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# **Exocytosis at the hair cell ribbon synapse apparently operates without neuronal SNARE proteins**

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**SNARE proteins mediate membrane fusion. Neurosecretion depends on neuronal SNAREs (SNAP-25, syntaxin-1, and synaptobrevin-1 or 2) and is blocked by neurotoxin-mediated cleavage or genetic ablation. We report that exocytosis in mouse inner hair cells (IHCs) is insensitive to neurotoxins and genetic ablation of neuronal SNAREs. We found mRNA but no synaptically localized protein of neuronal SNAREs in IHCs. Thus, IHC exocytosis is unconventional and may operate independently of neuronal SNAREs.**

Hearing in mammals relies on synchronous signaling at the ribbon synapses between cochlear IHCs and the afferent auditory fibers. The IHC synaptic machinery differs from conventional synapses. For example, IHC synapses lack synaptotagmins I-III<sup>1</sup> and complexins<sup>2,3</sup>, but express the ribbon-protein RIBEYE<sup>4</sup> and the multi-C<sub>2</sub>-domain protein otoferlin<sup>5</sup>. Previous studies indicated that the neuronal SNAREs (SNAP [Soluble NSF Attachment Protein] receptors) – SNAP-25, syntaxin-1, and synaptobrevin-1 – are

expressed in IHCs<sup>1,3</sup>. In analogy to most neurosecretory preparations investigated so far<sup>6</sup>, it has been assumed that these neuronal SNAREs are also required for vesicle fusion at the IHC ribbon synapse, but this assumption has not yet been tested.

To do so we initially employed the light chains of clostridial neurotoxins, which specifically cleave neuronal SNARE proteins<sup>6</sup>. Botulinum neurotoxin serotype E (BoNT/E) cleaves SNAP-25, whereas BoNT/C and BoNT/D target syntaxin-1/2/3 and synaptobrevin-1/2/3, respectively. We first verified biochemically that low concentrations of the light chains of BoNT/E (1 nM), BoNT/C (1  $\mu$ M), and BoNT/D (1 nM) efficiently cleave their respective substrates (**Supplementary Fig. 1a–c**). We then confirmed BoNT activity on large dense-core vesicle exocytosis in chromaffin cells by whole-cell patch-clamp recordings assessing exocytic membrane capacitance increments ( $\Delta C_m$ ). Application of active BoNT/E, BoNT/C, or BoNT/D via the patch-pipette strongly reduced  $\Delta C_m$ , as measured in response to 500 ms step depolarizations to -27 mV that were started 10 min after establishing the whole-cell configuration, but did not alter  $Ca^{2+}$  currents (**Supplementary Fig. 1d–g**). In contrast, chromaffin cells infused with the corresponding BoNTs rendered inactive by point mutations exhibited robust secretion.

Next, we applied the same set of BoNTs to IHCs (postnatal days 13-17) and measured exocytic  $\Delta C_m$ . We repeatedly probed exocytosis of the readily releasable pool of vesicles (RRP) by 20 ms step depolarizations<sup>7</sup>, starting 10 min after establishing the whole-cell configuration. Long inter-stimulus intervals (150 s) were chosen to provide sufficient time for RRP recovery and BoNT-mediated cleavage of un-complexed SNAREs. Still, IHCs infused with 2  $\mu$ M of active BoNT/E exhibited robust  $\Delta C_m$  during repetitive stimulation (**Fig. 1a–d**).

Exocytosis per unit of incoming  $Ca^{2+}$  ions ( $\Delta C_m/Q_{Ca}$ ;  $Q_{Ca}$ :  $Ca^{2+}$  current integral) from IHCs poisoned with BoNT/E was not significantly different from control values (BoNT-free solution, **Fig. 1d**). Both poisoned and control cells showed the same typical decline of responses during the time-course of the experiment (**Fig. 1d**). In comparison to the kinetics of toxin-mediated block of exocytosis at the calyx of Held<sup>8</sup>, this rundown, which we attribute to washout of essential factors from IHCs, appears sufficiently slow to reveal a potential toxin effect. BoNT-loading of IHCs was probed in a separate set of experiments by tracking the diffusion of Alexa488-conjugated BoNT/E. While the

fluorescence increase in IHCs indicated reliable loading, IHC exocytosis remained unaffected (**Supplementary Fig. 2**), despite the ability of the conjugated BoNT to cleave SNAP-25 *in vitro* and in chromaffin cells (**Supplementary Fig. 1,3**).

To ensure complete SNAP-25 cleavage we performed a set of experiments at near-physiological temperature (increasing enzymatic activity) in which we probed RRP exocytosis following a longer waiting time (20 min after break-in; **Supplementary Fig. 4**). Neither in these experiments nor upon stronger stimulation (500 ms depolarizations [**Supplementary Fig. 5**] or flash-photolysis of caged  $\text{Ca}^{2+}$  [**Fig. 1e**: only the slower secretory component remained present after 8 min of waiting in the whole-cell configuration]) did we observe an inhibition of exocytosis by BoNT/E. In contrast, non-specific intracellular proteolysis by trypsin caused a reduction of exocytosis evoked by  $\text{Ca}^{2+}$  uncaging (**Fig. 1e**). When we repeated the experimental protocol of **Fig. 1a–d** with BoNT/C and BoNT/D, we again failed to detect significant BoNT effects on  $\Delta C_m/Q_{\text{Ca}}$ , although tracking of Alexa488-conjugated BoNT/C and BoNT/D confirmed BoNT loading of the IHCs (**Supplementary Fig. 6**). We note that the cleavage of SNARE proteins in chromaffin cells would probably occur faster than in IHCs (approximate ratio of syb over BoNT/D after 5 minutes of infusion: 1.3 in chromaffin cell vs.  $\sim 15$  in IHCs, see **Supplementary Table 1**). However, given the *in vitro* turnover numbers ( $k_{\text{cat}}$ ) for BoNT/D-LC and BoNT/E-LC of  $25/\text{s}^9$  and  $21.8/\text{s}^{10}$ , respectively, the majority of substrate should be cleaved within seconds (supposed the ratios between BoNT and substrate are  $\sim 15:1$ ) or within less than a minute (if the ratio was larger by an order of magnitude).

In view of the unexpected resistance of IHC exocytosis to BoNTs, we investigated the SNARE dependence of IHC secretion using SNARE-deficient mice (**Fig. 1f–i**). Because synaptobrevin-1 had been described originally as one of the SNARE components in IHCs<sup>1</sup>, we first studied exocytosis in IHCs of *lethal-wasting* mice, which carry a loss-of-function mutation in the *synaptobrevin-1* gene<sup>11</sup>. In agreement with the lack of BoNT/D effect described above, we failed to detect any significant differences between IHCs of wild-type and *lethal-wasting* mice regarding RRP size, sustained exocytosis (approximated as exocytic rate between 20 and 100 ms of depolarization), or  $\text{Ca}^{2+}$  current integrals (**Fig. 1f,g**). Mice lacking synaptobrevin-2 and -3<sup>12</sup> or SNAP-25<sup>13</sup> die at birth. Therefore, we studied IHC exocytosis in organotypic cultures of the organ of

Corti on embryonic day 18. IHCs from synaptobrevin-2/3 double knock-out mice showed robust exocytosis, consistent with the resistance of IHC exocytosis to BoNT/D (**Fig. 1h,i**). IHCs from SNAP-25 knock-out mice displayed exocytic  $\Delta C_m$ , albeit at reduced amplitude. The reduction of exocytosis, which did not reach significance, is likely related to a decrease of the  $Ca^{2+}$  current. Indeed, the  $\Delta C_m/Q_{Ca}$  ratios were not altered in SNAP-25 knock-out mice indicating that the amount of exocytosis per  $Ca^{2+}$  influx was not affected (**Fig. 1i**).

In view of our functional data indicating that IHC exocytosis does not depend on neuronal SNAREs, we re-examined SNARE expression in the organ of Corti using single-IHC PCR, genetic labeling and immunohistochemistry. Real-Time PCR using nested primers on slightly pre-amplified amplicons detected mRNAs of all neuronal SNAREs in IHCs (**Supplementary Fig. 7, Supplementary Table 2**), albeit at lower levels than otoferlin or parvalbumin mRNAs. To study neuronal SNARE protein expression in IHCs, we initially took advantage of a knock-in mouse line that we generated (**Supplementary Fig. 8**), which expresses pHluorin-tagged synaptobrevin-1 instead of the wild-type protein<sup>14</sup>. We did not detect obvious GFP-fluorescence in knock-in IHCs (**Supplementary Fig. 9**) even when depolarizing them, dequenching GFP (by addition of 50 mM  $NH_4Cl$ ) or immunolabeling for GFP. Underneath the knock-in IHCs, faint GFP-fluorescence spots were detected, most likely representing efferent nerve terminals. Subsequently, cochlear cryosections and whole mounts of apical cochlear coils were analyzed immunohistochemically using multiple antibodies and labeling protocols. Staining for Vglut3 or otoferlin (**Fig. 2, Supplementary Fig. 10**) resulted in strong and diffuse cytosolic immunofluorescence, the typical localization pattern of synaptic vesicle proteins in IHCs. As illustrated in **Fig. 2** and **Supplementary Fig. 10**, we did not detect labeling for synaptobrevin-1 (two different antibodies), synaptobrevin-2 (two different antibodies) or synaptobrevin-3 (one antibody). Moreover, we did not observe immunolabeling of the afferent synapses for SNAP-25 (four different antibodies) and syntaxin-1 (four different antibodies). The efferent olivocochlear nerve terminals below the IHCs served as a positive control within the same specimen for most of these antibodies.

Collectively, the results of molecular interference with SNAREs by BoNTs and

genetic knock-out as well as the data on SNARE protein expression in IHCs argue against a canonical regulation of vesicle fusion by neuronal SNAREs in IHCs. Based on our data, we cannot rule out unequivocally that IHCs express very low levels of neuronal SNARE proteins, that SNARE proteins in IHCs are shielded by interacting proteins, which might prevent detection by antibodies or cleavage by BoNTs, or that the function of genetically ablated SNAREs is compensated by other SNAREs. The most likely explanation of the present data is that IHCs, being epithelial cells, make use of other SNARE proteins for synaptic vesicle exocytosis than neurons, which remain to be identified in future studies.

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## AUTHOR CONTRIBUTIONS

The study was designed by R.N. and T.M. The experimental work was performed by R.N., J.N., A.V.B., E.R., T.P., T.F., and S.S., and T.M., R.N., J.N., E.R., T.P., N.B., and T.B. prepared the manuscript.

## FIGURE LEGENDS

**Figure 1 Exocytosis in inner hair cells is resistant to BoNT attack and genetic ablation of neuronal SNAREs.** (a) Representative whole-cell  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}^{2+}}$ ),

membrane capacitance ( $C_m$ ), membrane conductance ( $G_m$ ) and series conductance ( $G_s$ ) traces. Recordings from IHCs were performed as described<sup>7</sup>. Ten minutes after gaining chemical access (BoNT/E (grey) or control (without toxins, black) IHCs were repetitively stimulated by 20 ms depolarizations to -27 mV. IHCs (b) Average exocytic  $\Delta C_m$  (upper panel) and  $Ca^{2+}$  current integrals ( $Q_{Ca^{2+}}$ ; lower panel), (c)  $\Delta C_m$  per  $Q_{Ca^{2+}}$ , (d)  $\Delta C_m/Q_{Ca^{2+}}$  ratio normalized to the first response plotted against each 20 ms step depolarization trial in BoNT/E poisoned (grey) and toxin-free IHCs (black). The number of IHCs is indicated in (b). (e) Average  $\Delta C_m$  responses evoked by  $Ca^{2+}$ -uncaging in IHCs<sup>15</sup> infused with trypsin (25.2  $\mu$ M, dark grey), BoNT/E (2  $\mu$ M, light grey) or control solution (without enzyme, black). On average, the UV flash was delivered 8.2 minutes after break-in (indicated as time 0) and postflash  $[Ca^{2+}]_i$  levels were comparable for the 3 conditions. The  $\Delta C_m$  amplitudes of control and trypsin-infused cells as well as of BoNT/E- and trypsin-infused cells differed (Mann-Whitney U-test,  $p = 0.003$  for both). (f) Representative perforated patch recordings of  $I_{Ca^{2+}}$ ,  $C_m$ ,  $G_m$  and  $G_s$  in lethal-wasting mutant (*Lew*, grey) and wild-type (Wt, black) littermate IHCs in response to 20 ms of maximal  $Ca^{2+}$  current. (g) Exocytic  $\Delta C_m$  and  $Q_{Ca^{2+}}$  versus duration of depolarization for *Lew* (grey) and wild-type (black) IHCs. (h) Representative  $I_{Ca^{2+}}$ ,  $C_m$ ,  $G_m$  and  $G_s$  perforated-patch recordings from IHCs of C57BL/6 (black), synaptobrevin-2/3 double knock-out (*Syb 2/3 dko*, dark grey) and SNAP-25 knock-out (*SNAP25 ko*, light grey) embryonic day 18 organ of Corti cultures in response to 200 ms of maximal  $Ca^{2+}$  current ( $[Ca^{2+}]_e:10$  mM). (i)  $\Delta C_m/Q_{Ca^{2+}}$  of C57BL/6, *Syb-2/3 dko* and *SNAP-25 ko* for 100 to 500 ms step depolarization. All error bars represent s.e.m. For methods, composition of solutions, chemicals etc. see Supplementary Information.

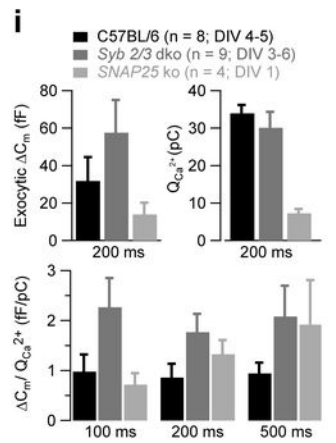
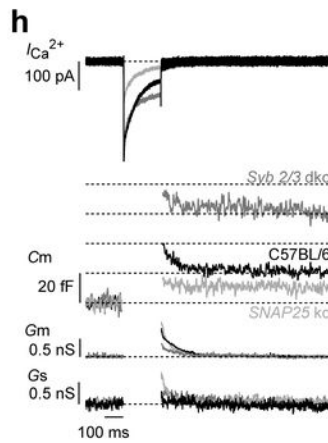
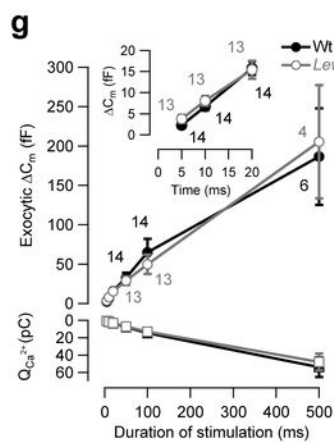
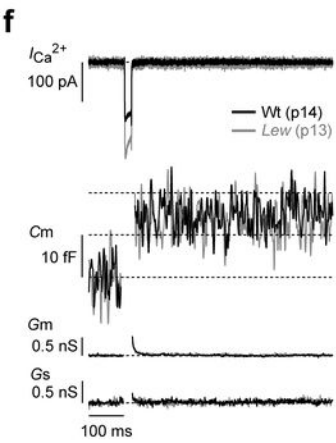
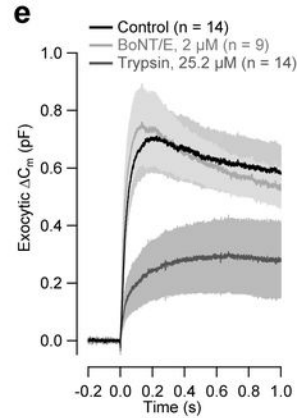
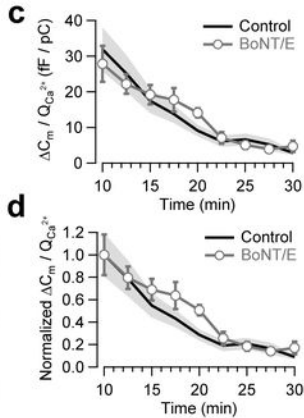
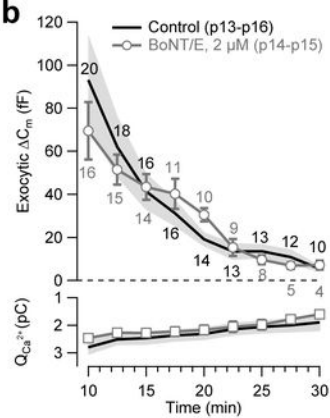
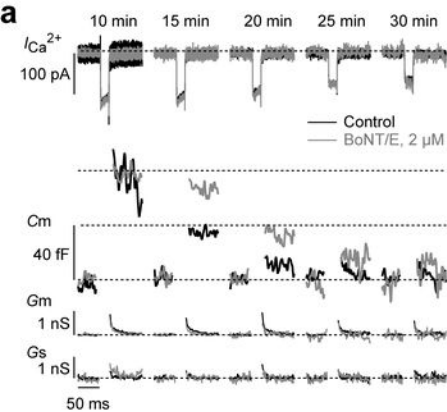
**Figure 2 Neuronal SNARE proteins are abundant in efferent nerve terminals but not detected in IHCs by immunohistochemistry.** (a-d) Inner hair cells in cochlear cryosections were labeled with a Vglut3 (Synaptic Systems 135203) or otoferlin antibody (Abcam ab53233; both magenta in the left panels) and the respective antibodies against neuronal SNAREs (synaptobrevin-1 [Synaptic Systems 104002] in a, total number of analyzed cryosections  $n=5$ ; synaptobrevin-2 [Synaptic Systems 104211] in b,  $n=8$ ; SNAP-25 [Covance SMI-81R] in c,  $n=12$ ; and syntaxin-1 [Sigma S0664] in d,  $n=10$ ; all

green in the left panels and grey in the right panels). The cartoon in the inset illustrates the afferent and efferent connectivity (**a**). Scale bars: 10  $\mu$ m.

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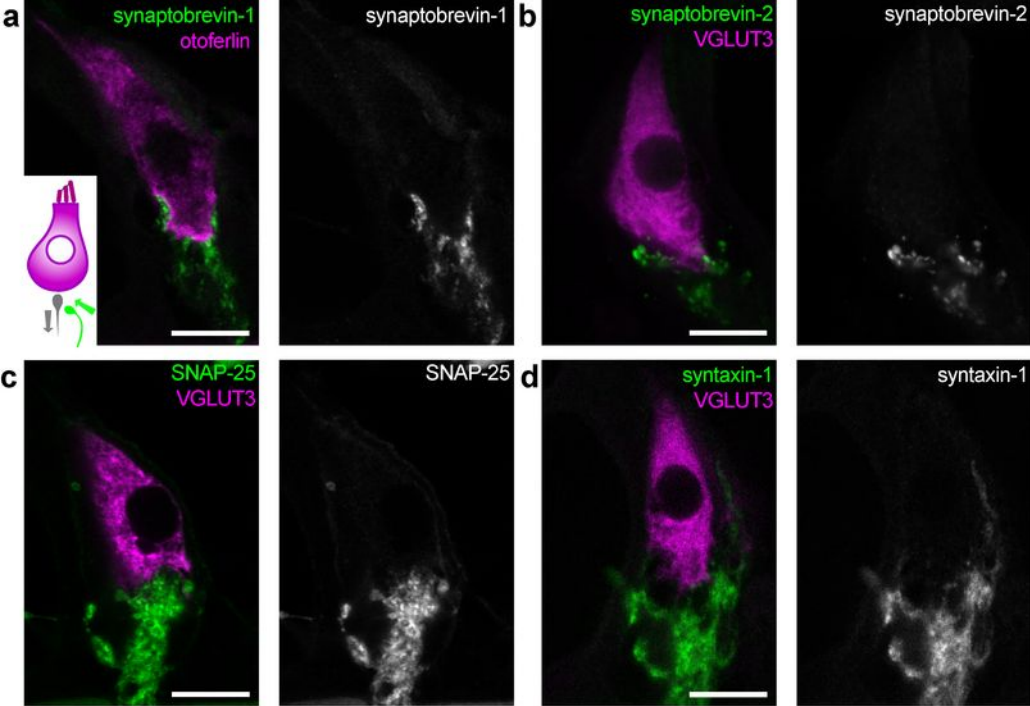


Figure-2 Moser