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REVIEW ARTICLE

Balancing presynaptic release and endocytic membrane retrieval at hair cell ribbon synapses

Tina Pangrsic[1] and Christian Vogl[2]

1 Synaptic Physiology of Mammalian Vestibular Hair Cells Group, Institute for Auditory Neuroscience and InnerEarLab, Auditory Neuroscience Group, Max Planck Institute of Experimental Medicine, University Medical Center Göttingen, Germany
2 Presynaptogenesis and Intracellular Transport in Hair Cells Group, Institute for Auditory Neuroscience and InnerEarLab, Auditory Neuroscience Group, Max Planck Institute of Experimental Medicine, University Medical Center Göttingen, Germany

Correspondence
T. Pangrsic, Synaptic Physiology of Mammalian Vestibular Hair Cells Group, Institute for Auditory Neuroscience and InnerEarLab, Auditory Neuroscience Group, Max Planck Institute of Experimental Medicine, University Medical Center Göttingen, Hermann-Rein-Str. 3, 37075 Goettingen, Germany
Fax: +49 0551 39-12950
Tel: +49 0551 39-61945
E-mail: tpangrs@gwdg.de
or
Christian Vogl, Presynaptogenesis and Intracellular Transport in Hair Cells Group, Institute for Auditory Neuroscience and InnerEarLab, Auditory Neuroscience Group, Max Planck Institute of Experimental Medicine, University Medical Center Göttingen, Hermann-Rein-Str. 3, 37075 Goettingen, Germany
Fax: +49 0551 39-12950
Tel: +49 0551 39-61945
E-mail: christian.vogl@med.uni-goettingen.de

Tina Pangrsic and Christian Vogl contributed equally to this article.

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The timely and reliable processing of auditory and vestibular information within the inner ear requires highly sophisticated sensory transduction pathways. On a cellular level, these demands are met by hair cells, which respond to sound waves – or alterations in body positioning – by releasing glutamate-filled synaptic vesicles (SVs) from their presynaptic active zones with unprecedented speed and exquisite temporal fidelity, thereby initiating the auditory and vestibular pathways. In order to achieve this, hair cells have developed anatomical and molecular specializations, such as the characteristic and name-giving 'synaptic ribbons' – presynaptically anchored dense bodies that tether SVs prior to release – as well as other unique or unconventional synaptic proteins. The tightly orchestrated interplay between these molecular components enables not only ultrafast exocytosis, but similarly rapid and efficient compensatory endocytosis. So far, the knowledge of how endocytosis operates at hair cell ribbon synapses is limited. In this Review, we summarize recent advances in our understanding of the SV cycle and molecular anatomy of hair cell ribbon synapses, with a focus on cochlear inner hair cells.

Keywords: auditory hair cell; endocytosis; exocytosis; ribbon synapse

Abbreviations
AZ, active zone; CAZ, cytomatrix at the AZ; CIE, clathrin-independent endocytosis; CME, clathrin-mediated endocytosis; CtBP2, C-terminal binding protein 2; EM, electron microscopy; EPSC, excitatory postsynaptic current; ER, endoplasmic reticulum; IHC, inner hair cell; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; RIM-BPs, RIM-binding proteins; RIMs, Rab3-interacting molecules; RRP, readily-releasable pool of SVs; SGN, spiral ganglion neuron; SV, synaptic vesicle; TRC40, transmembrane recognition complex 40; WRB, tryptophan-rich basic protein.

In the cochlea, synaptic sound encoding occurs at the monosynaptic connections between sensory inner hair cells (IHCs) and afferent spiral ganglion neurons (SGNs). These synapses are designed for exquisitely high, temporally precise, and indefatigable synaptic vesicle (SV) release rates that can drive peak action potential firing rates of hundreds of hertz in the auditory nerve for prolonged periods of time [1]. This impressive release capacity is due to the highly specialized molecular anatomy of IHC ribbon-type presynaptic active zones (AZs), which allows not only for ultrafast exocytosis, but also provides the molecular scaffold for rapid release site clearance, compensatory membrane retrieval and vesicular recycling. In fact, the coordinated and efficient interplay of these processes is fundamental for hearing as well as precise sound localization and dysfunction of this tightly controlled system leads to severe hearing impairment or deafness.

On the presynaptic side, hair cell synapses are ultrastructurally characterized by electron-dense proteinaceous projections – so-called 'synaptic ribbons'. Ribbon synapses cannot only be found in cochlear hair cells, but also in other sensory and neurosecretory cell types, such as vestibular hair cells, neuromasts of the lateral line system of fish, retinal photoreceptors and bipolar cells, as well as pinealocytes. Synaptic ribbons are mainly composed of the structural cytomatrix protein 'RIBEYE' [2]. Despite serving homologous functions in sensory neurotransmission, it is important to keep in mind that distinct ribbon types exhibit partly divergent molecular and structural compositions to cater for the specific needs of the system they are operating in. For example, while retinal ribbons exert a rather plate-like structure, hair cell ribbons are more compact and rather ovoid to spherical in appearance [3], indicative of differential regulation and/or expression patterns of accessory AZ components between these two systems.

Generally, ribbons are considered to provide molecular scaffolding at the AZ, tether a large complement of SVs and, hence, are thought to facilitate continuous vesicular replenishment to the release site. Moreover, ribbons are important for clustering presynaptic Ca^{2+} channels [4–7] and may act as physical Ca^{2+} diffusion barriers to produce spatially tightly confined Ca^{2+} hotspots [8]. Over recent years, extensive efforts have been invested into the molecular characterization of the exocytic machinery that mediates temporally precise, ultrafast, and indefatigable SV release at IHC ribbon synapses (reviewed in detail in Refs [9–12]). Here, it is well established that bassoon – a large multidomain cytomatrix protein also found at conventional synapses – plays a critical role in anchoring the ribbon to the presynaptic density [13–15]. In addition, in IHCs, bassoon promotes a large complement of presynaptic Ca^{2+} channels and facilitates SV replenishment [4,14,16,17]. Surprisingly, IHCs utilize the unconventional vesicular glutamate transporter Vglut3 for the loading of SVs with neurotransmitter molecules [18,19] and do not express core exocytic proteins such as synaptophysins, synapsins, or complexins [20,21]. Likewise, essential neurosecretory soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins – including synaptobrevins/VAMPs 1–3, syntaxins 1–3 and SNAP25 – are either not expressed or functionally redundant for SV exocytosis in IHCs, as evident from cell-physiological analyses of genetic deletion mutants and the lack of obvious effects of clostridial neurotoxins on presynaptic release [22]. Similarly, SV priming factors of the Munc13 and CAPS families – evolutionary highly conserved molecules that are fundamentally required for neurotransmitter release in various secretory systems of invertebrates and vertebrates alike – could not be detected in IHCs [23]. Moreover, the neuronal vesicular Ca^{2+} sensors synaptotagmins 1 and 2 are only transiently expressed during the early developmental stages [24,25]. Finally, while neurons mainly employ P/Q- and N-type $Ca_V2.1$ and 2.2 Ca^{2+}-channels for Ca^{2+}-influx–secretion coupling [26], IHC AZs utilize L-type $Ca_V1.3$ channels for this purpose [27–30]. Recent work revealed that the number, activation kinetics and open probability of these channels not only differ based on their tonotopic position along the cochlear axis [31–34], but also depend on their subcellular AZ localization [33–35], thereby suggesting the location-dependent presence of yet-to-be-identified regulatory proteins. In this context, $Ca_V1.3$ channels have been shown to be regulated by a number of modulatory proteins, including Gipc3 and CaBP2 [35,36] (for a recent extensive review on this topic see Ref. [37]).

While our understanding of the exocytic key players orchestrating stimulus–secretion coupling in IHCs continues to grow, the molecular machinery governing compensatory endocytosis at these synapses remains largely elusive. Hence, this short review aims to provide a brief update on the current state of knowledge concerning presynaptic exocytosis at IHC ribbon-type AZs, before focusing on the molecular machinery facilitating release site clearance, endocytic membrane retrieval, and SV reformation (Fig. 1). While our main objective is the discussion of cochlear ribbon synapses, we will also include available information on retinal

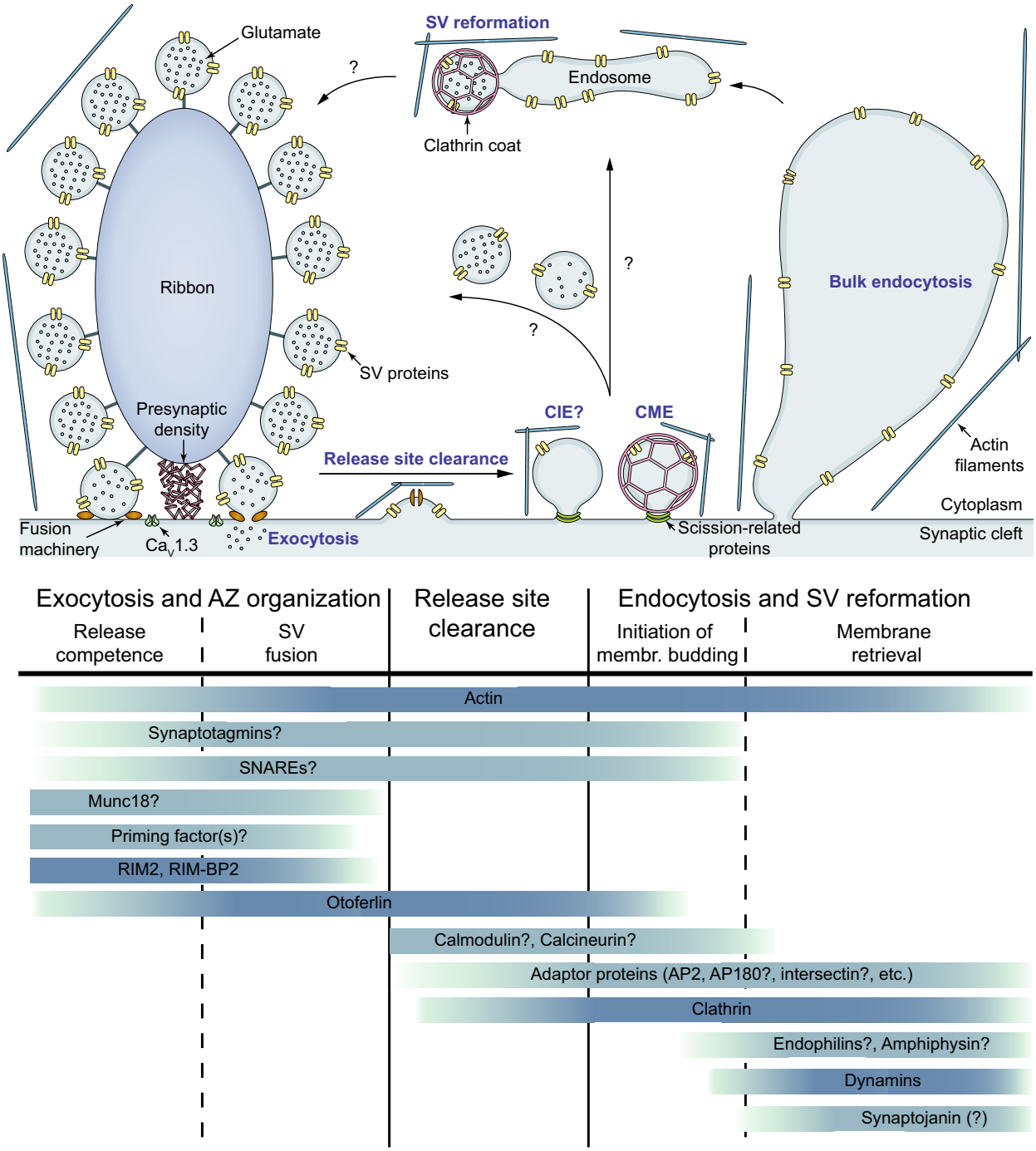

Fig. 1. The synaptic vesicle (SV) cycle at inner hair cell (IHC) ribbon synapses. IHC ribbons tether a large number of SVs that undergo exocytosis upon depolarization and subsequent Ca^{2+} influx through voltage-gated $Ca_V1.3$ channels. After fusion with the plasma membrane, previously used 'release slots' have to be cleared of exocytosed material before they can be re-occupied by new SVs. The exocytosed phospholipids and proteins of the exocytic machinery are then recycled into the cytosol *via* endocytosis. In IHCs, there is evidence for the presence of clathrin-mediated (CME) and bulk endocytosis, whereas the existence of clathrin-independent endocytosis (CIE) has not been confirmed yet. After membrane retrieval, endocytosed material likely fuses with large endosomal compartments in close proximity of the ribbons and new SVs may be formed in a clathrin-dependent pathway. The molecular machinery of exocytosis and endocytosis of IHC SVs has remained largely unknown. The table lists the most important identified and still elusive synaptic proteins potentially operating at IHC AZs and indicates in which processes of the SV cycle they may be involved. A question mark at the protein name indicates that it has been identified and described at conventional neuronal synapses, but its presence and/or function at the IHC ribbon synapse are unknown or controversial.

ribbon synapses wherever deemed appropriate. However, we would like to highlight here that both systems have their individual molecular, structural, and functional peculiarities (reviewed in Ref. [3]) and hence, comparisons remain difficult in the absence of direct experimental evidence.

The unconventional auditory IHC synaptic release machinery – an update

Synaptic ribbons tether a large complement of SVs [38] and have therefore been suggested to act as 'conveyor belts' for efficient SV replenishment to the presynaptic release site [6,39–42]. Yet, the underlying molecular mechanisms facilitating SV translocation to the AZ membrane remain elusive but have been shown to involve Ca^{2+} [43,44]. Considering SV trafficking, several distinct scenarios have been suggested to take place at retinal ribbons, ranging from passive, diffusional 'crowd-surfing' models [45] to active and targeted transport pathways – potentially involving the cytoskeleton and molecular motors such as KIF3a [15,46] – but are still lacking definitive experimental confirmation. In IHCs, these processes are even less clear and require further in-depth analysis. However, it is noteworthy that both, actin and tubulin have been shown to directly associate with RIBEYE in cochlear and retinal samples [47,48] and, hence, may indeed not only contribute to the structural integrity of the ribbon, but also facilitate SV recruitment and/or movement to the release site.

Apart from SV replenishment, additional structural and functional roles of the ribbon have been proposed, including (i) the clustering and functional modulation of presynaptic $Ca_V1.3$ Ca^{2+} channels at the AZ [4–7], (ii) SV priming – either directly or *via* recruitment of other priming factors – [42], (iii) spatial confinement of Ca^{2+} diffusion to ensure tightly localized presynaptic Ca^{2+} hotspots [8] – although this hypothesis has recently been challenged [7,49] – and finally, (iv) the coordination of multivesicular release and compound fusion of SVs prior to exocytosis [50–53].

Morphologically, AZ-tethered SVs are usually classified into two main populations, the membrane-proximal and the ribbon-associated pool of SVs [38,54]. Functionally, patch-clamp recordings of depolarization-evoked changes in IHC membrane capacitance similarly suggest multiple pools of SVs. The fast, exponential component of exocytosis, which is usually recruited by brief depolarizations, is believed to represent the fusion of the readily-releasable pool of SVs (RRP) [55–57]. This RRP might be further subdivided into two kinetically distinct SV pools according to postsynaptic recordings of SGN excitatory postsynaptic currents (EPSCs) [58]. While the morphological correlate of the RRP is likely represented by the membrane-proximal SVs, the sustained component of exocytosis or slowly-releasable pool of SVs may reflect continuous replenishment of ribbon-associated SVs after initial RRP depletion [4,55–57,59]. Data from turtle hair cells additionally suggest the presence of another, superlinear phase of exocytosis occurring after prolonged stimulation [44]; however, whether this latter phenomenon also plays a role in cochlear sound encoding in mammals requires further investigation.

In the following, we want to mainly focus on integrating the most recent findings regarding ribbon function and the roles of some of its tightly associated proteins into the current picture of ribbon synapse morphology and physiology. For a broader discussion of the molecular anatomy of these synapses as well as the controversially-debated release mechanism operating at IHC synapses (i.e., the uni- vs. multivesicular release hypotheses), we refer the interested reader to more exhaustive reviews on these topics [9–11,60,61].

RIBEYE

RIBEYE is the main constituent of the ribbon, contributing ~ 67% to the ribbon volume [62] and ~ 10–15% of the total molar quantity of the ribbon complex at retinal photoreceptor AZs [48]. Molecularly, RIBEYE consists of two main functional parts: (i) a unique amino-terminal A-domain, which does not exhibit any significant sequence homology to other known genes and (ii) a B-domain that is identical to the transcriptional repressor C-terminal-binding protein 2 (CtBP2), though lacking its initial twenty N-terminal amino acids [2]. Individual RIBEYE molecules auto-assemble to form highly organized structural aggregates that can reach considerable sizes of several hundreds of nanometers once attached to the presynaptic AZ membrane (reviewed in Ref. [63]). This auto-assembly occurs *via* multiple homo- and heterotypic interactions between proximal A- and B-domains of adjacent RIBEYE molecules [64]. In this framework, the A-domains are thought to provide structural support, whereas the B-domains have been shown to be involved in phosphatidic acid generation, which might play a role in facilitating SV fusion and/or retrieval [65].

In zebrafish lateral line neuromast hair cells, loss of both *ribeye* genes produces a rather striking phenotype as presynaptic release sites are characterized by mislocalized, electron-translucent 'ghost ribbons' of reduced size that still tether a full set of slightly smaller SVs

[66]. Here, the authors proposed that low residual Ribeye expression in these mutants may suffice to form a basic ribbon-like scaffold. Subsequent functional analysis revealed a significant increase in whole-cell Ca^{2+} currents and concomitant exocytosis in the mutant hair cells; a finding likely attributable to compensatory homeostatic plasticity in response to the structural AZ perturbation. Strikingly, these functional impairments did not translate into any detectable *in vivo* phenotype in the afferent neurons.

In contrast, genetic deletion of *RIBEYE-A* in mice resulted in the complete loss of presynaptic dense bodies from retinal photoreceptors, rod bipolar cells, and cochlear hair cells [6,7,49]. Interestingly, the functional consequences differed substantially between the latter two systems: while retinal neurotransmission was majorly impaired due to the mislocalization of presynaptic Ca^{2+} channels, loss of membrane-proximal SVs, and subsequently strongly reduced EPSC amplitudes [6], the cochlear phenotype of these mice was surprisingly mild. Here, two independent studies [7,49] thoroughly characterized the presynaptic morphology and function of cochlear IHCs. Apart from unchanged synapse counts, Jean and colleagues reported extensive presynaptic developmental compensation that produced multiple 'ribbonless' AZs at each individual synaptic contact, thereby maintaining or even increasing the presynaptic complement of releasable SVs [7]. Additionally, both studies consistently reported an increased size of postsynaptic densities and GluA3 receptor clusters [7,49], indicative of extensive pre- and postsynaptic homeostatic plasticity mechanisms aiming to compensate the loss of presynaptic release capacity. This structural reorganization in the mutants led to a minor exocytosis deficit in response to mild depolarizations that was lost upon stronger stimulation. However, single unit recordings from auditory nerve fibers revealed that, while firing thresholds were increased, peak as well as adapted firing rates were significantly reduced [7]. Moreover, the temporal jitter of firing and the time course of adaptation were increased and the recovery from adaptation was slowed. Taken together, these functional alterations indicated a role of the ribbon in regulating the voltage-dependence and spatial organization of Ca^{2+} channels and confirmed its importance for efficient vesicle replenishment. However, given the remarkable capacity for developmental compensation, the function of the ribbon in IHC presynaptic release has likely been underestimated in the constitutional *RIBEYE* knock-out. Hence, future studies should employ inducible *RIBEYE* deletion mutants to investigate the consequences of acute ribbon elimination upon normal synapse formation and presynaptic development in an otherwise unperturbed system.

Piccolo/aczonin

Piccolo/aczonin is the largest (~ 550 kDa) multi-domain scaffold protein expressed at conventional AZs [67,68]. Here, in concert with bassoon and Rab3-interacting molecules (RIMs), it acts as a key organizer in the assembly and maintenance of the cytomatrix at the AZ (CAZ). In addition, piccolo/aczonin facilitates presynaptic SV clustering, exocytosis, and replenishment [69–72], presumably through its direct interactions with core CAZ proteins, such as Munc13, RIMs, bassoon, and CAST/ELKS [73–75]. Moreover, piccolo/aczonin regulates activity-induced F-actin assembly at the presynapse [76,77], likely *via* its established interactions with various actin-binding proteins including Abp1, GIT1, profilins, and Daam1 [67,77–79].

Classically, the long isoforms of piccolo/aczonin have been considered to form an integral part of sensory ribbon complexes [13–15,80]; however, recent work challenges this idea and rather suggests that ribbons solely express a short (~350 kDa) C-terminally truncated piccolo/aczonin splice variant termed 'piccolino' [81]. Piccolino lacks most common CAZ–protein interaction domains but, interestingly, appears to retain all functional domains contributing to actin interaction [81]. AAV-mediated knockdown of *Piccolino* in retinal photoreceptors resulted in striking morphological alterations of ribbon shape: at the expense of the normally plate-shaped ribbons, predominantly spherical ribbons – reminiscent of ribbon precursor spheres and hence, indicative of either majorly delayed development or synapse degeneration – could be observed residing at the AZs [82]. It is therefore conceivable that piccolino still serves a multifunctional role at ribbon synapses by not only providing molecular scaffolding, but – analogous to its function at conventional synapses – also regulating presynaptic actin dynamics and dynamin-dependent SV recycling *via* its putative interactions with GIT1 and Abp1 [83–85]. Yet, the definitive functional role of piccolino at ribbon-type AZs remains elusive.

Rab3-interacting molecules (RIMs) and RIM-binding proteins (RIM-BPs)

RIMs are a family of multi-domain proteins that serve as key regulators of presynaptic release and Ca^{2+} channel density at conventional AZs [86–89]. In

addition, recent data suggest RIMs to further mediate SV tethering to the presynaptic density [90] and facilitate SV priming *via* the formation of a tripartite Rab3/RIM/Munc13 complex and subsequent activation of Munc13. This latter function is an essential prerequisite to establish SV fusion competence [91,92].

At retinal photoreceptors, RIM1 and RIM2 have classically been assumed to both be expressed, but have been suggested to exert distinct expression patterns at the ribbon and arciform density, respectively [2,15]; however, a recent study casts doubt on the expression of RIM1 and rather argues for a RIM1/Munc13-1-independent priming mechanism that exclusively utilizes RIM2 [93]. Similarly, at IHC ribbon-type AZs, recent work conclusively confirmed the absence of RIM1 from these synapses and identified RIM2α and RIM2β as the dominant RIM isoforms operating at IHC AZs [94]. Here, RIM2s localize to the presynaptic density at the base of the ribbon, where they regulate presynaptic Ca^{2+} channel clustering and additionally participate in tethering SVs to the AZ membrane. Apart from RIM2α/β, IHCs have also been shown to express RIM2γ and the two shorter RIM isoforms RIM3β and RIM3γ. Analogous to RIM2α, RIM3γ physically interacts with $Ca_V1.3$ channels and boosted its membrane insertion in a heterologous expression system; however, biophysical analysis of IHCs from *RIM3*γ-KO mouse mutants did not identify any obvious roles of RIM3γ in the regulation of IHC presynaptic Ca^{2+}-channel abundance, SV exocytosis, or hearing [95]. The apparent absence of RIM1 from sensory ribbon synapses is consistent with previous work suggesting a Munc13-independent SV priming mechanism to take place at photoreceptor synapses [96,97] and the complete lack of all currently described Munc13s and CAPS1/2 vesicular priming factors from IHCs AZs [23]. Taken together, these data suggest an unconventional and unique SV priming mechanism that remains to be identified, but may involve the ribbon itself [42] and/or otoferlin, a Ca^{2+}-binding, multi-C_2-domain protein essential for IHC exocytosis [59,98,99].

Finally, the synaptic function of deletion mutants for RIM-binding protein 2 (RIM-BP2) and RIM-BP1/2 have recently been analyzed in IHCs and photoreceptors, respectively [100,101]. Both studies reported a contribution of RIM-BPs to presynaptic Ca^{2+} channel clustering at the AZ – likely by acting as a molecular linker between RIM, bassoon, and voltage-gated Ca^{2+} channels [102]. Additionally, RIM-BP2 appears to promote SV recruitment to the release site during ongoing stimulation in IHCs and its absence causes a mild auditory synaptopathy [100].

Actin

Actin is one of the most abundant proteins at neuronal presynaptic AZs, where it colocalizes with the SV cluster and has been suggested to serve a multifunctional role including (i) functional scaffolding [103–105], (ii) the restriction of SV mobility within the terminal [106], (iii) the formation of a SV fusion barrier [107,108], (iv) a contribution to SV loading of release sites, docking and priming [103,109–112], (v) the promotion of SV fusion [113] and finally (vi) SV endocytosis and recycling [114–118].

Proteome analysis of ribbon complexes indicates the presence of actin in the core ribbon complex, suggestive of an involvement in ribbon structure and – potentially – release site-directed SV movement [47,48]. In support of this latter hypothesis, the actin-based motor protein myosin 6 has been shown to localize to IHC synaptic ribbons and other AZ substructures, where it physically interacts with the putative Ca^{2+}-sensor and SV priming factor otoferlin [119]. Hence, analogous to its suggested role in neurons [120], myosin 6 may play an extended – either direct or indirect – role in SV replenishment, release site clearance, and/or SV reformation at IHC AZs that relies on the presence of the presynaptic actin meshwork. Ribbons of *Myo6*-mutant Snell's Waltzer mice [121] display morphological and functional deficits that are indicative of delayed developmental maturation [119]; thus, it is tempting to speculate that myosin 6-dependent transport of ribbon material (or accessory proteins) along actin filaments is crucial for ribbon assembly and/or maintenance. Yet, in this context, it is important to consider that myosin 6 is not exclusively expressed within the IHC presynaptic compartment, but also mediates cargo transport of stereociliar components within the pericuticular necklace [122]. Hence, the developmental phenotype observed on the synaptic level might be of secondary nature and – at least partly – result from the severe stereociliar abnormalities described for Snell's Waltzer mutants [123].

Recently, two independent studies have investigated the functional role of presynaptic actin in neurotransmitter release at auditory ribbon synapses [124,125]. Here, pharmacological disruption of the actin cytoskeleton led to an apparent increase in the Ca^{2+}-efficiency of SV fusion for depolarizations exceeding the amplitude of the RRP. This observation is consistent with the previously proposed 'SV fusion barrier' hypothesis, where actin destabilization would relieve the spatial restriction imposed by the cortical actin meshwork. Indeed, actin has been suggested to form

'cages' around individual presynaptic AZs in IHCs and appears to support the tight association of Ca^{2+}-channel clusters and synaptic ribbons [124]. Actin disruption led to an increase in the distance between ribbons and $Ca_V1.3$-immunoreactive patches, but did not result in an attenuation of Ca^{2+}-influx [124,125]. Alternatively, the observed increase in the SV fusion rate could also result from an additional molecular priming mechanism enabling the recruitment of so far unavailable SVs [110,111], but further in-depth analysis is required to clarify this issue.

Otoferlin

Otoferlin plays an essential role in the late steps of IHC exocytosis, where it has been proposed to act as a priming factor and Ca^{2+} sensor for exocytosis [59,99,126–129]. This latter hypothesis is supported by the findings that at least four to five of otoferlin's C_2-domains bind Ca^{2+} and/or phospholipids [59,130–132]. In addition, various lines of evidence suggest a direct interaction of otoferlin with neuronal SNAREs [131,133,134]; however, considering that neither the genetic ablation of various SNAREs, nor clostridial neurotoxin application produced any measurable detrimental effect on IHC exocytosis [22], the physiological role of these interactions will have to be determined in future studies.

Apart from its role in exocytosis, recent evidence further suggests a prominent involvement of otoferlin in efficient release site clearance and SV reformation [59,126–128,135]. Interestingly, a point mutation in the otoferlin C_2C domain renders this protein instable and highly temperature-sensitive (an increase of core body temperature of 1 °C suffices to induce temporary deafness in humans [136]). In mice, this mutation significantly reduces otoferlin abundance in the plasma membrane [126]. Indeed, IHC exocytosis seems to scale with the plasma membrane abundance of otoferlin, but it is not clear whether this is an essential requirement for its function or merely a consequence of it. Additionally, otoferlin may aid the formation of short tethers connecting SVs to the plasma membrane, thereby likely promoting SV priming [23]; however, it does not represent their main constituent as tethers still do form even in the absence of otoferlin [23]. Thus, the molecular composition of these filaments still awaits identification.

Otoferlin belongs to a family of tail-anchored proteins, which contain their transmembrane domain at the COOH-terminus. Therefore, otoferlin can only be inserted into the endoplasmic reticulum (ER) post-translationally (for review see Ref. [137]). The main post-translational membrane insertion pathway that is required for proper insertion of otoferlin into the membrane employs the transmembrane recognition complex 40 (TRC40) and tryptophan-rich basic protein (WRB) [127], which – in concert with calcium-modulating cyclophilin ligand – constitutes the TRC40 ER receptor [138,139]. The absence of WRB from IHCs results in significantly reduced intracellular levels of otoferlin, thus causing a hair cell synaptic disruption and hearing impairment, which mimics the phenotype of some *otoferlin* mutations [127]. Likewise, human pathogenic mutations in the otoferlin transmembrane domain have been identified that likely impair posttranslational ER targeting of otoferlin and cause hearing impairment [127]. Despite the presence of a number of tail-anchored proteins whose biogenesis is likely also affected by the lack of WRB, the synaptic malfunction is the first obvious cellular defect observed in WRB-deficient IHCs. This may suggest a high turnover rate of otoferlin, which would thus be most strongly affected by the dysfunctional membrane insertion pathway. Alternatively, otoferlin may be less capable of utilizing alternative pathways as compared to other tail-anchored proteins, making it more vulnerable to the loss of the TRC40-dependent pathway.

Despite extensive experimental efforts, the exact role(s) of otoferlin have not been entirely clarified yet, partially due to its large size and structural complexity: otoferlin contains 6–7 C_2 domains as well as a Fer and a coiled-coil domain (for review see Ref. [9]), whose individual functions and interaction partners remain largely elusive to date. To clarify these issues, extensive site-directed mutagenesis studies with complete biochemical and physiological analysis will be required. This however has remained challenging due to various methodological limitations. For example, the size of *Otof* cDNA exceeds the maximum packaging size of commonly used AAV vectors that reliably transduce IHCs, hence hindering largescale *in situ* analysis of knock-in mutations. Otoferlin interaction analysis in heterologous expression systems is difficult to interpret without the context of the native cytoplasmic environment. Moreover, there are several technical challenges connected with the structural and biochemical analysis of otoferlin's functional domains (i.e., difficult purification, crystallization, etc. of individual C_2 domains).

Endocytosis at IHC ribbon synapses

Modes of endocytosis at IHC ribbon synapses

Regulated exocytosis at single IHC AZs can sustain exceedingly high rates for prolonged periods of time

[4,59]. In fact, SV release at this synapse occurs tonically, as we rarely experience complete silence. In order to preserve structural integrity of the AZ and assure unperturbed release capacity, endocytosis must quantitatively – and rapidly – balance exocytosis, either by matching rate or engulfing larger pieces of membrane at a time (the so-called 'bulk endocytosis'). Using electrophysiological membrane capacitance measurements upon cell depolarization, a fast exponential and a slow linear phase of endocytosis could be identified in IHCs [128,135,140]. These phases may represent clathrin-mediated endocytosis (CME; for review see Ref. [141]) and bulk endocytosis, respectively (Fig. 1). Recently, 'flash and freeze electron microscopy' enabled detection and description of ultrafast endocytosis in mammalian neurons and at *C. elegans* neuromuscular junctions [116,142]. In this system, ultrafast endocytosis occurred within 50–100 ms after cell depolarization and is mediated by actin and dynamin. In IHCs, this mode of endocytosis still awaits to be demonstrated.

Consistent with the findings from other tissues (reviewed in Refs [143,144]), brief depolarizations that predominantly recruit the RRP, activate a slow mode of endocytosis, which declines linearly with a rate of approximately 1–2 fF/s and is limited in speed [140]. In IHCs, this component of endocytosis can be selectively targeted (but not completely inhibited) by pharmacological inhibitors of CME and a missense mutation in *dynamin1*. Prolonged depolarizations additionally activate a faster mode of endocytosis – likely a rapid form of bulk membrane retrieval – that results in an exponential decay of ΔC_m with a tau of approximately 6 s and an amplitude that scales with the amplitude of the preceding exocytosis. Apart from IHCs, bulk retrieval has also been described for ribbon synapses of retinal bipolar cells and frog saccular hair cells, where cisterna-like structures could be observed that were slowly disassembled into small SVs [145–147]. Moreover, based on the findings from (i) electron microscopy (EM [38,135,140,147]), (ii) pHluorin as well as mCLING imaging [135,140,148] and (iii) EM analysis of labeled endocytic structures after photo-oxidation of the styryl dye FM1-43 [149], both modes of endocytosis appear to predominantly occur in close proximity to the AZs.

How are SVs reformed from endocytosed material? It seems that SVs do not derive directly from clathrin-coated vesicles, as these were found to be larger than SVs [i.e., approx. 50–60 nm vs 30–40 nm (using conventional fixation) [140,147]]. More likely, endocytosed vesicles fuse into larger endocytic compartments (e.g., endosomes), from which new SVs then pinch off. Alternatively, SVs could be reformed from membrane invaginations and cisterns in a distinct but complementary pathway. In this context, it is interesting to consider a potential role of the ribbon in SV reformation: endosomes often reside in close proximity to the ribbon [135,140] and the RIBEYE B domain has been shown to exert enzymatic activity to convert lysophosphatidic acid into phosphatidic acid in an NADH-dependent process [65]. Both of these phospholipids are known to affect membrane curvature [150,151]; hence, it is tempting to speculate that this spatial proximity is not coincidental. Moreover, RIBEYE B may also provide a physical link between the ribbon and SVs, as suggested in a recent study [152].

Is 'kiss-and-run' exo- and accompanying endocytosis (reviewed in Ref. [153]) taking place at the IHC AZ? Analysis of spontaneous EPSCs measured at synaptic boutons of SGNs contacting immature or mature IHCs revealed that ~ 30–50% of the events have complex shapes (as compared to transient events with single exponential decay times) [154–156]. Consistent with the *kiss-and-run* hypothesis and based on the results from mathematical modeling of these events, one study argued that these bizarre, multiphasic EPSCs, which are usually characterized by a lower amplitude, but similar charge to that of simple EPSCs, might stem from fusion-pore-flickering of a transiently fusing single SV [154]. Although compelling, this is difficult to prove experimentally: SVs of cochlear IHCs are small in size, measuring ~ 30–50 nm in diameter, depending on the fixation method [7,140,154], which equates to approximately 45 aF in membrane capacitance [157]. Monitoring the flickering of such small SVs requires cell-attached patch-clamp recordings, amperometry or *in vivo* fluorescent imaging of single SVs, which are all technically extremely challenging methods – in particular when applied within a tightly-structured and dense tissue such as the organ of Corti. Future use of these approaches as well as flash-and-freeze EM should assist in providing answers to some of the above-mentioned critical questions.

Molecular components of the endocytic machinery operating at IHC AZs

Whereas the mechanisms underlying exocytosis at IHC ribbon synapses have been studied extensively in recent decades, the processes determining regulated endocytosis and membrane recycling are far from clear, mainly due to the fact that experimental analysis of endocytic events at this synapse is technically highly challenging. For example, FM dyes [149,158,159] and imaging of

pHluorin-expressing SVs [140] have limited usability at the IHC ribbon synapse, capacitance recordings required for endocytic measurements are difficult to achieve due to the required long-term stability and have a limited resolution, etc.

Moreover, CME operates *via* the coordinated and sequential interplay of more than 50 distinct proteins, which makes the individual contribution of a given protein to the process particularly difficult to identify. In addition, developmental compensation and functional redundancy within this network can render experimental outcomes difficult to interpret at times. Several of the 'classical' endocytosis mutants (e.g., *endophilin* triple knock-out, *synaptojanin 1* knock-out etc.) are not viable, thus only very few of them could be analyzed with regard to their hearing or vestibular phenotypes to date. In agreement with a functional role for CME in IHC endocytosis, immunohistochemical analysis revealed the expression of several endocytic proteins in IHCs of hearing mice [140], including (i) the scaffold protein clathrin, (ii) the scission-related proteins dynamin (a large GTPase), and (iii) amphiphysin, which recruits dynamin to sites of CME (for review see Refs [141,160]).

Studies from zebrafish mutants indicate that synaptojanin 1, a lipid phosphatase, is required for the maintenance of SV pools and proper SV recycling in zebrafish neuromast hair cells, especially during sustained stimulation, which might activate bulk endocytosis [161]. Synaptojanin 1 regulates the turnover of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) [162] – the main binding partner of endocytic adaptor proteins [163] – and thus enables the shedding of the clathrin coat from internalized endocytic vesicles [164]. Synaptojanin 1 is recruited by endophilin [165,166] and the latter seems also to be relevant to endocytosis in mammalian IHCs (J. Kroll, L.M.J. Tobón, C. Vogl, J. Neef, I. Kondratiuk, M. König, N. Strenzke, C. Wichmann, I. Milosevic & T. Moser, unpublished observations). In neurons, both, amphiphysin and endophilins, mediate the constriction of the invagination neck and recruitment of dynamin to the site of scission, which is finally governed by all three proteins (for review see Ref. [141]). Recently, synaptojanin 1 together with endophilin A has additionally been suggested to be required for the rapid constriction of the neck of endocytic pits during ultrafast endocytosis [167]. Constitutive deletion of synaptojanin 1 in mice is lethal, thereby hampering studies of the role of this protein in mammalian IHCs.

Hearing is moderately perturbed in *fitful* mutant mice, which carry a missense mutation in the region of dynamin 1 that is responsible for its oligomerization and thus proper vesicle scission [168]. In IHCs, the *fitful* mutation significantly reduces the linear – presumably clathrin-mediated – component of endocytosis [140]. Unlike complete deletion of dynamin 1 in cultured cortical neurons [169], the *fitful* mutation does not result in massive endocytic vesicle arborizations (i.e., accumulation of interconnected clathrin-coated buds) in IHCs [140]. Hence, it is likely that other dynamin isoforms (potentially dynamins 2 and 3) or other proteins may be involved in pinching off endocytic structures from the plasma membrane and compensate impaired dynamin 1 function.

Finally, the heterotetrameric clathrin-adaptor protein complex AP-2 has also been detected in IHCs [135,170], where it may be responsible for lateral clearance of synaptic proteins from the AZ and clathrin-dependent SV reformation ([135]; see next chapter).

Exocytosis–endocytosis coupling at IHC ribbon synapses

The amount of membrane retrieval must match the extent of exocytosed membrane to keep the plasma membrane area constant in size and avoid cell swelling during ongoing SV fusion. Hence, compensatory endocytosis must follow exocytosis in a timely and highly efficient manner to clear vesicular release sites of exocytosed material and prevent larger presynaptic structural changes that may inhibit continuous exocytosis. This may be a particularly challenging task at the high-throughput ribbon synapses of auditory IHCs. How this is achieved is one of the crucial unresolved questions at synapses in general. It has been proposed that the presynaptic Ca^{2+} signal that triggers exocytosis may also control the timing of compensatory endocytosis, as increased $[Ca^{2+}]$ accelerates endocytosis [171–174]. The identity of the Ca^{2+} sensor for endocytosis is not entirely clear. At neuronal synapses, this role may be fulfilled by calmodulin ([172], but a rather modulatory role in endocytosis has been reported as well [175]). One of the calmodulin targets is calcineurin, which may activate endocytosis by dephosphorylating a number of endocytic proteins [176–178]. However, the involvement of calcineurin in the activation of endocytosis remains controversial [179–182]. Calmodulin may further regulate the activity of the bin-amphiphysin-rvs domains in endocytic accessory proteins (including endophilins and amphiphysins; [183]), which promote membrane curvature before the final constriction and scission (for review see Refs [141,160]). Moreover, a role for synaptotagmins in triggering endocytosis has also been implied from studies on human fibroblasts, the fly neuromuscular

junction, and mouse cortical neurons [184–187]; however, synaptotagmins 1 and 2 are absent from mature IHCs [24,25]. Whereas, the kinetics of endocytosis are influenced by the amplitude of the intracellular [Ca^{2+}] increase, the amplitude of endocytosis seems independent of the levels of intracellular [Ca^{2+}] [173], suggesting that other mechanisms must be involved in controlling the amount of compensatory endocytosis. Furthermore, the Ca^{2+} signal does not activate endocytosis in the absence of preceding exocytosis [188,189], implying that this requires additional – so far unidentified – factors. In this context, recycling exocytic proteins or membrane lipids, such as PIP_2, which are required for both, exo- and endocytosis, may regulate the amount of endocytosis (for recent reviews see Refs [190,191]). After SV fusion, exocytic (vesicular) proteins in the plasma membrane may attract endocytic adaptor proteins and thus initiate assembly of the clathrin coat components. Alternatively, by binding endocytic proteins, they may activate or simply bring them closer to retrieval sites. Moreover, some exocytic proteins with their 'curved shape' might even facilitate membrane bending [150]. How exactly any of these mechanisms contribute to regulating exo–endocytosis coupling at IHC ribbon synapses and which exocytic proteins may be involved remains to be addressed in the future. Neuronal SNAREs may fulfil the task of binding and attracting endocytic components to the membrane to be retrieved at conventional synapses [171,192–195], but their apparent absence from IHC ribbon synapses [22] suggests the involvement of different proteins in this process here.

Otoferlin, as a central exocytic protein at IHC AZs, is an obvious candidate for regulating endocytosis and/or exo–endocytosis coupling. This large multi-C_2-domain protein may harbor binding sites for several endocytic proteins and has previously been shown to directly interact with the endocytic adaptor protein AP-2 at multiple sites [135,170]. AP-2 complexes bind $PI(4,5)P_2$, protein cargo and clathrin, and thus are likely involved in the initiation of vesicle budding from the plasma membrane or endosomal vacuoles (for review see for example [141]). Recent data from conventional synapses suggests that endocytic membrane retrieval *per se* does not absolutely require AP-2 or clathrin, which however are both critically required for SV reformation from endosome-like vacuoles [196]. In line with results obtained in AP-2μ-deficient hippocampal neurons, disruption of AP-2 does not significantly perturb membrane retrieval at IHC ribbon synapses [135]. Upon strong stimulation, loss of AP-2 leads to the accumulation of endosome-like vacuoles, which – together with fewer clathrin-coated pits as well as reduced counts of ribbon-attached SVs (i.e., at the distal part of the ribbon) – indicates a requirement of AP-2 for SV reformation. Intriguingly, upon prolonged stimulation, the number of membrane-proximal SVs is unaltered; however, the speed of exocytosis decreases significantly in the mutants, suggesting impaired release site clearance that prevents the membrane-proximal SVs to reach fusion competence [135]. Otoferlin levels in AP-2-deficient IHCs are dramatically reduced and there is an indication that the remaining otoferlin might be more strongly present on the plasma membrane. Thus, it has been suggested that AP-2 acts as a sorting factor for otoferlin, mediating assembly and lateral clearance of used otoferlin from release sites. Thereby, AP-2 enables re-occupation of these sites with 'fresh' release-competent SVs [135]. Yet, a potential active role of otoferlin in AP-2 recruitment to the AZ needs to be further addressed. However, since perturbations of otoferlin structure usually lead to relatively strong impairment of exocytosis [59,99,126,128], this latter point remains difficult to address. In IHCs of *otoferlin* mutants that show partially preserved RRP exocytosis *in vitro*, the kinetics of endocytosis (membrane retrieval) upon cell depolarization or Ca^{2+} uncaging seems remarkably unaffected [59,140]. Thus, there is so far no evidence for otoferlin involvement in endocytic membrane retrieval, but further work with novel *otoferlin* mutations is required to fully address this question.

Perspectives

In the last decades, we have learned a lot about the SV cycle at IHC ribbon synapses; however, some critical basic knowledge is still missing. For example, what is the exact role of the ribbon in IHC exocytosis? Answering this question will require acute ribbon ablation in an otherwise completely undisturbed system. Which SNAREs – if any – are expressed and facilitate SV fusion at these synapses? Here, inner ear gene expression databases, such as the Shared Harvard Inner-Ear Laboratory Database (https://shield.hms.harvard.edu) [197], have been made publicly available in recent years and offer a wealth of gene expression data that currently await in-depth functional and morphological analyses. Hence, such data repositories should be used as guidelines for future projects aiming to identify key molecules in IHC exo–endocytosis coupling, membrane retrieval, and SV reformation. Moreover, it will be of great interest to elucidate the role of

otoferlin in these processes and determine which of its functional domains as well as molecular interactors may be involved in this framework.

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