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Clustered Ca^{2+} Channels Are Blocked by Synaptic Vesicle Proton Release at Mammalian Auditory Ribbon Synapses

Philippe F.Y. Vincent¹, Soyoun Cho^{2,3}, Margot Tertrais¹, Yohan Bouleau¹, Henrique von Gersdorff^{3,4,5,*}, and Didier Dulon^{1,4,*}

¹Université de Bordeaux, Bordeaux Neurocampus, Equipe Neurophysiologie de la Synapse Auditive, Inserm U1120, 33076 Bordeaux, France

²Center for Sensory Neuroscience, Boys Town National Research Hospital, Omaha, NE 68131, USA

³The Vollum Institute, Oregon Health and Science University, Portland, OR 97239, USA

⁴Senior author

⁵Lead Contact

SUMMARY

A Ca^{2+} current transient block (I_{CaTB}) by protons occurs at some ribbon-type synapses after exocytosis, but this has not been observed at mammalian hair cells. Here we show that a robust I_{CaTB} occurs at post-hearing mouse and gerbil inner hair cell (IHC) synapses, but not in immature IHC synapses, which contain non-compact active zones, where Ca^{2+} channels are loosely coupled to the release sites. Unlike I_{CaTB} at other ribbon synapses, I_{CaTB} in mammalian IHCs displays a surprising multi-peak structure that mirrors the EPSCs seen in paired recordings. Desynchronizing vesicular release with intracellular BAPTA or by deleting otoferlin, the Ca^{2+} sensor for exocytosis, greatly reduces I_{CaTB} , whereas enhancing release synchronization by raising Ca^{2+} influx or temperature increases I_{CaTB} . This suggests that I_{CaTB} is produced by fast multivesicular proton-release events. We propose that I_{CaTB} may function as a submillisecond feedback mechanism contributing to the auditory nerve's fast spike adaptation during sound stimulation.

Graphical Abstract

*Correspondence: vongersd@ohsu.edu (H.v.G.), didier.dulon@inserm.fr (D.D.).

AUTHOR CONTRIBUTIONS

H.v.G. and D.D. designed the research. P.F.Y.V., M.T., and D.D. carried out and analyzed the electrophysiological recordings in the *ex vivo* explants of organ of Corti from mice and gerbils. S.C. and H.v.G. carried out the electrophysiological recordings and data analysis from frog hair cell synapses. Y.B. performed the immunofluorescent confocal microscopy. D.D. performed and analyzed data on PSTH from mouse ANFs *in vivo*. D.D. and H.v.G. wrote the paper.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.11.072>.

DECLARATION OF INTERESTS

The authors declare no competing interests.

In Brief

Mature inner hair cells in the cochlea contain compact active zones where clustered Ca^{2+} channels are tightly coupled to the sites of vesicle fusion. Vincent et al. show that vesicle exocytosis leads to the release of protons into the synaptic cleft, which transiently blocks a subset of Ca^{2+} channels.

INTRODUCTION

Synaptic transmission depends on the spatial organization of Ca^{2+} channels and synaptic vesicles at the presynaptic active zone (Wang and Augustine, 2015). At auditory hair cell synapses, the active zone is distinguished by the presence of an electron-dense synaptic ribbon that overhangs two rows of vesicles that are closely docked at nanometer distances from a compact one- to three-row array of ~ 100 Ca^{2+} channels (Roberts et al., 1990; Brandt et al., 2005; Graydon et al., 2011; Vincent et al., 2014; Rutherford, 2015). At these specialized synapses, large monophasic and multiphasic excitatory postsynaptic currents (EPSCs) are observed, suggesting multivesicular release (MVR; Glowatzki and Fuchs, 2002; Goutman and Glowatzki, 2007). However, this interpretation has been challenged by a proposal that fusion pore flickering during single-vesicle exocytosis may account for multiphasic EPSCs (Chapochnikov et al., 2014).

At amphibian hair cell synapses, evidence for MVR has come from the observation that EPSC amplitudes are dependent on presynaptic membrane potential, and small EPSCs were interpreted as being due to univesicular release (UVR), whereas the large EPSCs are likely due to MVR (Keen and Hudspeth, 2006; Li et al., 2009). Additional evidence for MVR has come from the observation of a fast Ca^{2+} current transient block (I_{CaTB}) by protons released during exocytosis (Cho and von Gersdorff, 2014). The simultaneous release of multiple synaptic vesicles was required to explain I_{CaTB} because the proton content of a single vesicle was calculated to be insufficient to significantly acidify the synaptic cleft

microenvironment of the Ca^{2+} channel cluster. Similar proton regulation of Ca^{2+} current was first shown at retinal ribbon synapses (DeVries 2001; Palmer et al., 2003).

I_{CaTB} has not yet been observed in mammalian auditory hair cells, perhaps because release may be dominated by UVR, so the amount of H^+ released may be too low (Chapochnikov et al., 2014). Moreover, amphibian papilla hair cell synapses exhibit afferent fiber connectivity that differs greatly from the mammalian inner hair cells (IHCs), because a single large calyx-like afferent fiber receives the input of 30–40 ribbons (Graydon et al., 2014), whereas at IHCs a small afferent fiber bouton receives the input of a single ribbon. This configuration may not allow I_{CaTB} , because released protons may quickly diffuse out of the small synaptic cleft of IHCs ribbon synapse. Alternatively, I_{CaTB} may not have been reported in IHCs, because it is blocked by extracellular HEPES, a commonly used fast pH buffer. To address this possibility, we investigated I_{CaTB} in mouse and gerbil IHC synapses using the physiological pH buffer bicarbonate.

Our results show that a fast I_{CaTB} occurs in mature post-hearing IHCs at physiological temperatures. Importantly, I_{CaTB} was not observed in immature pre-hearing postnatal day 6–9 IHCs, which have non-compact active zones with many Ca^{2+} channels that reside distal to synaptic ribbons. I_{CaTB} is also absent in central synapses with non-compact conventional active zones, such as the calyx of Held synapse, although these synapses can release glutamate via fast Ca^{2+} nanodomain-triggered exocytosis (Meinrenken et al., 2002; Nakamura et al., 2015). Furthermore, using otoferlin-knockout mice, we propose an additional role for this multi-C2 domain Ca^{2+} sensor for exocytosis (Roux et al., 2006; Michalski et al., 2017); namely, otoferlin promotes a highly synchronous form of MVR at IHCs.

These findings suggest that a compact active-zone topography of Ca^{2+} -channel rows flanked vis-à-vis by two rows of docked vesicles promotes a Ca^{2+} nanodomain-triggered MVR, which produces a prominent I_{CaTB} at IHC synapses. Moreover, unlike I_{CaTB} in amphibian hair cells, which display a smooth “notch” after Ca^{2+} current peak activation, I_{CaTB} in mammalian IHCs displayed a remarkably noisy multi-peak notch followed by isolated “spikelets” that resembled the large EPSCs triggered by step depolarizations of hair cells in paired recordings (Goutman and Glowatzki, 2007). I_{CaTB} thus constitutes perhaps a more readily accessible way to study transmitter release from mature mammalian IHCs than the more challenging direct recordings from the small afferent fiber boutons. Finally, our *in vivo* recordings of sound-evoked spikes in mature mouse auditory nerve indicate that many fibers display a primary-like-with-notch structure and a fast adaptation component followed by a slower component. We thus propose that the submillisecond kinetics of I_{CaTB} promote a rapid reduction in synaptic vesicle exocytosis that may contribute to the fast spike adaptation of the auditory nerve.

RESULTS

Desynchronizing Release with BAPTA Reduces I_{CaTB} in Adult Bullfrog Hair Cells

To investigate the relationship between synchronous release and the transient block of presynaptic Ca^{2+} currents (I_{CaTB}) we first performed recordings from the bullfrog

amphibian papillae, in which paired recordings of Ca^{2+} currents and EPSCs from adult calyx synapses are less challenging than from mammalian IHC boutons. When the auditory hair cells are depolarized from -60 to -30 mV in an external bicarbonate-based solution, their Ca^{2+} currents exhibit I_{CaTB} when 2 mM EGTA is used as intracellular Ca^{2+} buffer (Cho and von Gersdorff, 2014). To examine the mechanisms of the H^+ and glutamate vesicular release, we used 10 mM of the fast Ca^{2+} chelator BAPTA in the hair cell intracellular recording solution. In these conditions, we found that the initial hair cell Ca^{2+} current recorded within 1 min after whole-cell break-in still showed I_{CaTB} (Figures 1A1 and 1A2, black trace). However, as the BAPTA was dialyzed into the hair cell, I_{CaTB} was gradually decreased and removed in the later recordings measured at more than 4 min after break-in (Figures 1A1 and 1A2, green and red traces). In parallel, the corresponding EPSCs were strongly reduced (Figures 1A1 and 1A2, green and red). In addition to reducing total EPSC charge, 10 mM BAPTA delayed the first evoked EPSC and dispersed the EPSC events during the stimulation (Figure 1A2, red trace), which is consistent with the effect of BAPTA on EPSCs in rat IHC synapses (Goutman and Glowatzki, 2007). Note that 10mMBAPTA also delayed the onset of I_{CaTB} (Figure 1A2, green trace). The average total EPSC charge was significantly decreased from 16.8 ± 4.5 pC ($n = 7$) in the initial recordings (measured within 1 min after break-in) to 2.0 ± 0.7 pC ($n = 7$) in the later recordings (measured after 4 min from break-in; Figure 1B; $p < 0.01$, paired t test). The size of I_{CaTB} strongly correlated with EPSC amplitudes. The ratio of I_{CaTB} to the peak of Ca^{2+} current decreased significantly in the later recordings (>4 min; $n = 7$; Figure 1C; $p < 0.01$, paired t test). These results show that 10 mM BAPTA in adult auditory hair cells strongly reduces the amplitude of EPSCs and I_{CaTB} . We suggest that this occurs because BAPTA desynchronizes and blocks the Ca^{2+} nanodomain-triggered multivesicular exocytosis of adult hair cell synapses.

Proton Release Triggers I_{CaTB} in Post-hearing Gerbil and Mouse IHCs

Mammalian IHCs make synaptic contacts with 10–20 separate bouton-type afferent fibers. I_{CaTB} may require intact IHC synapses because it is absent in acutely dissociated cells (Palmer et al., 2003). To better preserve the afferent synaptic boutons, the whole cochlear apical turn of the organ of Corti, attached to the afferent fibers and spiral ganglion neurons, was dissected and mounted intact in the recording chamber (Figures 2A and 2B). We also noticed that keeping the tectorial membrane intact above the organ of Corti greatly improved the survival of the afferent boutons (see STAR Methods). During continuous perfusion with a bicarbonate-based external solution containing 5 mM extracellular Ca^{2+} at room temperature (22°C – 24°C), the Ca^{2+} currents recorded in either IHCs from P20–P30 gerbils or P14–P18 mice (both post-hearing animals) systematically showed a fast I_{CaTB} right after the peak Ca^{2+} current (Figures 2C and 2D). The depolarization evoked a capacitance jump (C_m) due to synaptic vesicle exocytosis (Moser and Beutner, 2000).

The amplitudes of I_{CaTB} in gerbil and mouse IHCs were similar (Figures 2E and 2F). The kinetics of the transient upward block of the Ca^{2+} current (Figure 2C, inset) could be fitted with a single exponential with time constant τ of 2.62 ± 0.48 ms ($n = 7$) and 2.28 ± 0.17 ms ($n = 13$) ($p = 0.14$, unpaired t test) in gerbil and mouse IHCs, respectively. The amplitudes of I_{CaTB} , measured for a voltage step from -80 to -10 mV, were 28.0 ± 7.1 pA, equivalent to $13.7\% \pm 3.5\%$ of the peak Ca^{2+} current of 204.3 ± 5.6 pA ($n = 9$) for gerbil IHCs, and 29.3

± 3.2 pA, equivalent to $15.4\% \pm 1.7\%$ of the peak Ca^{2+} current of 190.0 ± 5.6 pA ($n = 17$) for mice IHCs ($p = 0.1$, unpaired t test). The exocytotic responses were also found to have similar amplitudes in gerbil and mouse IHCs: 27.0 ± 2.7 and 31.9 ± 1.9 fF, respectively (Figures 2E and 2F; $p = 0.1$, unpaired t test; see also Johnson et al., 2017).

Lowering the concentration of the pH buffer of the extracellular solution, from 25 to 10 mM bicarbonate, increased I_{CaTB} from $15.4\% \pm 1.7\%$ ($n = 17$) to $21.9\% \pm 3.4\%$ of the peak Ca^{2+} current ($n = 4$ mouse IHCs; $p = 0.06$; data not shown). Conversely, I_{CaTB} was abolished in both gerbil and mouse IHCs when 10 mM of the fast pH buffer HEPES was added to the external solution (Figure 2, red lines), indicating that it was produced by H^+ release during exocytosis. With added HEPES, the eC_m jump was unchanged in gerbil ($p = 0.5$) and only slightly reduced in mice compared with bicarbonate conditions (Figures 2E and 2F, $p < 0.05$).

Perfusing the IHCs for up to 30 min with a bicarbonate solution containing 100 μM DL-TBOA, a potent blocker of glutamate transporters (Kanai et al., 2013), or 100 μM amiloride, a potent blocker of H^+ -activated ASIC channels (Chu et al., 2011), did not affect the amplitude of I_{CaTB} (26.6 ± 4.6 pA, $n = 5$, $p = 0.74$, and 25.8 ± 3.6 pA, $n = 3$, $p = 0.7$, respectively; data not shown), suggesting that the transient H^+ block originates exclusively from a block of Ca^{2+} current.

I_{CaTB} Is Temperature Dependent

The timing and strength of evoked transmitter release is highly dependent on temperature (Kushmerick et al., 2006; Nouvian, 2007). We thus recorded I_{CaTB} in mouse IHCs (P14–P18) in near physiological conditions: IHCs held at -65 mV with 1.3 mM extracellular Ca^{2+} and at 32°C (Figures 3A and 3B). In these conditions, we found that both I_{CaTB} ($6.2\% \pm 1.0\%$ versus $18.9\% \pm 2.0\%$) and exocytosis (25.6 ± 4.8 versus 54.3 ± 6.4 fF) are greatly increased compared with IHCs at 22°C in 1.3 mM extracellular Ca^{2+} ($p < 0.01$). Also, the kinetics of I_{CaTB} were faster at 32°C ($n = 10$, $\tau = 2.3 \pm 0.4$ ms versus 4.4 ± 0.9 ms; $n = 14$; $p < 0.05$; data not shown). The latency or synaptic delay (t), defined as the time between the onset of the Ca^{2+} current and the first rising point of I_{CaTB} (Figure 3A1, black arrowheads), decreased from 22°C (8.3 ± 0.9 ms, $n = 10$) to 32°C (2.1 ± 0.3 ms, $n = 16$). Overall, the I_{CaTB} responses (amplitude and kinetics) obtained at 32°C in 1.3 mM extracellular Ca^{2+} were very similar to those obtained with 5 mM extracellular Ca^{2+} at room temperature (Figure 2F; $p > 0.05$). Therefore, in the rest of this study, all IHC recordings (unless otherwise indicated) were performed at room temperature and with 5 mM extracellular Ca^{2+} .

I_{CaTB} Decreases with Synaptic Depression

To further investigate the relationship between exocytosis and I_{CaTB} , we stimulated mouse post-hearing IHCs (P14–P18) with a paired pulse consisting of two consecutive depolarizing steps of 100 ms, from -80 to -10 mV, separated by 100 ms (Figure 3C). We found that C_m and I_{CaTB} displayed a similar paired-pulse depression of $60.3\% \pm 5.9\%$ and $53.4\% \pm 5.1\%$, respectively ($n = 12$; $p = 0.38$; Figures 3C and 3D). A similar correlation between C_m and I_{CaTB} depression was obtained with a train of pulses (Figures S1A and S1B). Plotting C_m

versus the corresponding I_{CaTB} , for each pulse revealed a linear relationship (Figure S1C). These results indicate that I_{CaTB} is caused by H^+ exocytosis. Interestingly, the width of the various I_{CaTB} was correlated with their respective amplitude (Figure S1D), suggesting that larger release events had a more prolonged H^+ block of nearby Ca^{2+} channels.

Voltage Dependence of I_{CaTB}

We next explored the relationship between C_m and I_{CaTB} in mouse IHCs (P14–P18) by varying the number of open Ca^{2+} channels during depolarizing voltage steps from -80 to -10 mV. As previously reported in extracellular HEPES (Brandt et al., 2005; Johnson et al., 2005; Beurq et al., 2010), we found that the amplitude of C_m increased linearly with Ca^{2+} currents in bicarbonate conditions (Figure S2). This linear relationship can be explained by an organization of independent nanodomains between Ca^{2+} channels and release sites at the IHC active zone (Brandt et al., 2005). I_{CaTB} also displayed a linear correlation with exocytosis (Figure S2E). Notably, a significant I_{CaTB} could be measured at potentials as low as -45 mV, a potential near the estimated physiological resting membrane potential of -55 to -60 mV of IHCs (Palmer and Russell, 1986). The amplitude of I_{CaTB} peaked at about -20 mV, a voltage close to the peak of the Ca^{2+} currents (Figures S2B and S2C). Taken together, these results indicated that the amplitude of I_{CaTB} was linearly related to the open probability of Ca^{2+} channels and the probability of vesicular fusion at the release sites.

Ca^{2+} Dependence of I_{CaTB}

We next investigated the cooperativity of the exocytotic Ca^{2+} sensor through I_{CaTB} measurements by recording Ca^{2+} currents and C_m while varying the concentration of external Ca^{2+} ($[Ca^{2+}]_{ext}$) from 0.5 to 10 mM at constant voltage step depolarization from -80 to -10 mV (Figures 3E–3H). Under these conditions, the peak amplitude of the Ca^{2+} currents increased linearly with the concentration of extracellular Ca^{2+} from 107.6 ± 9.1 pA ($n = 9$) at 0.5 mM to 237.8 ± 10.5 pA ($n = 13$) at 10 mM (Figure 3E). However, I_{CaTB} and Ca^{2+} current amplitudes displayed a nonlinear relationship that was best fit by a power function with an index $n = 3$ (Figure 3F). A similar nonlinear relationship was found between the charge integral of I_{CaTB} and the Ca^{2+} currents (data not shown). Likewise, the C_m jump also had a nonlinear relationship with the Ca^{2+} currents (Figure 3H). This nonlinear Ca^{2+} dependence of I_{CaTB} and C_m , when only varying the flux of Ca^{2+} , likely reflected the cooperativity of the putative exocytotic Ca^{2+} sensor of exocytosis otoferlin (Roux et al., 2006; Beurq et al., 2010). Such a cooperative superlinear relationship was also described between the presynaptic Ca^{2+} flux and the EPSC amplitudes (Goutman and Glowatzki, 2007). We note that the large I_{CaTB} (5.37 ± 0.41 pC, $n = 13$) we observed with 10 mM $[Ca^{2+}]_{ext}$ was 10 times larger than the I_{CaTB} (0.53 ± 0.41 pC, $n = 9$) with 0.5 mM external Ca^{2+} . The time to peak of I_{CaTB} (Figure 3E, inset), which presumably reflects release synchrony, decreased exponentially with the amplitude of the Ca^{2+} currents (Figure 3G), suggesting that release synchronization is Ca^{2+} dependent.

I_{CaTB} Requires Mature Ca^{2+} Nanodomains at Release Sites

We also investigated I_{CaTB} in immature pre-hearing IHCs at postnatal days 7–9 (P7–P9). In these IHCs, exocytosis is known to have a nonlinear Ca^{2+} dependence and a low Ca^{2+} efficiency, because of a loose spatial organization between Ca^{2+} channels and the sites of

release (Johnson et al., 2005; Beurq et al., 2010; Wong et al., 2014; Pangrsic et al., 2018). Using confocal microscopy, we explored the distribution of $\text{Ca}_v1.3$ channels, the most prominent Ca^{2+} channel isoform in IHCs (Platzter et al., 2000; Dou et al., 2004), and CtBP2 (the B domain of ribeye, a marker of synaptic ribbons) in P7–P9 IHCs and post-hearing P25 IHCs (Figures 4A–4D). Although in post-hearing IHCs, the immuno-labeling of $\text{Ca}_v1.3$ and CtBP2 overlapped at $89\% \pm 2\%$, with a mean surface area for the $\text{Ca}_v1.3$ patches of $0.15 \pm 0.01 \mu\text{m}^2$ ($n = 217$ ribbons; Figure 4C), this percentage was only $37\% \pm 5\%$, with a twice larger mean surface area of the $\text{Ca}_v1.3$ patches ($0.30 \pm 0.02 \text{ mm}^2$; $n = 169$ ribbons; Figure 4D) in immature P7–P9 IHCs. This indicated that a large proportion of $\text{Ca}_v1.3$ channels are extra-synaptic and at large distances from the ribbon in immature IHCs, a result compatible with the large sensitivity of exocytosis to 5mM intracellular EGTA in these cells (Beutner and Moser, 2001).

For a 100 ms voltage step depolarization from -80 to -10 mV, the Ca^{2+} currents of pre-hearing IHCs were twice as large as those of post-hearing IHCs (334.1 ± 26.9 pA [$n = 8$] and 190.0 ± 5.6 pA [$n = 17$] in pre- and post-hearing IHCs, respectively; $p < 0.05$, unpaired t test). However, the C_m responses were similar (28.5 ± 3.5 fF [$n = 14$], compared with 31.9 ± 1.9 fF in post-hearing IHCs [$n = 17$]; $p = 0.6$, unpaired t test). Nevertheless, in the pre-hearing IHCs, we observed no significant I_{CaTB} in the Ca^{2+} current (Figures 4E–4F1). The Ca^{2+} currents of pre-hearing IHCs showed a marked slow Ca^{2+} dependent inactivation (Grant and Fuchs, 2008). This inactivation was not due to a block by H^+ exocytosis, as it was still observed in extracellular HEPES (Figure 4F). We hypothesize that the absence of I_{CaTB} in immature IHCs was likely due to the loose spatial organization between Ca^{2+} channels and release sites. In the immature IHCs, we propose that the release of H^+ during exocytosis will be buffered in the synaptic cleft before the H^+ ions reach the distant Ca^{2+} channel open pore.

Nifedipine-Resistant Ca^{2+} Currents Display I_{CaTB}

A nifedipine-resistant Ca^{2+} current, whose activation is sufficient for the exocytosis of the readily releasable pool (RRP) of vesicles, has been proposed to be due to short C-term $\text{Ca}_v1.3$ channels (Vincent et al., 2017). With extracellular bicarbonate, the addition of 20 μM nifedipine reduced Ca^{2+} currents by more than 50% in P15–P18 IHCs, from 190.2 ± 5.6 pA (control, $n = 17$) to 94.6 ± 8.1 pA ($n = 8$; $p < 0.05$; Figure S3). However, this did not affect the C_m when stepping the cells for 100 ms from -80 to -10 mV (36.6 ± 5.9 fF [control] and 31.9 ± 1.9 fF [nifedipine], $p = 0.33$; Figure S3). The calculated Ca^{2+} efficiency of exocytosis increased from 1.59 to 3.10 fF/pC with nifedipine (Figure S3C; $p < 0.05$). Remarkably, we found that the residual Ca^{2+} current, resistant to nifedipine, displayed I_{CaTB} with kinetics and amplitudes comparable with controls: 26.0 ± 2.3 pA with $\tau = 1.9 \pm 0.4$ ms versus 29.0 ± 2.6 pA and $\tau = 2.3 \pm 0.2$ ms in controls ($p = 0.49$ and $p = 0.38$, respectively; Figure S3C). The percentage block of the peak nifedipine-resistant Ca^{2+} current reached a mean value of 26% compared with less than 15% in absence of nifedipine (Figure S3C). Interestingly, we also found shorter synaptic delay in the presence of 20 μM nifedipine (2.0 ± 0.7 ms, $n = 8$) compared with control (3.7 ± 1.6 ms, $n = 16$; $p < 0.01$; data not shown). Overall, these results suggested that only part of the total Ca^{2+} current in IHCs,

driven by nifedipine-resistant isoforms of $\text{Ca}_v1.3$ channels, contributes to exocytosis and I_{CaTB} .

Desynchronizing Release with BAPTA: I_{CaTB} Mini-events

After switching the intracellular Ca^{2+} buffer from 1 mM EGTA to 5 mM BAPTA, post-hearing IHCs displayed greatly reduced C_m from 31.9 ± 1.9 fF ($n = 17$) to 9.1 ± 2.1 fF ($n = 6$) ($p < 0.05$, unpaired t test) and reduced I_{CaTB} , although the peak amplitudes of the Ca^{2+} currents were unchanged (Figures 5A and 5D). The small residual amount of exocytosis in 5 mM BAPTA conditions produced a small block of $3.5\% \pm 0.6\%$ of the Ca^{2+} current ($\text{I}_{\text{CaTB}} = 7.0 \pm 1.5$ pA, $n = 12$) compared with $15.4\% \pm 1.7\%$ ($\text{I}_{\text{CaTB}} = 29.3 \pm 3.2$ pA, $n = 17$) in control EGTA conditions ($p < 0.05$, unpaired t test; Figure 5B1). When applying a train of five 100 ms stimulations (as described in Figure S1 for EGTA conditions), there was no correlation between I_{CaTB} and exocytosis depression with 5 mM BAPTA (Figures S5C and S5D). This shows that, similar to bullfrog hair cells (Figure 1), intracellular BAPTA reduces I_{CaTB} in mouse IHCs by blocking and desynchronizing vesicle exocytosis.

With 1 mM intracellular EGTA, I_{CaTB} of post-hearing mouse IHCs (P14–P18) consisted of an initial large notch that was followed by smaller notches (mini-events) riding on the Ca^{2+} current (Figure 5C). The amplitude of the mini-events displayed a bimodal distribution with two peaks at 6.4 ± 0.3 and 9.3 ± 0.3 pA ($n = 193$ mini-events, $n = 11$ IHCs; Figure 5G). The precise origin of this bimodal distribution is unknown, but it argues for an MVR mechanism (see also the bimodal EPSC amplitude distributions of Grant et al., 2010). The addition of 10 mM HEPES in the extracellular solution abolished both the initial large notch and the secondary mini-events in the Ca^{2+} current (Figures 2C, 2D, and 5C). The mini-events, contrary to the first large I_{CaTB} event, were found to be Ca^{2+} independent, as their amplitude did not change when varying the Ca^{2+} flux at various $[\text{Ca}^{2+}]_{\text{ext}}$ (Figure S4).

With 5 mM intracellular BAPTA, the secondary mini-events were still observed (Figures 5A and 5D) and showed a mono amplitude distribution centered at 9.15 ± 0.3 pA ($n = 113$ events in six IHCs), a value similar to the larger peak in EGTA conditions ($p = 0.2$, unpaired t test; Figure 5G). The mean charge integral of the mini-events was similar in 1 mM EGTA and 5 mM BAPTA (0.25 ± 0.01 and 0.26 ± 0.02 pC, respectively, $p = 0.2$). These results suggest that the mini-events, unaffected by BAPTA, are perhaps due to Ca^{2+} -independent asynchronous release events, whereas the first large I_{CaTB} in EGTA conditions, with a charge of 2.03 ± 0.35 pC, was due to MVR. A similar Ca^{2+} independence of the EPSC amplitudes during a sustained IHC depolarization was found in rat IHC afferent boutons (Goutman and Glowatzki, 2007).

Otoferlin Is Essential for Synchronous I_{CaTB}

Post-hearing (P14–P18) mouse IHCs lacking otoferlin ($\text{Otof}^{-/-}$ IHCs) displayed significantly reduced exocytosis, down to 9.6 ± 0.7 fF ($n = 10$; Figures 5B and 5B1) in extracellular bicarbonate. Accordingly, they displayed a greatly reduced I_{CaTB} of $1.9\% \pm 0.5\%$ (3.2 ± 0.2 pA, $n = 10$), for a 100 ms voltage step depolarization from -80 to -10 mV (Figure 5B). When varying the level of the small residual otoferlin-independent exocytosis during a train-pulse depression (as in Figure S1), the amplitude of the first I_{CaTB} event

following the Ca^{2+} current onset in *Otof*^{-/-} IHCs did not correlate with the amount of exocytosis (Figure S5A). Whereas wild-type IHCs showed significant I_{CaTB} spanning from 5% to 10%, *Otof*^{-/-} IHCs displayed greatly reduced exocytosis (from 2 to 12 fF) and I_{CaTB} with amplitude spanning from 1% to 4% (Figure S5B). These results suggested that similar to the recordings obtained with 5 mM intracellular BAPTA (Figures S5C and S5D), the absence of otoferlin desynchronized vesicular H^+ release.

Remarkably, small asynchronous mini-events were still visible in *Otof*^{-/-} IHCs (Figure 5E), but they had smaller amplitudes compared with wild-type control IHCs (mean amplitude of 5.5 ± 0.1 pA, mean of 67 events in 10 *Otof*^{-/-} IHCs, out of a noise level, determined in HEPES conditions, averaging 3.36 pA; $p < 0.05$; Figures 5F and 5G). The mini-events were distinguished from background noise by using the “ideal observer” criteria (see Abdi, 2007), so that mini-events below 5.04 pA (1.5×3.36 pA) were considered likely contaminated by noise and were therefore rejected (Figure 5F). On the basis of this noise correction, mini-events of *Otof*^{-/-} IHCs had a mean amplitude of 6.0 ± 0.1 pA and a distribution peak that was smaller than the peak of the wild-type IHCs (Figures 5F and 5G). Importantly, these small mini-events were not seen in *Otof*^{-/-} IHCs bathed in an extracellular solution with additional 10 mM HEPES, indicating that they are due to the H^+ effect on Ca^{2+} current (Figure 5E).

In Vivo Fast Spike Adaptation in the Mouse Auditory Nerve

Does the fast I_{CaTB} in IHCs leave a signature on the firing discharge pattern of the auditory nerve fibers (ANFs)? To address this, we analyzed the sound-evoked discharge properties of a large number of ANFs from 2- to 3-month-old mice. The peri-stimulus time (PST) histograms, measured at characteristic frequency (CF) and 30 dB above threshold (with a binning of 0.5 ms) as previously described by Taberner and Liberman (2005), showed a fast rise in discharge rate at tone-burst onset. The first spike latency (FSL mode) averaged 2.76 ± 0.06 ms (not corrected for the acoustic delay), and the coefficient of variation (CV) of interspike intervals was 0.63 ± 0.01 ($n = 44$ ANFs). These values of FSL and CV fall well into the criteria for distinguishing ANFs from cochlear nucleus units (Taberner and Liberman, 2005). The peak rate of the PST histograms averaged 862 ± 37 spikes/s ($n = 44$) and decayed rapidly to a mean steady-state value of 232 ± 7.5 spikes/s (Figure 6). After averaging the PST histograms (PSTHs) across multiple units, this decay or adaptation of the firing rate could be fitted with a double-exponential function (time constants of 0.5 ± 0.1 and 12.5 ± 1.4 ms; Figure 6B). Remarkably, during the first millisecond of adaptation, a notch corresponding to a fast and transient decrease in firing was observed in 33 of the 44 recorded ANFs (Figure 6C). The notch in the ANF firing was observed in C57BL/6J and Sv/129 mice (8 of 10 and 25 of 34 ANFs, respectively). In these fibers with a notch, the decay of the firing rate could also be fit with two time constants (0.6 ± 0.1 and 9.3 ± 0.8 ms; Figure 6D). The mean amplitude of the notch corresponded to a transient rebound in firing rate of 160 ± 11 spikes/s lasting 1–2 ms. This fast spike adaptation produced a mean drop of 655 spikes/s from the peak discharge rate (i.e., a reduction of $76\% \pm 3.5\%$ of the maximum firing rate; $n = 33$). The notch displayed similar amplitudes in high- and low-discharge rate (DR) fibers: for fibers with $\text{DR} > 1,000$ spikes/s, the notch rebound averaged 180 ± 37 spikes/s ($n = 5$) and 210 ± 24 spikes/s ($n = 5$) for fibers with $\text{DR} < 750$ spikes/s ($p < 0.01$, unpaired t test).

The notch was observed in fibers with low and high spontaneous rate (SR). In ANF with SR < 5, 5 < SR < 50, and SR > 50 spikes/s, the notch was observed in 7 of 12, 3 of 24, and 8 of 8 PSTHs, respectively. Finally, the notch was observed in both the low-frequency and high-frequency coding ANFs: CF < 10 kHz (2 of 4), 10 kHz < CF < 20 kHz (11 of 14), and CF > 20 kHz (20 of 26).

DISCUSSION

Fast H⁺ Block of Ca²⁺ Channels in Mammalian IHCs

Our study shows that a fast H⁺-mediated I_{Ca}TB occurs during exocytosis in post-hearing gerbil and mouse IHCs. Because IHC recordings were obtained from the apical coil of the cochlea corresponding to a frequency coding region spanning from 0.5 to 1 kHz in the gerbil and from 8 to 16 kHz in the mouse, our results show that I_{Ca}TB occurs in both low-frequency coding gerbil IHC with ANFs displaying phase locking (Versteegh et al., 2011) and middle-frequency coding mouse IHCs with ANFs that lack phase locking (Taberner and Liberman, 2005). Because I_{Ca}TB was also described in low-frequency tuned amphibian hair cells (Cho and von Gersdorff, 2014; Li et al., 2014), this regulation appears to be a general characteristic of auditory hair cells tuned to low and middle frequencies.

I_{Ca}TB Requires MVR within a Compact Active Zone

The I_{Ca}TB we observed in mammalian IHCs was surprisingly “noisy.” It contained several mini-events that were never observed in the smooth I_{Ca}TB of bullfrog hair cells (compare Figures 1 and 7A). What is the reason for this striking difference? We suggest that this may stem in part from the large number of synaptic ribbons in bullfrog amphibian papilla hair cells (~45–65 ribbons per hair cell; Graydon et al., 2014) compared with mouse IHCs (10–20 ribbons; Meyer et al., 2009). In bullfrog hair cells, fluctuations in I_{Ca}TB will be averaged over many ribbon active zones, producing a smoother I_{Ca}TB, whereas the relatively small number of ribbons of IHCs may not allow this smoothing of I_{Ca}TB. Furthermore, each synaptic ribbon in IHCs, receiving a single afferent terminal, may have different thresholds for exocytosis (Ohn et al., 2016). This will tend to desynchronize the I_{Ca}TB events in IHCs, whereas bullfrog hair cell synaptic ribbons may be more homogeneous in their exocytosis properties. In addition, it is possible that the density of carbonic anhydrase may be higher in the synaptic cleft of bullfrog hair cells.

Can the release from a single vesicle (UVR), with a flickering open fusion pore of varying size, account for I_{Ca}TB in post-hearing IHCs, as proposed by Chapochnikov et al. (2014)? The C_m up-step size distribution reflecting single vesicle fusion at the calyx of Held has been shown to vary greatly from 20 to nearly 200 aF (He et al., 2006), suggesting that UVR could possibly explain the large variance of the amplitude of I_{Ca}TB in hair cells. However, one major argument against UVR comes from the intrinsic nature of the proton block, because only Ca²⁺ channels near to the fusion site can be efficiently blocked by the released H⁺. Because the number of free H⁺ released by a single vesicle is estimated to be relatively small (about 50–100 protons; Cho and von Gersdorff, 2014), we consider that only the combined acidification stemming from MVR would efficiently overwhelm the extracellular

pH buffers near the Ca^{2+} channels. Our results with BAPTA suggest that only large and highly synchronous MVR produce a large I_{CaTB} (Figures 1 and 5A).

An intriguing finding in our study was that H^+ exocytosis blocked only a small part of the Ca^{2+} currents in mature mouse IHCs (15%–30%; Figure 2) and adult bullfrog hair cells (30%; Figure 1C). Because I_{CaTB} was insensitive to nifedipine, the H^+ block may only affect a small part of the Ca^{2+} channel sub-types, namely, the short C-term $\text{Ca}_v1.3$ isoforms that has been proposed to trigger the RRP release (Vincent et al., 2017). Thus, we propose that only Ca^{2+} channels tightly coupled to docked vesicles exhibit I_{CaTB} .

I_{CaTB} has not been observed in CNS synapses such as the calyx of Held synapse in the auditory brainstem. Figure 7B shows I_{Ca} recorded from a P16 mouse calyx nerve terminal at 34°C (Renden and von Gersdorff, 2007). It displays no I_{CaTB} , although the P-type Ca^{2+} channels are organized in nanodomains near the release sites (Nakamura et al., 2015). What is the reason for the absence of I_{CaTB} given that H^+ release has recently been observed at the calyx of Held (González-Inchauspe et al., 2017) and P-type Ca^{2+} channels are highly sensitive to extracellular H^+ (Cens et al., 2011)? One possible explanation comes from the spatial organization of the Ca^{2+} channels at these active zones. The Ca^{2+} channels appear to be organized in several topographically dispersed clusters that have a rather large inter-cluster distance of several hundreds of nanometers (Nakamura et al., 2015). Synaptic vesicles are presumably docked in the periphery of these clusters, with each cluster triggering the fusion of only one to four vesicles over an extended stimulation period. This UVR at each separate cluster likely prevents an efficient and synchronous acidification of a large percentage of the open Ca^{2+} channels (Figure 7C). An alternative model of Ca^{2+} channels organization has been proposed at the calyx of Held synapse (Keller et al., 2015). In this model, Ca^{2+} domains overlap in the active zone, and Ca^{2+} channels are located at a certain distance of the docked vesicles. This would also explain the absence of I_{CaTB} during release at this central synapse. Furthermore, the absence of I_{CaTB} in immature IHC synapses suggests that the Ca^{2+} channels need to be tightly clustered at the active zone to sense H^+ release via synchronous MVR (Figures 4B and 4D). The specificity of the mature ribbon synapse for I_{CaTB} therefore appears to arise from a very tight clustering of the Ca^{2+} channels that are surrounded by two rows of ready-to-fuse docked vesicles and an overarching ellipsoid ribbon (Figures 4C and 7C; Rutherford, 2015). This confined and compact active zone thus promotes the I_{CaTB} .

Synchronous MVR at IHCs Requires Otoferlin

The large reduction of I_{CaTB} by the fast Ca^{2+} chelator BAPTA suggests that this process involves a Ca^{2+} -dependent synchronized MVR. The secondary I_{CaTB} mini-events, which persist in the presence of BAPTA, are likely due to asynchronous release. This agrees with EPSC recordings from P9-P11 rat IHC synapses (Goutman and Glowatzki, 2007), which showed that the first large synchronous EPSCs was Ca^{2+} dependent (suppressed by BAPTA), while the amplitudes of the secondary asynchronous EPSCs, measured in the last 100 ms of stimulation, were apparently Ca^{2+} independent.

Our study also sheds new light on the role of otoferlin. This protein has been proposed to be a Ca^{2+} sensor for vesicular fusion (Roux et al., 2006) and for vesicular recruitment (Pangrsic

et al., 2010; Michalski et al., 2017). In IHCs lacking otoferlin, we found that the first large synchronous I_{CaTB} was abolished, but small asynchronous events persisted. However, their mean size was about half the size of those in wild-type IHCs. Although these remaining small events agree with the persistence of small EPSCs in vestibular and cochlear hair cell synapses from *Otof*^{-/-} mice (Dulon et al., 2009; Pangrsic et al., 2010), it remains to be determined whether they reflect the fusion of single vesicles. However, the fact that the large I_{CaTB} of IHCs is absent in *Otof*^{-/-} IHCs (Figure 5B) suggests that the multi-C2 domain protein otoferlin is essential to synchronize MVR at hair cell active zones.

I_{CaTB} and the Fast Spike Adaption of the Auditory Nerve

Mouse ANF respond to a tone burst with a fast increase in their spike rate at stimulus onset that rapidly declines in a double exponential decay (Westerman and Smith, 1984; Taberner and Liberman, 2005; Buran et al., 2010). We found that the first submillisecond phase of adaptation showed a notch in about 70% of the ANF. This notch corresponded to a fast and transient reduction of firing immediately following the onset peak of firing. A similar firing adaptation with notch was recorded from the chinchilla ANF (Ruggero, 1992) and gerbil ANF (Lütkenhöner and Smith, 1986). In these earlier studies, the notch was observed in PSTHs recorded at high sound intensities and computed with small bins of 0.1 or 0.2 ms. The fact that the notch was not systematically observed in our study, and by other groups, suggested that during our 0.5 ms binning period, we may have missed this brief event in some of the PSTHs. Moreover, we emphasize that the notch may be observed more clearly at intense sound stimuli, more than 30 dB above threshold, likely a level at which highly synchronous multivesicular proton release occurs.

Several possible origins can explain the fast firing adaptation of the ANFs: (1) The refractoriness of the auditory fibers. However, this factor has been suggested to be constant and independent of the discharge rate (Li and Young, 1993). Furthermore, because the relative refractory period decreases with increasing discharge rate, our observation of a similar notch in both high- and low-discharge rate fibers strongly argues against the contribution of only the refractoriness period to the notch. (2) The desensitization of the postsynaptic AMPA receptors. However, the fast depression of the auditory fiber activity is still present with cyclothiazide, an agent that removes AMPA desensitization (Goutman and Glowatzki, 2007). (3) The mechano-electrical adaptation at the hair bundles. However, the IHC receptor potential does not show fast adaptation (Russell and Sellick, 1978; Palmer and Russell, 1986). (4) The fast depletion of the RRP of synaptic vesicles from the ribbons (Furukawa and Matsuura, 1978; Moser and Beutner, 2000; Buran et al., 2010). However, an adaptation process based solely on vesicular exhaustion would be limited by vesicle pool replenishment rates during incoming sound stimulations. (5) Fast Ca^{2+} current inactivation, due to intracellular Ca^{2+} -dependent inactivation (Grant and Fuchs, 2008; Johnson and Marcotti, 2008; Joiner and Lee, 2015) of short C-term $Ca_v1.3$ isoforms (Vincent et al., 2017; Pangrsic et al., 2018), could also contribute to ANF firing adaptation. However, this Ca^{2+} -dependent inactivation can contribute only within a slower range of tens of milliseconds to the ANF firing adaptation.

We speculate that I_{CaTB} could underlie the fast component of the ANF firing adaptation and account for the notch seen in the PSTHs. Although the kinetics and amplitude of the transient notch observed in the PSTHs *in vivo* were much faster and larger than those measured for I_{CaTB} in IHCs *in vitro* (0.6 versus 2.0 ms and 70% block versus 15%–30%, respectively), these differences could be explained by a more efficient acidification of the synaptic cleft *in vivo*, by the higher *in vivo* body temperature (Figures 3A and 3B) and by the non-linear Ca^{2+} -dependent exocytosis and spike-triggering machinery of the ANF. I_{CaTB} thus provides a fast feedback mechanism that reduces Ca^{2+} current, thereby reducing hair cell and ANF energy consumption, while increasing the overall efficiency of exocytosis. In conclusion, we propose that the submillisecond ANF firing adaptation occurring during sound stimulation may result, at least in part, from a fast H^+ block of Ca^{2+} channels clustered at the compact IHC active zones.

STAR★METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Henrike von Gersdorff (vongersd@ohsu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Models—This study has been performed on adult female or male bullfrogs (*Rana catesbeiana*), gerbils (P20-P30) and female or male wild-type C57Bl6 mice both young (P6-P9) and adult (P14-P18). The otoferlin knock out mouse (*Otof^{-/-}*) was a gift from Christine Petit (Pasteur institute). All animal protocols were performed in accordance with the OHSU Institutional Animal Care and Use Committee (IACUC) for the use of bullfrogs and with the guidelines of the Animal Care Committee of INSERM and the ethics committee of the University of Bordeaux for the use of gerbils and mice.

METHOD DETAILS

Hair cell recordings from the adult bullfrog papilla—Amphibian papillae of adult female or male bullfrogs (*Rana catesbeiana*) were carefully dissected as described by Li et al. (2009). Amphibian papillae were placed in a recording chamber with artificial perilymph containing (in mM): 95 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 3 Glucose, 1 creatine, 1 Na-pyruvate, pH adjusted to 7.30 with NaOH, and continuously bubbled with 95% O_2 and 5% CO_2 (230 mOsm). Semi-intact preparations of hair cells and their connecting afferent fibers were viewed with an Olympus BX51WI microscope equipped with a 60× water-immersion objective lens (Olympus) and digital CCD camera (QImaging). For whole-cell recordings, patch pipettes of borosilicate glass were pulled to resistances of 6 to 7 MΩ for hair cells and 8 to 9 MΩ for afferent fibers. The control intracellular solution contained (in mM): 77 Cs-gluconate, 20 CsCl, 1 $MgCl_2$, 10 TEA-Cl, 10 HEPES, 2 EGTA, 3 Mg-ATP, 1 Na-GTP and 5 Na_2 -phosphocreatine (adjusted to pH 7.3 with CsOH). For internal solution containing 10 mM BAPTA instead of 2 mM EGTA, the amount of Cs-gluconate was reduced to match osmolarity of 230 mOsm. Oxygenated artificial perilymph continuously perfused (2–3 ml/min) during the recordings. Whole-cell voltage clamp recordings were performed with an EPC-10/2 (HEKA) patch-clamp amplifier and Patchmaster software

(HEKA) at room temperature. The resting holding potential of hair cells was -60 mV and that of afferent fibers was -90 mV. Membrane potentials were corrected for a liquid junction potential of 10 mV. Whole-cell calcium currents were not leak-subtracted. The averaged uncompensated series resistances in whole-cell recordings were 12.3 ± 1.0 M Ω for hair cells ($n = 7$) and 19.6 ± 2.9 M Ω for afferent fibers ($n = 7$). Whole-cell recordings were electronically compensated by 0% to 63% depending on the uncompensated series resistance. The average whole-cell resting membrane capacitance of hair cells was 12.6 ± 0.8 pF ($n = 7$).

Recordings from the mouse calyx of Held nerve terminal—Recordings of Ca^{2+} current and C_m from the mouse calyx of Held nerve terminal were performed as described by Renden and von Gersdorff (2007). For postnatal day P14-17 mice, a depolarization of 10 ms from -80 mV to 0 mV elicits a Ca^{2+} current with charge of 12.4 ± 1.5 pC and a $C_m = 241 \pm 34$ fF ($n = 9$) at room temperature. Both the Ca^{2+} current and C_m were 60% larger at 34°C (Renden and von Gersdorff, 2007). Nevertheless, this exocytosis at physiological temperature does not produce an I_{CaTB} (Figure 7B). The absence of I_{CaTB} could result from the functional organization of the giant nerve terminal, which has ~ 600 independent active zones, and at any one point in time a Ca^{2+} channel cluster triggers the release of one or two vesicles (Nakamura et al., 2015).

Hair cell recordings from gerbil and mouse organ of Corti—All mice (C57BL6 of either sex) and gerbil were anesthetized by intraperitoneal injection of xylazine (6 mg/ml) and ketamin (80 mg/ml) mixture (Sigma Aldrich, St Louis, USA) diluted in physiological saline. In experiments using $\text{Otof}^{f/f-}$ mice (C57BL6 genetic background), controls were littermate ($\text{Otof}^{f/+}$ or $\text{Otof}^{+/+}$) mice of either sex (Beurg et al., 2010). Electrophysiological recordings from IHCs were obtained in whole-mount organs of Corti (OC) from the apical cochlea containing intact afferent projections and spiral ganglion neurons (Figure 2A-B). The OC was freshly dissected under binocular magnification in an extracellular solution maintained at 4°C containing (in mM): NaCl 135 ; KCl 5.8 ; CaCl_2 1.3 ; MgCl_2 0.9 ; NaH_2PO_4 0.7 ; glucose 5.6 ; Na pyruvate 2 ; HEPES 10 , pH 7.4 , 305 mOsm. The OC was placed in a recording chamber and the hair cells were observed with a $60\times$ water immersion objective (CFI Fluor 60X W NIR, WD = 2.0 mm, NA = 1) attached to an upright Nikon FN1 microscope. The OC was continuously perfused at a rate of 1 ml/min by using a Masterflex L/S peristaltic pump (model 7519-20, Cole-Palmer Instrument) with a bicarbonate solution, continuously bubbled with carbogen gas (95% O_2 and 5% CO_2). This solution had the following composition: NaCl 135 ; KCl 5.8 ; CaCl_2 5 ; MgCl_2 0.9 ; Glucose 5.6 ; Na pyruvate 2 ; NaHCO_3 25 mM, pH 7.4 , 320 mOsm. This solution was complemented with 0.5 μM of apamin (Latoxan, Valence, France), 0.2 μM of XE-991 (Tocris Bioscience, Lille, France) and 0.05 μM TTX (Sigma) to block SK channels, KCNQ4 channels and Na^+ channels, respectively. In some experiments either 100 μM amiloride (Sigma, A7410) or 100 μM DL-TBOA (Tocris, 1223) was added to the extracellular medium to respectively block ASIC channels and glutamate transporters.

To preserve the afferent dendrites and improve the survival rate of the afferent synaptic boutons connecting the base of the IHCs, the OC was dissected with its spiral ganglion

neurons (Figure 2A-B). Also, we did not peel off the tectorial membrane on top of the OC, because its removal had the tendency to damage the IHCs and their synaptic boutons. In our preparation, we gently slid the patch recording electrode below the tectorial membrane in order to reach the IHCs. All patch-clamp experiments were carried out at room temperature ($22 \pm 1^\circ\text{C}$) in 5 mM external calcium, except when otherwise indicated as in Figure 3A-B where experiments were performed at $32 \pm 1^\circ\text{C}$ and in 1.3 mM extracellular Ca^{2+} . To reduce an increased leak current at 32°C , IHCs were step depolarized from -65 mV instead of -80 mV. In these latter conditions, the OC was placed in a HW-104 heating stage connected to a low noise HW-30 temperature controller (Dagan Corporation, Minneapolis, USA).

The mice IHC recordings were performed in the 20%–40% normalized distance from the apex, an area coding for frequencies ranging from 8 to 16 kHz. Gerbil IHCs were recorded from the apical turn low frequency region coding from 0.5 to 1 kHz. Whole cell patch-clamp recordings were performed by using an EPC10 amplifier controlled by Patchmaster software (HEKA Elektronik, Germany). Patch pipettes were pulled with a micropipette Puller P-97 Flaming/Brown (Sutter Instrument, Novato, CA, USA) and fire-polished with a Micro forge MF-830, (Narishige, Japan) to obtain a resistance range from 3 to 5 M Ω . The average whole-cell series resistance was 7.0 ± 1.0 M Ω (no electronic compensation was used). Patch pipettes were filled with an intracellular solution containing (in mM): CsCl 145; MgCl_2 1; HEPES 5; EGTA 1; TEA 20, ATP-Mg 2, GTP 0.3, pH 7.2, 310 mOsm. In a few experiments, the slow Ca^{2+} chelator EGTA was increased to 5 mM or replaced by 5 mM BAPTA. The measurements of the whole-cell membrane capacitance (C_m) from hair cells were performed under voltage-clamp with the “Sine + DC” method (Lindau and Neher, 1988; Gillis, 2000) using an EPC-10/2 (HEKA) patch-clamp amplifier and Patchmaster software (HEKA). A 2 kHz sine wave was superposed on the holding potential and the resulting current response was used to calculate C_m via a Patchmaster software emulator of a lock-in amplifier (see Vincent et al., 2014). The average resting C_m for mouse IHCs was 10.8 ± 0.1 pF ($n = 60$).

Immunohistochemistry fluorescence—Cochleae of P7 and P25 mice were rapidly perfused with 100% methanol at -20°C for 30 min and washed with cold phosphate buffered saline (PBS). The P25 cochleae were then incubated for 24 hours in PBS solution containing 10% EDTA for decalcification. The organs of Corti of P7 and P25 were dissected and the tectorial membrane. Tissues were first incubated with PBS containing 30% normal horse serum for 1 hour at room temperature (RT). Then, they were incubated with primary antibodies diluted with PBS (1:200) containing 5% horse serum and 0.1% Triton X-100. Synaptic ribbons and $\text{Ca}_v1.3$ channels were simultaneously labeled with anti-CtBP2 (Goat polyclonal, Santa Cruz, USA; cat # SC-5966), anti- $\text{Ca}_v1.3$ (Rabbit polyclonal, Alomone labs, Jerusalem, Israel; cat # ACC-005). The organs of Corti were then washed with PBS and incubated in two steps with secondary antibodies at 1:500: first Donkey anti-Goat Fluorophore 547H (Interchim; cat # FP-SB2110), second after PBS rinse with Goat anti-Rabbit Alexa Fluor 488 (Invitrogen; cat # A-11008). Surface preparations of organs of Corti (apical turns) were then mounted on Superfrost-Plus glass slides (Kindler GmbH, Freiburg, Germany) in a Prolong-Antifade medium (Molecular Probes, Invitrogen, Oregon USA) and kept in the dark at -20°C until observation.

Fluorescence images of organs of Corti were acquired and analyzed using a confocal laser scanning microscope Leica SP8 with a 63X oil immersion objective (NA = 1.4) and white light laser (470 to 670 nm) (Bordeaux Imaging Center). Wavelengths were selected by an acousto-optical tunable filter (AOTF) associated with a photonic crystal fiber. This combination allowed precise selection of multiple wavelengths simultaneously. Stack images were acquired with the following parameters: laser power 40%, scan rate 700 Hz, scans averaged per XY section 4 times, step size 250 nm, pixel size 80 nm giving a X-Y image size of $41 \times 41 \mu\text{m}$ (512×512 pixels). Images were then processed for three-dimensional (3D) blind deconvolution with AutoQuant X2 (MediaCybernetics). After deconvolution, images were processed under ImageJ software (NIH, Bethesda, USA).

Single fiber recordings from the mouse ANFs—Recordings from single ANFs were performed in C57BL/6J and Sv/129 mice (2 to 3 month old) as previously described in detail (Taberner and Liberman, 2005; Buran et al., 2010). Mice were anesthetized with xylazine (5 mg/kg) and urethane (1.32 mg/kg) and placed in a sound-proofed, electrically shielded room, with body temperature maintained at 37°C. After occipital bone removed, and a partial cerebellar aspiration performed to expose the cochlear nucleus, recording glass microelectrodes, filled with 2 M KCl, were advanced through the cochlear nucleus into the auditory nerve using a stereotaxic apparatus. Criteria for distinguishing ANFs from cochlear nucleus neurons included electrode depth ($> 1000 \mu\text{m}$ re brainstem surface) and response properties to tone bursts (Taberner and Liberman, 2005): a first spike latency < 3 ms (values not corrected for the acoustic delay) and a coefficient of variation for interspike interval > 0.5 . Post-stimulus onset time and interspike-interval histograms were computed from responses to tone bursts (50 ms) at CF and 30 dB above threshold.

QUANTIFICATION AND STATISTICAL ANALYSIS

Mean values are shown with mean $\pm$ SEM. HEKA data files were analyzed with Igor Pro (Wavemetrics) and OriginPro2017 and Origin 9 (OriginLab). Asynchronous I_{CaTB} events were analyzed with Mini Analysis Program (Synaptosoft). Statistical significance was assessed with paired or unpaired Student's *t* tests. Data are expressed as mean $\pm$ SEM. The symbols *, **, *** indicate $p < 0.05$; $p < 0.01$; $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Abdi H (2007). Signal detection theory In Encyclopedia of Measurement and Statistics, Salkind NJ, ed. (Thousand Oaks, CA: Sage), pp. 886–889.
- Beurg M, Michalski N, Safieddine S, Bouleau Y, Schneggenburger R, Chapman ER, Petit C, and Dulon D (2010). Control of exocytosis by synaptotagmins and otoferlin in auditory hair cells. *J. Neurosci.* 30, 13281–13290. [PubMed: 20926654]
- Beutner D, and Moser T (2001). The presynaptic function of mouse cochlear inner hair cells during development of hearing. *J. Neurosci.* 21, 4593–4599. [PubMed: 11425887]
- Brandt A, Khimich D, and Moser T (2005). Few $\text{Ca}_v1.3$ channels regulate the exocytosis of a synaptic vesicle at the hair cell ribbon synapse. *J. Neurosci.* 25, 11577–11585. [PubMed: 16354915]
- Buran BN, Strenzke N, Neef A, Gundelfinger ED, Moser T, and Liberman MC (2010). Onset coding is degraded in auditory nerve fibers from mutant mice lacking synaptic ribbons. *J. Neurosci.* 30, 7587–7597. [PubMed: 20519533]
- Cens T, Rousset M, and Charnet P (2011). Two sets of amino acids of the domain I of $\text{Ca}_v2.3 \text{ Ca}^{2+}$ channels contribute to their high sensitivity to extracellular protons. *Pflugers Arch.* 462, 303–314. [PubMed: 21611731]
- Chapochnikov NM, Takago H, Huang CH, Pangršič T, Khimich D, Neef J, Auge E, Göttfert F, Hell SW, Wichmann C, et al. (2014). Uniquantal release through a dynamic fusion pore is a candidate mechanism of hair cell exocytosis. *Neuron* 83, 1389–1403. [PubMed: 25199706]
- Cho S, and von Gersdorff H (2014). Proton-mediated block of Ca^{2+} channels during multivesicular release regulates short-term plasticity at an auditory hair cell synapse. *J. Neurosci.* 34, 15877–15887. [PubMed: 25429130]
- Chu XP, Papasian CJ, Wang JQ, and Xiong ZG (2011). Modulation of acid-sensing ion channels: molecular mechanisms and therapeutic potential. *Int. J. Physiol. Pathophysiol. Pharmacol.* 3, 288–309. [PubMed: 22162785]
- DeVries SH (2001). Exocytosed protons feedback to suppress the Ca^{2+} current in mammalian cone photoreceptors. *Neuron* 32, 1107–1117. [PubMed: 11754841]
- Dou H, Vazquez AE, Namkung Y, Chu H, Cardell EL, Nie L, Parson S, Shin HS, and Yamoah EN (2004). Null mutation of $\alpha1D \text{ Ca}^{2+}$ channel gene results in deafness but no vestibular defect in mice. *J. Assoc. Res. Otolaryngol.* 5, 215–226. [PubMed: 15357422]
- Dulon D, Safieddine S, Jones SM, and Petit C (2009). Otoferlin is critical for a highly sensitive and linear calcium-dependent exocytosis at vestibular hair cell ribbon synapses. *J. Neurosci.* 29, 10474–10487. [PubMed: 19710301]
- Furukawa T, and Matsuura S (1978). Adaptive rundown of excitatory post-synaptic potentials at synapses between hair cells and eight nerve fibres in the goldfish. *J. Physiol.* 276, 193–209. [PubMed: 650439]
- Gillis KD (2000). Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. *Pflugers Arch.* 439, 655–664. [PubMed: 10764227]
- Glowatzki E, and Fuchs PA (2002). Transmitter release at the hair cell ribbon synapse. *Nat. Neurosci.* 5, 147–154. [PubMed: 11802170]
- González-Inchauspe C, Urbano FJ, Di Guilmi MN, and Uchitel OD (2017). Acid sensing ion channels activated by evoked released protons modulate synaptic transmission at the mouse calyx of Held synapse. *J. Neurosci.* 37, 2589–2599. [PubMed: 28159907]
- Goutman JD, and Glowatzki E (2007). Time course and calcium dependence of transmitter release at a single ribbon synapse. *Proc. Natl. Acad. Sci. USA* 704, 16341–16346.
- Grant L, and Fuchs P (2008). Calcium- and calmodulin-dependent inactivation of calcium channels in inner hair cells of the rat cochlea. *J. Neurophysiol.* 99, 2183–2193. [PubMed: 18322004]
- Grant L, Yi E, and Glowatzki E (2010). Two modes of release shape the postsynaptic response at the inner hair cell ribbon synapse. *J. Neurosci.* 30, 4210–4220. [PubMed: 20335456]
- Graydon CW, Cho S, Li GL, Kachar B, and von Gersdorff H (2011). Sharp Ca^{2+} nanodomains beneath the ribbon promote highly synchronous multivesicular release at hair cell synapses. *J. Neurosci.* 31, 16637–16650. [PubMed: 22090491]

- Graydon CW, Cho S, Diamond JS, Kachar B, von Gersdorff H, and Grimes WN (2014). Specialized postsynaptic morphology enhances neurotransmitter dilution and high-frequency signaling at an auditory synapse. *J. Neurosci.* 34, 8358–8372. [PubMed: 24920639]
- He L, Wu XS, Mohan R, and Wu LG (2006). Two modes of fusion pore opening revealed by cell-attached recordings at a synapse. *Nature* 444, 102–105. [PubMed: 17065984]
- Johnson SL, and Marcotti W (2008). Biophysical properties of $\text{Ca}_v1.3$ calcium channels in gerbil inner hair cells. *J. Physiol.* 586, 1029–1042. [PubMed: 18174213]
- Johnson SL, Marcotti W, and Kros CJ (2005). Increase in efficiency and reduction in Ca^{2+} dependence of exocytosis during development of mouse inner hair cells. *J. Physiol.* 563, 177–191. [PubMed: 15613377]
- Johnson SL, Olt J, Cho S, von Gersdorff H, and Marcotti W (2017). The coupling between Ca^{2+} channels and the exocytotic Ca^{2+} sensor at hair cell ribbon synapses varies along the tonotopically along the mature cochlea. *J. Neurosci.* 37, 2471–2484. [PubMed: 28154149]
- Joiner ML, and Lee A (2015). Voltage-gated Cav1 channels in disorders of vision and hearing. *Curr. Mol. Pharmacol.* 8, 143–148. [PubMed: 25966695]
- Kanai Y, Clmenon B, Simonin A, Leuenberger M, Lochner M, Weisstanner M, and Hediger MA (2013). The SLC1 high-affinity glutamate and neutral amino acid transporter family. *Mol. Aspects Med.* 34, 108–120. [PubMed: 23506861]
- Keen EC, and Hudspeth AJ (2006). Transfer characteristics of the hair cell's afferent synapse. *Proc. Natl. Acad. Sci. USA* 103, 5537–5542. [PubMed: 16567618]
- Keller D, Babai N, Kochubey O, Han Y, Markram H, Schrmann F, and Schneggenburger R (2015). An exclusion zone for Ca^{2+} channels around docked vesicles explains release control by multiple channels at a CNS synapse. *PLoS Comput. Biol.* 11, e1004253. [PubMed: 25951120]
- Kushmerick C, Renden R, and von Gersdorff H (2006). Physiological temperatures reduce the rate of vesicle pool depletion and short-term depression via an acceleration of vesicle recruitment. *J. Neurosci.* 26, 1366–1377. [PubMed: 16452660]
- Li J, and Young ED (1993). Discharge-rate dependence of refractory behavior of cat auditory-nerve fibers. *Hear. Res.* 69, 151–162. [PubMed: 8226336]
- Li GL, Keen E, Andor-Ard D, Hudspeth AJ, and von Gersdorff H (2009). The unitary event underlying multiquantal EPSCs at a hair cell's ribbon synapse. *J. Neurosci.* 29, 7558–7568. [PubMed: 19515924]
- Li GL, Cho S, and von Gersdorff H (2014). Phase-locking precision is enhanced by multiquantal release at an auditory hair cell ribbon synapse. *Neuron* 83, 1404–1417. [PubMed: 25199707]
- Lindau M, and Neher E (1988). Patch-clamp techniques for time-resolved capacitance measurements in single cells. *Pflgers Arch.* 411, 137–146. [PubMed: 3357753]
- Ltkenhner B, and Smith RL (1986). Rapid adaptation of auditory-nerve fibers: fine structure at high stimulus intensities. *Hear. Res.* 24, 289–294. [PubMed: 3793645]
- Meinrenken CJ, Borst JGG, and Sakmann B (2002). Calcium secretion coupling at calyx of Held governed by nonuniform channel-vesicle topography. *J. Neurosci.* 22, 1648–1667. [PubMed: 11880495]
- Meyer AC, Frank T, Khimich D, Hoch G, Riedel D, Chapochnikov NM, Yarin YM, Harke B, Hell SW, Egner A, and Moser T (2009). Tuning of synapse number, structure and function in the cochlea. *Nat. Neurosci.* 12, 444–453. [PubMed: 19270686]
- Michalski N, Goutman JD, Auclair SM, Boutet de Monvel J, Tertrais M, Emptoz A, Parri A, Nouaille S, Guillon M, Sachse M, et al. (2017). Otoferlin acts as a Ca^{2+} sensor for vesicle fusion and vesicle pool replenishment at auditory hair cell ribbon synapses. *eLife* 6, e31013. [PubMed: 29111973]
- Moser T, and Beutner D (2000). Kinetics of exocytosis and endocytosis at the cochlear inner hair cell afferent synapse of the mouse. *Proc. Natl. Acad. Sci. USA* 97, 883–888. [PubMed: 10639174]
- Nakamura Y, Harada H, Kamasawa N, Matsui K, Rothman JS, Shigemoto R, Sliver RA, DiGregorio DA, and Takahashi T (2015). Nanoscale distribution of presynaptic Ca^{2+} channels and its impact on vesicular release during development. *Neuron* 85, 145–158. [PubMed: 25533484]
- Nouvian R (2007). Temperature enhances exocytosis efficiency at the mouse inner hair cell ribbon synapse. *J. Physiol.* 584, 535–542. [PubMed: 17717016]

- Ohn TL, Rutherford MA, Jing Z, Jung S, Duque-Afonso CJ, Hoch G, Picher MM, Scharinger A, Strenzke N, and Moser T (2016). Hair cells use active zones with different voltage dependence of Ca^{2+} influx to decompose sounds into complementary neural codes. *Proc. Natl. Acad. Sci. USA* 113, E4716–E4725. [PubMed: 27462107]
- Palmer AR, and Russell IJ (1986). Phase-locking in the cochlear nerve of the guinea-pig and its relation to the receptor potential of inner hair-cells. *Hear. Res* 24, 1–15. [PubMed: 3759671]
- Palmer MJ, Hull C, Vigh J, and von Gersdorff H (2003). Synaptic cleft acidification and modulation of short-term depression by exocytosed protons in retinal bipolar cells. *J. Neurosci.* 23, 11332–11341. [PubMed: 14672997]
- Pangrsic T, Lasarow L, Reuter K, Takago H, Schwander M, Riedel D, Frank T, Tarantino LM, Bailey JS, Strenzke N, et al. (2010). Hearing requires otoferlin-dependent efficient replenishment of synaptic vesicles in hair cells. *Nat. Neurosci.* 13, 869–876. [PubMed: 20562868]
- Pangrsic T, Singer JH, and Koschak A (2018). Voltage-gated calcium channels: Key players in sensory coding in the retina and the inner ear. *Physiol. Rev.* 98, 2063–2096. [PubMed: 30067155]
- Platzter J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, and Striessnig J (2000). Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca^{2+} channels. *Cell* 102, 89–97. [PubMed: 10929716]
- Renden R, and von Gersdorff H (2007). Synaptic vesicle endocytosis at a CNS nerve terminal: faster kinetics at physiological temperatures and increased endocytotic capacity during maturation. *J. Neurophysiol.* 98, 3349–3359. [PubMed: 17942618]
- Roberts WM, Jacobs RA, and Hudspeth AJ (1990). Co-localization of ion channels involved in frequency-selectivity and synaptic transmission at presynaptic active zones of hair-cells. *J. Neurosci* 10, 3664–3684. [PubMed: 1700083]
- Roux I, Safieddine S, Nouvian R, Grati M, Simmler MC, Bahloul A, Perfettini I, Le Gall M, Rostaing P, Hamard G, et al. (2006). Otoferlin, defective in a human deafness form, is essential for exocytosis at the auditory ribbon synapse. *Cell* 127, 277–289. [PubMed: 17055430]
- Ruggero MA (1992). Physiology and coding of sound in the auditory nerve In *The Mammalian Auditory Pathway: Neurophysiology*. Springer Handbook of Auditory Research, Popper AN and Fay RR, eds. (New York: Springer), pp. 34–93.
- Russell IJ, and Sellick PM (1978). Intracellular studies of hair cells in the mammalian cochlea. *J. Physiol.* 284, 261–290. [PubMed: 731538]
- Rutherford MA (2015). Resolving the structure of inner ear ribbon synapses with STED microscopy. *Synapse* 69, 242–255. [PubMed: 25682928]
- Taberner AM, and Liberman MC (2005). Response properties of single auditory nerve fibers in the mouse. *J. Neurophysiol.* 93, 557–569. [PubMed: 15456804]
- Versteegh CP, Meenderink SW, and van der Heijden M (2011). Response characteristics in the apex of the gerbil cochlea studied through auditory nerve recordings. *J. Assoc. Res. Otolaryngol.* 12, 301–316. [PubMed: 21213012]
- Vincent PF, Bouleau Y, Safieddine S, Petit C, and Dulon D (2014). Exocytotic machineries of vestibular type I and cochlear ribbon synapses display similar intrinsic otoferlin-dependent Ca^{2+} sensitivity but a different coupling to Ca^{2+} channels. *J. Neurosci.* 34, 10853–10869. [PubMed: 25122888]
- Vincent PF, Bouleau Y, Charpentier G, Emptoz A, Safieddine S, Petit C, and Dulon D (2017). Different $\text{Ca}_v1.3$ channel isoforms control distinct components of the synaptic vesicle cycle in auditory inner hair cells. *J. Neurosci.* 37, 2960–2975. [PubMed: 28193694]
- Wang LY, and Augustine GJ (2015). Presynaptic nanodomains: a tale of two synapses. *Front. Cell. Neurosci.* 8, 455. [PubMed: 25674049]
- Westerman LA, and Smith RL (1984). Rapid and short-term adaptation in auditory nerve responses. *Hear. Res.* 15, 249–260. [PubMed: 6501113]
- Wong AB, Rutherford MA, Gabrielaitis M, Pangrsic T, Göttfert F, Frank T, Michanski S, Hell S, Wolf F, Wichmann C, and Moser T (2014). Developmental refinement of hair cell synapses tightens the coupling of Ca^{2+} influx to exocytosis. *EMBO J.* 33, 247–264. [PubMed: 24442635]

Highlights

- A proton-mediated Ca^{2+} current transient block (I_{CaTB}) occurs at hair cell synapses
- I_{CaTB} is absent from immature inner hair cell synapses with non-compact active zones
- I_{CaTB} has a multi-peak structure that is eliminated in otoferlin-KO mice
- I_{CaTB} may contribute significantly to fast *in vivo* spike adaptation at afferent fibers

Figure 1. Paired Recordings Reveal that BAPTA Decreases Exocytosis and the Transient Block of Presynaptic Ca^{2+} Currents (I_{CaTB}) in Mature Bullfrog Auditory Hair Cells

(A1) Representative trace of paired whole-cell voltage-clamp recordings of presynaptic hair cell and a post-synaptic afferent fiber. The hair cell intracellular pipette solution included 10 mM BAPTA, and the external solution contained 25 mM bicarbonate (HCO_3^-) as a pH buffer. The hair cell was depolarized from -60 to -30 mV for 20 ms after 10 s (black), 3 min (green), and 5 min (red) from the whole-cell break-in. The multiquantal EPSC had a large amplitude that correlates with the proton inhibition of the Ca^{2+} currents. As 10 mM BAPTA dialyzed into the hair cell, the EPSC amplitude decreased, and the proton effect on the Ca^{2+}

current was removed (red trace), in contrast with recordings obtained 10 s after the whole-cell break-in (black).

(A2) An expanded timescale of (A1) shows that 10 mM BAPTA delays the first EPSC event (green) and strongly decreases the EPSC amplitude.

(B) The average EPSC charge of the later recordings (>4 min after the break-in, 2.0 ± 0.7 pC) was significantly decreased in comparison with the initial recordings (<1 min after the break-in, 16.8 ± 4.5 pC, $n = 7$ pairs; $p < 0.01$, paired t test).

(C) The ratio of transient block (%) of the Ca^{2+} current was measured from the amplitude of transient block (b) divided by the amplitude of peak Ca^{2+} current (a). BAPTA (10 mM) significantly decreased the transient block from $30.5\% \pm 5.3\%$ in the initial recordings (<1 min after the break-in, black, $n = 7$) to zero in the later recordings (>4 min after the break-in, red, $n = 7$; $p < 0.01$, paired t test). All data are expressed as mean $\pm$ SEM.

Figure 2. I_{CaTB} in Post-hearing Inner Hair Cells with Intact Bouton-type Ribbon Synapses

(A) Fluorescent labeling of the afferent fibers and postsynaptic boutons contacting the basal pole of the inner hair cells (IHCs; dotted line) in an *in vitro* living organ of Corti preparation that was freshly dissected from a postnatal day 15 (P15) mouse cochlea. In this preparation, the tectorial membrane and the spiral ganglion neurons were preserved along with the organ of Corti. The mouse was injected at P3 with an AAV2/8-CAG-GFP to label the spiral ganglion neurons and their afferent fibers. Note the presence of the intact afferent boutons on the IHC's basal pole.

(B) A differential interference contrast (DIC) image of the well-preserved afferent boutons contacting the IHC. Scale bar, 5 μ m.

(C and D) Simultaneous recordings of Ca^{2+} currents (I_{Ca}) and capacitance jump (C_m) in a P30 gerbil IHC (C) and a P17 mice IHCs (D) in the presence of 25 mM bicarbonate (HCO_3^-), a weak H^+ buffer in the extracellular solution. Red traces show examples of recordings in gerbil and mouse IHCs at the same age that lack I_{CaTB} when 10 mM HEPES was added to the extracellular HCO_3^- solution. Cells were depolarized from -80 to -10 mV for 100 ms (top). Note that I_{CaTB} was “noisy” compared with the smooth I_{CaTB} in bullfrog hair cells. Middle inset shows how we determined the percentage of I_{CaTB} and its onset kinetics. Recording conditions were room temperature (22°C) and 5 mM external Ca^{2+} .

(E and F) The histograms display the C_m (mean $\pm$ SEM) and I_{CaTB} amplitudes, measured in gerbil (E) and mouse (F) IHCs in the presence of 25 mM HCO_3^- alone (black) or with an additional 10 mM HEPES (red) in the extracellular solution. Note that C_m and I_{CaTB} values of the two mammalian species fall within a similar range. Data are expressed as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ (unpaired t test).

Figure 3. Relationship between Exocytosis and I_{CaTB} during Temperature Shifts, Synaptic Depression, and Changes in External Ca^{2+} Concentration

Post-hearing mouse IHCs were perfused with a HCO_3^- external solution.

(A and B) I_{CaTB} and exocytosis in P14–P18 mice IHCs at 22°C (A, blue traces) and 32°C (A, pink traces). The extracellular Ca^{2+} was 1.3 mM. The IHCs were stepped from a holding potential of -65 to -10 mV. (B) shows a quantitative comparison of I_{CaTB} and exocytosis at 22°C ($n = 13$) and 32°C ($n = 16$). Data are expressed as mean $\pm$ SEM. $**p < 0.01$ (unpaired t test).

(A1) The latency (t) of the I_{CaTB} at 32°C (pink trace) was shorter than at 22°C (blue trace). Data are expressed as mean $\pm$ SEM. $**p < 0.01$ (unpaired t test).

(C) Example of Ca^{2+} currents and C_m in response to a 100 ms paired-pulse stimulation from -80 to -10 mV, with each step separated by a 100 ms interval. Recording conditions were room temperature (22°C) and 5 mM external Ca^{2+} .

(D) Histograms comparing the mean depression of I_{CaTB} and exocytosis during paired-pulse stimulation as in (C) (P1, first pulse; P2, second pulse). Note the comparable depression of I_{CaTB} and C_m for the second pulse. I_{CaTB} and exocytosis data showed similar coefficient of variation (CV): CV = 0.33 and 0.24 at P1 and CV = 0.44 and 0.53 at P2, respectively. Data are expressed as mean $\pm$ SEM. $***p < 0.001$ (paired t test).

(E) The extracellular Ca^{2+} dependence of I_{CaTB} . Mouse IHCs were perfused with a solution containing 0.5 mM (blue; $n = 9$), 1.3 mM (light red; $n = 12$), 5.0 mM (light green; $n = 7$), or 10 mM (orange; $n = 13$) extracellular Ca^{2+} ($[Ca^{2+}]_{ext}$). Ca^{2+} currents (top) and C_m responses (bottom) were recorded during a 100 ms constant voltage step from -80 to -10 mV. The inset indicates how the maximum amplitude of the Ca^{2+} current ($I_{Ca} max$), the amplitude of I_{CaTB} , and the time to peak of I_{Ca} were analyzed.

(F) The relationship between I_{CaTB} amplitude and I_{Ca} peak amplitude was best fit with a nonlinear power function with power index 3.

(G) The time to peak of I_{CaTB} decreases exponentially when Ca^{2+} flux is increased.

(H) The C_m responses versus I_{Ca} were fit with a nonlinear power function with power index 3.

Note that for (F)–(H), the colored points represent each individual cell recording, and the black points are the mean $\pm$ SEM for each $[Ca^{2+}]_{ext}$ condition.

Figure 4. I_{CaTB} Is Absent at Immature (Pre-hearing) Mouse IHCs

(A and B) Confocal images from averaged z stack projections (20 slices of 0.25 μ m) in the basal synaptic area below the nucleus of P25 IHCs (A) and P7 IHCs (B) labeled for Ca_v1.3 Ca²⁺ channels and CtBP2 (ribbon). Scale bar represents 5 μ m.

(C and D) Line scan of fluorescent intensity profiles through a Ca_v1.3 cluster and its associated CtBP2 immunoreactive patch in a P25 IHC (C) and a P7 IHC (D). Scale bar represents 0.5 μ m.

(E and F) Recordings of Ca^{2+} current and exocytosis in P17 IHCs (E) and P7 IHCs (F). Immature wild-type P7 IHCs do not display a significant I_{CaTB} (orange), although they show large exocytotic responses and Ca^{2+} current amplitudes. The superimposed purple trace shows an example of a P7 IHC in 10 mM external HEPES. Note that the slow inactivation of the immature I_{Ca} currents was similar in both conditions, indicating that it was not related to the H^+ block. Recording conditions were room temperature (22°C) and 5 mM external Ca^{2+} . Data are expressed as mean $\pm$ SEM; *** $p < 0.001$, unpaired t test.

Figure 5. Decreasing and Desynchronizing Exocytosis with BAPTA or with Otoferlin Knockout Strongly Reduced I_{CaTB}

IHCs were step depolarized from -80 to -10 mV for 100 ms.

(A) In blue, I_{CaTB} was strongly reduced when 5 mM BAPTA, a fast Ca^{2+} buffer, was present in the intracellular recording solution. Exocytosis was also greatly reduced. The superimposed black trace shows I_{CaTB} recorded with 1 mM intracellular EGTA as Ca^{2+} buffer and with 25 mM external HCO_3^- .

(B) Exocytosis and I_{CaTB} were strongly reduced in *Otof*^{-/-} IHCs (otoferlin-KO mice; red). The superimposed black trace shows I_{CaTB} recorded from a P17 wild-type (WT) IHC in 1 mM intracellular EGTA and with 25 mM external HCO_3^- .

(B1) The histograms show the exocytosis (C_m) and I_{CaTB} (%) in control (bicarbonate; black), with intracellular 5 mM BAPTA (blue) and in *Otof*^{-/-} IHCs (red). Data are expressed as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, unpaired t test.

(C) An example of I_{CaTB} in a WT IHC: asynchronous small I_{CaTB} -like events (asterisks) can be seen after the first large (synchronous) response (arrowhead) in 25 mM bicarbonate (top trace, black line). In the presence of 10 mM HEPES, both the first large and small events are abolished (bottom trace, green line).

(D) I_{CaTB} in a WT IHC recorded with 5 mM intracellular BAPTA: the first large synchronous event is greatly reduced (arrowhead, top trace, blue line), but the small secondary events are preserved (asterisks). In 10 mM extracellular HEPES, all I_{CaTB} -like events are abolished (bottom trace, light blue line).

(E) I_{CaTB} in an *Otof*^{-/-} IHC: asynchronous I_{CaTB} -like events are reduced in amplitude (top trace, red line). In 10 mM extracellular HEPES, all I_{CaTB} events are abolished. Recording conditions were room temperature (22°C) and 5 mM external Ca^{2+} .

(F) Synchronous and asynchronous I_{CaTB} events: the first large I_{CaTB} event (synchronous event, arrowhead) was followed by secondary small notches (asynchronous events, asterisks). These small events were measured following the onset of the Ca^{2+} currents evoked by a 100 ms step depolarization from -80 to -10 mV and plotted over time in WT (black) and *Otof*^{-/-} IHCs (red). Note the absence of the first large I_{CaTB} immediately following the onset of the Ca^{2+} currents in *Otof*^{-/-} IHC, suggesting that synchronous multivesicular release was abolished. Secondary asynchronous and very small I_{CaTB} events were still observed in *Otof*^{-/-} IHC. Using the “ideal observer” criteria for signal-to-noise ratio, the small events below 5.04 pA were rejected.

(G) Top: examples of asynchronous mini- I_{CaTB} events when recorded with 1 mM intracellular EGTA (black) in WT IHCs, with 5 mM intracellular BAPTA in WT IHCs (blue) or with 1 mM intracellular EGTA in *Otof*^{-/-} IHC. Bottom: amplitude distribution of the asynchronous mini- I_{CaTB} events for each condition. Data points were fitted with a Gaussian function using a binning of 1 pA. In control condition (black), the distribution displayed two peaks at 6.33 ± 0.06 and 9.04 ± 0.05 pA (114 events). In the presence of 5 mM BAPTA (blue), the distribution displayed a single peak at 9.15 ± 0.02 pA (80 events). The amplitude of the mini- I_{CaTB} events was largely decreased in otoferlin-deficient IHCs (red). This *Otof*^{-/-} IHC distribution had a single peak at 3.54 ± 0.15 pA (93 events). At right, cumulative frequency plots of asynchronous mini events of WT IHCs (EGTA or BAPTA) were different from noise with $p < 0.001$ (Kolmogorov-Smirnov test). Recording conditions were room temperature (22°C) and 5 mM external Ca^{2+} .

Figure 6. *In Vivo* Fast Adaptation in the Discharge Rate of the Mouse Auditory Nerve Fibers

(A) Example of a PST histogram recorded from an *in vivo* fiber with the firing characteristics of a primary-like without notch (CF = 17.96 kHz, SR = 3.7 spikes/s, FSL = 2.6 ms, and CV = 0.64). The 50 ms tone burst was set at CF and 30 dB above threshold. Histogram bin was set at 0.5 ms.

(B) Mean $\pm$ SEM. PST histograms recorded from 11 fibers from 16 to 25 kHz without notch. The fast firing adaptation could be described as the sum of two exponential functions with time constants 0.5 ± 0.1 ms (blue line) and 12.5 ± 1.4 ms (green line).

(C) Example of PST histogram with a notch recorded from a low-threshold fiber (CF = 18 kHz, SR = 64 spikes/s, threshold = 25 dB SPL, FSL = 2.5 ms, CV = 0.71).

(D) Mean $\pm$ SEM. PST histograms recorded from 16 fibers with notch with CF ranging from 16 to 25 kHz. The inset is an enlargement of the notch seen in the mean PST histogram. The adaptation could be fitted with two exponential functions with time constants 0.6 ± 0.1 ms (blue line) and 9.3 ± 0.8 ms (green line).

Figure 7. A Compact Ca^{2+} Channel Cluster Surrounded by Docked Vesicles at Mature IHC Ribbon Synapses Produces I_{CaTB}

(A) A voltage-clamp step from -80 to 0 mV to a mature mouse IHC in bicarbonate-based external solution reveals a prominent initial I_{CaTB} notch that is followed by several smaller events (asterisks). The initial large notch is due to the synchronous exocytosis of multiple vesicles, whereas the subsequent smaller events are due to asynchronous exocytosis events, which may also be multivesicular.

(B) The presynaptic I_{Ca} evoked by a voltage-clamp step from -80 to 0 mV in bicarbonate-based external solution and at 34°C displays no I_{CaTB} notch at a mouse (postnatal day P16) calyx of Held nerve terminal, which contains ~ 600 active zones.

(C) Top left: schematic of an active zone at the mature calyx of Held. Depolarization opens several Ca^{2+} channels, and Ca^{2+} ions flowing through open Ca^{2+} channels trigger the exocytosis of nearby docked synaptic vesicles that release their glutamate and H^{+} content into the synaptic cleft. The docked vesicles are located around a non-compact Ca^{2+} channel cluster. During a strong depolarization, only two to five vesicles fuse per active zone, which is not enough to block a large percentage of the open Ca^{2+} channels. Thus, the topographically dispersed vesicle fusions within the active zone, leads to a very inefficient acidification of the synaptic cleft. Top right: in an immature IHC ribbon synapse, a large number of Ca^{2+} channels and vesicles are not tightly organized in nanodomain rows of Ca^{2+} channels and docked vesicles. This loose organization increases the distance between H^{+} release and open Ca^{2+} channels, which does not produce I_{CaTB} . Bottom: in a mature IHC ribbon synapse, the acidification of the synaptic cleft produced by a topographically constrained and large multivesicular release at the ribbon leads to a very efficient transient block of a significant fraction ($\sim 30\%$) of the open Ca^{2+} channels at 32°C (see Figure 3A).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-CtBP2	Santa Cruz, USA	Cat#sc-5966; RRID: AB_2086771
Rabbit polyclonal anti-Cav1.3	Alomone labs, Israel	Cat#ACC-005; RRID: AB_10785202
Donkey anti-Goat Fluoprobe 547H	Interchim	Cat#FP-SB2110
Goat anti-Rabbit Alexa Fluor 488	Invitrogen	Cat#A-11008; RRID: AB_143165
Chemicals, Peptides, and Recombinant Proteins		
Cadmium	Sigma-Aldrich	Cat#C3141
EGTA-AM	Biomol	Cat#ABD-21005
TEA	Sigma-Aldrich	Cat#T2265
TTX	Sigma-Aldrich	Cat#554412
Strychnine	Sigma-Aldrich	Cat#S2880
Xylazine/ketamine mixture	Sigma-Aldrich	Cat#K4138
Apamin	Latoxan	Cat L8407
XE-991	Tocris Bioscience	Cat#2000
Amiloride	Sigma-Aldrich	Cat#A7410
DL-TBOA	Tocris Bioscience	Cat#1223
Experimental Models: Organisms/Strains		
Mouse (C57BL/6J)	Charles River Lab, FR	C57BL/6J
Mouse: Otoferlin Knock out (<i>Otof^{-/-}</i>)	Pasteur Institute, FR	MGI: 1894247
Gerbils	Charles River Lab, FR	GERSINIS
<i>Rana catesbeiana</i> : American Bullfrogs	Rana Ranch, Idaho	N/A
Software and Algorithms		
IgorPro 6	Wavemetrics	RRID: SCR_000325
OriginPro 2017	OriginLab	RRID: SCR_015636
Mini Analysis 6.0.3	Synaptosoft	RRID: SCR_002184
CorelDraw X7	Corel	RRID: SCR_014235