Impairs Sound Onset Encoding.** What are the consequences of RIM2 disruption for hearing? The thresholds of auditory brainstem responses (ABRs) were mildly but significantly elevated in the RIM mutants (Fig. 6B and D; by 5 dB SPL in RIM1/2 cDKO mice and by 16 dB in RIM2a SKO mice compared with their respective littermate controls; both $P < 0.0001$). As the presence of otoacoustic emissions with normal amplitudes (SI Appendix, Fig. S7 A–D) indicates normal mechatroncalectrical transduction and cochlear amplification, this suggests a synaptopathic hearing impairment (44).

![Figure 4](image-url)

**Fig. 4.** Disruption of RIM2a affects the ultrastructure of IHC ribbon synapses. (A) Exemplary virtual electron tomographic sections of RIM2a con and RIM2a SKO ribbon synapses: normal ribbon (R) size and shape and intact anchorage in RIM2a SKO IHCs can be observed. Different classes of synaptic vesicles are found in the membrane proximity: tethered (red arrowhead) and non-tethered (yellow asterisk). For RIM2a con $n = 10$ tomograms from one animal and two organs of Corti, for RIM2a SKO $n = 10$ tomograms from two animals and two organs of Corti were analyzed. (Scale bar: 100 nm.) (B) Front view of rendered models of whole-ribbon synapse in RIM2a con and RIM2a SKO IHCs with tethered (dark green) and non-tethered (light green) synaptic vesicles in the ribbon-associated pool. Tethered (orange) and non-tethered (yellow) membrane-proximal vesicles and other synaptic structures such as ribbon (red), presynaptic density (pink), and AZ membrane (blue) are indicated. For simplicity, tethers are not reconstructed. (C) Illustration depicting the quantification criteria of the membrane-proximal vesicle pool (not drawn to scale). (D) Top view of rendered models of RIM2a SKO and RIM2a con ribbon synapse AZs with tethered (orange) and non-tethered (yellow) membrane-proximal vesicles. Other synaptic structures such as ribbons and ribbon-associated vesicles are removed for clarity. (Scale bar: 100 nm.) (E) The ratio of the number of membrane-tethered vesicles to the total number of membrane-proximal vesicles (fraction of tethered SV) is significantly reduced in RIM2a SKO IHCs. (F) The average number of membrane-proximal vesicles and (G) the ribbon height are unchanged in RIM2a SKO IHCs compared with RIM2a con.

Corresponding integrated $\text{Ca}^{2+}$ current ($Q_{\text{Ca}}$; Fig. 5C) reduced the discrepancy of the exocytic responses between the RIM2a SKO and control IHCs, indicating that the reduction of presynaptic $\text{Ca}^{2+}$ influx explains impaired exocytosis to a large extent.

As an interaction partner of Rab3, which is present in hair cells (42), and of the $\text{Cav}_{1.3}$ channel (13), RIM2a is a candidate molecular linker between $\text{Ca}^{2+}$ channel and vesicular release site at the IHC AZ. Hence, we studied $\text{Ca}^{2+}$ influx-exocytosis coupling by estimating the apparent $\text{Ca}^{2+}$ cooperativity $m$ of exocytosis upon manipulation of the single $\text{Ca}^{2+}$ channel current via corresponding integrated $\text{Ca}^{2+}$ current ($Q_{\text{Ca}}$; Fig. 5C) reduced the discrepancy of the exocytic responses between the RIM2a SKO and control IHCs, indicating that the reduction of presynaptic $\text{Ca}^{2+}$ influx explains impaired exocytosis to a large extent.
RIM2 synthesis in the cochlear nucleus appears to be involved in tethering synaptic vesicles to the AZ. In addition, RIM2 seems to serve as the only membrane. Disruption of RIM2 channel, although we and SI Appendix showed a nonsignificant trend toward smaller values. Both spontaneous (SI Appendix, Fig. S8B) and evoked rates (Fig. 6F) also tended to be smaller in putative SGNS of RIM2α SKO mice, although differences did not reach significance. The increase of average spike rate with sound intensity was significantly less steep, and the dynamic range tended to be broader in RIM2α SKO SGNS compared with SGNS of littermate controls (SI Appendix, Fig. S8 C–E). The time course of recovery from adaptation (likely reflecting RRP recovery from depletion; SI Appendix, Fig. S8F) was normal in RIM2α SKO SGNS, as was the temporal precision of coding, assessed as the jitter of the first spike after sound onset (SI Appendix, Fig. S8G) and as the synchronicity of firing to the sound envelope of amplitude-modulated tones (SI Appendix, Fig. S8H). Similar to SGNS, neurons of the cochlear nucleus in RIM2α SKO mice showed only subtle alterations in their spontaneous and sound-evoked spiking activity, such as a small but significant reduction of peak rates in putative bushy cells and in multipolar cells (SI Appendix, Fig. S8 I and J). Taken together, RIM2 disruption caused a mild sound-encoding phenotype that reached significance only in RIM2α cDKO SGNS, compatible with the stronger impairment of the presynaptic function of their IHCs compared with RIM2α SKO IHCs. RIM2α disruption at the central auditory synapses of RIM2α SKO mice was reflected by reduced peak firing rates in bushy cells and alteration of the central ABR peaks.

Discussion

Here we identified RIM2α and RIM2β as the long RIM isoforms at the afferent IHC synapse of hearing mice. RIM2α and RIM2β positively regulate the number of synaptic CaV1.3 Ca2+ channels in IHCs, likely by clustering them at the AZ. In addition, RIM2α seems to be involved in tethering synaptic vesicles to the AZ membrane. Disruption of RIM2α and RIM2β diminishes IHC exocytosis primarily via reducing synaptic Ca2+ influx and mildly impairs synaptic sound encoding.

AZs of Cochlear IHCs of Hearing Mice Use Multiple RIM Isoforms.

The molecular composition of the IHC ribbon synapse is highly specialized to suit the needs of fast and indefatigable transmitter release and deviates from "conventional" synapses of the CNS in several ways (28–30). Here we show that IHCs of hearing mice express RIM2α, RIM2β, and RIM3γ, which corrects a previous notion of a developmental loss of RIM expression in IHCs (13). However, unlike many other synapses studied so far (14, 18, 47–49), IHC ribbon synapses seem to lack RIM1. Obviously, negative single-cell PCR and immunohistochemistry do not rule out weak RIM1 expression. However, the normal ABR in RIM1α SKO mice and the indistinguishable presynaptic dysfunction of IHCs from RIM2 cDKO and RIM1/2 cDKO mice support the notion that IHC synapses operate without RIM1. Therefore, RIM2α seems to serve as the only α-RIM capable of interacting with the vesicle-associated protein Rab3, which is also expressed in hair cells (42). Beyond the Rab3 interaction, the long isoforms RIM2α and RIM2β have overlapping interaction partners such as Munc13, ELKS, and Ca2+ channels. The further reduction of synaptic Ca2+ influx by additional deletion of RIM2β likely reflects additive and potentially nonoverlapping roles of RIM2α and RIM2β in clustering synaptic Ca2+ channel, although we cannot definitively exclude a compensatory up-regulation of tern, with spike rate peaking at sound onset and then gradually declining [short-term adaptation, likely reflecting partial RRP depletion (2, 46)] to an adapted rate that was maintained throughout stimulation (Fig. 6E and F). SGNS of RIM1/2 cDKO mice showed a significantly reduced peak rate (Fig. 6E; P = 0.02, paired t test) and a delayed first spike latency (P = 0.02, paired t test), whereas the jitter of the first spike was unaltered (P = 0.45 for comparison of the variance of first spike latency). Steady-state spike rates (Fig. 6F) and spontaneous spike rates (SI Appendix, Fig. S8B) showed a nonsignificant trend toward smaller values. Both spontaneous (SI Appendix, Fig. S8B) and evoked rates (Fig. 6F) also tended to be smaller_tential Ca2+ channels of the CNS in IHCs, likely by clustering them at the AZ. In addition, RIM2 seems to serve as the only membrane. Disruption of RIM2 channel, although we and SI Appendix showed a nonsignificant trend toward smaller values. Both spontaneous (SI Appendix, Fig. S8B) and evoked rates (Fig. 6F) also tended to be smaller in putative SGNS of RIM2α SKO mice, although differences did not reach significance. The increase of average spike rate with sound intensity was significantly less steep, and the dynamic range tended to be broader in RIM2α SKO SGNS compared with SGNS of littermate controls (SI Appendix, Fig. S8 C–E). The time course of recovery from adaptation (likely reflecting RRP recovery from depletion; SI Appendix, Fig. S8F) was normal in RIM2α SKO SGNS, as was the temporal precision of coding, assessed as the jitter of the first spike after sound onset (SI Appendix, Fig. S8G) and as the synchronicity of firing to the sound envelope of amplitude-modulated tones (SI Appendix, Fig. S8H). Similar to SGNS, neurons of the cochlear nucleus in RIM2α SKO mice showed only subtle alterations in their spontaneous and sound-evoked spiking activity, such as a small but significant reduction of peak rates in putative bushy cells and in multipolar cells (SI Appendix, Fig. S8 I and J). Taken together, RIM2 disruption caused a mild sound-encoding phenotype that reached significance only in RIM2α cDKO SGNS, compatible with the stronger impairment of the presynaptic function of their IHCs compared with RIM2α SKO IHCs. RIM2α disruption at the central auditory synapses of RIM2α SKO mice was reflected by reduced peak firing rates in bushy cells and alteration of the central ABR peaks.

Fig. 6. Mild impairment of sound encoding in RIM1/2 cDKO and RIM2α SKO mice. (A) ABR waveforms of RIM1/2 cDKO mice (gray, n = 8) and RIM1/2 con (black, n = 12) in response to 80 dB (pe) click stimulation were generally preserved, but in RIM1/2 cDKO, the latency of peak I was prolonged (SI Appendix, Fig. S7F). (B) Average ABR thresholds of RIM1/2 cDKO mice (gray, n = 8) were significantly elevated compared with RIM1/2 con (black, n = 11). (C) ABR in RIM2α SKO mice (gray, n = 15) were preserved but had prolonged latencies of peaks II–V compared with RIM2α con (black, n = 15) littermate controls (SI Appendix, Fig. S7E). (D) Average ABR thresholds of RIM2α SKO mice (gray, n = 11) were significantly elevated compared with littermate controls (black, n = 14). (E) Mean peristimulus time histogram of RIM1/2 cDKO (gray, n = 10) and RIM1/2 con (black, n = 22) SGNS to tone burst stimulation at the characteristic frequency of each fiber, 30 dB above threshold. Shadowed areas indicate the SEM. (Inset) Quantification of peak rates at sound onset (maximum, 0.5 ms bin) and steady state rates (averaged between 37 and 47 ms after sound onset) of individual fibers. Peak rates were significantly reduced in RIM1/2 cDKO (P = 0.02), and sustained rates tended to be reduced (n.s.); the overall response pattern was preserved. (F) Mean peristimulus time histogram of RIM2α SKO (gray, n = 32) and RIM2α con (black, n = 42) SGNS, illustrated as in E. There was a nonsignificant trend to lower spike rates in the RIM2α SKO SGNS.

The significant delay of ABR waves 2–5 (Fig. 6C and SI Appendix, Fig. S7E; P < 0.0001 for each) and the reduction in ABR wave 3 amplitude in RIM2α SKO mice (Fig. 6D; P < 0.0001) indicate an additional impairment of synaptic transmission deficit at central auditory synapses. Consistent with the above notion that the IHC synapse seems to operate without RIM1, RIM1α SKO mice showed normal ABR thresholds and waveforms (SI Appendix, Fig. S7 G and H), also indicating that the sole deletion of RIM1α does not impair transmission at central auditory synapses. Next, we performed extracellular recordings from individual SGNS, each of which is driven by a single AZ (45), using glass microelectrodes targeted toward the entry point of the auditory nerve into the anteroventral cochlear nucleus (46). SGNS were distinguished from cochlear nucleus neurons on the basis of their response patterns and the electrode position (Methods) (46). Sound thresholds (SI Appendix, Fig. S8A) and frequency tuning of SGNS were comparable between RIM mutant mice and their respective littermate controls. Responses of SGNS to suprathreshold tone bursts followed an overall normal pat-
RIM2β in RIM2α SKO IHCs. However, neither a compensatory expression of RIM1 nor an up-regulation of RIM2β can fully substitute for the lost RIM2α in the regulation of synaptic Ca2+ channels in IHCs. These findings contrast recent observations at the calyx of Held synapse of the central auditory pathway, in which RIM1 and RIM2 isoforms can largely replace each other in regulating the abundance of presynaptic Ca2+ channels (50). The localization of RIM3γ at the IHC AZ was unexpected, given that γ-RIMs assume postsynaptic roles in CNS neurons (51, 52). Future studies on RIM3γ knockout mice will be required to confirm the specificity of RIM3γ immunolabeling at IHC AZs and to elucidate its relevance for IHC synaptic transmission and hearing.

Positive Regulation of Presynaptic Ca2+ Signaling of IHCs by RIM2α and RIM2β. Here we identified the IHC ribbon synapse as a system that requires RIM2α and RIM2β for normal function. Importantly, the functional synaptic deficit arose despite a normal presence of synaptic ribbons, which is different from the Bassoon disruption that also reduced synaptic Ca2+ channels but, in addition, impaired the anchorage of synaptic ribbons (2, 9). Immunohistochemistry and patch-clamp recordings of Ca2+ influx and confocal Ca2+ imaging consistently demonstrated a reduction of synaptic Ca2+ channels on disruption of RIM2α. Similar to previous findings on Bassoon-deficient IHCs (2), the reduction of synaptic Ca2+ channels was greater than that of the total number of channels, which likely indicates that RIM2 primarily recruits and/or retains CaV1.3 channels at the AZ. In addition, RIM2 may have a role in CaV1.3 trafficking to the plasma membrane, but the extent to which this mechanism contributes is less clear, given that palmitoylated CaV1.2α, which is likely expressed in IHCs (35), might take this role. Unlike in Bassoon-deficient IHCs that primarily lack the population of AZs with a large Ca2+ channel complement (2, 9), the cumulative distribution function of CaV1.3 immunofluorescence intensities of RIM2α SKO AZs is overall shifted toward smaller values. The shape of the Ca2+ channel clusters at RIM2α SKO IHC AZs remained stripe-like, indicating that channel localization at the AZ is maintained on the tens of nanometer scale, whereas clusters are disintegrated into two to three round spots on Bassoon-disruption. Moreover, we found a generally well-preserved synaptic ultrastructure in IHCs of RIM2α SKO mice. Most IHCs with disrupted ribbons, unlike the ribbons in RIM2β-deficient IHCs (8), were similarly anchored and displayed a normal morphology in RIM2β-deficient IHCs. Hence, although both RIM (14, 16) and Bassoon (53) may use binding to RIM-binding protein to interact with Ca2+ channels, the effects of RIM2α and Bassoon disruption on CaV1.3 Ca2+ channel clustering at the IHC AZ differ. This may reflect a stronger perturbation of the AZ in Bassoon-deficient IHCs, which is also evident from the additional loss of the synaptic ribbon. Most likely, RIM2 also indirectly interacts with the CaV1.3 Ca2+ channel complex via binding to the CaV1β subunits (13); for instance, to CaV1β2, the predominant β-subunit in IHCs (35). Future studies of RIM interactions with the CaV1.3 channel complex will be required to explore additional binding mechanisms and evaluate the relative contribution of the interactions for mediating the positive regulation of the number of synaptic Ca2+ channels by RIM2α and RIM2β.

Impaired Exocytosis and Hearing Upon RIM2 Disruption. Given the concurrent reduction of Ca2+ influx and exocytosis observed in the present study for both RIM2α- and RIM2β-deficient IHCs, we favor the interpretation that RIM2 disruption reduces exocytosis primarily by diminishing synaptic Ca2+ channels. The reduced presynaptic exocytosis underlies the impaired sound encoding that was more pronounced on deletion of both RIM2α and RIM2β, which also caused a stronger reduction of presynaptic Ca2+ influx and exocytosis. This view is in line with previous studies of IHCs with fewer channels resulting from the deletion of CaV1β2 (35) and CaV1.3a (6), where we also observed a linear reduction of exocytosis with the Ca2+ current (by 70% and 90% in CaV1β2- and CaV1.3-deficient IHCs, respectively). The apparent Ca2+ cooperativity of exocytosis (Fig. 5C) and Ca2+ efficiency (Fig. 5D) suggests that coupling of Ca2+ influx to exocytosis (1, 4, 54) was largely unaltered in RIM2α-deficient IHCs, which contrasts findings at the in RIM1,2-deficient IHCs calyx of Held (18), which might reflect compensation by other molecular links between channels and release sites at the IHC AZ. Alternatively, a subtle alteration of Ca2+ channel position relative to the vesicular Ca2+ sensor on the scale of few nanometers might have gone undetected in our measurements of exocytic membrane capacitance changes. Our morphological analysis did not reveal major structural deficits of RIM2α-deficient synapses; synapse number, ribbon anchorage and the shape of the Ca2+ channel cluster appeared intact, and the number of ribbon-associated and membrane-proximal vesicles was not changed. This contrasts the synaptic phenotype elicited by disruption of two other presynaptic scaffold proteins, CAST and Bassoon, for which a reduction in ribbon size in rod photoreceptors (55) and a loss of the ribbon, respectively, from AZs of photoreceptors (56) and hair cells (8) was reported that led to a reduction of membrane-proximal synaptic vesicles at the presynaptic AZ in each case. Our observation of a reduced fraction of membrane-tethered vesicles adds further support to a role of RIMs in regulating vesicle tethering to the membrane of the AZ (27), although RIM2α seems not to be essential for this process. The contribution of the impaired tethering to reduced exocytosis and the consequences of deleting all RIM isoforms on synaptic ultrastructure remains to be probed in future studies. Deletion of all long RIM isoforms reduced the number of docked synaptic vesicles at AZs of both cultured neonatal hippocampal neurons (14) and the calyx of Held (18). Interestingly, neither the isolated deletion of RIM1 nor RIM2 isoforms affected synaptic transmission at the calyx of Held in vitro (50), and ABR recordings suggest normal auditory signaling in RIM1 or knockout mice (SI Appendix, Fig. S7). In contrast, delayed latencies of central auditory ABR peaks and extracellular recordings from single cochlear nucleus neurons suggested a mild impairment of transmission at central auditory synapses in the absence of RIM2α. Thus, there seems to be a requirement for RIM2 at central auditory synapses for normal hearing. Future in vivo recordings from the central auditory pathway on cell-specific RIM deletion mutants will help reconciling these data sets.

Methods

Animals. Generation of RIM2α knockout mice (RIM2α SKO) (48) and conditional RIM1/2 knockout mice (RIM1/2 cDKO) (14) was described earlier. Generation of Vglut3-Cre mice is described in SI Appendix, Fig. S1. Most experiments compared RIM1/2 DKO and RIM2 cSKO with their Cre-negative littermate controls (RIM1/2 con and RIM2 con), or RIM2α SKO with their wild-type littermates (RIM2α con, respectively). C57BL/6 mice were used for some experiments related to Fig. 1 and for controlling for possible effects of the Vglut3-Cre expression. All experiments complied with national animal care guidelines and were approved by the University of Göttingen Board for animal welfare and the animal welfare office of the state of Lower Saxony.

Immunohistochemistry and Immunofluorescence Microscopy. Acipalic cochlear turns of p14–p18 mice were fixed for 25 min in methanol at −20 °C. Primary antibodies were mouse anti-CtBP2 (1:200, BD Biosciences), rabbit anti-GluA2/3 (1:200, Chemicon), rabbit anti-CaV1.3 (1: 50, Alomone Labs), mouse anti-GluA2 (1:75 Chemicon), RIM2 (HS140103 recognizing the P5995/discs large/ZO-1 (PDZ) domain, 1:200; Synaptic Systems), RIM1 (1:200; Synaptic Systems), RIM3 (1:100) (52), and Vglut3 (1:500; Synaptic Systems). Secondary antibodies were AlexaFluor488, 594, and 647 (1:200; Molecular Probes). Each presented immunolabeling was repeated with similar result at least three times. Images were acquired with an SP5 confocal microscope (Leica Microsystems) with a 63× oil-immersion objective (NA = 1.4). Each preparation yielded several images, each containing a row of six IHCs. For
semiquantitative analysis of immunofluorescence, samples of mutant and control were processed identically and in parallel throughout immunohistochemistry, imaging, and analysis. Intensity per synapse was estimated in the optical section with peak intensity. Immunofluorescence intensity was measured as the integral of the pixel values under the 2D Gaussian after subtraction of the background (estimated by fitting a linear function to the region outside of the 2D Gaussian, and subsequently subtracting this function from the entire image (2)). The average voxel intensity of the stack was subtracted as background. As the absolute immunofluorescence intensity varied between the sets of three (Ca2,1 and RIBEYE) and four (GLuA2/3) experiments, the analysis was presented for one experiment each, but the findings in mutant relative to control were similar in all experiments.

Two-color STED images were acquired on a custom-built setup, as described previously (57). A pair of pulsed laser diodes at 595 and 640 nm wavelength were used to excite the dyes Alexa488 (Atto-Tec) and PKH26 (Invitrogen) before placing onto the sample. Samples were frozen immediately using the HPM100 (Leica) and rapidly transferred into liquid nitrogen for storage. Freeze substitution was performed in an EM AFS2 (Leica). Samples were incubated with 4.4% in 0.1% tannic acid solution and subsequently washed three times for 1 h in acetone. Before increasing the temperature from −90 °C to −20 °C (5 °C) samples were stained in acetone to remove the osmium tetroxide and warmed to room temperature (RT). Samples were infiltrated with 20% (vol/vol) methanol and 20% (vol/vol) propylene oxide and warmed to room temperature (RT). Samples were infiltrated with 20% (vol/vol) propylene oxide and 80% (vol/vol) methanol and subsequently washed three times for 1 h in acetone. Before increasing the temperature from −90 °C to −20 °C (5 °C), 2% (wt/vol) osmium tetroxide in acetone was added and incubated for 16 h at −20 °C. Finally, the temperature was raised to 4 °C (10 °C) and samples were washed in acetone to remove the osmium tetroxide and warmed to room temperature (RT). Samples were infiltrated and embedded in Epon resin. After thin embedding, 200-nm sections for electron tomography were obtained on an Ultrotom E (Leica) with a diamond knife (Diatome). Sections were applied to Formvar-coated copper mesh grids and posttained with 4% (wt/vol) uranyl acetate and Reynolds’s lead citrate.

Electron Tomography. To both sides of the stained grids, 10-nm gold particles (British Bio Cell) were applied. Single-tilt series were acquired using a JEOL JEM2100 transmission electron microscope at 200 kV from −60 to +60 with 1° increment, using the Serial-EM software. The tomograms were generated using the IMOD package etomo, and models were rendered using 3dmod (bio3d.colorado.edu/imod).

Quantitative image analysis was performed blinded, using the ImageJ software package as follows: The height of the ribbon was determined as the maximum extent from the presynaptic density in vertical sections. For ribbon-associated vesicles, the first row of vesicles with a maximal distance of 80 nm from the ribbon to the vesicle membrane was counted per tomogram. Membrane-proximal synaptic vesicles were defined as vesicles with the membrane-to-membrane distance to the AZ of maximal 50 nm. For tether length analysis, the coordinates of the tether starting (X1, Y1, Z1) and ending (X2, Y2, Z2) points were defined in vertical sections, using the ImageJ software package, and length was calculated according to Tether Length = √((X2 − X1)² + (Y2 − Y1)² + (Z2 − Z1)²) (see also SI Appendix, Fig. S6).

Patch-Clamp and Confocal Ca2+ Imaging in IHCs. p14–p15 IHCs from apical coils of freshly dissected organs of Corti were patch-clamped as described (41). The standard pipette solution contained (in mM) 130 Cs-glutamate, 13 TEA-Cl, 20 CsOH Hepes, 1 MgCl2, 2 MgATP, 0.3 NaGTP, 2 EGTA, 0.4 Fluo-SN (Penta-K+ salt; invitrogen), and carboxyfluorescein-carboxyfluorescein-conjugated RIBEYE-binding dimer peptide (2 μM [59]) for Ca2+ imaging. The pipette solution for perforated patch experiments contained (in mM) 135 Cs-gluconate, 10 TEA-Cl, 10 4-aminoypyridine, 10 CsOH Hepes, 1 MgCl2, and 300 μM amphotericin. The extracellular solution contained (in mM) 104 NaCl, 35 TEA-Cl, 2.8 KC1, 2 CaCl2, 1 MgCl2, 10 NaOH Hepes, 11.3 D-glucose at pH 7.3. Extracellular [Ca2+] was varied between nominally Ca2+-free to 5 mM for experiments manipulating the single Ca2+ channel current and was 10 mM for fluctuation analysis. In all cases, NaCl concentration was adjusted for osmolality of solutions with different CaCl2 concentrations. Currents were low-pass filtered at 2.9 kHz (8.5 kHz for fluctuation analysis) and sampled at 50 kHz (100 kHz for fluctuation analysis). Cells with holding current greater than −50 pA were discarded. An EPC-9 amplifier and ‘Pulse’ or an EPC-10 amplifier and a custom two-microelectrode system (HEKA Elektronik) were used for measurements. Whole-cell capacitance measurements were performed in perforated-patch configuration, as previously described (41), except that ΔCm was estimated as the difference of the mean Cm over 100 ms after the end of the depolarization (the initial 80 ms were skipped). All voltages were corrected for liquid-junction potentials and voltage drops across series resistance. Ca2+ currents were further isolated from background current, using a Pip protocol. Confocal Ca2+ imaging was performed essentially as described (37). In brief, presynaptic Ca2+-signal of IHCs were observed as changes of Ca2+ indicator fluorescence in line scans at the ribbon location, using (20 ms) step depolarizations to −7 mV.

Recordings of Auditory Brainstem Response and Distortion Product Otoacoustic Emissions. Animals aged 5–9 wk were anesthetized intraperitoneally with a combination of ketamine (125 mg/kg) and xilazine (2.5 mg/kg). The core temperature was maintained constant at 37 °C, using a rectal temperature-controlled heating blanket (Hugo Sachs Elektronik-Handarzehn Apparatus GmbH). For stimulus generation, presentation, and data acquisition, we used the TDT System III (Tucker-Davis-Technologies) run by BioSig32 software (Tucker-Davis-Technologies). Sound pressure levels are provided in dB SPL (toral stimuli) or dB SPL peak equivalent (PL, clicks) and were calibrated using a Bruel & Kjaer microphone (Type 4190). Two 500-Hz tone bursts (3-ms plateau, 1 ms rise/fall, stimulus rate 40 Hz) or clicks of 0.03 ms (stimulus rate 20 and 100 Hz) were presented in the free field ipsilaterally using a JBL 2402 speaker (JBL GmbH & Co.). The difference potential between vertex and mastoid subdermal needles was amplified (50,000 times), filtered (low pass, 4 kHz; high pass, 400 Hz) and sampled at a rate of 50 kHz. Two sound-pressure levels were used: 0 and 20 dB SPL. Two additional 10- or 20-s recording interval was added, respectively. Next, we determined the instantaneous rate-intensity function to the region outside of the 2D Gaussian, and subsequently sub-

Extracellular Recordings from Single. SGNs were performed as previously described (9, 46). In brief, mice aged 4–12 wk were anesthetized by i.p. injection of urethane (1.32 mg kg−1), xilazine (5 mg kg−1), and buprenorphine (0.1 mg kg−1). An occipital approach with partial removal of the cerebellum was taken to advance a glass microelectrode during noise burst stimulation, aiming to identify SGNs near the auditory nerve’s entry zone into the cochlear nucleus and avoiding the posterior region of the anteroverentral cochlear nucleus, where spherical bushy cells are mainly located. Putative SGNs were distinguished from primary cochlear nucleus units by their characteristic afferent nerve conduction velocity (8 ms entry zone into the cochlear nucleus) and their primary-like response characteristic to 200 suprathreshold 50-ms tone bursts presented at the characteristic frequency of the SGN, 30 dB above threshold. The irregular firing pattern observed in SGNs was confirmed by a coefficient of variation of interspike intervals of adapted responses greater than 0.5. We first determined the spontaneous rate in a 10-s interval, and if it was below 10 or 2 Hz, an additional 10- or 20-s recording interval was added, respectively. Next, we determined the tuning curve by varying the frequency and intensity of 15-ms tone bursts by an automatically adapting procedure (9). For tuning curves, spike detection was performed online based on a manually set trigger, whereas all other data were analyzed offline using custom-written Matlab routines using waveform-based spike detection. For rate-intensity function, we varied the intensity of 50-ms tone bursts at characteristic frequency (CF) in 5-dB steps between 20 dB below and 50 dB above threshold with 25 repeats for each intensity; the dynamic range was calculated as in ref. 46.

Data Analysis and Statistical Tests. Data are presented as mean ± SEM unless otherwise specified. Normality was assessed with the Jarque-Bera test. F test was used to assess equality of variance in normally distributed data sets. The unpaired, two-tailed Wilcoxon rank sum test (also known as Mann-Whitney U test) was used to compare data of nonnormal distribution or when vari-

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to compare two samples (*P < 0.05). Comparison of dispersion was performed with a modified Levine’s test [Brown-Forsythe test (60)], using median instead of mean for improved robustness under nonnormality. One-way ANOVA followed by Tukey’s test was used to detect differences in multiple comparisons for fluctuation analysis. Comparison of ABR thresholds between 6 and 32 kHz and of ABR latencies and amplitudes in response to click stimulation at 10–100 dB was done by two-way ANOVA.

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