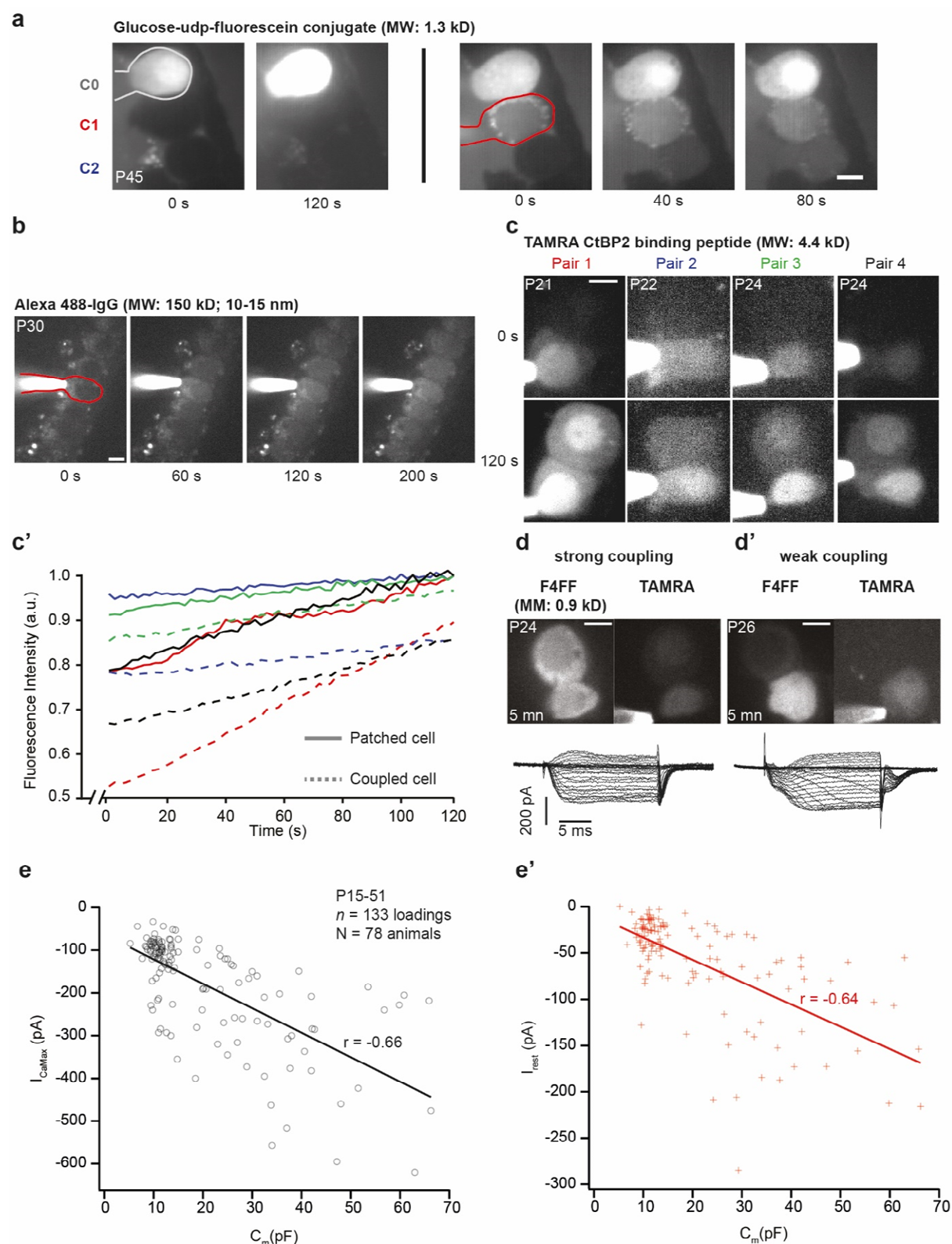
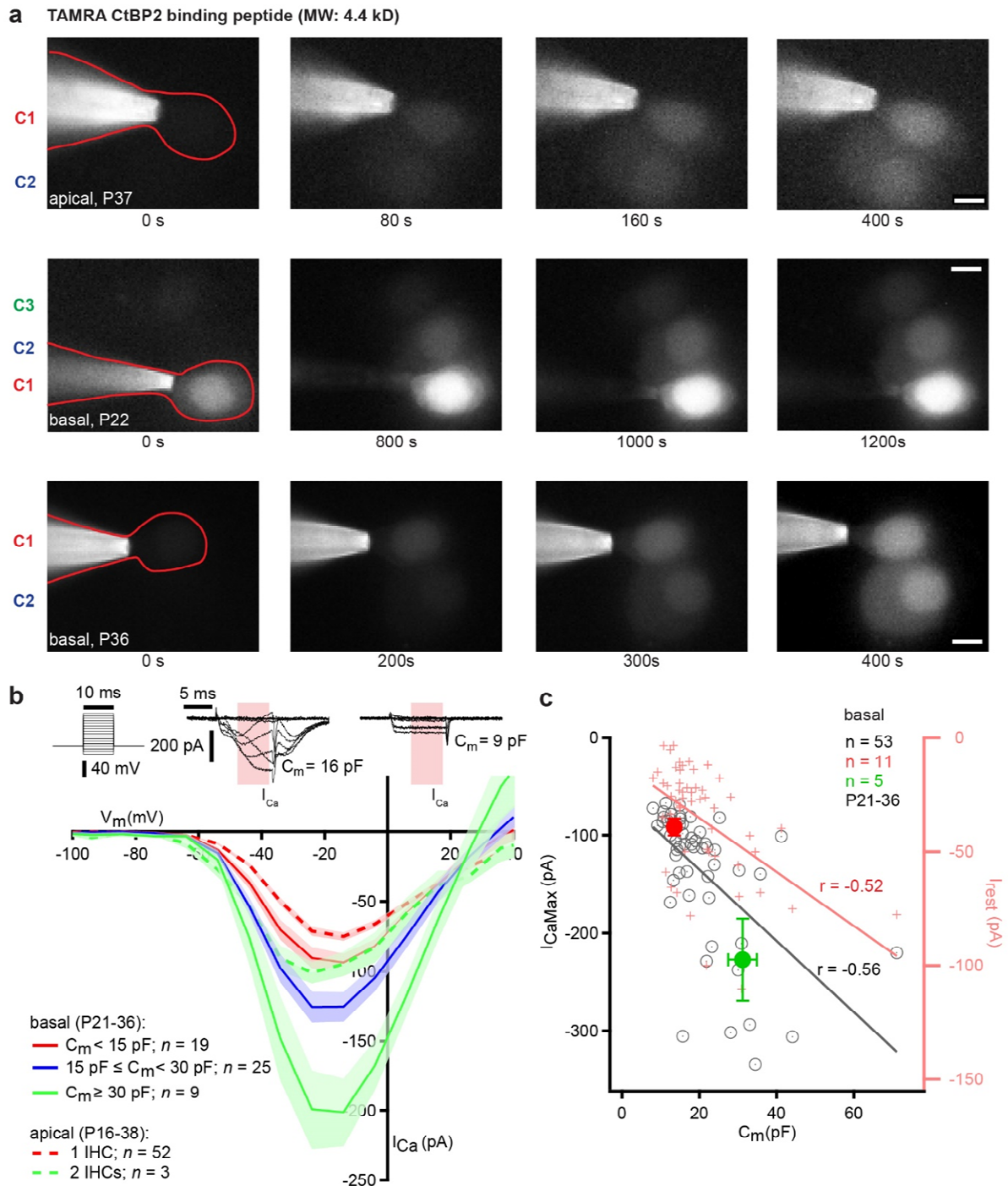


Supplementary figures



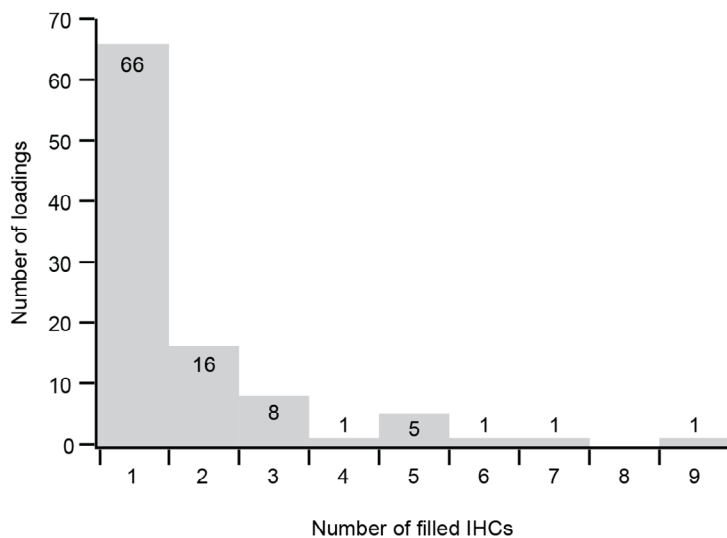
Supplementary figure 1. Different levels of molecular and electrical coupling exist between IHCs. (a) Time series of fluorescently labeled glucose loading in a single, non-coupled IHC (C0, no spread to other IHCs) and coupled IHC (C1) that shared glucose with the coupled IHC (C2). **(b)** Time series of the Alexa 488-IgG loading among 7 coupled IHCs. The patched IHC is delineated in red. **(c)** The pictures illustrate the TAMRA-peptide loading of four different pairs of IHCs. **(c')** Mean fluorescence intensities of the patched IHCs (solid line) and their coupled IHCs (dashed line) over time from the loadings in (b). The fluorescence is normalized to the maximal fluorescence of the

patched cell (line). **(d)** Example picture of a strong IHC-IHC coupling where the F4FF and TAMRA peptide are equally loaded in both coupled IHCs after 5 min, resulting in a monophasic activation of the whole-cell Ca^{2+} -current from depolarization steps. **(d')** The weak IHC-IHC coupling example shows much weaker intensities for F4FF and TAMRA in the non-patched cell, with a whole-cell Ca^{2+} -current exhibiting multiple phases of activation. **(e-e')** Scatter plot and line fits relating I_{CaMax} (e) and I_{rest} (e') currents from step depolarizations against C_m values across all age groups from ruptured-patch experiments in 1.3, 2 or 5 $[\text{Ca}^{2+}]_e$. **Abbreviations:** a. u., arbitrary units. **Scale bars:** 5 μm .

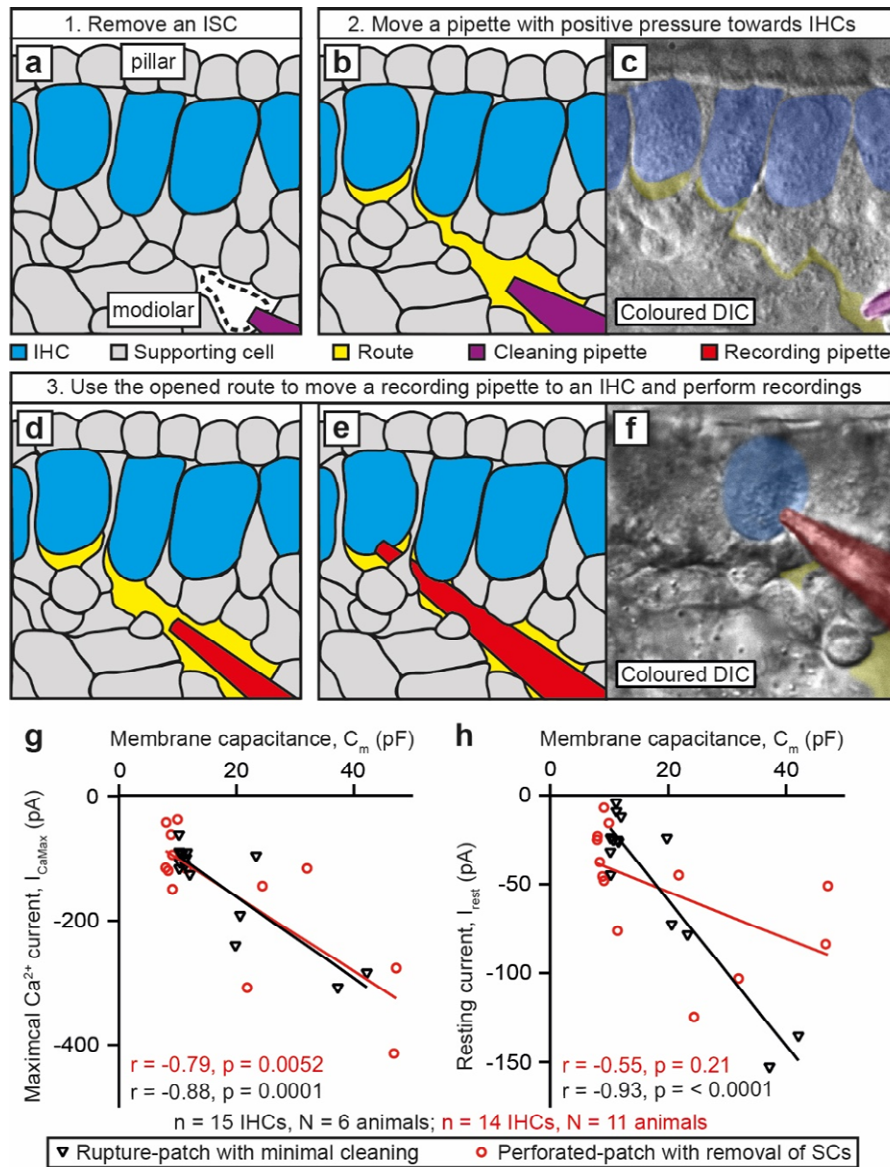


Supplementary figure 2. Macromolecular and electrical IHC-coupling in the high- and low-frequency cochlea of the gerbil. **(a)** Fluorescence images taken during loading of TAMRA-peptide through the patch pipette into the IHC. In addition to the patched cell (C1), one IHC (first row; C2) in the apical cochlea and two (second row; C2-3) IHCs or one (third row; C2) neighboring IHC in the basal cochlea were also loaded with the fluorescent dye, demonstrating a molecular coupling between

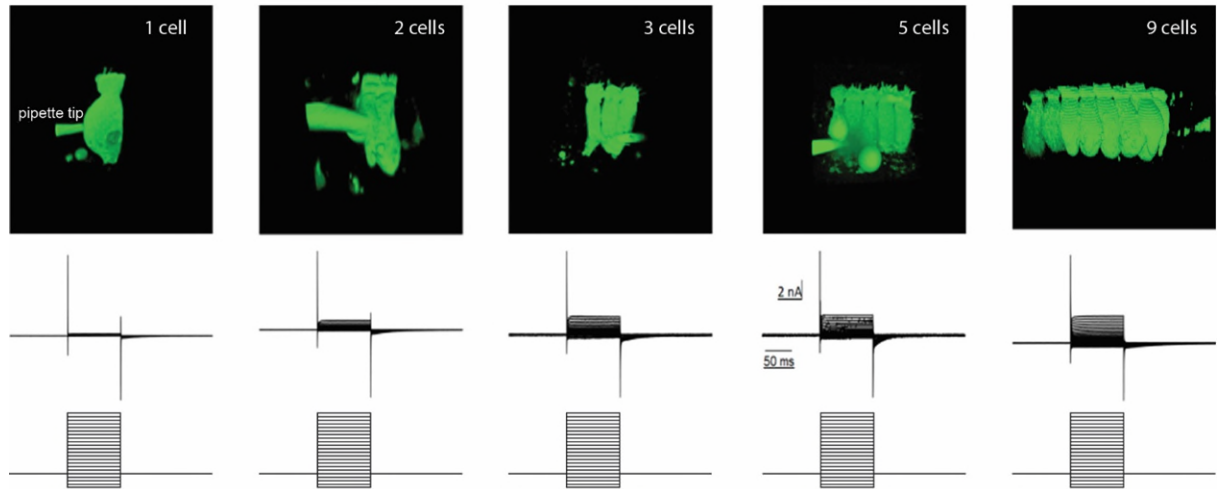
these cells. Note that in the right three panels of the second row, the pipette is slightly out of focus due to drift. In the basal cochlea, the mean C_m of non-coupled, single IHCs (as demonstrated by the lack of TAMRA-fluorescence spread) was 13.5 ± 0.7 pF ($n = 11$ loadings). When the dye loaded into 2 or 3 neighboring cells, the C_m read-out was 31.0 ± 4.0 pF ($n = 5$ loadings). In the apical part the C_m values for non-coupled IHCs were 14.3 ± 0.3 pF ($n = 52$ loadings) and 29.8 ± 4.6 pF ($n = 3$ loadings) for coupled IHCs. Values are shown as mean $\pm$ S.E.M. **(b)** Current-voltage relationship obtained by 10-ms long depolarizations of increasing voltages (10 mV-increments) from IHCs of either basal or apical turn ($N_{\text{animals}} = 42$ (basal turn recordings) and $N_{\text{animals}} = 17$ (apical turn recordings), respectively). Ca^{2+} currents were significantly larger in dye-filled coupled cells or cells with C_m values from 15-30 pF and above 30 pF (likely representing coupled cells) as compared to cells with C_m values smaller than 15 pF. In some cells with large C_m , the Ca^{2+} current activation displayed multiple time constants (middle insert), likely due to a delayed channel activation in the incompletely clamped neighboring cells. **(c)** The relation between the resting membrane capacitance C_m and the maximal depolarization-evoked Ca^{2+} current I_{CaMax} (gray circles, Pearson correlation coefficient: $r = -0.56$, $p < 0.0001$), as well as the resting current I_{rest} (red crosses, Pearson correlation coefficient: $r = -0.52$, $p < 0.0001$) in the basal cochlea ($n = 53$ basal cells from the panel b). Mean values for C_m and I_{CaMax} and their respective S.E.M. for single-dye-filled (red; $n = 11$) and coupled-dye-filled (green; $n = 5$) IHCs are presented as filled dots with error bars. Data was obtained across all age groups (P16-38) using ruptured patch experiments with 1.3 mM extracellular Ca^{2+} . *Abbreviations: IHC, inner hair cell Scale bars: 5 μm .*



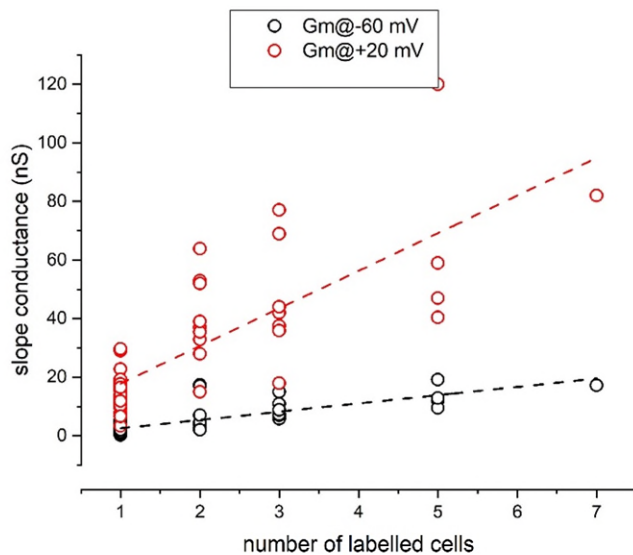
Supplementary figure 3: Distribution of dye-filled IHC numbers in the temporal bone preparation. Histogram displaying the number of dye-filled IHCs per loading at P26-100, with a prevalence for coupled cells of 33%. *Abbreviations: IHC, inner hair cell.*



Supplementary figure 4. IHC coupling can be observed with least invasive recording approaches. Figures **a-f** illustrate the ruptured-patch of IHCs with minimal cleaning. (**a**) After removing a single inner sulcus cell, (**b**) a cleaning pipette with applied positive pressure is gently moved in between SCs so that a tunnel-like route that reaches IHCs is formed. (**c**) Coloured DIC image of a formed route. (**d-e**) By carefully moving a recording pipette through the route to an IHC, ruptured-patch recordings can be performed. (**f**) Coloured DIC image of a successful ruptured-patch. (**g-h**) In addition to typical IHCs with membrane capacitance (C_m) ~ 9 pF, IHCs with atypically high Ca^{2+} currents (I_{CaMax} in **g**), resting membrane currents (I_{rest} in **h**), and C_m can be observed in ruptured-patch recordings with minimal cleaning (black triangle, $n = 5$ out of 15 IHCs; $N_{\text{animals}} = 6$, P16-P24) and with perforated-patch recordings with removal of SCs (red circle, $n = 5$ out of 14 IHCs, $N_{\text{animals}} = 11$, P15-P36). Linear fits are displayed (Pearson correlation coefficient for ruptured-patch with minimal cleaning: I_{CaMax} : $r = -0.88$, $p = 0.0001$, I_{rest} : $r = -0.93$, $p < 0.0001$; for perforated-patch with minimal cleaning: I_{CaMax} : $r = -0.79$, $p = 0.0052$, I_{rest} : $r = -0.55$, $p < 0.21$). *Abbreviations:* DIC, differential interference contrast, IHC, inner hair cell; ISC, inner sulcus cell; SC, supporting cell.

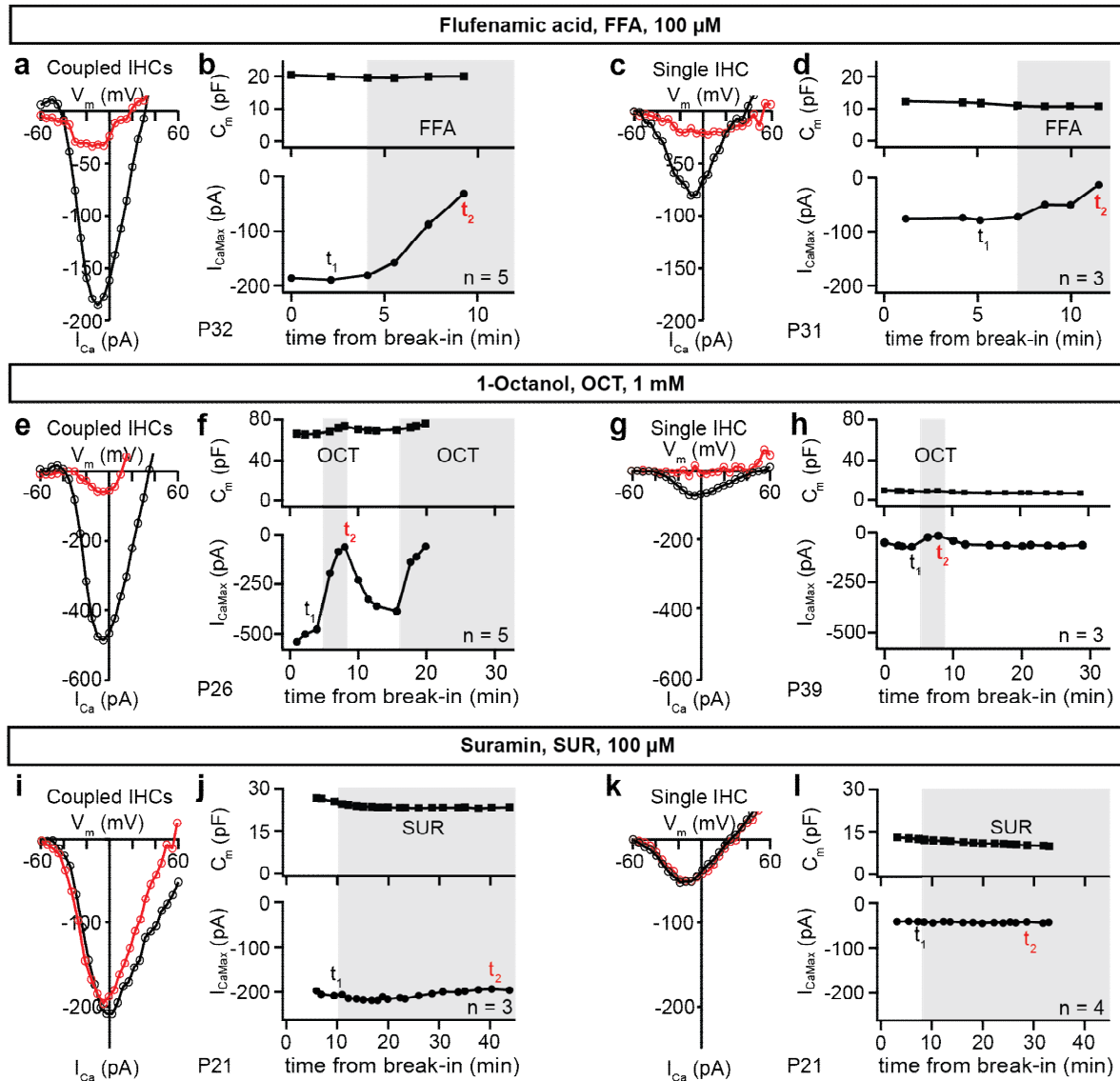


Supplementary figure 5. Cesium outward currents increase with the number of dye-coupled IHCs. Analyzing dye and electrical IHC coupling in temporal bone preparation, using 2PLSM imaging of OGB5N-loaded IHCs (top panel) and IHC recordings of Cs^+ -mediated outward currents (middle panel) in response to 100 ms long depolarizations (in 10 mV increments, bottom panel) from a holding potential of -60 mV. As shown for a sample of $n = 56$ distinct experiments and quantified in Fig. S6, the maximal Cs^+ current increases with the number of dye-filled IHCs, indicating a partial summation over the currents of the coupled IHCs. We note that the recordings underestimate the true currents due to voltage drops over the series resistance to the patched IHC and the junctional resistance among the coupled cells.

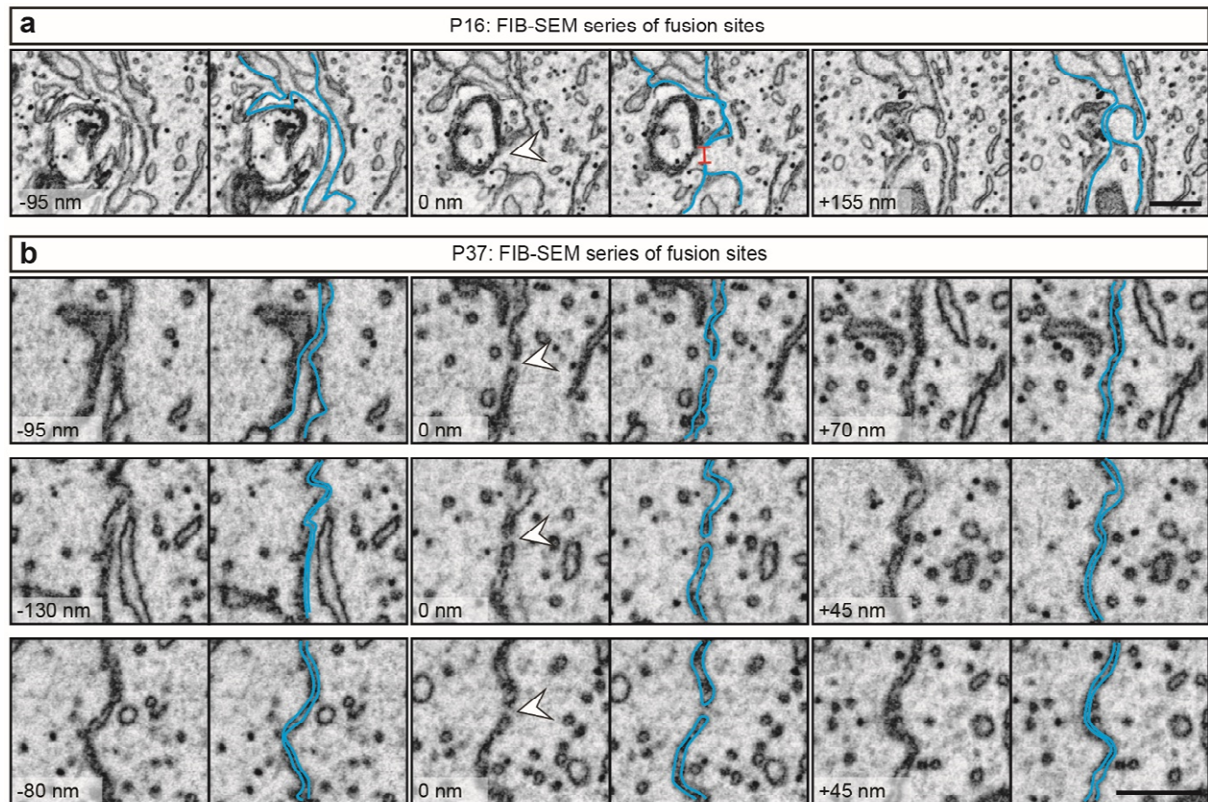


Supplementary figure 6. IHC membrane conductances increase with the number of dye-coupled IHCs. Membrane slope conductances (G_m) at the holding potential -60 mV and at +20 mV from I-Vs for positively identified uncoupled and coupled cells. All the data was collected within 60 s of whole cell break-in with Cs^+ as the major pipette cation. The membrane slope conductance at -60 mV, increasing with the number of coupled cells, is a good estimator of the effect of coupling. In this sample, using internal Cs^+ , $G_m = 2.05 \pm 1.25$ nS (mean $\pm$ SD, $n = 25$ uncoupled cells) at -60 mV. Preliminary data using K^+ as the major pipette cation did not permit the sufficient voltage-clamp control to estimate G_m at +20 mV. Dashed lines show regression fits, with slopes significantly

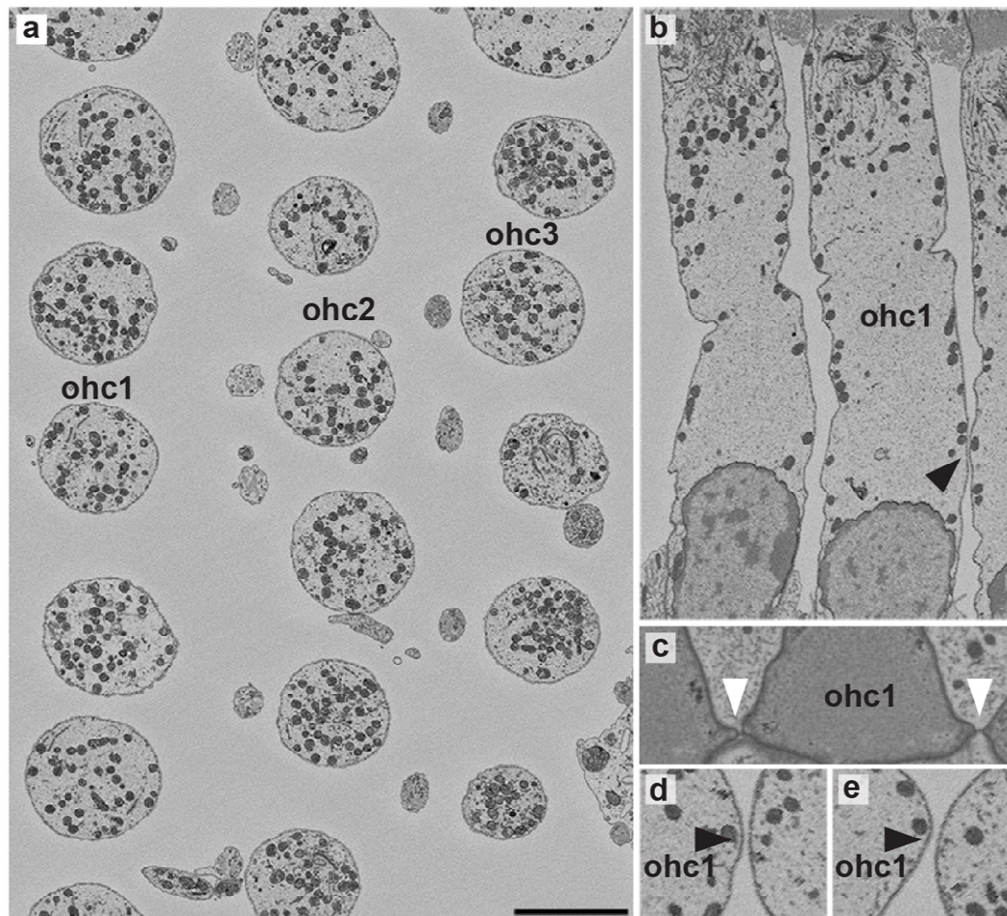
different from 0 nS/#cell (Pearson correlation coefficient: $r = -0.78$ @ -60mV and -0.77 @ $+20\text{mV}$, $p < 0.05$).



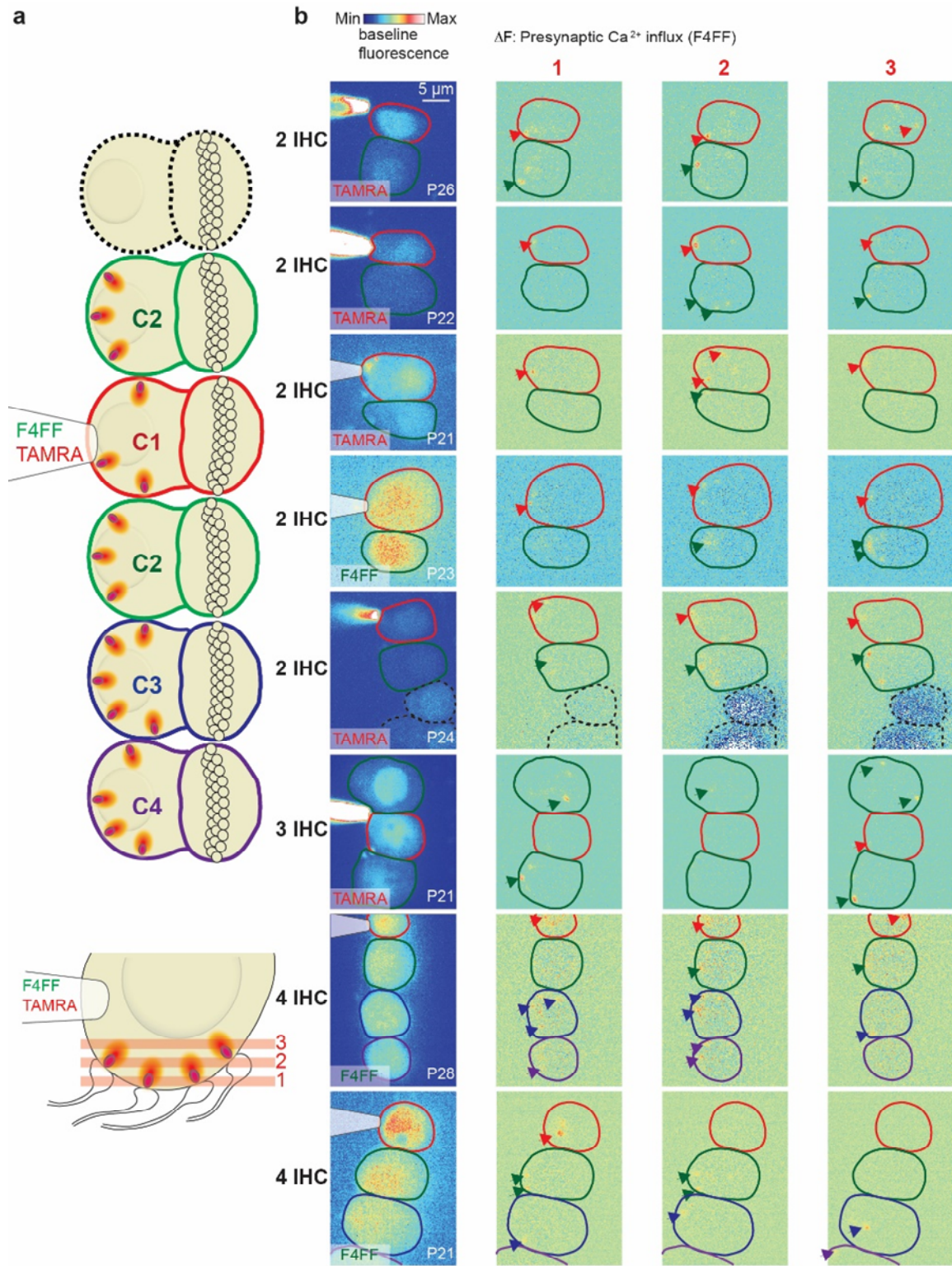
Supplementary figure 7. Electrical IHC-coupling is not prevented by GJ blockers and suramin. Ca^{2+} -current voltage (IV) curves (a, c, e, g, i, k) were obtained by depolarizing IHCs from the holding voltage (-60mV) with 5mV steps before and during bath application of blockers. The presence of the blockers is marked with gray in the membrane capacitance (C_m)-time and maximal I_{Ca} (I_{CaMax})-time plots (b, d, f, h, j, l). The recording time points of the presented IV curves are marked with t_1 (for black curve) and t_2 (for red curve) in the I_{CaMax} -time plots. The experiments were stopped when the patched IHC was lost. **(a-h)** FFA (a, b) and OCT (e, f) inhibit I_{Ca} of coupled IHCs without changing C_m , as seen in these representative experiments. As similar results were obtained with uncoupled IHCs (c, d, g, h), the observed reduction of I_{Ca} is not due to inhibition of IHC-coupling, but potentially due to the blockade of IHC Ca^{2+} channels. **(i-l)** As can be seen from the I_{Ca} - V_m curves, C_m -time, and I_{CaMax} -time plots of representative experiments, suramin does not have an obvious effect on C_m or I_{CaMax} of coupled (i, j) and single IHCs (k, l). *Abbreviations:* IHC, inner hair cell, FFA, flufenamic acid; OCT, 1-octanol, SUR, suramin.



Supplementary figure 8. FIB-SEM datasets containing putative fusion sites. Depicted are series of putative IHC fusion sites occurring in P16 (**a**) and P37 (**b**) IHCs. Single FIB-SEM micrographs show the opposing IHC membranes (highlighted in blue) forming contacts (P16, from a filopodium; P37, from flat contacts) that contain IHC fusion sites (white arrowheads). n FIB-SEM run (P34/37) = 3, $N_{\text{animal}} = 2$; n FIB-SEM run (P15/16) = 3, $N_{\text{animal}} = 2$. For each age group 2 independent embeddings were performed. *Abbreviations:* IHC, inner hair cell. *Scale bar* = 500 nm.



Supplementary figure 9. No direct contacts between P22 OHCs. (a-b) Oblique slices through a serial block-face electron microscopic dataset show only rare occasions of closely apposed basolateral membranes of OHCs (black arrowhead). (c) OHCs of the first row are closely apposed near their apical junctions and (d,e) occasionally at their basolateral membranes (black arrowheads). No direct OHC-OHC contacts were observed in two independent datasets. *Abbreviations:* OHC, outer hair cell *Scale bar* (depicted in a) = 5 μm in a,b; 3.3 μm in c; 2.6 μm in d,e.



Supplementary figure 10. Collective synaptic activity in IHC mini-synctia.

a) Coupled IHCs (delineated in color) were patch-clamped and loaded with the calcium indicator Fluo-4FF (F4FF) and the TAMRA-fluorescent CtBP2-binding peptide. C1 (red) is the patched IHC, C2 (green) are coupled IHCs neighbouring C1, C3 (blue) is coupled to C2 and C4 (violet). Red lines show the imaging planes at bottom (1), middle (2) and top sections (3) of the synaptic IHC pole. **(b)** Left column: basal fluorescence indicating the cells in either the red (TAMRA peptide) or green (F4FF) channels. The pipette was drawn in the case it was not in the imaging plane. Right columns: $\Delta F/F_0$ images from single planes showing localized Ca^{2+} signals (arrowheads) in the coupled IHCs. Non-coupled IHCs that have been previously patched are delineated in dashed lines.

Supplementary Note 1

Encoding of auditory signals at IHC-SGN synapses entails 1) mechanoelectrical transduction at the tips of the stereocilia generating the receptor potential and 2) the coupling of the receptor potential to glutamate release (stimulus-secretion-coupling) at the ribbon synapses. Both processes involve relatively small numbers of signaling proteins: around 40 mechanotransducer channels per hair cell (13) and around 10 release sites per synapse (14). So, although neighboring IHCs receive virtually identical mechanical stimuli, because basilar membrane tuning is much wider than an IHC (see Supplementary Text 2), each IHC is subject to different realizations of stochastic mechanotransduction currents and hence they show different membrane voltage trajectories. As a consequence, signal encoding precision will be limited by the count statistics of the stochastically operating elements. The low-conductance electric coupling between the IHCs in a mini-syncytium causes voltage averaging, which increases the signal to noise ratio. This can be shown by computational modeling two scenarios in the Neuron simulation environment (15), three non-coupled IHCs (Fig. 8, left panel) and a mini-syncytium of three IHCs (Fig. 8, right panel), during weak stimulation driving the opening of on average 2.7 of the 40 mechanotransducer channels in an IHC (Fig. 8). Note how weakly the mechanotransducer activity is correlated and how correlations downstream of mechanotransduction are increased by IHC-coupling. Ca^{2+} channel gating seems more representative of the input in coupled than in non-coupled IHCs, demonstrated by the tendency toward higher correlation between mechanical input and Ca^{2+} channel open probability (Pearson correlation coefficient: 0.88 for coupled IHCs vs. 0.82 for non-coupled IHCs).

A simple estimation shows how coupling improves the signal to noise ratio. Assuming unmodulated weak input, the number of open mechanotransducer channels would fluctuate around an average value, e.g. four out of the 40 per IHC. At any time, the actual number of open channels is random, following a binomial distribution $\mathcal{B}(N = 40, p = 0.1)$ with a standard deviation of $SD = \sqrt{N \cdot p \cdot (1 - p)} = 1.90$. In the extreme case of three completely fused cells, a common membrane voltage would be controlled by all 120 mechanotransduction channels. The open channel count would follow $\mathcal{B}(N = 120, p = 0.1)$, with 12 channels open on average and an SD of 3.29. Due to the coupling, the total number of mechanotransducer channels controlling the syncytium's membrane

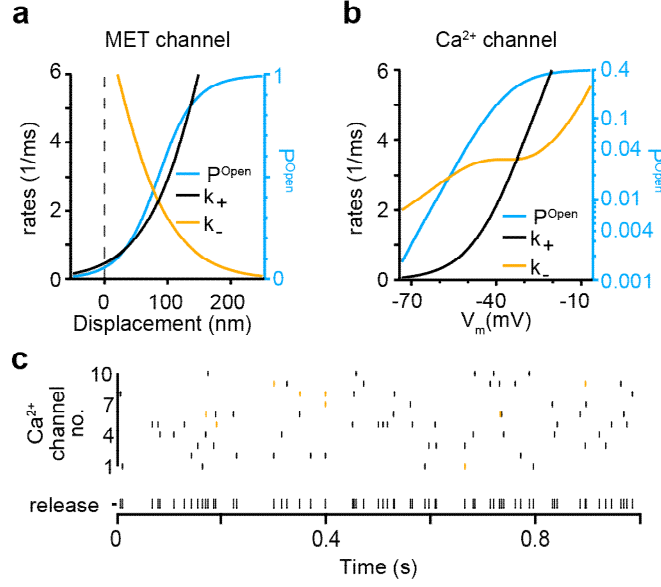
voltage becomes less noisy, the ratio between standard deviation and average drops from 0.47 to 0.27. The improved signal to noise ratio carries over to the fluctuations in the membrane voltage, and Ca^{2+} channel opening. Therefore, the timing of vesicle release would be more synchronized across the synapses of three coupled IHCs as compared to synapses of neighboring but non-coupled IHCs. We determined the magnitude of this effect by simulation of mechanotransduction and Ca^{2+} channel gating in non-coupled and coupled IHCs using stochastic ion channel models in the Neuron simulation environment (all code is published on the Neuron database).

Model layout

The simulation comprises of several IHCs, which are either isolated from each other or coupled by fusion sites. The passive properties of the IHC and the connections follow standard properties: the specific membrane capacitance, $c_m = 10 \text{ fF}/\mu\text{m}^2$; intracellular resistivity $r_i = 120 \text{ Ohm}\cdot\text{cm}$ and the specific membrane resistance, $r_m = 250 \text{ Ohm}\cdot\text{cm}^2$ of the IHCs is chosen to obtain an effective membrane time constant of $250 \mu\text{s}$ (16). This basal conductance subsumes all channels that are open at rest. IHC-IHC fusion sites are approximated by 100 nm long, 78 nm wide tubes. This size is chosen to approximate a junctional resistance, R_j of 25 MOhm. For the numerical simulations, a cell is represented by 11 segments, a joint by 3 segments. Joints on different sides of an IHC are longitudinally offset so that they do not connect to the same segment. Stochastic gating of mechanotransducer channels and $\text{Ca}_v1.3$ voltage dependent Ca^{2+} channels, are simulated with a variant of the Gillespie-Algorithm (17) that was adapted to work with the fixed time step of neuron simulations. To obtain statistics over many realizations of the $\text{Ca}_v1.3$ gating, a two-step process is used. During the first run, the mechanotransducer channels are driven by the mechanical stimulus and gate stochastically. The number of open mechanotransducer channels is recorded at every time step. During the next thousands of runs, those open channel counts are played back while the gating of $\text{Ca}_v1.3$ follows independent stochastic realizations. The output of these simulations, i.e. $\text{Ca}_v1.3$ open events, are further processed to obtain release events and AP times, as described below.

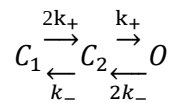
Mechanotransducer channels and voltage-gated Ca^{2+} channels

Forty mechanotransducer channels mediate mechanosensitive influx of cations (mostly potassium) at the top end of each cell. They are driven by an external sinusoidal stimulus representing mechanical displacement in units of nanometers. A mechanotransducer channel has two states (O, C), the speed and mechano-dependence of the transition are chosen to obtain properties as described in experimental studies (13, 18), i.e. sub-millisecond opening and closing transitions and an open probability that increases sigmoidally with the displacement d . The range of displacement for which the open probability changes, is over-estimated in experiments using glass probes and it is under-estimated when using the fluid jet to stimulate (13). Therefore, the value used here lies in between those experimental estimates. The opening and closing rates in ms^{-1} are $k_+ = k_{\max} / \left(1 + \exp\left((s_{1/2}^o - d)/k_o\right)\right)$; $k_- = k_{\max} / \left(1 + \exp\left((d - s_{1/2}^c)/k_c\right)\right)$ given a displacement d . The parameters are $k_{\max} = 16.9 \text{ ms}^{-1}$; $s_{1/2}^o = 179 \text{ nm}$; $s_{1/2}^c = -10 \text{ nm}$; $k_o = 50.6 \text{ nm}^{-1}$; $k_c = 51.4 \text{ nm}^{-1}$. Rates and open probability are visualized in Fig. S11 We aimed to use a realistic model that closely reproduces experimental results, even though the exact details of the mechanotransducer model are in no way crucial to the outcome of the simulation. The key aspect, the stochasticity of the voltage trajectory of individual cells, results from two main determinants: the low overall number of mechanotransducer channels and their sub-millisecond gating kinetics. Both parameters are experimentally documented in multiple studies.



Supplementary figure 11: Implementation of ion channels and release event pruning. (a) The model for mechanotransducer channels is designed to account for published experimental result. (b) Ca_v1.3 channels are implemented as described earlier (19), for details see text. (c) Approximate times of release events were determined by long Ca_v1.3 channel openings (600 μs and longer), for which Ca²⁺ influx is expected to be sufficient to trigger release in the Ca²⁺ nanodomain-like scenario (19). The top ten rows show time stamps at which an opening of the respective channel (#1 to 10) exceeded a duration of 600 μs. All ten channels are from the same cell, this is, all experienced the same voltage time course. Note that only a small fraction of openings (3%) last longer than this the 600 μs cut-off. When all events from the ten channels are combined to approximate release events and then postsynaptic action potentials at the IHC ribbon synapse, nine out of 71 events are pruned (orange) when post-synaptic refractoriness is considered. The postsynaptic spike rate for this weak stimulus is on average 64 action potentials/s.

The Ca_v1.3 Ca²⁺ channel gating parameters are obtained from experiments (19–21), derived as described before. In short, the channel has two closed and one open state with the following transitions:



with opening and closing rates in ms⁻¹:

$$k_+ = \frac{\exp(-b \cdot V)}{a} \sqrt{p_{max} / \left(1 + \exp\left((V_{1/2} - V)/w\right)\right)} ; k_- = \frac{\exp(-b \cdot V)}{a} - k_+$$

with $a = 0.2565/3^{(T-23^\circ C)/10K}$ ms; $b = -0.0295$ mV⁻¹; $p_{max} = 0.4$; $V_{1/2} = -36.2$ mV ; $w = 6.8$ mV; $T = 37^\circ C$. Rates and open probability are visualized in Fig. S11b).

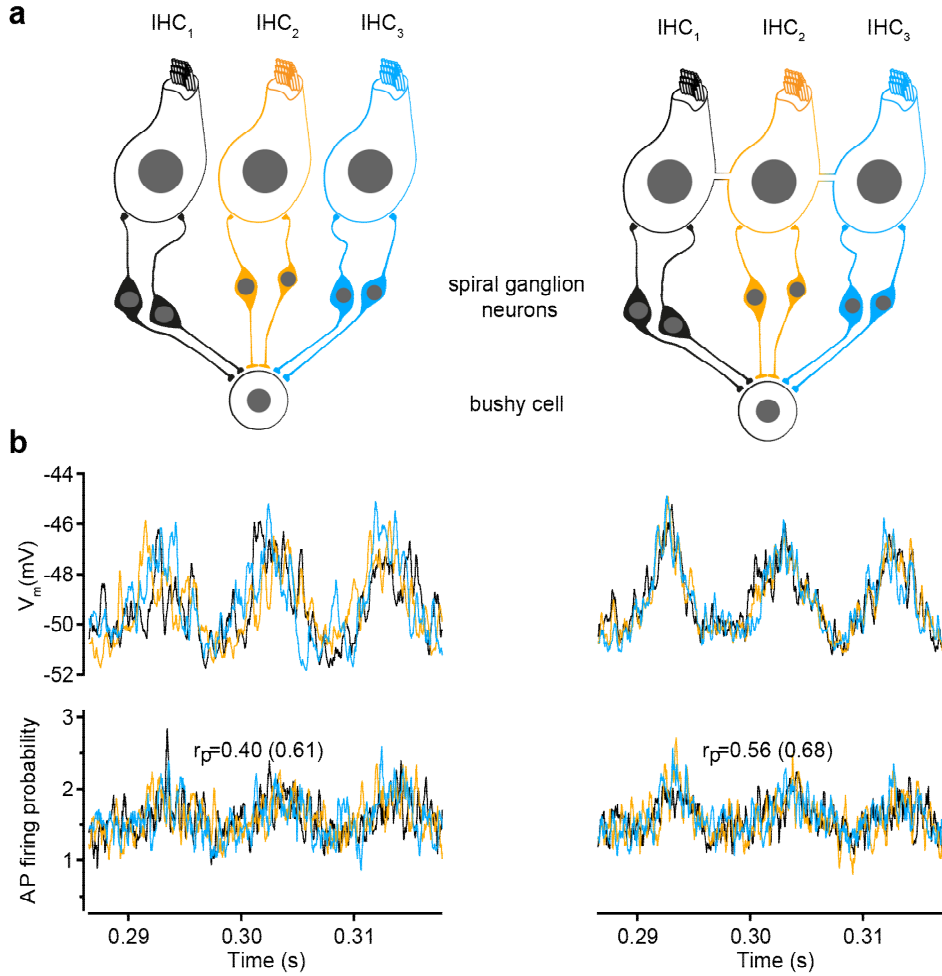
Stimulus-secretion coupling

Ca^{2+} enters through open Ca^{2+} channels and binds to the fusion machinery of a nearby release-competent synaptic vesicle (SV), triggering its fusion. In mature IHCs, this stimulus-secretion coupling operates mostly in the Ca^{2+} nanodomain-like regime (19, 22, 23), i.e. the opening of one or very few Ca^{2+} channels controls the fusion of a given vesicle. These processes are not explicitly modelled. Instead, we use the functional properties of Ca^{2+} nanodomain signaling and approximate the occurrence of release events with the occurrence of long Ca^{2+} channel openings. Channel open times beyond a certain cut-off time are considered effective to trigger fusion of the nearby vesicle. The exact duration of this cut-off had no influence on the outcome. This was established using cut-off values between 100 microseconds and 800 microseconds. For cut-offs beyond 800 microseconds the collection of a sufficient number of events was beyond the available processor time. The results below are derived with a cut-off of 600 microseconds.

We previously estimated the number of independent vesicle release sites per IHC ribbon synapse to be around 10, based on electron microscopy studies and quantitative analysis of release rate dynamics in response to sound stimulation (4, 14). Therefore, 10 Ca^{2+} -channels were considered to simulate the 10 Ca^{2+} channel-release units of a synapse. Release events occurring at any of those 10 units were considered as inputs to a single postsynaptic SGN. In mammals, most SGNs receive input from only one ribbon synapse. Release events occurring in close succession were pruned by a refractory process with an absolute refractory period of 0.6 ms and a relative refractory period of 0.65 ms as we used earlier (24, 25), based on our experimental estimates (26). In this way, 10 independent trials of single $\text{Ca}_v1.3$ channels yield one realization of an action potential sequence from a synapse (Fig. S11c. In order to obtain not only a single realization, but rather an approximation of the instantaneous rate of action potentials under the given realization of mechanotransduction, we simulated 35,000 trials equaling to 3,500 synapses (Fig. 8g).

For all simulations in this study, we restrict ourselves to weak stimuli, i.e. causing low release rates. Therefore, the release sites are rarely depleted and the dynamics of release site refilling is of little influence. Hence, to simplify the model, we ignored the time required to refill the release site. We feel

this is further justified, because the improved signal to noise in the syncytium is driven by averaging of the mechanotransducer currents, i.e. events upstream of vesicle release.



Supplementary figure 12: Coupling of three IHCs increases the correlations between action potential firing by SGNs. (b) In the first configuration of the model, six SGNs relay auditory information from three IHCs to a bushy cell, which fires only if sufficiently coincident input arrives from the SGNs. **(b)** A short section of membrane voltage and action potential frequency (average event rate within 200 μ s, across 4,500 synapses) are shown for uncoupled (left) and coupled IHCs with a junctional resistance of 15 MOhm (right) - compare to the dataset for 25 MOhm in Fig. 8. Note how the coupling improves correlations between the action potential rate for SGNs originating at neighboring IHCs as well as the correlation between the mechanical stimulus and the action potential rate (in parentheses).

In summary, our model includes a deterministic sinusoidal stimulus that controls stochastic mechanotransducer channels. The resulting current drives excursions of the membrane voltage, which in turn controls Ca^{2+} channel gating and long single Ca^{2+} channel openings are considered to trigger release events. Pruned by refractoriness, release events translate to action potentials in spiral ganglion neurons. The simulation shows that coupling through 25 MOhm junctions strongly increases the

correlations between the auditory input and the voltage in the coupled IHCs and also the correlations the release events (Fig. 8).

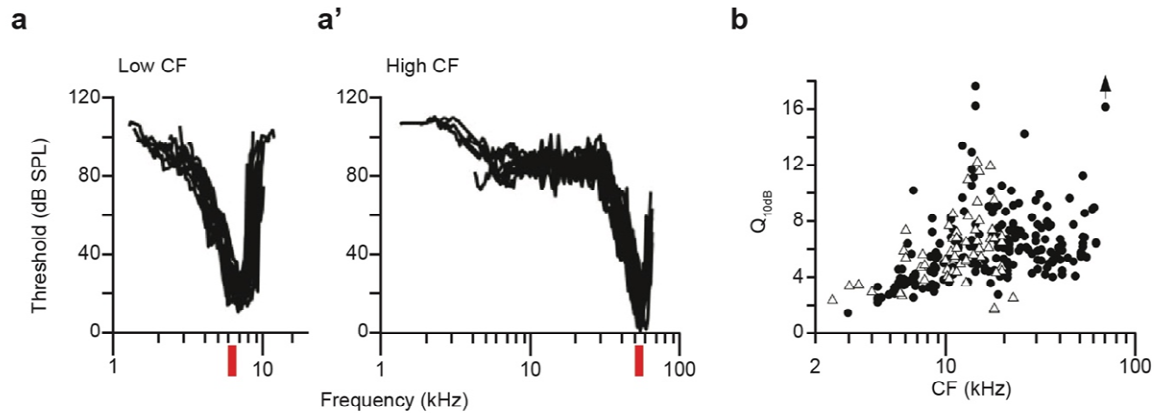
Coincidence detection

Although the functional benefit of coupling is clear from the increased correlation between IHC input and SGN output, we wanted to take the analysis a step further in the auditory pathway. In the cochlear nucleus multiple SGNs contact each bushy cell, which only fires, if a sufficient number of fibers is firing coincidently within a narrow time window. For example, bushy cells in the anteroventral cochlear nucleus fire an action potential, when a number of SGNs fire action potentials within a short coincidence window and, as a result, show improved precision of spike timing when compared to the spiral ganglion neurons (27, 28). Each bushy cells receive multiple inputs of SGNs with similar characteristic frequency and spontaneous firing rate (29), which allows us to use one parameter set to simulate such a set of incoming action potential times. The number of coincidences required depends on the bushy cell type and species. It is considered to range from a few fibres up to 20 or even more. The coincidence window has no fixed width, it depends on the activity status, i.e. the depolarization history of the bushy cell (30). Typical bushy cell membrane time constants and spike jitter (28) suggest coincidence window times of 50 to 200 μ s. We find that different assumptions about this coincidence window had no impact on the outcome of the model (as described below) at the cochlear nucleus (CN) because the bandwidth of signals from the IHC is already restricted by the IHC membrane time constant and the rise time of the Ca^{2+} currents.

The probability of two coincident action potentials from two SGNs within a 200 μ s coincidence window simply corresponds to the product of the individual firing probabilities (Fig. S12). The average value of this product depends on the origin of the signals of the SGNs. If they received input from two synapses of the same IHC, coincidences are slightly more likely. To our knowledge, there is no information yet about the precise connectivity between IHCs and bushy cells. We studied two different configurations in which six SGNs converge onto a bushy cell, where the coincidences are detected. In the first configuration, three IHCs provide two SGN inputs each. In the second configuration, each of the six SGNs originates at a different IHC. In each case, we studied two

possibilities: either all three/six IHCs are non-coupled or all three/six IHCs are coupled with each other.

For relatively weak stimuli (25 nm deflections), which one could consider close to threshold, we find typically that IHC coupling causes a subtle increase of around 2% in the coincidence rate for the three IHCs configuration and of 3% for the six IHC configuration. Interestingly, at very small mechanical stimuli, 2 nm sinusoidal deflection, when the correlation between the stimulus and the mechanotransducer signal drops below 0.06, the coupling between IHC does not cause an increase, but rather a small (1%) decrease in the coincidence rate. When the mechanotransducer channels open mostly unrelated to the stimulus, the averaging across coupled IHCs reduces the impact of the spurious channel openings on the coincidence rate. Together, the slight reduction at subthreshold stimuli and the slight increase at weak, supra-threshold inputs, serves to suppress thermal noise of the mechanotransduction by a slight increase of the detection threshold, i.e. the stimulus intensity, at which the coincidence rate crosses a certain threshold. This small effect size of a few percent, resulting from our simulations, is not unexpected given the identical mechanical input to the neighboring IHCs. It can be expected to increase, if basolateral K^+ channels were added to the simulation of IHC conductances. These adaptation currents would provide a high-pass filter that reduces the average voltage and thereby increases the ratio between voltage modulation and signal average. The simulations presented here are kept deliberately simple to illustrate the essence of the coupling effect: a reduction in the noise contributions from the limited number of mechanotransducer channels.



Supplementary figure 13. Frequency resolution of sound coding by auditory nerve fibers. (a-a') Tuning curves of high spontaneous, low threshold auditory nerve fibers recorded from CBA/J mice taken from (31) and modified: **a**, auditory nerve fibers with relatively low characteristic frequency (CF) of around 7 kHz; **a'**, auditory nerve fibers with relatively high characteristic frequency (CF) of around 50-60 kHz. Red boxes indicate the frequency range covered by the largest observed mini-syncytium (9 IHCs: 83.7 μ m, approximately 1.29% of the length of the organ of Corti or 0.06 octaves). **(b)** Quality of frequency tuning assessed as Q_{10dB} (see below) of auditory nerve fibers recorded from CBA/J (circles) and C57Bl/6 (triangles) mice taken (31).

Supplementary note 2

Does IHC-coupling degrade the frequency resolution of cochlear sound coding?

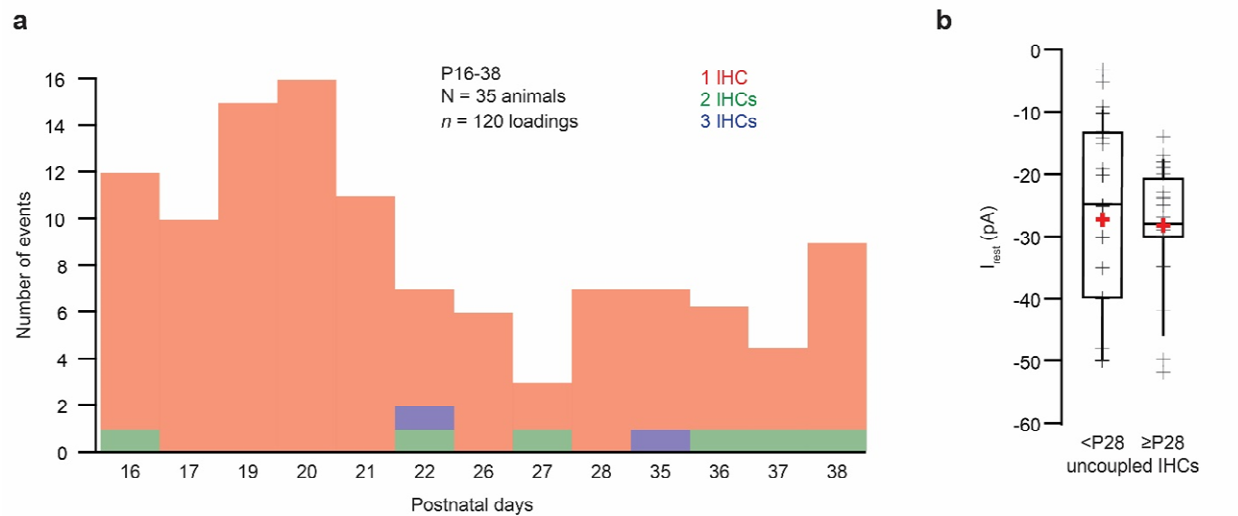
IHC coupling with large-conductance intercellular coupling would be expected to degrade frequency resolution of sound coding if it was not limited to within the mini-syncytia that are embedded in the row of IHCs of which most (70%) are non-coupled. In our data set on P30-45 mice, the average number of cells per syncytium was 3 (3.05 ± 0.17 (S.D. = 1.53)). The mouse cochlea (with differences between strains and methods) is approximately 6-7 mm long (32–34), covers approximately 5 octaves (35) and contains 660-760 IHCs (32, 33), each of them approximately 9 μ m in diameter. Here, assuming the above parameters being 6.5 mm, 5 octaves and 700 IHCs (i.e. 9.3 μ m per IHC) we calculate that the average mini-syncytium (27.9 μ m) covers 0.43% of the length or 0.02 octaves. In the most extreme case observed, i.e. the largest block (9 IHCs, 83.7 μ m) would cover approximately 1.29% of the length or 0.06 octaves.

Next, we compared those estimates to the physiological estimates of frequency resolution at the level of the basilar membrane and auditory nerve fibers. For the basilar membrane, the Q_{10} dB (characteristic frequency of basilar membrane displacement at threshold, i.e. the frequency for which the least sound pressure is required to elicit the displacement, divided by the bandwidth at 10 dB above threshold) amounts to 8.7 ± 4.3 in the high frequency range of the cochlea of CBA/J mice (50-56 kHz,

(36)). This reflects a bandwidth at the level of the basilar membrane of approximately 5.75 kHz at 50 kHz or 0.17 octaves, approximately 3-times larger than the frequency range that is represented by the largest observed syncytium (0.06 octaves).

The quality of frequency tuning of auditory nerve fibers quantified as Q10dB (ratio of the characteristic frequency at threshold, i.e. the frequency for which the least sound pressure is required to increase the firing rate, and the frequency bandwidth of the tuning curve 10 dB above threshold) typically varies between 2 and 12 in the mouse cochlea (26, 31), Fig. S13). In the characteristic frequency range from 8 kHz to 16 kHz, for which we directly demonstrated IHC-coupling in young C57Bl/6J mice, the average Q10 dB was approximately 4 (26, 31) Fig. S13). For 12 kHz this reflects a bandwidth at the level of the nerve of 3000 Hz, or 0.37 octave approximately 6-times larger than represented by the broadest observed syncytium (0.06 octaves).

For a comparison to psychophysical estimates of frequency resolution, we relied on human data (37) which states that humans can discriminate down to 7 cents of a musical semitone (cent: percentage of a semitone). We also used a model of the human cochlea (38) with a cochlear length of 33 mm containing 3500 IHCs of approximately 9 μm per IHC. The mid-cochlear quarter of a turn (263-407 Hz) should then hold approximately 245 IHCs. Making a linear approximation of the frequency distribution within the mid-cochlear quarter of a turn we calculated that there are approximately 30 IHCs per a musical semitone (ST). This means each IHC “would cover” approximately 3 cents on the tonotopic map. Now, let us assume a similar situation of IHC coupling for the human cochlea as we observed experimentally in the mouse cochlea. Then, even for the case that the entire cochlea was tiled by mini-syncytia of the average size of 3 IHCs reported here, this would indicate a best possible frequency resolution of 9 cents, which is close to the lowest psychophysical estimates (7 cent, (37)). Therefore, we conclude that low prevalence and small size of mini-syncytia avoids trading off frequency resolution against detection sensitivity.



Supplementary figure 14: Coupling probability and resting currents as a function of age in gerbils.

(a) Distribution of the number of IHCs labelled during a dye-loading experiment as a function of age.

(b) I_{rest} of un(non)-coupled IHCs before 28 and from P28 was not statistically different (<P28: -26.79 ± 2.32 pA, $n = 39$, $N = 15$; $\geq$ P28: -28.16 ± 1.96 pA, $n = 24$, $N = 9$, Mann-Whitney-Wilcoxon test, $p = 0.47$)

Age group	Membrane contacts/ Fusion sites	Section	Contact range in z (brackets: in xy in fusion sites)
P15 (FIB-run #1, animal 1)	1. Flat contact IHC 1 with IHC 2 2. Filopodial contact IHC 1 with IHC 2 3. Filopodial contact IHC 1 with IHC 2 4. Filopodial contact IHC 1 with IHC 2 5. Filopodial contact IHC 1 with IHC 2 6. Filopodial contact IHC 1 with IHC 2	571-580 929-934 1778-1796 1781-1792 1955-1994 1973-1981	45 nm 25 nm 90 nm 55 nm 195 nm 40 nm
	1. Flat contact IHC 2 with IHC 3 2. Filopodial contact IHC 2 with IHC 3 3. Filopodial contact IHC 2 with IHC 3 4. Filopodial contact IHC 2 with IHC 3	1549-1556 1620-1726 1813-1846 2024-2028	35 nm 530 nm 165 nm 20 nm
P15 (FIB-run #2, animal 1)	1. Filopodial contact IHC 1 with IHC 2 2. Filopodial contact IHC 1 with IHC 2 3. Filopodial contact IHC 1 with IHC 2 4. Filopodial contact IHC 1 with IHC 2 5. Filopodial contact IHC 1 with IHC 2 6. Filopodial contact IHC 1 with IHC 2 7. Filopodial contact IHC 1 with IHC 2 8. Filopodial contact IHC 1 with IHC 2 9. Filopodial contact IHC 1 with IHC 2 10. Filopodial contact IHC 1 with IHC 2	3341-3360 3569-3581 3412-3454 3418-3457 3453-3459 3569-3580 3666-3677 3672-3705 3730-3747 3805-3812	95 nm 60 nm 210 nm 195 nm 30 nm 55 nm 55 nm 165 nm 85 nm 35 nm
	1. Filopodial contact IHC 2 with IHC 3 2. Filopodial contact IHC 2 with IHC 3 3. Filopodial contact IHC 2 with IHC 3 4. Filopodial contact IHC 2 with IHC 3 5. Filopodial contact IHC 2 with IHC 3 6. Filopodial Contact IHC 2 with IHC 3 7. Filopodial contact IHC 2 with IHC 3 8. Filopodial contact IHC 2 with IHC 3 9. Filopodial contact IHC 2 with IHC 3 10. Filopodial contact IHC 2 with IHC 3	1073-1088 1263-1285 1348-1375 1383-1407 1906-1928 1933-1938 2060-2148 2300-2328 2616-2656 2688-2696	75 nm 110 nm 135 nm 120 nm 110 nm 25 nm 88 nm 140 nm 200 nm 40 nm
P16 (FIB-run #3, animal 2)	1. Flat contact IHC 2 with IHC 3 2. Fusion site between IHC 2 and IHC 3 3. Filopodial contact IHC 2 with IHC 3 4. Filopodial contact IHC 2 with IHC 3 5. Filopodial contact IHC 2 with IHC 3 6. Filopodial contact IHC 2 with IHC 3	17-64 738-745 1110-1135 1245-1260 2775-2785 2820-2870	47 nm 35 nm (xy: 135.83 nm) 125 nm 75 nm 50 nm 250 nm
P34 (FIB-run #1, animal 1)	1. Flat contact IHC 1 with IHC 2 2. Flat contact IHC 1 with IHC 2	1357-1558 1615-1726	1 μ m 555 nm
	1. Flat contact IHC 2 with IHC 3 2. Flat contact IHC 2 with IHC 3 3. Flat contact IHC 2 with IHC 3 4. Flat contact IHC 2 with IHC 3	11-76 453-1687 586-634 2129-2200	325 nm 6.17 μ m 240 nm 355 nm
P34 (FIB-run #2, animal 1)	1. Flat contact IHC 1 with IHC 2 2. Flat contact IHC 1 with IHC 2	1-766 1144-1411	3.83 μ m 1.335 μ m

Age group	Membrane contacts/ Fusion sites	Section	Contact range in z (brackets: in xy in fusion sites)
P34 (FIB-run #2, animal 1)	3. Fusion site between IHC 1 and IHC 2	1233-1340	535 nm (xy: 124.81 nm)
	1. Flat contact IHC 2 with IHC 3	1-1112	5.56 μ m
	2. Fusion site between IHC 2 and IHC 3	115-121	30 nm (xy: 101.98 nm)
	3. Fusion site between IHC 2 and IHC 3	519-526	35 nm (xy: 146.82 nm)
	4. Fusion site between IHC 2 and IHC 3	1171	5 nm (xy: 123.69 nm)
	5. Filopodial contact IHC 2 with IHC 3	1730-1768	190 nm
P37 (FIB-run #3, animal 2)	1. Flat contact IHC 1 with IHC 2	11-22	55 nm
	1. Flat contact IHC 2 with IHC 3	12-25	65 nm
	2. Filopodial contact IHC 2 with IHC 3	234-275	205 nm
	3. Flat contact IHC 2 with IHC 3	1717-1862	725 nm
	1. Flat contact IHC 3 with IHC 4	304-314	50 nm
	2. Flat contact IHC 3 with IHC 4	1445-2128	3.42 μ m
	3. Fusion site between IHC 3 and IHC 4	1653-1661	40 nm (xy: 30.15 nm)
	4. Fusion site between IHC 3 and IHC 4	1706-1709	15 nm (xy: 14.42 nm)
	5. Fusion site between IHC 3 and IHC 4	1821-1824	15 nm (xy: 15.23 nm)
	6. Fusion site between IHC 3 and IHC 4	2233-2244	55 nm (xy: 52.80 nm)

Table S1. Quantification of contact sites and putative IHC fusion sites in the FIB data. IHC-IHC membrane contacts are categorized into contact sites via a filopodium touching the neighboring IHC (filopodial contact) and flat membrane contacts. Both contact categories can contain perforations, likely due to the fusion of membranes of two neighboring IHCs (putative IHC fusion sites). In P34/37 IHCs, the flat contacts as well as putative IHC fusion sites are more prevalent than at P15/16 IHCs, when the filopodial contacts prevail (Table 1 for summary). The length of the individual contact sites in z is calculated via the number of sections, each section accounts for 5 nm. The xy length of the syncytial contacts was measured in the section with the largest extend of the perforation in 3dmod. In each dataset, one full IHC was visible in the region of interest, flanked by neighboring IHCs that were only partially visible, but showing the full membrane contact (see also Fig. 5). n FIB-SEM run (P34/37) = 3, N_{animals} = 2; n FIB-SEM run (P15/16) = 3, N_{animals} = 2. In total, 3 full IHCs with their contacts to neighboring IHCs were analysed per age group. Per age group 2 independent embeddings were performed. Note that 2 FIB-runs from P15 and 1 FIB-run from P34 do not show putative fusion sites.

Figure	Data	n (replicates) N (animals)	Statistical test	Significance at 0.05
Figure 2b,c	Distribution of number of dye-filled IHCs	P21-28 (n = 365 loadings, N = 113) P30-45 (n = 155 loadings, N = 59)	Kolmogorov-Smirnov test	p < 0.00001
Figure 2e	I _{Camax} of 1 IHC vs 3 and 4 IHCs	1 IHC (n = 21 recordings, N =9) 3 and 4 IHCs (n = 4 recordings, N = 4 , for each)	Dunn-Holland-Wolfe non-parametric multiple comparison test	p < 0.05
Figure 2e	I _{Camax} vs C _m	n = 42 recordings, N = 26	Pearson correlation coefficient	r = -0.66 p < 0.00001
Figure 2e'	I _{rest} of 1 IHC vs 3 and 4 IHCs	1 IHC (n = 21 recordings, N =9) 3 and 4 IHCs (n = 4 recordings, N = 4 , for each)	Dunn-Holland-Wolfe non-parametric multiple comparison test	p < 0.05
Figure 2e'	I _{rest} vs C _m	n = 42 recordings, N = 26	Pearson correlation coefficient	r = -0.74 p < 0.00001
Figure 7c	$\Delta F_{max}/F_0$ of C1 vs C2	C1: 37 spots in 8 IHCs, N = 8 C2: 43 spots in 9 IHCs, N = 8	Two-tailed Mann-Whitney-Wilcoxon test	p = 0.31
Figure 7c	V _h of C1 vs C2	C1: 17 spots in 7 IHCs, N = 7 C2: 30 spots in 8 IHCs, N = 8	Two-tailed Student t-test	p = 0.96
Supplementary Figure 1e	I _{Camax} vs C _m	n =133 recordings, N = 78	Pearson correlation coefficient	r = -0.66 p < 0.00001
Supplementary Figure 1e'	I _{rest} vs C _m	n =133 recordings, N = 78	Pearson correlation coefficient	r = -0.64 p < 0.00001
Supplementary Figure 2c	I _{Camax} vs C _m	n =53 recordings, N = 42	Pearson correlation coefficient	r = -0.56 p < 0.00001
Supplementary Figure 2c	I _{rest} vs C _m	n =53 recordings, N = 42	Pearson correlation coefficient	r = -0.52 p < 0.00001
Supplementary Figure 4g	I _{Camax} vs C _m perforated-patch	n = 14 recordings, N = 11	Pearson correlation coefficient	r = -0.79, p < 0.01
Supplementary Figure 4g	I _{Camax} vs C _m ruptured-patch	n = 15 recordings, N = 6	Pearson correlation coefficient	r = -0.88, p < 0.001
Supplementary Figure 4h	I _{Camax} vs C _m perforated-patch	n = 14 recordings, N = 11	Pearson correlation coefficient	r = -0.55, p = 0.21
Supplementary Figure 4h	I _{Camax} vs C _m ruptured-patch	n = 15 recordings, N = 6	Pearson correlation coefficient	r = -0.93, p < 0.001
Supplementary Figure 6	G _m at -60mV	n = 56 recordings, N = 56	Pearson correlation coefficient	r = 0.78, p < 0.05
Supplementary Figure 6	G _m at +20 mV	n = 56 recordings, N = 56	Pearson correlation coefficient	r = 0.77, p < 0.05

Table S2 Summary table of the statistical tests used

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Peer Review File

Reviewers' comments first round:

Reviewer #1 (Remarks to the Author):

This exceptionally interesting manuscript describes the findings that may significantly change current understanding of the cell-to-cell communications in the mammalian organ of Corti. According to the wisdom of the field, the major mechanosensory receptors of the inner ear – the inner hair cells (IHCs) – send their signals to the auditory nerve fibers separately and do not communicate to each other. However, the authors of this study discovered that, at the latest stages of postnatal development, IHCs form functional mini-syncytia interspersed between non-coupled IHCs along the tonotopic axis of the organ of Corti. The existence of these three-to-nine cells mini-syncytia was confirmed by dye diffusion, patch-clamp recordings, calcium imaging, and 3D electron microscopy. The data are of high-quality and quite convincing. Even though the functional significance of the IHC coupling remains purely speculative, the study may have a profound impact on our understanding of the mechanisms of sound encoding in the mammalian cochlea. I was very excited by this story and have only few minor comments.

As the authors correctly pointed out, the major concern is a potential artefactual IHC fusion due to cellular damage in an ex vivo preparation. The authors outlined in the Discussion several reasons to believe that such an artefact is unlikely. However, I would be more comfortable if the authors would show, on a separate figure, the data of a crucial experiment that they described as, "IHC recordings revealing Ca²⁺ current amplitudes worth multiples of the single IHC Ca²⁺ current were also observed ... without removing the contacting supporting cells."

Specific comments:

Figure 2f: Please clarify which values the data points (open circles and red crosses) represent. I assume I_{CaMax} and I_{rest}, correspondingly. If so, why I_{CaMax} data are presented separately for ruptured and perforated-patch experiments, while I_{rest} data are combined? Exactly which data were used for linear fits? There are three color-coded groups and three linear fits could be expected.

Figure S2c: Please clarify which values the data points (gray open circles and red crosses) represent. I assume I_{CaMax} and I_{rest}, correspondingly. What is the group with n=49? Is that a total number of the cells? Why is the number of cells in panel c not matching to the number of cells in panel b?

Legends to figure 3, 4, and 7 are missing the ages of the preparations.

Results, last paragraph: "...we estimate that a single IHC covers approximately 3 cents on the tonotopic map of the mid-cochlear quarter of a turn...". Please define "cent" here.

Reviewer #2 (Remarks to the Author):

The focus of this manuscript from Jean et al is to describe the presence of functional coupling between the sensory IHCs of the adult cochlea of mice and gerbils, which was previously unknown. Using a combination of approaches, including dye labelling, whole-cell patch clamp recordings, high-resolution imaging and computational modelling, the authors have described the presence of mini-syncytia among IHCs (between 2 and 9 cells) in post-hearing mice. These syncytia appear to increase from just 2% of IHCs during the third post-natal week to about 30% at 5-6 weeks of age. The different number of IHCs connected within a mini-syncytium was indicated by results including the diffusion of fluorescent dyes among the cells, a correspondingly large cell membrane capacitance and reduction in input resistance. The fact that gap-junction blockers did not close these cellular connections indicates that connexin gap junction channels are unlikely to be involved. The article also provides high-resolution microscopy data showing "putative" fusion sites

among IHCs, and a computational model aimed at showing the potential functional benefit of having such syncytia among IHCs. Overall, this work provides a descriptive investigation for the possible presence of IHC-IHC connections in the adult mammalian cochlea. Although the conclusions appear substantiated by some of the data presented, there are several aspects of the work that need some additional consideration in order to rule out the presence of any potentially confounding scenarios that could affect the interpretation of the data.

1) The description of the work is very superficial with minimal quantification and a complete absence of statistical analysis. Although the word "significance" is mentioned in some places, there is no mention of the P value anywhere in the text nor the specific test that was done. In addition, it is difficult to extrapolate how many experiments have been performed, including the number of biological replicates used for each set of data. For example, was the IHC coupling mainly found in some preparations/animals? This should be clearly described in the ms. This lack of information makes it very difficult to assess the robustness of the data provided. The methods section is also very poorly written, with minimal detail.

2) It is not clear to me why the mice have to be over 1 month old in order to show a substantial increase in the coupling between IHCs. From a purely functional point of view, if this phenomenon is so crucial for proper sound perception, why is it not present during the first month of life? Also, does the IHC-IHC coupling increase further at older ages? If so, then the interpretation is likely to change substantially.

3) Figure 2f is interesting but also highlights some concerns. The level of variability in the size of the calcium current recorded from an "uncoupled" IHCs is very variable (from about 50 pA to 450 pA), which is not consistent with work previously published from this lab. Is it possible that this large variability is due to differences in the general health of the patched IHCs? Is the correlation shown in Fig 2f significantly different from 0, given the large current size variability? It would be useful to show the Irest of the IHCs as a function of age –does it become larger?

4) If coupling between IHCs is present, then a similar variability in the outward K current size to that seen for IcaMax would be expected. Considering that an adult IHC showing a Cm of about 9pF and a IK of about 15nA (e.g. from your previous study: Sendin et al., 2007), IK would be expected to be about 130nA if 9 IHCs are connected? You should provide some examples of very large K currents to corroborate the calcium current results. This is important because K currents are less likely to be affected by the physiological state of the cells than the calcium currents.

5) The use of the perforated patch configuration is a crucial part of the present work. Despite this, there is no information regarding how it has been performed (one sentence in the Method section, which does not say very much), and which control experiments have been done to make sure whole-cell was not achieved.

6) The morphological images are very nice, although, as the authors mention in the text, they only provide limited evidence for a "putative" representation of the IHC-IHC fusion sites. It is very difficult to associate the small membrane gaps observed in very few images to the data presented in the first part of the paper. The dimensions of the fusion sites provided in Table 1 were obtained from 7 identified sites, however, there is no indication of how many IHCs/mice have been looked at in total. If 30% of IHCs are connected, then it would be reasonable to expect to find a much larger number of fusion sites. Unfortunately, as mentioned above, there is an underlying possibility that all changes observed are due to experimental artifacts, which are not fully excluded by the authors. Is it possible to obtain high-resolution images from the same IHCs used for dye labelling?

7) Assuming that the number of coupled IHCs does not change further in older animals (see above), the functional explanation for these syncytia is not very clear. Although, there are a lot of speculative hypotheses listed in the manuscript, there is not a clear understanding of the distribution of these "putative" syncytia that occurs in about 30% of IHCs. Their random distribution along the cochlea would be differentially "diluted" with the remaining 70% of IHCs that do not show any functional coupling, making it difficult to really appreciate the functional benefit. Moreover, it is difficult to reconcile why the afferent fibers undergo extensive pruning during development, in order to make one-to-one connections with the IHCs, and then after a couple of

weeks revert back to a configuration resembling that of the immature cochlea.

Reviewer #3 (Remarks to the Author):

The manuscript "Macromolecular and electrical coupling between inner hair cells in the rodent cochlea" by Jean and colleagues (NCOMMS-19-40978) is a very detailed and powerful study of a novel type of communication between cochlear inner hair cells (IHC), namely direct junctions in the membrane of adjacent IHC. The authors demonstrate the presence of these junctions, which couple up to 9 adjacent IHCs, using dye diffusion, patch-clamp electrophysiology and FIB-SEM reconstructions. They then demonstrate potential function of the junctions in hearing by demonstrating that activation of one IHC can cause pre-synaptic calcium increases at ribbons of neighboring coupled IHC using an especially elegant technique of imaging CtBP2 protein conjugated to a calcium sensor, which diffuses between neighboring cells. They conclude with a computational model demonstrating a modest improvement in coordination of IHC activity, with possible benefits for sound encoding. While this work raises many interesting questions about the function of these "mini-syncytia" in hearing for future studies, it is a thorough and well-done manuscript. A few concerns remain:

Major:

- The main concern with this work, which is partially, but not completely, addressed, is that of damage to the tissue during in vitro experiments. While in vivo measurement of this phenomenon is not currently possible, the authors should show clearly the results of their experiments in which techniques minimal tissue damage (supporting cell removal) were used. They are mentioned in the discussion as well as the supplementary methods. However, the results of these experiments were not mentioned (why?). Please move this data to the main results text, preferably with a figure detailing any differences between the two dissection conditions. This is especially important because the authors note an increase in mini-syncytia occurrence and # of IHC involved with age, but the cochlear tissue becomes more fragile with age as well. The authors notes that coupling between IHC and SCs was not observed, which supports their hypothesis, but SCs could have different membrane components, extracellular matrix molecules, etc that could make them more resistant to damage.
- The introduction details other forms of coupling among neurons. However, cochlear hair cells are not neurons, they are epithelial cells. A discussion of similar junctions (or lack thereof) in this cell type is warranted, and may help support the experimental results.
- In table 1, how many cells were imaged and analyzed, including cells with no fusion or filopodial sites?
- The experiments presented in figure 7 are excellent, showing that functional effect of connected IHCs in potentially stimulating each other's pre-synaptic active zones. Is there any difference in the number of spots activated, or the fluorescence intensity of the spots, with distance from the patched IHC? This is not required for the main body of the manuscript, but could be added to the supplement. This data could give an idea of the effect of distance in determining function of the connected cells.

Minor:

- Please add a clock to the supplementary movies
- Page 4, 5th line from bottom (line numbering would help), "sound born" = "sound borne"
- Page 4, 3rd line from bottom: delete "already"
- Figure 2f – define the red 'plus' symbols in the legend

Reviewer #4 (Remarks to the Author):

The article from Jean et al reports that about 30% inner hair cells in rodents appear to form a syncytium. The experimental breadth includes classical neurobiology methods as well as three-dimensional light and electron microscopy.

I am here to judge the quality of the imaging, and technically from my perspective, there are no

problems. The choice to perform TEM tomography is correct as the junctions and the membrane contacts are difficult to demonstrate with lower resolution approaches. The preparation protocol is standard and in my opinion appropriate to the task. maybe the presence of tannic acid is a little excessive given the fine details understudy, but overall I do not see it as a blocking issue.

As an outsider to the field, I question the general interest of the study, which is from my perspective very limited and more appropriate to a neurobiology journal.

Rev#1

This exceptionally interesting manuscript describes the findings that may significantly change current understanding of the cell-to-cell communications in the mammalian organ of Corti. According to the wisdom of the field, the major mechanosensory receptors of the inner ear – the inner hair cells (IHCs) – send their signals to the auditory nerve fibers separately and do not communicate to each other. However, the authors of this study discovered that, at the latest stages of postnatal development, IHCs form functional mini-syncytia interspersed between non-coupled IHCs along the tonotopic axis of the organ of Corti. The existence of these three-to-nine cells mini-syncytia was confirmed by dye diffusion, patch-clamp recordings, calcium imaging, and 3D electron microscopy. The data are of high-quality and quite convincing. Even though the functional significance of the IHC coupling remains purely speculative, the study may have a profound impact on our understanding of the mechanisms of sound encoding in the mammalian cochlea. I was very excited by this story and have only few minor comments.

First, we would like to thank the reviewer for the appreciation of our multidisciplinary study. The constructive evaluation of our work has helped us to further improve the manuscript. We have addressed all concerns raised by the reviewer.

As the authors correctly pointed out, the major concern is a potential artefactual IHC fusion due to cellular damage in an ex vivo preparation. The authors outlined in the Discussion several reasons to believe that such an artefact is unlikely. However, I would be more comfortable if the authors would show, on a separate figure, the data of a crucial experiment that they described as, “IHC recordings revealing Ca^{2+} current amplitudes worth multiples of the single IHC Ca^{2+} current were also observed ... without removing the contacting supporting cells.”

Done, as requested we provide an additional Supplemental figure (new Fig. S4). We now state in page 8:

“Similar observations were made i) from ruptured-patch recordings from IHCs of the excised apical and basal gerbil organ of Corti (P14-38, Fig. S2), ii) from perforated-patch recordings of mouse IHCs in the excised organ of Corti ($n = 14$ IHCs, $N_{\text{animals}} = 11$, P15-P36), and iii) from ruptured-patch recording of IHCs when only one inner sulcus cell was removed for access of the pipette in the excised apical mouse organ of Corti ($n = 15$ IHCs; $N_{\text{animals}} = 6$, P16-P24, Fig. S4).”

Specific comments:

Figure 2f: Please clarify which values the data points (open circles and red crosses) represent. I assume I Ca_{Max} and I rest , correspondingly. If so, why I Ca_{Max} data are presented separately for ruptured and perforated-patch experiments, while I rest data are combined? Exactly which data were used for linear fits? There are three color-coded groups and three linear fits could be expected.

Correct: open circles for the I_{CaMax} , crosses for I_{rest} . We had combined the ruptured-patch and perforated-patch data for both I_{CaMax} and I_{rest} and the black and red lines represent fits to the C_m relationships of I_{CaMax} and I_{rest} , respectively. For clarity, and in response to the reviewer's concern, we have modified Fig. 2e now plotting I_{CaMax} and former 2f (now 2e') now plotting I_{rest} of single and dye coupled IHCs (recorded at 5mM $[Ca^{2+}]_e$) against their respective membrane capacitance. The former Fig. 2f combining different conditions has now become Fig. S1e,e', where we reduced the heterogeneity of the recording conditions and only present the ruptured-patch data. The perforated patch-data are now shown in Fig. S4 in separation together with the minimal cleaning approach data. We have now clarified this in the text of the results section as well as in the figure legends.

"As expected, a correlation with C_m was found for I_{CaMax} ($r = -0.66$, $p < 0.00001$, Fig. 2e) as well as for I_{rest} (Fig 2e', $r = -0.74$, $p < 0.00001$). In a larger data set combining different $[Ca^{2+}]_e$ (1.3, 2 or 5 mM), correlations were also found for I_{CaMax} and I_{rest} with C_m (I_{CaMax} , $r = -0.66$, $p < 0.00001$, I_{rest} , $r = -0.64$, $p < 0.00001$, P15-51, Fig. S1e,e'). Similar observations were made i) from ruptured-patch recordings from IHCs of the excised apical and basal gerbil organ of Corti (P14-38, Fig. S2), ii) from perforated-patch recordings of mouse IHCs in the excised organ of Corti ($n = 14$ IHCs, $N_{animals} = 11$, P15-P36), and iii) from ruptured-patch recording of IHCs when only one inner sulcus cell was removed for access of the pipette in the excised apical mouse organ of Corti ($n = 15$ IHCs; $N_{animals} = 6$, P16-P24, Fig. S4)."

Figure S2c: Please clarify which values the data points (gray open circles and red crosses) represent. I assume I_{CaMax} and I_{rest} , correspondingly. What is the group with $n=49$? Is that a total number of the cells? Why is the number of cells in panel c not matching to the number of cells in panel b?

Correct: open circles for the I_{CaMax} , crosses for I_{rest} ! We have added this information in the figure legend. We would like to thank reviewer for spotting a mistake in numbers. The graphs have been updated and the numbers corrected now. The number of cells in panel c is matching the number of cells in panel b (19 cells with $C_m < 15$ pF, 25 cells with $15 \text{ pF} \leq C_m < 30$ pF, and 9 cells with $C_m \geq 30$ pF = 53 cells).

Legends to figure 3, 4, and 7 are missing the ages of the preparations.

We have now made sure the age is specified in results section, figure and legend.

Results, last paragraph: "...we estimate that a single IHC covers approximately 3 cents on the tonotopic map of the mid-cochlear quarter of a turn...". Please define "cent" here.

Done

"For the human cochlea, we estimate that a single IHC covers approximately 3 cents (1 cent is 1 percent of a semitone, one semitone is $1/12^{\text{th}}$ of an octave) on the tonotopic map of the mid-cochlear quarter of a turn."

The focus of this manuscript from Jean et al is to describe the presence of functional coupling between the sensory IHCs of the adult cochlea of mice and gerbils, which was previously unknown. Using a combination of approaches, including dye labelling, whole-cell patch clamp recordings, high-resolution imaging and computational modelling, the authors have described the presence of mini-syncytia among IHCs (between 2 and 9 cells) in post-hearing mice. These syncytia appear to increase from just 2% of IHCs during the third post-natal week to about 30% at 5-6 weeks of age. The different number of IHCs connected within a mini-syncytium was indicated by results including the diffusion of fluorescent dyes among the cells, a correspondingly large cell membrane capacitance and reduction in input resistance. The fact that gap-junction blockers did not close these cellular connections indicates that connexin gap junction channels are unlikely to be involved. The article also provides high-resolution microscopy data showing “putative” fusion sites among IHCs, and a computational model aimed at showing the potential functional benefit of having such syncytia among IHCs. Overall, this work provides a descriptive investigation for the possible presence of IHC-IHC connections in the adult mammalian cochlea. Although the conclusions appear substantiated by some of data presented, there are several aspects of the work that need some additional consideration in order to rule out the presence of any potentially confounding scenarios that could affect the interpretation of the data.

First, we would like to thank the reviewer for the appreciation of our multidisciplinary study. The constructive evaluation of our work has helped us to further improve the manuscript. We have addressed all concerns raised by the reviewer.

1) The description of the work is very superficial with minimal quantification and a complete absence of statistical analysis. Although the word “significance” is mentioned in some places, there is no mention of the P value anywhere in the text nor the specific test that was done. In addition, it is difficult to extrapolate how many experiments have been performed, including the number of biological replicates used for each set of data. For example, was the IHC coupling mainly found in some preparations/animals? This should be clearly described in the ms. This lack of information makes it very difficult to assess the robustness of the data provided. The methods section is also very poorly written, with minimal detail.

In brief, the dye and electrical coupling of IHCs was consistently observed throughout the study by multiple experimenters in different labs: P.J., T.A., M.GD.A., D.Ö., J.F.A., in mice from different breeding colonies and vendors over several years as well as in gerbils. The number of analyzed cells was provided throughout and in our view already reflected the robust data base for our report.

In response to the reviewer’s comment, we have now provided more details on how the data was acquired in the different labs. Specifically, we have placed the information on number of observations, cells, animals, statistical tests used at each instance (in addition to the methods section where the information had been summarized).

2) It is not clear to me why the mice have to be over 1 month old in order to show a substantial increase in the coupling between IHCs. From a purely functional point of view, if this phenomenon is so crucial for proper sound perception, why is it not present during the first month of life? Also, does the IHC-IHC coupling increasing further at older ages? If so, then the interpretation is likely to change substantially.

At present we do not have a sensible reply for why biology might employ less IHC coupling for hearing at earlier stages. Mechanistically, it seems that the extent of supporting cell separation declines during postnatal maturation which gives rise to more opportunities for IHCs to contact each other in the more mature state.

In order to address the reviewer's question we have revisited our data on mice and gerbils but did not observe a clear change of the incidence of IHC coupling in the older animals. Since the experiments were not designed to determine the progression of coupling with age in the oldest age group, this sample is not sufficiently large to determine a trend in this population.

Mice:

We use the temporal bone preparation in which the largest sample of recording in older mice was available. The 71 mice in the total sample of temporal bones contained approximately equal proportions, 30 females, 34 males and 7 unspecified. There was no obvious effect of gender. The remaining 29 mice in the coupling histogram were not specified either for age or sex. 15 of the age specified mice were older than P100 but still could be readily recorded in the 2P configuration. Of these, the oldest where 3 cells were found to be coupled was P350. These were C57BL6 animals that were donated and recorded more out of curiosity. At this stage, we have insufficient information to carry out any analysis in a systematic manner and so these data will be excluded here. The plot shows the numbers of loaded cells versus the age for mice with ages less than P100. The data can be replotted to show the age distribution for each set of coupled cells (from 1-8 cells). As can be seen coupling was found at all ages in this sample. Since the experiments were not designed to determine the progression of coupling with age, the sample is not sufficiently large to determine a trend in this population.

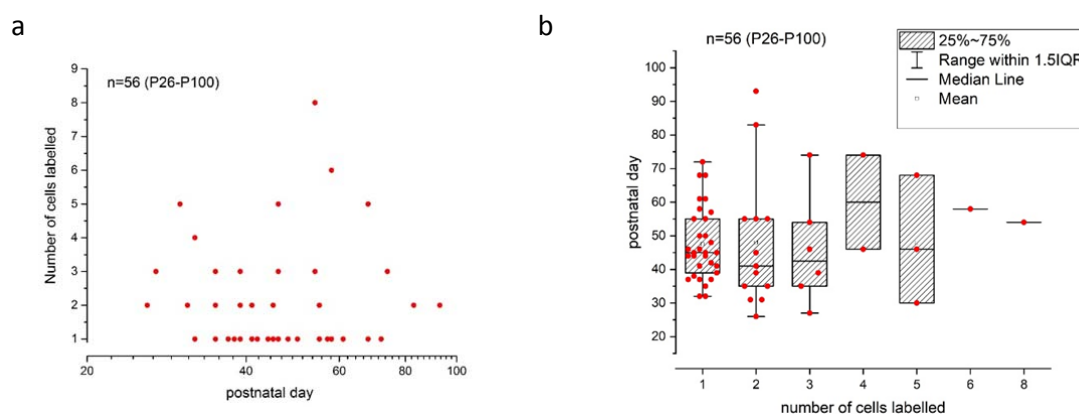


Figure 1 of this rebuttal letter: Coupling is found at all ages in mice.

(a) The plot shows the numbers of loaded IHCs versus the age for mice with ages less than P100. (b) The data can be replotted to show the age distribution for each set of labeled IHCs (from 1-8 IHCs). As can be seen coupling was found at all ages in this sample.

Data sample: IHC number in recording, rounded mean postnatal age (P), n of recordings
 1 IHC (non-coupled), P48, $n = 31$; 2 IHCs, P48, $n = 15$; 3 IHCs, P46, $n = 6$; 4 IHCs, P60, $n = 2$;
 5 IHCs, P48, $n = 3$; 6 IHCs, P58, $n = 1$; 8 IHCs, P54, $n = 1$

Gerbil (dissected organ of Corti)

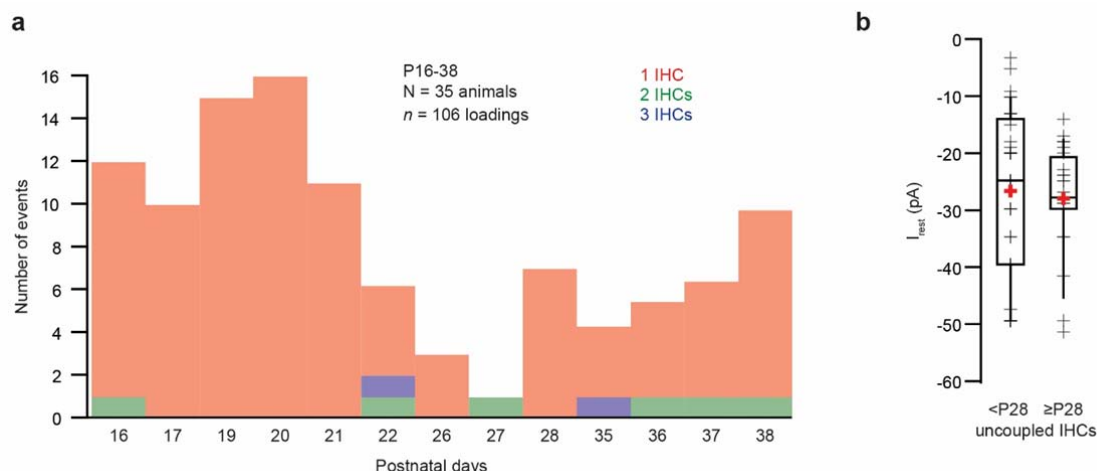


Figure 2 of this rebuttal letter: Coupling is found at all ages in Gerbils.

(a) distribution of the number of IHCs labelled during a dye-loading experiment as a function of age.

(b) I_{rest} of un(non)-coupled IHCs before 28 and from P28 was not statistically different (<P28: -26.79 ± 2.32 pA, $n = 39$, $N=15$; ≥P28: -28.16 ± 1.96 pA, $n = 24$, $N=9$, Mann-Whitney-Wilcoxon test, $p = 0.47$)

3) Figure 2f is interesting but also highlights some concerns. The level of variability in the size of the calcium current recorded from an “uncoupled” IHCs is very variable (from about 50 pA to 450 pA), which is not consistent with work previously published from this lab. Is it possible that this large variability is due to differences in the general health of the patched IHCs? Is the correlation shown in Fig 2f significantly different from 0, given the large current size variability? It would be useful to show the I_{rest} of the IHCs as a function of age –does it become larger?

We fear there was a misunderstanding of the reviewer regarding the maximal Ca^{2+} current amplitude: Fig. 2f plots Ca^{2+} current amplitude of non-coupled IHCs and IHCs coupled to various extents (as seen from the different membrane capacitance (C_m)). There is no case of a non-coupled IHC with a maximal Ca^{2+} current amplitude of 450 pA! In fact, even in 5 mM $[Ca^{2+}]_e$ the largest maximal Ca^{2+} current amplitude of a non-coupled IHC was 300 pA. The 4 coupled cells presenting a maximal calcium current of 450 pA for a capacitance of only 10 pF in Fig 2f, was due to an incomplete compensation of the slow capacitance often encountered with large mini-synctia of 3-4 cells.

Moreover, we would like to stress that these currents were recorded in different conditions and we combined them here to illustrate the robustness of the finding of the scaling of Ca^{2+}

current and holding current amplitudes with the extent of coupling. Specifically data of former Fig. 2f were acquired at different different age (postnatal days 14-56) and at extracellular $[Ca^{2+}]$ (1.3, 2 or 5 mM) in ruptured or perforated-patch recording configurations in different protocols (maximum current in protocols to establish the current-voltage relation or single step depolarization targeting the potential that elicits the maximal Ca^{2+} influx). This had been indicated in the figure legend and explains the substantial variability. It is despite this variability that we found a correlation of the maximal Ca^{2+} current amplitude with the membrane capacitance ($r = -0.66$, $p < 0.00001$) which further corroborates the notion of low-resistance electrical coupling.

The Ca^{2+} current data also argue strongly against the concern of the reviewer that IHC coupling might reflect a compromised physiological state. As pointed out by the reviewer in the following comment, compromised general health of the coupled cells would be expected to cause lower Ca^{2+} currents. However, instead, the recordings from coupled cells indicate a summation of normally sized Ca^{2+} currents in the limits of the expected poorer voltage clamp. Therefore, by the reviewer's argument #4, the fact that the Ca^{2+} current scales with the number of coupled IHCs in the mini-syncytium supports the good general health of the recorded IHCs, which is the standard in our recordings. For clarity, and in response to the reviewer's concern, we have modified Fig. 2e now plotting I_{CaMax} and former 2f (now 2e') now plotting I_{rest} of single and dye coupled IHCs against their respective membrane capacitance at 5mM $[Ca^{2+}]_e$. The former Fig. 2f combining different conditions has now become Fig. S1e,e', where we reduced the heterogeneity of the recording conditions and only present the ruptured-patch data. The single non-coupled IHC data is taken from a previous paper from the Göttingen lab (Jean et al., 2018) recorded by the same experimenter, using the same protocol, where the resting currents never exceeded -50 pA (mean of -22 pA). The perforated patch-data are now shown in Fig. S3 together with further ruptured-patch data obtained with the minimal cleaning approach.

In response to the reviewers suggestion to look at I_{rest} as a function of age, we refer to the Figure 2b of this rebuttal letter which shows based on gerbil recordings that there was no significant difference between recordings from IHCs before and from P28.

4) If coupling between IHCs is present, then a similar variability in the outward K current size to that seen for I_{CaMax} would be expected. Considering that an adult IHC showing a C_m of about 9pF and a I_K of about 15nA (e.g. from your previous study: Sendin et al., 2007), I_K would be expected to be about 130nA if 9 IHCs are connected? You should provide some examples of very large K currents to corroborate the calcium current results. This is important because K currents are less likely to be affected by the physiological state of the cells than the calcium currents.

We hope the reviewer will agree that the large size of the K^+ currents (as indicated by the reviewer) together with the series resistance (~ 5 -15 MOhm) and the coupling resistance (~ 10 -100 Mohm) would make it impossible to perform the proposed experiment. While the Ca^{2+} currents (hundreds of picoamperes) of the IHCs within a mini-syncytium could typically be recorded reasonably well, this would not be the case for the K^+ currents, which as the reviewer points out, are of nanoampere amplitude and can barely be clamped in the patched cell, let alone in the coupled cells.

In response to the reviewer's concern, we analyzed the membrane conductance as a function of the number of OGB5N-loaded IHCs in the temporal bone preparation. Using Cs^+ to minimize the conductance of K^+ channel we depolarized the patch-clamped IHCs to various levels (100 ms long depolarizations in 10 mV increments from a holding potential of -60 mV (Fig. S5)). The Cs^+ currents increased with the number of dye-filled IHCs, indicating a partial summation over the currents of the coupled IHCs. We note that the recordings underestimate the true currents due to the voltage drop over the pipette series resistance to the patched IHC and due to the junctional resistance among the coupled cells. In order to quantify this result, we analyzed the membrane slope conductance (g_m) of IHCs at -60 and +20 mV. There was a significant positive correlation of g_m and the number of coupled cells was observed at both potentials (-60 mV: $r = 0.78$; +20 mV: $r = 0.77$, both regression slopes significantly different from 0 at $p < 0.05$; Fig. S6). This corroborates our conclusion electrical IHC coupling.

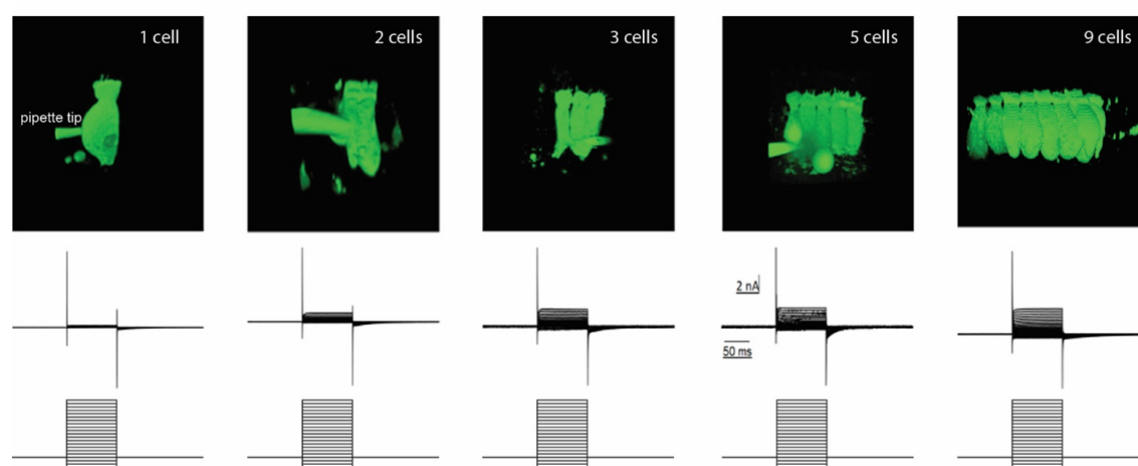


Figure S5. Cesium outward currents increase with the number of dye-coupled IHCs.

Analyzing dye and electrical IHC coupling in temporal bone preparation, using 2PLSM imaging of OGB5N-loaded IHCs (top panel) and IHC recordings of Cs^+ -mediated outward currents (middle panel) in response to 100 ms long depolarizations (in 10 mV increments, bottom panel) from a holding potential of -60 mV. As shown for a sample of $n = 56$ distinct experiments and quantified in Fig. S6, the maximal Cs^+ current increases with the number of dye-filled IHCs, indicating a partial summation over the currents of the coupled IHCs. We note that the recordings underestimate the true currents due to voltage drops over the series resistance to the patched IHC and the junctional resistance among the coupled cells.

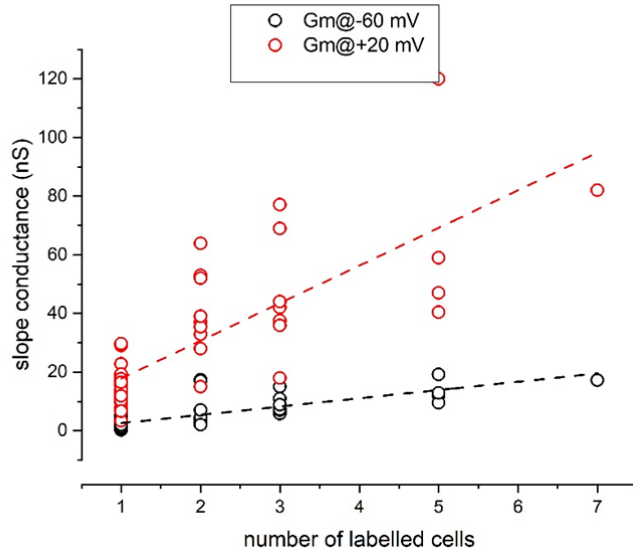


Figure S6. IHC membrane conductances increase with the number of dye-coupled IHCs. Membrane slope conductances (G_m) at the holding potential -60 mV and at +20 mV from I-Vs for positively identified uncoupled and coupled cells. All the data was collected within 60 s of whole cell break-in with Cs^+ as the major pipette cation. The membrane slope conductance at -60 mV, increasing with the number of coupled cells, is a good estimator of the effect of coupling. In this sample, using internal Cs^+ , $G_m = 2.05 \pm 1.25$ nS (mean $\pm$ SD, $n = 25$ uncoupled cells) at -60 mV. Preliminary data using K^+ as the major pipette cation did not permit the sufficient voltage clamp control to estimate G_m at +20 mV. Dashed lines show regression fits, with slopes significantly different from 0 nS/cell ($p < 0.05$).

5) The use of the perforated patch configuration is a crucial part of the present work. Despite this, there is no information regarding how it has been performed (one sentence in the Method section, which does not say very much), and which control experiments have been done to make sure whole-cell was not achieved.

Since we have reported these experiments in our first IHC study 20 years ago, the Göttingen laboratory has used the perforated patch-recordings for the reason of their less invasive and more stable nature. An accidental transition from perforated patch to ruptured patch is very rare for experienced patch-clampers as those contributing to the study and obvious as an instantaneous reduction in the series resistance that is regularly monitored by the membrane capacitance compensation circuitry of the EPC amplifiers used here. This was not observed in any of the recordings of this study.

In response to the reviewer's concern, we now offer a more extensive description here and in the methods section.

"For perforated-patch, the intracellular solution contained (in mM): 130 Cs-methanesulfonate, 10 TEA-Cl, 10 4-AP, 10 HEPES, and 1 MgCl_2 . Amphotericin B (250 $\mu\text{g}/\text{ml}$ Calbiochem, La Jolla, CA) was added from a 1000x stock in DMSO (final DMSO concentration not exceeding 0.1%) during sonication. Pipettes were tip-dipped in amphotericin-free pipette solution for 20-40 sec and then brought to the IHC within less than 1 min to avoid accidental spill of amphotericin which blocks seal formation. Perforation of the patch

membrane usually occurred within 5 min and recordings were started when the series resistance was lower than 30 MOhm. A sudden drop of series resistance, which would be indicative of a spontaneous rupturing of the patch membrane was not observed."

6) The morphological images are very nice, although, as the authors mention in the text, they only provide limited evidence for a "putative" representation of the IHC-IHC fusion sites. It is very difficult to associate the small membrane gaps observed in very few images to the data presented in the first part of the paper. The dimensions of the fusion sites provided in Table 1 were obtained from 7 identified sites, however, there is no indication of how many IHCs/mice have been looked at in total. If 30% of IHCs are connected, then it would be reasonable to expect to find a much larger number of fusion sites. Unfortunately, as mentioned above, there is an underlying possibility that all changes observed are due to experimental artifacts, which are not fully excluded by the authors. Is it possible to obtain high-resolution images from the same IHCs used for dye labelling?

Using FIB-SEM, we investigated the membranes of 3 IHCs to their respective neighboring IHCs (neighbors were not entered into the IHC count) right after the onset of hearing (2 IHCs from a P15 organ of Corti and 1 IHC from a P16 organ of Corti, $n = 3$ FIB-SEM runs, $N_{animals} = 2$) and 3 IHCs after full maturation (2 IHCs from a P34 organ of Corti and 1 IHC from a P37 organ of Corti, $n = 3$ FIB-SEM runs, $N_{animals} = 2$). This corresponds to a total of 6 FIB-SEM runs. We took this effort, because FIB-SEM is the only method that enables reconstruction of a large volume with close to isotropic and sufficiently small voxel size (3 nm in x/y and 5 nm in z) for faithful inspection of the IHC membrane. We see that the Table 1 and S1 figure were not completely clear on the IHC numbers. We investigated in each run 1 full IHC that makes contact to the two neighboring cells. The neighboring cells, however, are not fully included in the 3D volume and therefore not completely analyzed. We clarified this confusing statement now in the text:

"To identify a structural basis for the mechanism of IHC coupling, we employed 3D electron microscopy (focused ion beam—scanning electron microscopy (FIB-SEM): voxel size: 3 nm in x/y and 5 nm in z). We focused our imaging on the perinuclear basolateral membranes of mouse IHCs that appeared to be in contact in more mature preparations (Figs. 5 and 6; Fig. S8; Movie S4). We investigated the membranes of 3 IHCs to their respective neighboring IHCs (neighbors not being included into the IHC count) right after the onset of hearing (2 IHCs from a P15 organ of Corti and 1 IHC from a P16 organ of Corti, $n = 3$ FIB-SEM runs, $N_{animals} = 2$) and 3 IHCs after full maturation (2 IHCs from a P34 organ of Corti and 1 IHC from a P37 organ of Corti, $n = 3$ FIB-SEM runs, $N_{animals} = 2$)."

Further, we have now prominently included all that information into the results section and specify N to state $N_{animals}$.

We have used the electron microscopy to provide structural insight into the cell biological mechanism underlying the IHC-coupling. While gap junctions were not observed in the basolateral region, we found 8 events with morphological attributes of putative IHC-IHC fusion sites.

The connection between our electrophysiological estimates of contact (12 to 75 MOhm coupling resistance, equivalent to 13 to 80 nS coupling conductance) are in line with the EM

results. The dimensions of the putative intercellular bridges can be converted to resistances assuming a standard intracellular resistance of 150 Ohm · cm. For the bridges listed in table S1 (excluding the outlier), the following tables lists the estimated individual resistances and conductances, as well as the resulting values for all the bridges between two connected cells. Those combined resistances are the inverse of the summed conductances, because they act in parallel to each other.

FIB Run	cells	diameter (nm)	length (nm)	Resistance (MOhm)	Conductance (nS)
P16 #3	IHC2-IHC3	135	35	3.7	272.6
P34 #2	IHC2-IHC3	30	102	216.5	4.6
		35	147	229.2	4.4
		5	124	9472.9	0.1
				110	9.1
P37 #3	IHC3-IHC4	30	40	84.9	11.8
		14.4	15	138.2	7.2
		15.23	15	123.5	8.1
		52.8	55	37.7	26.5
				19	53.7

The resulting resistances between neighboring cells are close to the range estimated with electrophysiology, if you account for an additional resistance of several MOhm across the cells from pipette to pipette.

We think that this counters the reviewer’s argument that the coupling should be reflected in a “much larger number of fusion sites”.

7) Assuming that the number of coupled IHCs does not change further in older animals (see above), the functional explanation for these syncytia is not very clear. Although, there are a lot of speculative hypotheses listed in the manuscript, there is not a clear understanding of the distribution of these “putative” syncytia that occurs in about 30% of IHCs. Their random distribution along the cochlea would be differentially “diluted” with the remaining 70% of IHCs that do not show any functional coupling, making it difficult to really appreciate the functional benefit. Moreover, it is difficult to reconcile why the afferent fibers undergo extensive pruning during development, in order to make one-to-one connections with the IHCs, and then after a couple of weeks revert back to a configuration resembling that of the immature cochlea.

This, to our knowledge is the first report of an unprecedented phenomenon. The finding is robust across various mouse breeds, recording conditions, several experimenters in two labs and generalizes to another rodent species. We have carefully discussed the potential functional implications of IHC-coupling. Future work will be required to further elucidate mechanisms, prevalence and functional role in mice and other species.

The manuscript “Macromolecular and electrical coupling between inner hair cells in the rodent cochlea” by Jean and colleagues (NCOMMS-19-40978) is a very detailed and powerful study of a novel type of communication between cochlear inner hair cells (IHC), namely direct junctions in the membrane of adjacent IHC. The authors demonstrate the presence of these junctions, which couple up to 9 adjacent IHCs, using dye diffusion, patch-clamp electrophysiology and FIB-SEM reconstructions. They then demonstrate potential function of the junctions in hearing by demonstrating that activation of one IHC can cause pre-synaptic calcium increases at ribbons of neighboring coupled IHC using an especially elegant technique of imaging CtBP2 protein conjugated to a calcium sensor, which diffuses between neighboring cells. They conclude with a computational model demonstrating a modest improvement in coordination of IHC activity, with possible benefits for sound encoding. While this work raises many interesting questions about the function of these “mini-syncytia” in hearing for future studies, it is a thorough and well-done manuscript. A few concerns remain:

First, we would like to thank the reviewer for the appreciation of our multidisciplinary study. The constructive evaluation of our work has helped us to further improve the manuscript. We have addressed all concerns raised by the reviewer.

1. Major:

The main concern with this work, which is partially, but not completely, addressed, is that of damage to the tissue during *in vitro* experiments. While *in vivo* measurement of this phenomenon is not currently possible, the authors should show clearly the results of their experiments in which techniques minimal tissue damage (supporting cell removal) were used. They are mentioned in the discussion as well as the supplementary methods. However, the results of these experiments were not mentioned (why?). Please move this data to the main results text, preferably with a figure detailing any differences between the two dissection conditions. This is especially important because the authors note an increase in mini-syncytia occurrence and # of IHC involved with age, but the cochlear tissue becomes more fragile with age as well. The authors notes that coupling between IHC and SCs was not observed, which supports their hypothesis, but SCs could have different membrane components, extracellular matrix molecules, etc that could make them more resistant to damage.

We are well aware of the potential caveats of the explanted organ of Corti preparation and clearly, the submittal reflects our longer-standing effort to move to more mature preparations in order to elucidate the physiology of mature inner hair cells and to better relate the *ex vivo* synaptic physiology to the *in vivo* recordings e.g. from auditory nerve fibers. In the present study, the data were collected by experienced hair cell patch-clampers who applied high quality criteria to their preparations: e.g. DIC imaging showing well preserved hair bundles, lack of membrane blebs (apically or basolaterally), maintained shape/size of IHC and lack of obvious “Brownian motion” inside the IHC. In a subset of experiments, minimal cleaning of supporting cells was used and comparable results were obtained (Fig. S4).

In order to further address the concern of the reviewer we have i) compared parameters that are sensitive to IHC viability and ii) included experiments in a more intact preparation of the cochlea.

i) Findings that speak to the viability of the coupled cells are that the Ca^{2+} current and the resting current of couples of two did not exceed double that of non-coupled cells (1 IHC: $I_{\text{CaMax}} = -161 \pm 15 \text{ pA}$, $I_{\text{rest}} = -22.05 \pm 2.23 \text{ pA}$, $n = 21$; 2 IHCs: $I_{\text{CaMax}} = -241 \pm 28 \text{ pA}$, $I_{\text{rest}} = -32.15 \pm 4.39 \text{ pA}$, $n = 13$, Fig. 2e). Lower viability of coupled cells would have likely produced more leak (greater resting current) and the poorer condition would have reduced the Ca^{2+} current which is very sensitive (e.g. ref. 1). The resting currents were not significantly different between non-coupled IHCs of $<P28$ and $\geq P28$ gerbils ($<P28$: $-26.79 \pm 2.32 \text{ pA}$, $n = 39$ IHCs, $N_{\text{animals}} = 15$; $\geq P28$: $-28.16 \pm 1.96 \text{ pA}$, $n = 24$, $N_{\text{animals}} = 9$, Mann-Whitney-Wilcoxon test, $p = 0.47$). These findings suggest that the developmentally increased probability of coupling does not reflect artificial IHC-IHC fusion due to poorer conditions of the preparation. Instead, we propose that this developmental increase of IHC-coupling results from the extended membrane-membrane contact area due to the reduced separation of IHCs by the supporting cells.

ii) Coupling was also found in the least invasive IHC recording approach in the dissected mouse organ of Corti when only one inner sulcus cell was removed for access of the pipette in the excised apical mouse organ of Corti and IHC membranes were approached with applying positive pressure to the patch pipette ($n = 15$ IHCs; $N = 6$ animals, P16-P24, Fig. S4).

iii) An experimental cochlea preparation with least mechanical alteration of the cochlea was contributed by the Ashmore lab, which worked in an *in situ* preparation of the temporal bone. The temporal bone containing the semicircular canals and the cochlea was removed from the skull and placed in a perilymph like extracellular solution. The bone covering the organ of Corti was carefully opened and Reissner's membrane was removed to gain access to the scala media, exposing a small opening to observe the structures. Patch-clamp of IHCs avoided removal of supporting cells from all but the cells being recorded as no other access to 'clean' membrane for patching was possible. In this set of experiments the same findings were obtained: dye- and electrical coupling, activation of presynaptic Ca^{2+} influx at the active zones of all IHCs of the syncytium, upon depolarization.

We also took all measures possible to exclude the possibility of histological artifacts in EM experiments: dissections were performed by an experienced researcher and we further carefully selected the samples to fulfil our requirements for high quality embeddings and excluded beforehand samples that did not meet our criteria. Further, three researchers independently analysed the data sets and the listed putative fusion sites are the result of this independent analysis. Conventional, fixative based embeddings of the organ of Corti are established in the Wichmann laboratory for many years. Regarding the artefacts of this specific high-contrast embedding, in our view, the 3 criteria employed take additional care of selecting the putative fusion sites and distinguish them from non-traceable membranes that could be present due to the embedding for FIB-SEM: Fusion sites were defined when at least 2 out of 3 criteria were fulfilled: i) a lack of traceable opposing IHC-IHC membranes, ii) seamless transition of the membranes of both IHCs, forming iii) a cytoplasmic bridge. The notion of cytoplasmic bridges was in some cases further supported by the presence of cellular structures such as ribosomes. Therefore, we are confident that the flat cell-cell

contacts are not an artifact due to a specific embedding method. Moreover, flat contacts in older animals were also observed upon high-pressure freezing followed by freeze substitution and subsequent electron tomography, which preserves the tissue in a near-to-native state. This way, we can exclude that some kind of collapse or shrinkage took place causing the flat cell-cell apposition, which appears in older animals and forms the prerequisite for fusion sites. However, this method is not suitable to check for membrane-to-membrane fusion sites in a systematic manner, because 250 nm semithin-sections need to be performed for tomography and therefore only small regions can be covered.

2. The introduction details other forms of coupling among neurons. However, cochlear hair cells are not neurons, they are epithelial cells. A discussion of similar junctions (or lack thereof) in this cell type is warranted, and may help support the experimental results.

done

3. In table 1, how many cells were imaged and analyzed, including cells with no fusion or filopodial sites?

This information was contained in Table S1 and has now been moved to results section and table 1.

4. The experiments presented in figure 7 are excellent, showing that functional effect of connected IHCs in potentially stimulating each other's pre-synaptic active zones. Is there any difference in the number of spots activated, or the fluorescence intensity of the spots, with distance from the patched IHC? This is not required for the main body of the manuscript, but could be added to the supplement. This data could give an idea of the effect of distance in determining function of the connected cells.

The fluorescence intensity of the spots with distance to the patched IHC is already depicted by Fig 7c. Indeed, this panel shows the averaged spot intensity in C1 (patched IHC), C2 (adjacent IHCs), C3 (two cells away), and C4 (three cells away). However, the number of spots is insufficient to answer whether there is a distance dependent activation, which is a very interesting point. In response to the reviewer's comment we have now compared the amplitude and voltage-dependence of activation of the spots in C1 and C2 (left and right inset of Figure 7c, respectively), and no significant differences were found. We have added the following sentence in the manuscript:

"We did not find differences of the synaptic Ca^{2+} -signals between the patched IHC (C1) and its coupled neighbor(s) (C2, the number of spots analyzed from further distant coupled cells did not permit statistical comparisons). Neither their maximal amplitude ($\Delta F_{\text{max}}/F_0$: 1.48 ± 0.25 , $n = 37$ spots in C1 of 8 mini-syncytia, $N_{\text{animals}} = 8$ vs. 1.88 ± 0.28 , $n = 43$ spots in C2 of 8 mini-syncytia, $N_{\text{animals}} = 8$; $p = 0.31$, Mann-Whitney-Wilcoxon test) nor their voltage-dependence of activation (V_h : -23.66 ± 2.24 mV, $n = 17$ spots in C1 in 7 mini-syncytia, $N_{\text{animals}} = 7$ vs. -23.79 ± 1.59 mV, $n = 30$ spots in C2 in 8 mini-syncytia, $N_{\text{animals}} = 8$; $p = 0.96$, Student t-test; note that because of signal-to-noise requirements the analysis of voltage-dependence could only be performed on a subset of synapses) were different."

Regarding the number of spots activated, a full z-scan of the mini-syncytia would have been required and would have taken several minutes since the cells are not fully aligned in z. Given the difficulty of recording good quality Ca^{2+} signals in such syncytia, the protocol was engaged as fast as possible after breaking in where the most ribbons were observed in all cells.

A supplemental figure S10 has now been added, where we can appreciate more examples of synaptic Ca^{2+} signals in IHC syncytia at different z-coordinates from the data analyzed in Fig. 7. A supplemental Movie S7 has also been added where we see the appearance of Ca^{2+} signals upon depolarization in two coupled IHCs.

5.Minor:

Please add a clock to the supplementary movies

Done for Supplemental Movie 1 and new Supplemental Movie 7. Moreover, scale bars were added as well where appropriate.

- Page 4, 5th line from bottom (line numbering would help), “sound born” = “sound borne”

Done

- Page 4, 3rd line from bottom: delete “already”

Done

- Figure 2f – define the red ‘plus’ symbols in the legend

The figure has been changed for more clarity

Reviewer #4 (Remarks to the Author):

The article from Jean et al reports that about 30% inner hair cells in rodents appear to form a syncytium. The experimental breadth includes classical neurobiology methods as well as three-dimensional light and electron microscopy.

I am here to judge the quality of the imaging, and technically from my perspective, there are no problems. The choice to perform TEM tomography is correct as the junctions and the membrane contacts are difficult to demonstrate with lower resolution approaches. The preparation protocol is standard and in my opinion appropriate to the task. maybe the presence of tannic acid is a little excessive given the fine details understudy, but overall I do not see it as a blocking issue.

First, we would like to thank the reviewer for the appreciation of our study. We used tannic acid for our high-pressure frozen samples because in our experience it gives a superb contrast for our tissue to visualize all required structures. So far, we did not face any problems with this staining in combination with subsequent osmium application.

Peer Review File

Reviewers' comments second round:

Reviewer #2 (Remarks to the Author):

The revised MS has addressed some of my previous concerns, although I am still puzzled as to whether this new morphological observation, which only affects around 30% of IHCs, has any functional role in hearing. What if this observation is part of the slow and progressive degeneration of the aging cochlea? This could be an important point, but this is not even considered by the Authors.

As mentioned in my previous report, is difficult to reconcile why the afferent synapses undergo such extensive pruning and tonotopic refinement during development, only then to revert back to a configuration resembling that of the immature cochlea, a few weeks later. The random distribution of IHC interconnections along the cochlea would be dispersed within the remaining 70% of IHCs that do not show any functional coupling, diluting any functional role these could have. This makes it very difficult to appreciate any functional benefit these IHC connections could have.

It is clear that the Authors have done a large amount of work, and it is very commendable, but in its current format the ms describes a morphological phenomenon with unknown functional implications – and as such is unlikely to be of interest to a broad readership.

Considerations regarding my previous comments:

- 1) More details are now included in the text and Method section.
- 2) This point remains largely unanswered. From a physiological perspective, it does not make sense why such a crucial mechanism (as described by the authors) is absent during the first month of an adult mouse. The hearing ability of a mouse is fully developed well before postnatal day 30. So, why is this phenomenon only observed at later stages? Which sound encoding characteristics are improved or reduced by the appearance of this mechanism. From a general perspective, IHC coupling could also be interpreted as a cochlear dysfunction in a slowing aging mouse. This point is vital for the overall interpretation of the paper.
- 3) This has not been clarified in the revised ms.
- 4) This is another critical point that has not been fully addressed. The reason for not recording the K current is not justified. The authors could easily prove this concept by stepping the membrane potential to moderately depolarizing voltages (for example -40 mV). The data shown using Cs⁺ are impossible to judge due to the fact that the traces are compressed together.
- 5) Thank you for dealing with this point.
- 6) The additional explanation is very useful for interpreting the results.
- 7) This aspect has not been addressed at all in the revised ms. Without any clear understanding of the physiological and functional implications of the findings (see also point 2 above), the ms represents a very descriptive/observational study of an unknown morphological phenomenon, which could have no implication at all in hearing. There is also the possibility, which is not discussed by the authors, that this unusual observation could be linked to cochlear dysfunction (see general comment above). As such, the impact of the work is reduced and does not substantially advance our current understanding of cochlear function or sound processing.

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns.

Rev#2

The revised MS has addressed some of my previous concerns, although I am still puzzled as to whether this new morphological observation, which only affects around 30% of IHCs, has any functional role in hearing. What if this observation is part of the slow and progressive degeneration of the aging cochlea? This could be an important point, but this is not even considered by the Authors. As mentioned in my previous report, is difficult to reconcile why the afferent synapses undergo such extensive pruning and tonotopic refinement during development, only then to revert back to a configuration resembling that of the immature cochlea, a few weeks later. The random distribution of IHC interconnections along the cochlea would be dispersed within the remaining 70% of IHCs that do not show any functional coupling, diluting any functional role these could have. This makes it very difficult to appreciate any functional benefit these IHC connections could have. It is clear that the Authors have done a large amount of work, and it is very commendable, but in its current format the ms describes a morphological phenomenon with unknown functional implications – and as such is unlikely to be of interest to a broad readership.

We would like to thank the reviewer for the continued interest in our study.

Considerations regarding my previous comments:

1) More details are now included in the text and Method section. We appreciate that this concern was appropriately addressed.

2) This point remains largely unanswered. From a physiological perspective, it does not make sense why such a crucial mechanism (as described by the authors) is absent during the first month of an adult mouse. The hearing ability of a mouse is fully developed well before postnatal day 30. So, why is this phenomenon only observed at later stages? Which sound encoding characteristics are improved or reduced by the appearance of this mechanism. From a general perspective, IHC coupling could also be interpreted as a cochlear dysfunction in a slowing aging mouse. This point is vital for the overall interpretation of the paper.

quoting previous point 2: “It is not clear to me why the mice have to be over 1 month old in order to show a substantial increase in the coupling between IHCs. From a purely functional point of view, if this phenomenon is so crucial for proper sound perception, why is it not present during the first month of life? Also, does the IHC-IHC coupling increasing further at older ages? If so, then the interpretation is likely to change substantially.”

At present we do not have a sensible reply for why biology might employ less IHC coupling for hearing at earlier stages. Mechanistically, it seems that the extent of supporting cell separation declines during postnatal maturation which gives rise to more opportunities for IHCs to contact each other in the more mature state. One might also speculate that immature IHCs experience a different means of synchronization which is mediated by supporting cells releasing ATP and/or K^{+1-3} thought to functionally group neighboring hair cells and their postsynaptic Spiral Ganglion Neurons (SGNs) for refinement of tonotopic organization throughout the pathway.

Finally, we would like to refer the reviewer to another means of cochlear information mixing that results from the electron microscopy demonstration of a branching of the SGN dendrites in 14% of the SGNs. These SGNs then receive input from more than one active zone of one IHC or even more often two IHCs⁴. SGN dendritic branching has also been described by Nadol and colleagues for the human cochlea⁵. Sampling from several active zones and hair cells is well established for the acoustic papilla in birds and reptiles^{6,7}.

3) This has not been clarified in the revised ms.

quoting previous point 3: "Figure 2f is interesting but also highlights some concerns. The level of variability in the size of the calcium current recorded from an "uncoupled" IHCs is very variable (from about 50 pA to 450 pA), which is not consistent with work previously published from this lab. Is it possible that this large variability is due to differences in the general health of the patched IHCs? Is the correlation shown in Fig 2f significantly different from 0, given the large current size variability? It would be useful to show the I_{rest} of the IHCs as a function of age –does it become larger?"

We are not sure that we understand this comment: we believe we have implemented this in the MS: we separated the recordings and now show them as Fig. 2e and Figs. S1e and S2.

"Macromolecular coupling is accompanied by low resistance electrical coupling of IHCs. Indeed, the maximal voltage-gated Ca^{2+} -influx (I_{CaMax}) and resting currents (I_{rest}) increase with the number of dye-filled IHCs (Fig. 2d,e,e', Fig. S2b). Both parameters were significantly larger for mini-syncytia of 3 and 4 dye-coupled IHCs than for single IHCs (1 IHC: $I_{CaMax} = -161 \pm 15$ pA, $I_{rest} = -22.05 \pm 2.23$ pA, $n = 21$ (data taken from Jean *et al.*, 2018 where similar recording conditions were used); 2 IHCs: $I_{CaMax} = -241 \pm 28$ pA, $I_{rest} = -32.15 \pm 4.39$ pA, $n = 13$; 3 IHCs: $I_{CaMax} = -356 \pm 67$ pA, $I_{rest} = -56.25 \pm 10.28$ pA, $n = 4$; 4 IHCs: $I_{CaMax} = -482 \pm 68$ pA, $I_{rest} = -50.75 \pm 6.99$ pA, $n = 4$ recordings; mean $\pm$ S.E.M, Dunn-Holland-Wolfe non-parametric multiple comparison test, $p < 0.05$ for 3 IHCs and 4 IHCs vs. 1 IHC). Interestingly, the strength of IHC coupling varied as also indicated by the variable spread of the TAMRA peptide (Fig. S1c,c'). Strongly dye-coupled IHCs, showing a rapid dye-spread, exhibited fast monophasic I_{Ca} activation kinetics. In the rare case of weakly dye-coupled IHCs, showing slow dye-spread, I_{Ca} exhibited a multiphasic activation, probably due to poorer voltage-clamp of the coupled IHCs via a larger intercellular resistance (Fig. S1d,d' and Fig. S2b). Likewise, membrane capacitance (C_m) estimates, derived from capacitive currents to voltage steps, increased with the number of dye-coupled IHCs again indicating low resistance connections in the majority of the mini-syncytia. As expected, a correlation with C_m was found for I_{CaMax} ($r = -0.66$, $p < 0.00001$, Fig. 2e) as well as for I_{rest} (Fig 2e', $r = -0.74$, $p < 0.00001$). In a larger data set combining different $[Ca^{2+}]_e$ (1.3, 2 or 5 mM), correlations were also found for I_{CaMax} and I_{rest} with C_m (I_{CaMax} , $r = -0.66$, $p < 0.00001$, I_{rest} , $r = -0.64$, $p < 0.00001$, P15-51, Fig. S1e,e'). Similar observations were made i) from ruptured-patch recordings from IHCs of the excised apical and basal gerbil organ of Corti (P14-38, Fig. S2), ii) from perforated-patch recordings of mouse IHCs in the excised organ of Corti ($n = 14$ IHCs, $N_{animals} = 11$, P15-P36), and iii) from ruptured-patch recording of IHCs when only one inner sulcus cell was removed for access of

the pipette in the excised apical mouse organ of Corti ($n = 15$ IHCs; $N_{animals} = 6$, P16-P24, Fig. S4)."

4) This is another critical point that has not been fully addressed. The reason for not recording the K current is not justified. The authors could easily prove this concept by stepping the membrane potential to moderately depolarizing voltages (for example -40 mV). The data shown using Cs⁺ are impossible to judge due to the fact that the traces are compressed together.

quoting previous point 4: "If coupling between IHCs is present, then a similar variability in the outward K current size to that seen for IcaMax would be expected. Considering that an adult IHC showing a C_m of about 9pF and a I_K of about 15nA (e.g. from your previous study: Sendin et al., 2007), I_K would be expected to be about 130nA if 9 IHCs are connected? You should provide some examples of very large K currents to corroborate the calcium current results. This is important because K currents are less likely to be affected by the physiological state of the cells than the calcium currents."

We are not sure that we understand this comment: We have presented the Cs⁺ currents in the supplement and the slope conductance estimates (Fig. S5 and S6) clearly support the notion of an increase of membrane conductance as a function of the number of IHCs in the syncytium and stated in the rebuttal: "Preliminary data using K⁺ as the major pipette cation did not permit the sufficient voltage clamp control...."

5) Thank you for dealing with this point.

We appreciate that this concern was appropriately addressed.

6) The additional explanation is very useful for interpreting the results.

We appreciate that this concern was appropriately addressed.

7) This aspect has not been addressed at all in the revised ms. Without any clear understanding of the physiological and functional implications of the findings (see also point 2 above), the ms represents a very descriptive/observational study of an unknown morphological phenomenon, which could have no implication at all in hearing. There is also the possibility, which is not discussed by the authors, that this unusual observation could be linked to cochlear dysfunction (see general comment above). As such, the impact of the work is reduced and does not substantially advance our current understanding of cochlear function or sound processing.

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difficult to really appreciate the functional benefit. Moreover, it is difficult to reconcile why the afferent fibers undergo extensive pruning during development, in order to make one-to-one connections with the IHCs, and then after a couple of weeks revert back to a configuration resembling that of the immature cochlea.”

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Rev#3

The authors have addressed my concerns.

We would like to thank the reviewer for the support of our study.

1. Tritsch, N. X., Yi, E., Gale, J. E., Glowatzki, E. & Bergles, D. E. The origin of spontaneous activity in the developing auditory system. *Nature* **450**, 50–55 (2007).
2. Sendin, G., Bourien, J., Rassendren, F., Puel, J.-L. & Nouvian, R. Spatiotemporal pattern of action potential firing in developing inner hair cells of the mouse cochlea. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1999–2004 (2014).
3. Johnson, S. L. *et al.* Position-dependent patterning of spontaneous action potentials in immature cochlear inner hair cells. *Nat Neurosci* **14**, 711–717 (2011).
4. Hua, Y. *et al.* Electron Microscopic Reconstruction of Neural Circuitry in the Cochlea. *bioRxiv* 2020.01.04.894816 (2020) doi:10.1101/2020.01.04.894816.
5. Nadol, J. B. Serial section reconstruction of the neural poles of hair cells in the human organ of Corti. I. Inner hair cells. *The Laryngoscope* **93**, 599–614 (1983).
6. Fischer, F. P. Quantitative analysis of the innervation of the chicken basilar papilla. *Hear. Res.* **61**, 167–178 (1992).
7. Sneary, M. G. Auditory receptor of the red-eared turtle: II. Afferent and efferent synapses and innervation patterns. *J. Comp. Neurol.* **276**, 588–606 (1988).

Description of Additional Supplementary Files

File Name: Supplementary Movie 1

Description: Macromolecular coupling between two IHCs. Diffusion of the fluorescent TAMRA-peptide pipette-loaded in one IHC to the neighboring IHC.

File Name: Supplementary Movie 2

Description: Z-stack of five coupled IHCs. Z-stack of TAMRA-peptide fluorescence from bottom to top of a mini-syncytia of five coupled IHCs. The patched IHC can be recognized by a hole at its basolateral membrane.

File Name: Supplementary Movie 3

Description: 3D-view of five coupled IHCs. 3D-view of TAMRA-peptide fluorescence in a mini-syncytia of five coupled IHCs. The patched IHC can be recognized by a hole at its basolateral membrane.

File Name: Supplementary Movie 4

Description: 3D reconstruction of FIB-SEM image stack of neighboring IHCs (P34) illustrating a flat membrane contact. 3D reconstruction shows the neighboring membranes (highlighted in blue and red) forming flat contacts.

File Name: Supplementary Movie 5

Description: Sequential FIB-SEM images and the 3D reconstruction of P34 IHCs revealing a putative membrane fusion site. Illustrated are neighbouring membranes (highlighted as blue and red 3D reconstructions) of two basolateral IHCs that form a contact leading to a putative fusion site.

File Name: Supplementary Movie 6

Description: Lack of physical contacts between outer hair cells. 3D

reconstruction of serial block-face electron microscopic datasets demonstrates that the basolateral membranes of P22 mouse midcochlear outer hair cells (in blue) do not share physical contacts between each other. Outer hair cells and surrounding supporting cells are initially showed in yellow. Additional oblique slices of the used datasets are demonstrated in the supplemental figure 9.

File Name: Supplementary Movie 7

Description: Live calcium imaging of two coupled IHCs. Depicted are the calcium hotspots (indicated by arrows) that appear in the two coupled IHCs upon membrane depolarization. Bottom, middle and top sections of the IHCs are showed.

Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☐ ☒ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☐ ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

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Software and code

Policy information about [availability of computer code](#)

Data collection

Patch clamp data: PClamp 10.6, Pulse 8.8 or Patchmaster 2.90 softwares were used for electrophysiological measurements and images were acquired by Andor 4.21 or Till-Vision 4.0.1 softwares.
For tomography EM: the Serial-EM software 2.8.10 was used for image acquisition
For FIB-SEM 3D data: Atlas 3D 5.1 and SmartSEM 6.3 softwares were utilized

Data analysis

The data were analyzed using Fiji (Image J 1.8), microscopy image browser (MIB 2.3), Imaris 9.0, IMOD 4.9 and custom programs in Igor Pro 6.3 (Wavemetrics, Portland, OR, USA).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data is available from the corresponding authors (CW,TM) upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample-size calculations were performed. Since the phenomenon of interest was present in a subset of cells, sample size was set to the maximal amount the experimenter could handle.
Data exclusions	Dye loading and patch-clamp experiments: We excluded cells that were not stable electrophysiologically during the time of the dye-loading, and excluded recordings when the leak current was more than 50 pA for a single cell for ruptured-patch experiments. Electron microscopy experiments: We excluded one data point, from the P34 FIB-SEM data as an outlier, as the z-measurements of 535 nm clearly exceeds 1.5 times the interquartile range above the third quartile (75th percentile). The exclusion criteria were pre-established.
Replication	Results were replicated in different animals within the experimental groups, by several scientists from different laboratories. Since the phenomenon of interest was present in a subset of cells, replications were performed as much as possible.
Randomization	The study was not allocating the data in different experimental groups, therefore no randomization was used.
Blinding	The study was not allocating the data in different experimental groups, therefore no blinding was performed.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Alexa Fluor 488 goat anti-rabbit IgG and 568 anti-rabbit IgG (3% dilution in intracellular solution, A11008 and A11011 respectively, Life technologies, Germany)
Validation	Antibodies have been extensively tested by the manufacturer in applications such as cell imaging, flow cytometry and fluorescent western blotting. Robust QC procedures guarantee high performance for each individual product.

Animals and other organisms

Policy information about [studies involving animals](#): [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	Postnatal day (P) 14-100 mice from C57BL/6J, C57BL/6N, and CD1 background and Mongolian gerbils (P16-38) of either sex were used. The animals were either housed in local animal facilities in Göttingen (three different facilities) and London or imported from Charles River Germany straight into the experiment in Göttingen
Wild animals	No wild animals were used in this study.
Field-collected samples	No field collected samples were used in this study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

