Otoferlin: a multi-C$_2$ domain protein essential for hearing

T. Pangršič$^{1,*}$, E. Reisinger$^2$* & T. Moser$^{1,3,4,#}$

$^1$InnerEarLab, Department of Otolaryngology and Collaborative Research Center 889, University Medical Center Göttingen, Göttingen, Germany

$^2$Molecular Biology of Cochlear Neurotransmission group, Dept. of Otolaryngology and Collaborative Research Center 889, University Medical Center Göttingen, Göttingen, Germany

$^3$Bernstein Center for Computational Neuroscience, University of Göttingen, Göttingen, Germany

$^4$Center for Molecular Physiology of the Brain, University of Göttingen, Göttingen, Germany

* equal contribution

# corresponding author

Key words: hair cell, calcium, priming, fusion, synaptic vesicle, ferlins

Correspondence:

Tobias Moser

InnerEarLab, Dept. of Otolaryngology and Collaborative Research Center 889, University of Göttingen Medical Center, 37099 Göttingen, Germany

Tel.: 49-551-39-8968

Fax.: 49-551-3912950, tmoser@gwdg.de
Abstract

Sound is encoded at synapses between cochlear inner hair cells and the auditory nerve. These synapses are anatomically and functionally specialized to transmit acoustic information with high fidelity likely over a lifetime. The molecular mechanisms of hair cell transmitter release have recently attracted substantial interest. Here we review progress toward understanding otoferlin, a multi-C\(_2\) domain protein identified a decade ago by genetic analysis of human deafness. Otoferlin functions in hair cell exocytosis. Several otoferlin C\(_2\) domains bind to Ca\(^{2+}\), phospholipids, and proteins. Current research reveals requirements for otoferlin in priming and fusion of synaptic vesicles during sound encoding. Understanding the molecular mechanisms through which otoferlin functions also has important implications for understanding disease mechanisms that lead to deafness.

Introduction

The inner hair cells (IHCs) of the mammalian inner ear transduce mechanical stimuli into electrical signals and transmit them to auditory neurons via ribbon-type synapses. Physiologically, the IHC synapse features submillisecond precision and unprecedented high rates of release and replenishment of vesicles. Its morphological feature is the synaptic ribbon, a nanoscale proteinaceous complex tethering a halo of synaptic vesicles close to the active zone (AZ; see Glossary). Except for the presence of the ribbon and the use of L-type Ca\(^{2+}\)-channels for stimulus-secretion coupling, IHCs were thought to employ the same set of synaptic proteins as glutamatergic synapses of the central
nervous system (CNS; Box 1). However, already in 1999 immunohistochemistry and RT-PCR reported that synaptotagmin 1-3, synaptophysin and synapsin are absent from this synapse [1]. Subsequently, physiological and immunohistochemical studies indicated that it also lacks complexins [2,3] and seems to operate without neuronal soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins [4]. Instead, IHCs use other proteins such as the C2-domain protein otoferlin for exocytosis. We expect that future studies will further demonstrate that this synapse does not use the same molecules thought to be mandatory for docking, priming and fusion of vesicles in brain synapses. Such molecular differences allow for studies of disorders of the auditory synapse in an otherwise unaffected mouse model or in human patients. To date, mutations in otoferlin, the L-type voltage-dependent Ca\(^{2+}\)-channel Ca\(_{v}\)1.3 and the vesicular glutamate transporter 3 (Vglut3) have been identified to cause human deafness [5–7] as a consequence of defective synaptic transmission at the hair cell synapse [6,8–12]. These hearing disorders can therefore be referred to as “synaptopathies” [13–15].

Otoferlin, similarly to many presynaptic proteins like synaptotagmins (Syts), Munc13s and Rab interacting molecules (RIMs), contains several C\(_2\) domains (Fig. 1). It belongs to the family of ferlin proteins, which includes dysferlin and myoferlin of vertebrate myocytes, Fer-1 of sperm in invertebrates, and misfire in *Drosophila*. Ferlins have been generally implicated in membrane-membrane fusion events (e.g. [16]), but compared to other C\(_2\)-domain proteins involved in synaptic vesicle exocytosis, we have just begun to understand their precise functions.
**Otoferlin is required for synaptic transmission at the IHC ribbon synapse**

The analysis of otoferlin knockout mice gave first insights into the function of otoferlin [8]. Otoferlin-deficient IHCs display a severe reduction in Ca$^{2+}$-dependent exocytosis with normal numbers of ribbon-associated and docked vesicles [8] (Fig. 2, Box 2). Therefore, it was concluded that otoferlin is essential for a late step of exocytosis (e.g. vesicle priming and/or fusion [8]). Subsequently, otoferlin was shown to be required for synaptic exocytosis of immature outer hair cells and type I vestibular hair cells [17,18].

The function of the vestibular system was partially impaired when assessed by vestibular evoked potentials [18], while no gross vestibular deficit was detected in behavioral assays [8]. This is in line with the less severe exocytosis phenotype found in otoferlin-deficient vestibular hair cells [18].

In contrast to otoferlin deletion, abrogation of similar CNS synaptic proteins like Munc13-1 or Syt1 is lethal. The disruption of the priming factor Munc13-1 leads to an almost complete block of evoked and spontaneous transmitter release at most neuronal synapses [19,20]. Similarly, deletion of the Ca$^{2+}$-sensor protein Syt1 abolishes the synchronous component of evoked response [21], while the effects on asynchronous and spontaneous transmitter release vary among preparations [21–25]. How does this compare to the observed effects in IHCs lacking otoferlin? These cells show no detectable synchronous exocytosis [8,9] but slowly release transmitter regardless of stimulation [9]. Similarly to Syt, otoferlin is mainly found on synaptic vesicles but also on the plasma membrane [8,9] (Fig. 2b).
**Current hypotheses of otoferlin function in exocytosis**

The observed association between otoferlin and synaptic vesicles, the structural and biochemical similarities with Syt1, and the impairment of exocytosis despite abundant vesicles at the AZ, led to the hypothesis that otoferlin functions as a Syt1-like Ca\(^{2+}\)-sensor for fusion in IHCs [8]. A subsequent investigation of an Otof missense mutation indicated a role for otoferlin in vesicle replenishment and suggested priming as another site of action [9].

*Otoferlin: a Ca\(^{2+}\)-sensor for synaptic vesicle fusion?*

The direct interaction of otoferlin with the neuronal SNAREs, Syntaxin 1 and SNAP25, [8,26,27] supported the hypothesis that otoferlin may act as a Syt1-like Ca\(^{2+}\)-sensor for fusion. A reconstituted SNARE-mediated membrane fusion assay showed that, similar to Syt1, individual C\(_2\) domains of otoferlin (except C\(_2\)A) and also otoferlin fragments containing 3 consecutive C\(_2\) domains could stimulate SNARE-mediated liposome fusion in a Ca\(^{2+}\)-dependent manner [27]. An interaction of otoferlin and neuronal SNAREs in the organ of Corti was indicated [8] and the presence of SNAREs in hair cells was reported based on immunostaining and Western blotting (e.g. [1,3]). However, a recent study cast doubt on the physiological relevance of these interactions by indicating that neuronal SNAREs are not required for exocytosis in IHCs [4]. Obviously, otoferlin could still interact with as yet unidentified SNAREs or SNARE-like proteins mediating exocytosis in IHCs.
Ca\(^{2+}\)- and phospholipid binding by several of the C\(_2\) domains [8,26,27] further supports the Ca\(^{2+}\)-sensor hypothesis, though, the biochemical properties of these C\(_2\) domains are still under debate. Predicting Ca\(^{2+}\)- and phospholipid binding sites is challenging as otoferlin and non-ferlin C\(_2\) domains share only low sequence similarity (e.g. 31% amino acid identity between PKC\(\alpha\) C\(_2\) and otoferlin C\(_2\)D is one of the highest). Further, except for the C\(_2\)A domain, providing the first crystal structure of an otoferlin C\(_2\) domain [28] (Fig. 1), purification of otoferlin C\(_2\) domains expressed in E. coli is difficult. In some studies, protein aggregates were dissolved under denaturing conditions and proper protein folding after re-naturation was not ascertained [8,26]. Moreover, uncertainty about the extent of the C\(_2\) domains led to the design of different constructs for expression [9,26,27], possibly affecting the results of binding assays. In a recent comprehensive study on recombinant otoferlin C\(_2\) domains, the binding of Ca\(^{2+}\) was indicated for all C\(_2\) domains with the exception of C\(_2\)A [27]. This is consistent with other results on C\(_2\)A [28], C\(_2\)D [8], and C\(_2\)F domains [26], while a lack of Ca\(^{2+}\)-binding was reported for C\(_2\)F in another study [9]. Similarly, phospholipid binding has been indicated for all otoferlin C\(_2\) domains in [27], but not for C\(_2\)A in [28] and C\(_2\)F in [9]. Future studies solving the structure of the otoferlin C\(_2\) domains will ultimately reveal their borders and help resolve controversy about Ca\(^{2+}\)- and phospholipid binding sites.

The Ca\(^{2+}\)-sensor hypothesis has also been addressed in a study expressing Syt1 in otoferlin-deficient mouse IHCs [29]. Despite high viral transduction rates and proper Syt1 targeting to synaptic vesicles, hearing and IHC exocytosis (Box 2) could not be restored [29]. Further, otoferlin expressed in Syt1-deficient neuroendocrine cells and
hippocampal neurons failed to restore synchronous exocytosis. In the latter, a small increase in asynchronous response was found, which may be related to the slightly larger miniature excitatory postsynaptic potentials (EPSCs), potentially reflecting a subtle increase in the number of postsynaptic AMPA receptors. Together, the results argued against a simple functional equivalence of these structurally and biochemically related proteins. However, potential pitfalls need to be considered. First, otoferlin may not have been sufficiently targeted to the presynaptic terminals of hippocampal neurons. Second, the fusion with eGFP might have impaired its function. Third, failure of cross-rescue may have resulted from a lack of the relevant interaction partners of otoferlin and Syt1 in the respective cells.

Perhaps the strongest argument for the Ca\(^{2+}\)-sensor hypothesis comes from the observation that otoferlin-knockout IHCs never show synchronous exocytosis, not even after a long period of exocytic arrest such as long hyperpolarization [8,9]. Specifically, neither short Ca\(^{2+}\)-influx typically used to discharge the readily releasable pool of vesicles (RRP; Fig. 2a, Box 2) nor Ca\(^{2+}\)-uncaging (Fig. 2c) elicited fast exocytic responses, although small but significant exocytosis could be documented by capacitance changes during long depolarizations of IHCs and by postsynaptic recordings in the absence and presence of IHC stimulation. Therefore, although otoferlin is critical for vesicle replenishment (see below), impaired vesicle replenishment is unlikely to account for the complete lack of RRP exocytosis in otoferlin-deficient IHCs. Instead, together with the above-mentioned evidence, this finding supports a role of otoferlin in vesicle fusion. Future studies combining mutagenesis directed towards Ca\(^{2+}\)-binding sites in otoferlin C\(_2\)
domains - ideally sparing vesicle replenishment - together with flash photolysis of caged Ca$^{2+}$ (to probe for changes in the intrinsic Ca$^{2+}$-dependence of fusion, Box 2) will be required to ultimately test the Ca$^{2+}$-sensor of fusion hypothesis for otoferlin.

*Otoferlin: a priming factor enabling fast vesicle replenishment?*

A function of otoferlin in IHC synaptic vesicle priming was uncovered when analyzing the deaf *pachanga* mouse mutant [9] that carries a missense mutation in the C$_2$F domain of otoferlin elicited by random mutagenesis [30]. Mutant IHCs displayed normal synaptic Ca$^{2+}$-influx and exocytosis of the RRP (Fig. 2a), a small but fast exocytic response to Ca$^{2+}$-uncaging (Fig. 2c) and an unaltered intrinsic Ca$^{2+}$-dependence of exocytosis [9], supporting the view that this mutation does not impair the mechanism of Ca$^{2+}$-triggered fusion. However, a marked reduction of sustained exocytosis (Fig. 2a) and a slowed recovery of the RRP from depletion indicated an impaired vesicle replenishment of the release sites. This, together with an unchanged anatomical estimate of the synaptic vesicle complement, and normal endocytic membrane retrieval, suggested that otoferlin is involved in vesicle priming [9]. However, as these authors noted, a function of otoferlin in clearing exocytic material from the release site towards the perisynaptic sites of endocytosis [31] could not be excluded. Indeed, a role in coupling exocytosis to endocytosis has been suggested for of Syt1 [32–34] and, hence, potentially, another interesting parallel between otoferlin and Syt1 might exist. Vesicle replenishment in hair cells is Ca$^{2+}$-dependent [35–39] and it is tempting to speculate that otoferlin is a key effector of this process.
The finding of normal RRP exocytosis in vitro (Fig. 2a, Fig. 3a) seemed hard to reconcile with the profound hearing impairment in pachanga mice in vivo [9]. However, in vivo recordings from single auditory nerve fibers and brainstem neurons showed that lowering stimulus rates improved spiking at sound onset in pachanga mice [9] (Fig. 3b). It was then hypothesized that, in vivo, with given evoked and spontaneous vesicle release the impaired vesicle replenishment does not support the maintenance of a sufficient standing RRP (i.e. the AZ is chronically depleted of primed, release-ready vesicles or perhaps of reusable synaptic machinery; Fig. 3c). Longer interstimulus intervals partially restore a standing RRP (Fig. 3a), enabling some as yet limited neuronal spiking upon presentation of sounds. In contrast, during in vitro experiments, cells are kept at hyperpolarized potentials in-between stimuli, so that “spontaneous” transmitter release is blocked (see [40]). As a consequence, even the reduced rate of vesicle replenishment can establish a normal-sized RRP for exocytosis in IHCs of pachanga mice upon stimulation at the low rates of repetition (0.01-0.03 Hz) used in [9]. Figure 3 illustrates this hypothesis with the product of standing RRP and release probability defining the transmitter release (Fig. 3a) and ensuing spike rate (Fig. 3b).

Why might cochlear IHCs require otoferlin as a special priming factor? The answer may be related to the demands of synaptic encoding of sound, where each spiral ganglion neuron (SGN) can spike at hundreds of Hz during ongoing acoustic stimulation. Different from a bipolar cell of the retina or a vestibular ganglion neuron, each SGN is driven by a single IHC AZ [41], reviewed in [42]). Moreover, SGNs seem to only spike once for each presynaptic release event [43]. Therefore, in response to ongoing acoustic
stimulation, the SGN spike rate is probably limited by the rate of vesicle replenishment to the AZ. Assuming an adapted spike rate of 200 Hz, and one or more vesicles per release event, the vesicle replenishment rate must be 200 Hz and likely greater. If the average release event involves four [44] or seven [40] vesicles, then a replenishment of at least 800-1400 vesicle-quanta would be required per second per AZ. IHCs in vitro can sustain exocytic rates of 700 synaptic vesicles per second per AZ for at least 100 ms of strong stimulation [9]. Assuming approximately 10 release sites per AZ, this indicates that each ‘RRP slot’ can be replenished 70 times per second. These rates surpass estimates in other synapses that lack otoferlin. For example, the IHC vesicle supply rate exceeds that of rat bipolar cells by approximately an order of magnitude, despite comparable synaptic structure and only slightly different RRP size (70 vesicles/s/AZ or 7 vesicles/s/RRP slot [45]). On the other hand, vestibular hair cells express otoferlin too, but show lower replenishment rates, which are less strongly affected by disruption of otoferlin [18].

The search for interaction partners of otoferlin is an active topic of research. In addition to the above mentioned SNAREs, the unconventional Myosin VI [46,47], the small G-protein Rab8b [48], Ergic2 [49] and the CaV1.3 channel [26] were described to interact with otoferlin. However, three of the studies [47–49] used a yeast-two-hybrid bait covering half of the C2D domain (amino acids 904 to 1025 while C2D extends from 962 to 1095), which might reveal unspecific protein interactions due to misfolding of the incomplete domain. Further functional studies to demonstrate the relevance of these protein interactions would therefore be desirable.
How can the priming and Ca\textsuperscript{2+}-sensing functions (Fig. 3c, d) be selectively affected by different mutations of the otoferlin gene? We consider two possible scenarios. While no significant biochemical difference between the mutant and the wild type C\textsubscript{2}F domain has been described so far [9], the \textit{pachanga} mutation might alter binding site(s) for interaction(s) involved in priming but leave the molecular events leading to fusion intact. Alternatively, priming and fusion may require different amounts of proteins. For example, 30% of the normal levels of otoferlin, as detected in \textit{pachanga} IHCs, might suffice for intact fusion but not for proper priming. On the other hand, in the complete absence of otoferlin, fusion events might be so rare that priming is no longer the rate-limiting step, although it, too, is affected by the lack of otoferlin (see Fig. 3c). However, the dependence of priming and fusion on otoferlin levels has yet to be established. It is likely that the \textit{pachanga} mutation renders the protein product less stable, consequently only very little protein is detected by immunofluorescence (Fig. 2b). Interestingly, cross-breeding of \textit{pachanga} with knockout mice resulted in a further reduction of otoferlin protein levels in IHCs, leading to even less sustained exocytosis [9]. Human missense mutations in \textit{OTOF}, including the mutations that confer enhanced temperature-sensitivity to otoferlin (see below), might also act by destabilizing the protein. Research on mouse models and \textit{in vitro} biochemical studies of otoferlin and exocytosis at the IHC synapse will help to understand clinical findings described in patients with mutations in otoferlin.
Potential molecular mechanisms involving otoferlin

Compared to the putative Ca\textsuperscript{2+}-sensor of fusion (Syt1) and the putative priming factor (Munc13-1) acting at conventional synapses, little is known about the structure and function of otoferlin. Moreover, direct comparison is hampered by the unconventional exocytic machinery found in hair cells. Nevertheless, we provide some speculations on potential molecular mechanisms of otoferlin function: Considering vesicle replenishment and fusion, possible molecular mechanisms include (i) tethering of opposing membranes, potentially involving otoferlin oligomerization, (ii) promotion of the assembly of trans-SNARE complexes, (iii) Ca\textsuperscript{2+}-dependent approximation of vesicular and plasma membranes, (iv) interaction with other proteins mediating Ca\textsuperscript{2+}-triggered fusion, and (v) interaction with endocytic proteins to facilitate the clearance of the release site. Otoferlin’s potential involvement in tethering synaptic vesicles to the plasma membrane is an attractive hypothesis for its function in vesicle replenishment. Indeed, vesicles at IHC synapses are tethered to the synaptic ribbon and/or to the plasma membrane (e.g. [50]), but the molecular identity of the tethers remains unknown. Ca\textsuperscript{2+}-dependent phospholipid binding has been indicated for several otoferlin C\textsubscript{2} domains [26,27]. Therefore, it seems conceivable that otoferlin inserts into the cytosolic leaflet of the plasma membrane, acting as a cone or wedge that promotes membrane bending and brings the opposing membranes in closer apposition, as has been suggested for Munc13 C\textsubscript{2}B [51] and Syt C\textsubscript{2} domains [52,53]; reviewed in [54–57]). Alternatively, in analogy to predictions for Syt1, otoferlin might act to stabilize the yet unidentified SNARE complex (as reported using liposome fusion assays [27]) or help to
reduce electrical repulsion between the opposing membranes by way of the positive charges of bound Ca\(^{2+}\)-ions. Finally, the coiled-coil domain of otoferlin might form a superhelix either with itself or with other proteins, and the “zippering-together” of the helices could help to overcome the energy barrier for fusion of the two membranes.

**Human mutations and clinical findings**

Pathogenic mutations in *OTOF* underlie the nonsyndromic autosomal recessive deafness DFNB9 [5] and together with the non-pathogenic mutations [58–65] provide some indication for which protein domains are important for otoferlin function (Fig. 4). Non-pathogenic sequence variants are mostly found in the C\(_2\)A domain or in the linker regions between C\(_2\) domains. So far, more than 60 pathogenic mutations have been reported. Among those are 15 pathogenic missense mutations of which 10 are found in C\(_2\) domains C-F (Fig. 4, see also [63]). In addition to disabling the function of the protein at the synapse, missense mutations might lead to enhanced protein degradation, as reported for the deaf5 [47,66] and pachanga [9] mutations in mice.

The hearing impairment varies among subjects with different mutations in *OTOF*. In most patients, auditory brainstem responses are absent or highly abnormal despite the presence of otoacoustic emissions and/or cochlear microphonics [60–62,64,65,67] as also found in auditory neuropathy [68]. Together with the results of animal research this indicates impaired SGN activation by IHC transmitter release (synaptopathy) despite intact mechanoelectrical transduction and cochlear amplification [67]. Otoacoustic emissions can also disappear [60], presumably due to subsequent loss of outer hair cells.
Most pathogenic \textit{OTOF} mutations lead to profound prelingual deafness. The predominant one, the so-called “Spanish mutation” (Q829X), causes a premature stop codon and was found in 8% of the congenital non-syndromic deaf population with autosomal recessive inheritance of Spanish descent [58,63]. Missense mutations (Fig. 4) produce heterogeneous phenotypes. Some cause profound prelingual deafness, as do premature stop codons or frameshift-inducing deletions or insertions [63]. Others, like the L1011P mutation [69], leave residual high-frequency hearing, which suggests partial functionality of the mutated protein.

For some hearing-impaired individuals, their phenotypes are exacerbated at elevated body temperature. In these cases, an in-frame deletion or missense mutations in \textit{OTOF} have been identified and proposed to mediate the temperature-dependent hearing loss. To date, four such point mutations have been described which affect different sites of the protein (violet in Fig. 4), indicating a general mechanism like protein destabilization and subsequent degradation leading to a reduction in protein levels. Interestingly, when afebrile the individuals with the I515T, the G541S and the E1804del mutations have almost normal hearing thresholds in pure tone audiograms albeit with impaired speech recognition particularly in background noise [62,65,70]. In contrast, at 38.1°C they are profoundly deaf for low frequencies, severely hearing impaired for high frequencies, and they reported tinnitus. Case studies such as these have provided launch pads for the investigation of specific neurobiological mechanisms of hearing and deafness in the past decade.
Concluding remarks and future directions

Interdisciplinary studies over the past decade have characterized otoferlin as a hair cell specific protein essential for synaptic sound encoding. Following the discovery of OTOF as a deafness gene, its expression, clinical genotype-phenotype relationship, subcellular localization, protein biochemistry and structure, functional role in synaptic transmission, and disease mechanism(s) have been addressed. Otoferlin has become an important bait for fishing other members of the unconventional hair cell release machinery, but this search has just begun to yield results.

Recent studies have revealed the crystal structure of its C2A domain and demonstrated Ca\(^{2+}\)- and phospholipid binding of several other C\(_2\) domains. However, the precise topology of Ca\(^{2+}\)-binding to the structure of full-length otoferlin and the functional consequences are not clear (see Box 3). In vitro work in knockout mice and point mutants has played a major role in unraveling the function(s) of otoferlin. So far, it has helped to pinpoint the function of otoferlin to hair cell exocytosis. Hypothetical roles in Ca\(^{2+}\)-dependent vesicle priming and vesicle fusion are not mutually exclusive and will guide future morphological and physiological analyses. Mouse models with missense mutations in otoferlin will likely provide insights into disease mechanisms of moderate hearing phenotypes, including temperature-sensitive hearing impairment. The defective vesicle replenishment in IHCs of pachanga mice, which is possibly related to the reduced levels of otoferlin protein, could well be a common denominator of weaker otoferlin-related human hearing impairments. Establishing efficient gene transfer into IHCs will
enable detailed structure-function analysis in the otoferlin knockout background and also lay the foundation for future gene replacement therapy.

Acknowledgment

We would like to thank M. Rutherford, C. Dean, R. Jahn and S. Hallermann for critical reading of the manuscript. We thank Linda Hsu for preparing the artwork. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) through the Collaborative Research Center 889 “Cellular Mechanisms of Sensory Processing” (to T. M. and E. R.).
**Figure 1: Structure and function of various C2-domain proteins**

Schematic diagrams of the protein structure of a selection of ferlins (dysferlin, myoferlin and otoferlin) and other C2-domain proteins involved in synaptic exocytosis (synaptotagmin, Munc13, RIM; for reviews see [55,71,72]), along with their putative functions, are illustrated. Among the C2-domain proteins, ferlins possess the highest number of C2 domains known (up to seven). In addition, they contain a DysF and/or Fer domain (60-70 residue conserved motif), both specific for this family of proteins.

Proteins involved in membrane fusion events are often membrane anchored proteins and contain either an amphiphatic α-helix, which forms a superhelix with α-helices of interaction partners (as the SNARE motif in the SNARE complex), or domains which bind to phospholipid membranes in a Ca^{2+}-dependent manner. Otoferlin contains both types of domains: in addition to six C2 domains known for Ca^{2+} and phospholipid binding (ie. C2A-F), a coiled-coil domain (CC) is present in one region otherwise predicted to be unstructured. This CC domain might work in a similar manner as the SNARE motif, although the other players forming a superhelix like in the SNARE complex still await
identification. In otoferlin, myoferlin and dysferlin, a seventh C₂ domain is predicted to fold between the C₂D and the C₂E domains (ie. C₂de [73]). However, the sequence similarity between these ferlin C₂de domains and any other C₂ domain is much lower than the similarity amongst other ferlin C₂ domains. The tertiary structure of the C₂A domain (PDB ID: 3L9B, upper right; reproduced, with permission, from [28]) is shown. The crystal structure of otoferlin C₂A shows a similar folding as the Munc-13 C₂B domain, but has a shorter top loop [28]. Abbreviations: DUF, domain of unknown function; DysF, dysferlin domain; Fer, Ferlin-specific motif; MHD, Munc13 homology domain; PDZ, postsynaptic density-95/Discs large/Zona occludens-1 domain; ;; TM, transmembrane domain.

Figure 2: Exocytosis and otoferlin expression in IHCs of wild-type mice and otoferlin mutant mice

The schematic diagrams (insets to parts a and c) depict an IHC. Between 5 and 20 ribbon-type active zones are typically seen, although only 3 are depicted here.
Exocytosis was evoked by depolarizations of varying duration (in the case of part a) or by flash photolysis of caged Ca\(^{2+}\) (part b, for a fast and global elevation of cytosolic [Ca\(^{2+}\)]) measured as membrane capacitance increase (ΔC\(_m\)).

**a)** Two components of ΔC\(_m\) in response to depolarization of IHCs in wild-type mice (*black line*): readily-releasable pool (RRP; *gray line*) and the sustained component (*black dashed line*). While RRP exocytosis is preserved, the sustained component of release is strongly reduced in IHCs of *pachanga* mice (*C\(_2\)F missense mutants, *green line*). In otoferlin knockout (KO) mice, exocytosis is almost absent (*violet*) [9].

**b)** Immunofluorescence of otoferlin (otof: *green*) and vesicular glutamate transporter 3 (Vglut3: *violet*) expressed in IHCs of wild-type (*top*), *pachanga* (*middle*) and otoferlin KO mice (*bottom*). *Pachanga* IHCs show lower expression levels of otoferlin than the wild-type animals [9]. In the wild-type IHCs the immunofluorescence signal for otoferlin and Vglut3 largely overlap, while otoferlin shows a stronger staining than Vglut3 on the plasma membrane. Scale bar: 5 µm.

**c)** Upon Ca\(^{2+}\)-uncaging, a large pool of vesicles is released within a few ms in wild-type IHCs (*black*). In IHCs of *pachanga* mice (*green line*), this fast component is strongly reduced (*arrow*); however, its time constant is comparable to the fast component of the wild-type response [9]. The strong reduction in amplitude is currently not understood. It might reflect a reduction in the total number of synaptic vesicles or experimental conditions that do not allow sufficient priming in the mutant IHCs. In otoferlin-KO mice, only some residual slow release can be observed (*violet*) [9]. Adapted, with permission, from [9].
Figure 3: Proposed roles of otoferlin in IHC vesicle priming and fusion.

a) Schematic drawing of the rate of exocytosis upon depolarization in IHCs of wild-type (black) and pachanga (green) mice roughly to scale (assuming a vesicle capacitance of 45 attofarad [74]): the rate of sustained release is strongly reduced in mutant IHCs [9].

b) The spike rate of auditory nerve fibers and bushy cells over time (post-stimulus time histogram) upon presentation of suprathreshold acoustic stimuli (gray bar) in wild-type and pachanga mice [9]. The poor sound encoding in pachanga mice was proposed to be due to slowed vesicle replenishment, which was supported by in vitro data (a) and the finding that decreasing the rate of stimulus repetition (10 versus 0.5 Hz: green versus violet trace) improved sound encoding in mutant mice. In vitro, the mutant IHCs
sufficiently replenished the RRP of vesicles because synaptic release was inhibited for several seconds between consecutive stimuli. The rate of spiking of auditory fibers is driven by the rate of transmitter release from IHCs. In order to illustrate the replenishment hypothesis we relate spike rate to the rate of transmitter release from IHCs that is determined by the size of the standing RRP and the probability of neurotransmitter release \((P_r)\). For simplicity, we neglected the effects of refractoriness and large bin-width that lead to underestimation of the actual rate of release. The standing RRP differs between the resting (Rest) and stimulated (Stim) conditions, but is always much smaller at the AZ of the mutant IHCs. c) Schematic en-face view of the AZ with synaptic vesicles and \(\text{Ca}^{2+}\)-nanodomains in wild-type, *pachanga* and otoferlin-KO IHCs in *vivo*. Normal numbers of docked vesicles were reported in both mutants. The *pachanga* mutation spares fusion but impairs the priming of synaptic vesicles [9]. The loss of otoferlin in KO mice severely affects fusion and probably priming [8,9]. Because fusion events are extremely rare in otoferlin-KO IHCs, enough time is available for vesicles to reach fusion competence despite defective priming. d) Schematic model of the proposed role of otoferlin in vesicle priming and fusion; \(\text{Ca}^{2+}\) is critical for both steps.
Figure 4: Single amino acid mutations in OTOF

Protein domain structure of otoferlin with pathogenic missense mutations and in-frame deletions (top), and sequence variants (bottom), according to [58–65,69,70,75,76]. The numbers are based on the amino acid sequence of human otoferlin. C2 domains (C2A-F) are depicted according to analysis in silico [77] or the crystal structure [28]. A seventh putative C2 domain is also depicted [73] (see Fig 1 for details). Five potentially pathogenic mutations that occurred in the heterozygous state in hearing impaired patients - and for which the mutation in the second allele has not been identified - are labeled by §. The P490Q* mutation was found in cis with I515T [59]. Mutations in violet have been associated with cases of temperature-sensitive hearing loss [62,64,65,70].

Abbreviations: CC, coiled-coil domain; FerB, Ferlin-specific motif ; TM, transmembrane domain.

Glossary:

Active zone (AZ): the site of the presynaptic plasma membrane where neurotransmitter is released opposite to the postsynaptic density.
**C₂ domains:** β-sandwich domains consisting of a pair of four-stranded β-sheets (see Fig. 1). They are found in protein kinase C, phospholipases, and in many presynaptic proteins like synaptotagmins, Munc-13s, and RIMs. C₂ domains generally bind Ca²⁺ and phospholipids and/or interact with other proteins. They are involved in a plethora of cellular functions (i.e. exocytosis, regulation of GTPase activity, modification of lipids, protein phosphorylation etc). In C₂ domains within non-ferlin proteins (such as Synaptotagmins, Munc13s etc.), Ca²⁺ is coordinated by three to five negatively-charged aspartate residues located at specific positions in top loops 1 and 3. Phospholipid binding occurs either simultaneously with that of Ca²⁺ to the top loops or independently of Ca²⁺ to the β-groove (reviewed in [78]).

**Pathogenic mutations:** mutations in the genomic DNA that grossly affect the length, stability or function of the encoded protein. Deletions or insertions of nucleotides in the coding sequence that lead to a shift in the reading frame are always considered to be pathogenic. The same is true for point mutations leading to premature stop codons and therefore to truncated, mostly nonfunctional proteins, which are termed nonsense mutations. Mutations that interfere with the splice donor or acceptor site in the pre-mRNA are also mostly pathogenic. The exchange of one amino acid by another might be deleterious for the protein function or stability, in which case those are called “missense mutations”, or they do not impair the protein and are therefore termed “sequence variants”.

23
Profound hearing loss: a very severe form of hearing impairment (hearing loss greater than 90 decibels). Patients with such hearing impairment usually do not benefit from hearing aids, but can be aided by cochlear implantation if the auditory pathway is intact.

Readily-releasable pool of vesicles (RRP): population of vesicles that can immediately fuse upon increase in intracellular [Ca^{2+}] without any further preparatory step. In most synapses these vesicles appear to be morphologically docked.

Ribbon synapse: a specialized type of synapse, where the AZ contains a presynaptic electron-dense body, the ribbon, which is composed of ribeye and other scaffold proteins. Ribbon synapses are found in vertebrate hair cells, synaptic terminals of photoreceptors, bipolar cells, pineal glands and electroreceptors. Structurally similar synapses, known as T-bar synapses, exist in Drosophila, however, such synapses lack ribeye.
Box 1: Comparison of the excitatory CNS synapse and the IHC ribbon synapse

The conventional neuronal synapse and the IHC ribbon synapse differ in their molecular anatomy and physiology (Figure I). While the AZs of neurons reside in the presynaptic terminal remote from the soma, the AZs of IHCs are part of the epithelial soma. AZs commonly contain a mesh of scaffold proteins, termed the cytomatrix of the active zone (CAZ), that tether vesicles and position $\text{Ca}^{2+}$-channels at the sites of vesicle fusion (reviewed in [79–81]). Some scaffold proteins are conserved between CNS synapses and IHC ribbon synapses. However, the CNS AZs are usually small, and on average zero to two vesicles are released by a brief stimulus (an action potential). Knowledge on several proteins involved in vesicle maturation, docking, priming and release is quite detailed. The large IHC AZ is equipped with a specialized molecular anatomy, which enables the release of dozens to hundreds of vesicles per second in response to graded (receptor) potentials. Most prominently, it contains the synaptic ribbon, primarily composed of ribeye [82,83] tethering approximately 70-200 synaptic vesicles with approximately 10-20 of them also tethered to the plasma membrane. The scaffold protein bassoon and possibly other scaffolds link the ribbon to the AZ membrane. The ribbon and/or bassoon stabilize a large complement of $\text{Ca}^{2+}$-channels and a large RRP (10-20 vesicles) and contribute to rapid vesicle replenishment [50,83,84].
Box 1 Figure I legend: Schematic diagram

illustrating some of the main anatomical and molecular differences between conventional synapses of the mammalian CNS (left) and the IHC ribbon synapse (right).

Docked vesicles are shown in yellow, vesicles tethered to the CAZ or the ribbon are shown in blue. Several synaptic proteins important for vesicle docking, priming and fusion at CNS synapses seem to be absent from the IHC ribbon synapse, which instead uses proteins that are either specific to hair cells or found in only few other cell types.

The table lists references for either reviews or first report (in mammals) and functional characterization within the CNS (preference to mouse mutants) and IHC ribbon synapses. “None” indicates that a protein is likely absent; “?” indicates lack of published data for mature IHCs (or conflicting results). Otoferlin is found on synaptic vesicles and in the plasma membrane of hair cells (top right) and functions in vesicle priming and fusion.
Box 2: Properties of exocytosis at the IHC ribbon synapse

The presynaptic function of IHCs is often studied using patch-clamp measurements of Ca^{2+}-currents and membrane capacitance (C_m) (see Fig. 2a). Depolarization opens CaV1.3 channels [10,11,119] and consequently triggers exocytosis. The ensuing increase in surface area of the IHC plasma membrane can be detected as a C_m increment (ΔC_m). Measurements of ΔC_m reveal at least two components (fast and sustained) of exocytosis upon depolarization and the corresponding transmitter release has been revealed by paired pre- and postsynaptic recordings [40]. Paired recordings from frog hair cells and connecting afferent fibers showed a good correspondence between the ΔC_m and the postsynaptic EPSC charge transfer for short and long depolarizations [120], indicating that most if not all exocytosis occurs at synapses. The fast component of the exocytic C_m increase saturates with a time constant of a few milliseconds [35]. When expressed in units of fused vesicles it agrees well with counts of membrane-proximal or AZ-tethered vesicles by electron microscopy [9,38,50,83] and has been interpreted as the exocytosis of a limited RRP tethered near the Ca^{2+} channels [35,38,50,121,122]. The rate of sound-evoked spikes in afferent fibers can be predicted from in vitro RRP fusion rates, depletion kinetics, and RRP replenishment rates [50,84,123]. This strongly suggests that the RRP mediates sound encoding. The slower component of the C_m rise largely reflects the re-supply of vesicles into the RRP and their subsequent fusion with the plasma membrane. The slow component scales in amplitude with the number of synapses [122] and predicts auditory nerve fiber spiking with the same scale factor as needed for RRP exocytosis [84]. IHC exocytosis evoked by fast and spatially homogenous elevations of
intracellular $[\text{Ca}^{2+}]$ via UV-flash photolysis of caged $\text{Ca}^{2+}$ also shows two kinetically distinct phases (Fig. 2c). However, the total flash-evoked $C_m$ increment is very large amounting to a plasma membrane surface increase of approximately 15% and the fast $C_m$ component exceeds the size of the depolarization-evoked RRP exocytosis by 100-times (see Fig. 2a and c). This discrepancy is currently not understood, but likely involves extrasynaptic fusion of vesicles during homogenous elevations of intracellular $[\text{Ca}^{2+}]$ [124,125].

**Box 3. Outstanding Questions**

- Can deafness in otoferlin mutants be rescued by expression of wild-type otoferlin in IHCs?
- How is otoferlin distributed between various membranous organelles such as the various populations of synaptic vesicles and endosomes and the plasma membrane? To answer this question, further immunogold electron microscopy and super-resolution light microscopy are needed.
- Which interacting protein(s) of otoferlin are relevant to its function in priming and fusion?
- Does otoferlin act as the $\text{Ca}^{2+}$-sensor of fusion in hair cells? How precisely does it promote fusion?
- What is the precise molecular mechanism underlying the promotion of vesicle priming by otoferlin?
- Do ferlins employ a common mechanism in mediating membrane fusion reactions?
- How do the six (or seven) C2 domains co-operate in ferlin function?
- How precisely does Ca\(^{2+}\) concentration affect ferlin function?

**References**

22 Littleton, J. T. et al. (1994) Calcium Dependence of Neurotransmitter Release and Rate of Spontaneous Vesicle Fusions Are Altered in Drosophila Synaptotagmin Mutants. PNAS. 91, 10888–10892
30 Schwander, M. et al. (2007) A forward genetics screen in mice identifies recessive deafness traits and reveals that pejvakin is essential for outer hair cell function. J. Neurosci. 27, 2163–2175


89 Mukherjee, K. et al. (2010) Piccolo and bassoon maintain synaptic vesicle clustering without directly participating in vesicle exocytosis. *Proceedings of the National Academy of Sciences*. 107, 6504
92 Gregory, F. D. et al. (2011) Harmonin inhibits presynaptic Cav1.3 Ca2+ channels in mouse inner hair cells. Nat Neurosci. 14, 1109–1111
100 Wang, Y. et al. (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature. 388, 593–598
110 Perin, M. S. et al. (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature. 345, 260–263


Brandt, A. et al. (2005) Few CaV1. 3 channels regulate the exocytosis of a synaptic vesicle at the hair cell ribbon synapse. *J Neurosci.* 25, 11577


