Zusammenfassung des wissenschaftlichen Inhalts

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Hair cell synaptic dysfunction, auditory fatigue and thermal sensitivity in otoferlin Ile515Thr mutants

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Abstract

The multi-C2 domain protein otoferlin is required for hearing and mutated in human deafness. Some OTOF mutations cause a mild elevation of auditory thresholds but strong impairment of speech perception. At elevated body temperature, hearing is lost. Mice homozygous for one of these mutations, Otof215Thr/215Thr, exhibit a moderate hearing impairment involving enhanced adaptation to continuous or repetitive sound stimulation. In Otof215Thr/215Thr inner hair cells (IHCs), otoferlin levels are diminished by 65%, and synaptic vesicles are enlarged. Exocytosis during prolonged stimulation is strongly reduced. This indicates that otoferlin is critical for the reformation of properly sized and fusion-competent synaptic vesicles. Moreover, we found sustained exocytosis and sound encoding to scale with the amount of otoferlin at the plasma membrane. We identified a 20 amino acid motif including an RXR motif, presumably present in human but not in mouse otoferlin, which reduces the plasma membrane abundance of Ile515Thr-otoferlin. Together, this likely explains the auditory synaptopathy at normal temperature and the temperature-sensitive deafness in humans carrying the Ile515Thr mutation.

Keywords auditory neuropathy; hair cell; hearing loss; otoferlin; ribbon synapse

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

Mutations in OTOF, the gene coding for the multi-C2 domain protein otoferlin, cause human prelingual deafness DFNB9 (Yasunaga et al., 1999). Otoferlin is required for a late step in exocytosis in mouse inner hair cells (IHCs), as its absence nearly abolishes depolarization-induced exocytosis despite the presence of synaptic vesicles at the ribbon-type active zones (Roux et al., 2006). It was proposed that this phenotype reflects a role of otoferlin as a Ca2+ sensor of exocytosis (Roux et al., 2006; Johnson & Chapman, 2010), but this idea requires further experimental testing. Indeed, in the profoundly hearing impaired pachanga mouse model (Otof2/2), which carries a point mutation in otoferlin (Schwander et al., 2007), only sustained exocytosis is impaired, while fast exocytosis, reporting the fusion of the readily releasable pool (RRP), is not (Pangrsic et al., 2010). The finding of impaired vesicle replenishment led to the hypothesis that otoferlin also functions in vesicle priming, which was subsequently supported by a recent study showing a reduction in short tethers connecting synaptic vesicles with the active zone membrane in otoferlin knockout (Otof2/2) mice (Vogl et al., 2015). In addition to these putative roles in priming and fusion, otoferlin may be involved in exocytosis–endocytosis coupling via an interaction with the clathrin adapter protein AP-2 (Duncker et al., 2013; Jung et al., 2015).

While most mutations in human OTOF lead to profound deafness, a defined set of missense mutations cause a striking nonsyndromic recessive temperature-sensitive auditory synaptopathy (reviewed in Pangrsic et al., 2012) that is exceptional in several
aspects. First, at normal body temperature, patients have near-normal pure tone hearing thresholds but impaired speech recognition, especially in background noise (Starr et al., 1998). Second, psychoacoustic testing of some patients revealed severe abnormalities of loudness adaptation to continuous pure tone stimulation, also called “auditory fatigue” ( Wynne et al., 2013). Third, elevation of body temperature to \( \geq 38.1^\circ\text{C} \) due to physical activity or fever causes severe to profound deafness. In the five independent familial cases described so far, different missense mutations (Ile515Thr, Gly541Ser, Arg1607Trp and compound heterozygosity for Gly614Glu and Arg1080Pro) and an in-frame deletion (Glu1804del) were discovered ( Varga et al., 2006; Romano et al., 2009; Marlin et al., 2010; Wang et al., 2010; Matsunaga et al., 2012). Furthermore, three more missense mutations in otoferlin ( Pro1987Arg, Glu1700Gln and Ile1573Thr ) were described to cause moderate age-progressive hearing loss without evident temperature sensitivity ( Varga et al., 2003; Chiu et al., 2010; Yildirim-Baylan et al., 2014). Unfortunately, speech perception, auditory temporal processing, auditory fatigue and temperature sensitivity have not been tested on these patients so far.

The severity of the hearing impairment of otoferlin mutant mouse lines studied to date ( Roux et al., 2006; Longo-Guess et al., 2007; Pangrsic et al., 2010; Reisinger et al., 2011 ) has precluded further functional studies at the cellular, systems and behavioural level. To address this problem, we set out to generate a novel Otof mutant mouse model with an intermediate hearing defect, which would allow us to tackle current open questions regarding the function of otoferlin in synaptic sound encoding. We generated a knock-in mouse carrying the p.Ile515Thr point mutation (in NP_001274418), which was identified in one knock-in mouse carrying the p.Ile515Thr point mutation (in

# Results

## The Ile515Thr mutation lowers otoferlin protein levels

We introduced the Ile515Thr substitution in mouse Otof via targeted knock-in (Appendix Supplementary Methods). First, we investigated the abundance and localization of otoferlin by using immunohistochemistry and confocal fluorescence microscopy in organs of Corti of 14- to 15-day-old (P14-15) mice (Fig 1). Quantifying immunofluorescence per cell, we found a 65% reduction in otoferlin levels in Otof\(^{I515T} \)/\(^{+/+}\) IHCs compared to wild-type (Otof\(^{+/+}\)) controls (Fig 1A, B and G). In a parallel analysis of Otof\(^{I515T} \)/\(^{+/+}\) IHCs, a 69% reduction of protein levels was detected (Fig 1C and G), close to previous results (73%, Pangrsic et al., 2010). The reduction of otoferlin in the mutant genotypes was confirmed using another anti-otoferlin antibody (Appendix Fig S1A–D). The lower protein levels in Otof\(^{I515T} \)/\(^{+/+}\) IHCs are likely due to a faster degradation of mutated otoferlin (Appendix Fig S1E). In Otof\(^{I515T} \)/\(^{I515T}\) IHCs, the remaining otoferlin localized more towards the synaptic area below the midline of the nucleus of IHCs compared to Otof\(^{+/+}\), while it was found more apically in Otof\(^{Pga\_I515T} \)/\(^{+/+}\) IHCs (Fig 1H). In the IHCs of all genotypes, otoferlin immunoreactivity was found in the cytoplasm and at the plasma membrane. In Otof\(^{I515T} \)/\(^{I515T}\) IHCs, the otoferlin immunofluorescence at the plasma membrane relative to total cellular protein levels was unaltered as compared to Otof\(^{+/+}\) IHCs. In contrast, Otof\(^{Pga\_I515T} \)/\(^{+/+}\) IHCs showed an 85% lower relative membrane staining (Fig 1D–F and I). Taken together, the calculated absolute level of membrane-bound otoferlin was reduced by 66% in Otof\(^{I515T} \)/\(^{I515T}\) and by 97% in Otof\(^{I515T} \)/\(^{+/+}\) IHCs (Fig 1J).

We conclude that the Ile515Thr mutation lowers the absolute levels of otoferlin, but preserves the relative distribution between plasma membrane and cytoplasm.

### Auditory brainstem responses (ABRs) indicate a progressive hearing impairment in Otof\(^{I515T} \)/\(^{I515T}\) mice

Hearing was first assessed by ABR recordings. ABR thresholds were elevated by 20 dB for pure tones and 10 dB for click stimuli in young Otof\(^{I515T} \)/\(^{I515T}\) mice (Fig 2A). The amplitude of the spiral ganglion neuron (SGN) compound action potential, approximated as the amplitude of ABR wave I in response to clicks, was reduced to one-third of the Otof\(^{+/+}\) lissencephalic values, while subsequent waves were better preserved (Figs 2B and C, and EV1A and B). Together, population responses indicate a mild impairment of synchronous auditory signaling already in juvenile mice. We then cross-bred Otof\(^{I515T} \)/\(^{I515T}\) mice with deaf Otof\(^{+/–}\) mice in order to model the human OTOF\(^{I515T} \)/\(^{I515T}\) subjects ( Varga et al., 2006) even more closely. We found an additional elevation of ABR thresholds by 15 dB and a further reduction of ABR amplitudes in Otof\(^{I515T} \)/\(^{I515T}\) mice, indicating a gene dosage-dependent effect (Figs 2A–C and EV1A). Consistent with a primary defect of the IHC synapse, distortion product otoacoustic emissions (DPOAE) were present, which report active cochlear amplification and require intact mechanoelectrical transduction (Fig EV1C).

Like in some patients with age-progressive hearing loss due to OTOF mutations ( Varga et al., 2003; Chiu et al., 2010; Yildirim-Baylan et al., 2014), the hearing impairment, reflected by altered ABR thresholds and amplitudes, progressed rapidly during adolescence (Fig 2D–F), which correlated with a further reduction in otoferlin protein levels (Fig EV1D).

### Intact synaptic vesicle fusion but impaired vesicle replenishment in Otof\(^{I515T} \)/\(^{I515T}\) IHCs

Perturbations of otoferlin function have been shown to interfere with presynaptic function in IHCs ( Roux et al., 2006; Pangrsic et al., 2010). To test the effect of the Ile515Thr mutation, we performed perforated-patch recordings from IHCs in P14–P17 mice at room temperature (RT). The current–voltage relationship revealed a normal voltage dependence of activation and amplitude of Ca\(^{2+}\) currents in Otof\(^{I515T} \)/\(^{I515T}\) IHCs (Fig 3A and B). We then measured exocytosis as increments of plasma membrane capacitance (\(\Delta C_m\)) in
Figure 1. Otoferlin levels and cellular distribution are differentially affected in Otof\textsuperscript{I515T/I515T} and Otof\textsuperscript{Pga/Pga} IHCs.

A–C Immunofluorescence images (inverted intensity) of P14-P15 IHCs from indicated genotypes, revealing differences in otoferlin fluorescence intensity and distribution. Maximum projection of confocal stacks; scale bar, 10 µm.

D–F Upper panels, examples for IHCs, co-labelled for otoferlin (magenta) and Vglut3 (green) and position of the line for line scans; maximum projection of few optical sections; scale bars, 5 µm. Lower panels, for quantification of membrane staining, the fluorescence was normalized to the cellular fluorescence for each fluorophore, and then the average of five parallel line scans through the middle of the cells for the sum of both fluorescence values (black line) was used to determine the position of the basal membrane. At the most basal cellular point along this line which exceeds the threshold value of 2 (yellow diamond), the otoferlin-Vglut3 fluorescence difference (blue line) gave the value for relative otoferlin plasma membrane levels (orange diamond). Insets: enlargements of basal regions.

G Otoferlin protein levels were reduced in Otof\textsuperscript{I515T/I515T} IHCs (indicated numbers represent numbers of cells) and Otof\textsuperscript{Pga/Pga} IHCs compared to wild-type (Otof\textsuperscript{+/+}) controls (mean ± SEM).

H Ratio of apical/basal protein levels (above/below nuclear midline depicted as green line in (A) indicates an apical shift of otoferlin in Otof\textsuperscript{Pga/Pga} IHCs.

I Relative levels of membrane-bound otoferlin at the basal pole of IHCs (mean ± SEM).

J Absolute amount of otoferlin at the basal IHC plasma membrane, gained by multiplication of relative plasma membrane levels (I) × total cellular otoferlin protein levels (G) (mean ± SEM).

Data information: Cell numbers in (G) apply also to (H–J); Kruskal–Wallis test; ***P < 0.001.
response to step depolarizations to the voltage where maximal Ca$^{2+}$ currents are elicited (typically $-14$ mV). Depolarizations were followed by recovery periods of 30 to 60 s at $-84$ mV, which precludes exocytosis triggered by voltage-gated Ca$^{2+}$ influx. Comparable $\Delta C_m$ in response to short depolarizations (2–20 ms) indicated intact fusion of a normally sized RRP under these experimental conditions (Fig 3C and D). Consistently, the number of ribbon synapses was normal in mutant IHCs (Fig EV1E and F). However, when Otof$^{I515T/I515T}$ IHCs were depolarized for 50 ms or longer, exocytosis was significantly smaller than in controls (Fig 3C and D). The amplitude was significantly reduced in Otof$^{I515T}$ mice compared to Otof$^{+/+}$ mice (Fig EV1E and F; fast and slow components reduced to 40 and 63%, respectively). This indicates that the Ile515Thr mutation does not impair the Ca$^{2+}$-triggered fusion of vesicles to the plasma membrane itself, but instead impairs vesicle replenishment, potentially affecting priming, active zone clearance or yet another mechanism.

In vivo postsynaptic recordings reveal a use-dependent depression of sound encoding at Otof$^{I515T/I515T}$ synapses

In order to directly assess sound encoding at Otof$^{I515T/I515T}$ synapses in vivo, we performed extracellular recordings from individual SGNs, each driven by a single IHC active zone (Fig 4). We found spontaneous spiking (Fig 4A), sound thresholds and frequency tuning (Fig EV2A and B) of individual SGNs to be normal which corroborates our notion of intact cochlear amplification in Otof$^{I515T/I515T}$ mice. Upon stimulation with tone bursts at a stimulus rate of 2 Hz, Otof$^{I515T/I515T}$ SGNs showed a near-normal onset response with a
high rate of instantaneous spiking, but then underwent stronger spike rate adaptation, not reaching a steady state within the 50 ms of stimulation (Fig 4B and E; ratio of onset/adapted rates 5.2 ± 1.8 in OtofI515T/+ littermates compared to 3.5 ± 0.2 in Otof+/- SGNs, P = 0.03, t-test). Similar reduction in rate-intensity functions of individual SGNs was observed in OtofI515T/+ littermates (Fig EV2C). The rate at which evoked spiking was reduced was 2.7 times lower in OtofI515T/+ littermates (P < 0.001, t-test).

Figure 3. Sustained exocytosis is impaired in OtofI515T/I515T hair cells.
A, B No difference in voltage-dependent Ca2+ currents (A) and fractional activation of ICa channels (B) between OtofI515T/I515T IHCs (n = 16) and IHCs of Otof+/- littermates (n = 13; mean ± SEM).
C Exocytosis was recorded by measures of changes in membrane capacitance (ΔCm, lower panel) in response to depolarization (left, 20 ms, right, 100 ms) to the voltage where maximum Ca2+ currents were elicited (upper panel); typically −14 mV. Representative examples.
D Upper panel, while for stimuli up to 20 ms exocytosis from OtofI515T/I515T (n = 13) and Otof+/- (n = 11) IHCs was similar, sustained exocytosis, representing most likely the release of replenished vesicles, was significantly reduced in OtofI515T/I515T IHCs (mean ± SEM; *P < 0.01; **P < 0.001). Lower panel, Ca2+ current integrals were of similar size (mean ± SEM).
E Flash photolysis of caged Ca2+ elicited a smaller exocytic response in OtofI515T/I515T IHCs (mean ± SEM).
F The kinetics of the fast component from (E) was comparable between OtofI515T/I515T IHCs and Otof+/- littermates (black circles). Open circles represent previously published data on IHCs of hearing wild-type mice (Beutner et al, 2001; Pangrsic et al, 2010).

Impaired synaptic sound encoding in OtofI515T/I515T mice affects the perception of silent gaps in noise

Using prepulse inhibition of the acoustic startle response, we found an impairment of gap detection performance in OtofI515T/I515T mutants compared to Otof+/- littermates (Fig EV3A–C). We propose that this is consistent with the delayed recovery from adaptation (Fig 4I) and reflects the impaired vesicle replenishment during the gap. For a more sensitive method that approaches the physiological limit of gap detection abilities, we employed operant conditioning using the Audiobox system (de Hoz & Nelken, 2014). We conditioned mice to avoid drinking when continuous broadband noise was present. When the noise was interrupted by 90 ms silent gaps, access to the water bottles was denied and drinking attempts were punished by air puffs. After reaching > 30% discrimination performance, we introduced shorter gaps in a total of 8% of the trials. The two Otof+/- mice avoided drinking when gaps lasted 3 ms or more. This agrees well with descriptions of gap thresholds near 2 ms in CBA/J mice (Radziwon et al, 2009). In contrast, OtofI515T/I515T mice often attempted to drink in trials with short gaps (Figs 4J and EV3F). The interpolated 50% value of the normalized discrimination function was 2.7 ± 0.4 ms in Otof+/- mice and 17.2 ± 4.9 ms in OtofI515T/I515T.

In a second task, we conditioned mice to avoid drinking when they heard 12 kHz tone bursts. The responses to varying tone...
intensities indicate comparable hearing thresholds in two Otof<sup>I515T/I515T</sup> mice and one Otof<sup>+/+</sup> mouse (Fig EV3D and E).

Together, the impaired gap detection performance and normal sound sensitivity in Otof<sup>I515T/I515T</sup> mice are consistent with the results of in vivo recordings from SGNs that show normal sound sensitivity but impaired recovery from adaptation. Like in other mice with synaptic transmission deficits (e.g. Buran et al., 2010; Jung et al., 2015) and patients with auditory synaptopathy/
neuropathy, the deficit appears overly pronounced in ABR recordings which represent a combination of the sensitivity, the rate and the synchronicity of SGN firing.

**ABR amplitudes gradually decline at elevated temperature**

Our data show that Otof<sup>I515T/I515T</sup> mice reflect the auditory phenotype of human patients at normal body temperature very well. Thus, we assume that the synaptic disease mechanisms found in mice at or below physiological temperature likely describe the situation in human patients. We next assayed whether mice, like the human patients, experience an exacerbation of their hearing loss when their body temperature exceeds 38°C (Varga et al., 2006). We monitored the amplitude of ABR wave I in response to click stimulation while locally applying heat to the inner ear (Fig 5A and Appendix Fig S2). Here, we found a reversible linear decrease in ABR wave I amplitude in both Otof<sup>I515T/I515T</sup> and Otof<sup>+/+</sup> littermate mice, with comparable average regression slopes of 0.084 µV/°C and 0.081 µV/°C, respectively. The effect varied considerably between animals, presumably due to variable experimental conditions. In some animals (3/6 Otof<sup>I515T/I515T</sup> and 2/6 Otof<sup>+/+</sup>), ABR amplitudes did not recover after extensive heating (Appendix Fig S2B and C), presumably indicating permanent heat damage. Nonetheless, since ABRs in the mouse model never disappeared completely, we conclude that the elevated cochlear temperature did not fully abolish sound encoding in Otof<sup>I515T/I515T</sup> mice mutants, as it seems to be the case in human Otof<sup>I515T/I515T</sup> patients. Thus, as the heat-induced phenotype seems to be weaker in mice compared to human patients, the cell physiological or ultrastructural effect of fever found in our mouse model might also be weaker than in human patients.

**A 20 amino acid stretch present in human otoferlin reduces plasma membrane localization of Ile515Thr-otoferlin**

Looking at differences in the protein sequence of human and mouse otoferlin which might contribute to the less pronounced heat sensitivity in mice, we found an arginine-rich insertion including an RXR motif at position 1244–1263 in the long human reference sequences (e.g. variant e; NP_001274418). While there is currently no experimental evidence which splice isoform regarding this motif is expressed in human IHCs, PCRs on mouse organ of Corti cDNA revealed that the isoform lacking this RXR motif is predominant in mouse IHCs (Fig EV4). A similar motif was described to cause a temperature-dependent decrease in surface expression of an alpha-adrenergic receptor (Filipeanu et al., 2011, 2015). We subcloned the human RXR stretch into mouse otoferlin cDNA and performed biolistic transfection into Otof<sup>-/-</sup> IHCs (Fig SE–I). RXR-otoferlin showed a strong plasma membrane immunostaining (relative to total cellular otoferlin levels), which was comparable to the values from explanted Otof<sup>-/-</sup> cells (Fig SD and J). Otoferlin with both the Ile515Thr mutation and the RXR motif, however, displayed almost complete absence from the plasma membrane already at 37°C (Fig SH–J), which is in stark contrast to mouse Ile515Thr-otoferlin (Fig 1). Since the abundance of otoferlin at the plasma membrane seems to be most relevant for sound encoding in vivo, this might explain the more pronounced heat sensitivity in human patients.

When comparing to the hearing phenotype of our mouse models, hearing at normal and elevated temperature in human Ile515Thr patients would best be explained by a mixture of the splice variant with RXR and the splice variant without being expressed in human IHCs.

**Heat reduces exocytosis and otoferlin membrane levels**

We next tested for a synaptic dysfunction elicited by fever by performing patch clamp recordings of IHCs (Fig 6). We recorded ΔCm in IHCs induced by 5–100 ms depolarization steps, first at room temperature (RT), then at near physiological temperature (PT, 35–36.5°C), followed by high temperature (38.5–40°C). Consistent with a previous study (Nouvian, 2007), we found Ca<sup>2+</sup> currents and exocytosis from Otof<sup>-/-</sup> IHCs to increase when temperature was raised from RT to PT (Fig 6A and B). While Ca<sup>2+</sup> current integrals increased only by 27 and 15% in Otof<sup>-/-</sup> (n = 4) and Otof<sup>I515T/I515T</sup> (n = 5), respectively, exocytosis was more strongly enhanced by warming (Fig 6B) and increased 2.7- to threefold in both genotypes.

We next heated the bath to 38.5–40°C expecting a strong reduction of exocytosis for Otof<sup>I515T/I515T</sup> IHCs given the human fever-induced deafness. Recordings were started as soon as the temperature recorded in the bath close to the tissue was stable. Surprisingly, we found a strong decline in ΔCm for Otof<sup>-/-</sup> IHCs compared to PT, especially for sustained exocytosis which was halved (Fig 6C and F). This fever-induced reduction was weaker in Otof<sup>I515T/I515T</sup> (13% less than at PT; Fig 6G). As a result, ΔCm did not differ between Otof<sup>I515T/I515T</sup> and Otof<sup>-/-</sup> IHCs at high temperature for all depolarization durations tested (Fig 6C). However, we cannot exclude that a longer heating period might have unravelled a stronger synaptic dysfunction in Otof<sup>I515T/I515T</sup> IHCs. In fact, sustained exocytosis between the genotypes appeared slightly stronger in recordings after heating. At 2 min of recovery at < 29°C, exocytosis in Otof<sup>-/-</sup> IHCs was reduced to 52% relative to RT before heating for depolarization stimuli of ≥ 10 ms (n = 4), while in Otof<sup>I515T/I515T</sup> IHCs, it was reduced to 40% of initial RT values (n = 3; Fig 6D, F and G). Ca<sup>2+</sup> currents were of similar size as before heating and comparable between Otof<sup>-/-</sup> and Otof<sup>I515T/I515T</sup> IHCs. In few of these IHCs, exocytosis was tested also after a longer recovery period of 5–20 min, and we recorded additional cells only after heating (Fig 6E). Considering IHCs of both conditions, we found ΔCm levels for 100 ms depolarization in Otof<sup>-/-</sup> IHCs to be restored to 61% compared to observations at RT before heating (n = 7 from 4 cells). In contrast, in Otof<sup>I515T/I515T</sup> IHCs, sustained exocytosis remained reduced (37% of initial values for 100 ms depolarization, n = 8 from 4 cells).

Based on the loss of exocytic capacity in wild-type and mutant IHCs between PT and ≥ 38.5°C, we propose that part of the synaptic machinery is thermally unstable. The poorer recovery from heat in Otof<sup>I515T/I515T</sup> IHCs and the temperature-sensitive phenotype of patients with different OTOF mutations suggest that otoferlin itself is the heat-sensitive protein and that heat instability of otoferlin is enhanced by certain mutations like Ile515Thr.

Indeed, 3D structure predictions show that the isoleucine 515 points towards the hydrophobic core of the C<sub>C</sub> domain (Appendix Fig S3), suggesting that an exchange into the more
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Since we also did not find a change in otoferlin mRNA hydrophilic threonine potentially decreases the stability of the protein. Since we also did not find a change in otoferlin mRNA levels in Otos/•+/ mice at P4 we transfected by GeneGun with otoferlin cDNA and eGFP and immunostained at DIV20. (E) Example cell transfected with wild-type mouse otoferlin cDNA. (F, G) Representative cells transfected with mouse otoferlin including the 20 amino acids of the RXR motif (see Fig EV4). Cells in (G) were incubated at 38.5°C for 30 min prior to fixation. The RXR-otoferlin transfected cells (F, G) show a membrane localization of otoferlin surrounding the Vglut3 immunofluorescence/eGFP fluorescence at the basal pole of the cells (arrows), similar as in controls (D, E). (H, I) Both the human RXR motif and the ile515Thr mutation were introduced in mouse cDNA, and cells were incubated at 37°C (H) or for 30 min at 38.5°C (I) before fixation. Here, green fluorescence surrounds the otoferlin immunofluorescence (arrows), suggesting loss of otoferlin from the plasma membrane.

Quantification of the relative plasma membrane levels of otoferlin (as in Fig 1) revealed normal plasma membrane abundance when the human RXR motif was present, but a strong reduction for otoferlin with RXR and Ile515Thr/individual cells; mean ± SEM; t-test; **P < 0.01.

Figure 5. Apparently lower thermal sensitivity of hearing in mice than in humans, likely due to a human RXR motif reducing plasma membrane localization of Ile515Thr-otoferlin.

A Exemplary ABR traces (click 100 dB) from one Otof/•+/ mouse recorded at indicated bulla temperatures during local heating. Note that ABRs never disappeared completely, but wave I amplitude changed reversibly with temperature.

B, C ABR wave I amplitudes in Otos/•+/ (B, click 100 dB) and Otos/+/+ mice (C, click 80 dB) decreased with increasing temperature in the bulla. Each colour represents a different experiment, and dashed lines are line fits. Open symbols indicate the beginning of the experiments; subsequent recordings are connected by lines. The four indicated data points in (B) correspond to ABRs in [A].

D Explanted organ of Corti from an Otos/+/+ mouse at P4 after 10 days in vitro (DIV2) at 37°C immunostained against otoferlin (magenta) and Vglut3 (green). Note the intense immunostaining of otoferlin at the plasma membrane. Scale bar, 10 μm.

E–I Explanted organs of Corti from Otos/•+/ mice at P4 were transfected by GeneGun with otoferlin cDNA and eGFP and immunostained at DIV2. (E) Example cell transfected with wild-type mouse otoferlin cDNA. (F, G) Representative cells transfected with mouse otoferlin including the 20 amino acids of the RXR motif (see Fig EV4). Cells in (G) were incubated at 38.5°C for 30 min prior to fixation. The RXR-otoferlin transfected cells (F, G) show a membrane localization of otoferlin surrounding the Vglut3 immunofluorescence/eGFP fluorescence at the basal pole of the cells (arrows), similar as in controls (D, E). (H, I) Both the human RXR motif and the Ile515Thr mutation were introduced in mouse cDNA, and cells were incubated at 37°C (H) or for 30 min at 38.5°C (I) before fixation. Here, green fluorescence surrounds the otoferlin immunofluorescence (arrows), suggesting loss of otoferlin from the plasma membrane.

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after heterologous expression in HEK293T cells, we found hardly any degradation within 2 or 24 h even at elevated temperature, similar as for wild-type otoferlin (Appendix Fig S4B and C). Although the lifetime of mutated otoferlin might be shorter in IHCs (see Appendix Fig S1E), the degradation seems too slow to explain hearing loss due to elevation of the body temperature. We hypothesize that loss of otoferlin from the plasma membrane in addition to
Figure 6. Temperature alters exocytosis, otoferlin protein levels and plasma membrane abundance.

A–E Capacitance increments recorded from *Otof*+/+ (black) and *Otof*515T/515T (red) IHCs. Individual cells recorded at indicated temperatures (light circles), mean ± SEM (filled circles) for perforated-patch clamp experiments.

F, G Summary of the capacitance changes and Ca²⁺ current integrals for *Otof*+/+ (F) and *Otof*515T/515T (H) IHCs (G) at the different temperatures illustrates the drastic increase in exocytosis for physiological temperature. Significant differences compared to RT measurements are indicated with colours of the respective temperature, and between PT and high temperature in violet.

H–J Otoferlin immunofluorescence in *Otof*+/+ IHCs (upper panel) and *Otof*515T/515T IHCs (middle and lower panels) of explanted organs of Corti at P7–P8 after incubation at indicated temperatures for 24 h, maximum projections of 2-z-stacks, inverted images; scale bar, 10 μm. The same imaging settings have been applied in all experiments, and the same lookup table was applied for the upper and middle panels. Images of lower panels are enhanced compared to middle panels (lookup table covering full data range of only this genotype) to visualize otoferlin distribution.

K Quantification of otoferlin immunofluorescence in *Otof*+/+ IHCs (black bars) and *Otof*515T/515T IHCs (red bars) indicates reduced protein levels with increasing temperature.

L Apical/basal otoferlin protein distribution, revealing a significant apical shift of otoferlin and Vglut3 for *Otof*515T/515T IHCs (red symbols) at 38.5°C compared to *Otof*+/+ (grey/black symbols).

M Relative levels of membrane-bound otoferlin were lowered with increasing temperature.

N Absolute otoferlin membrane immunostaining strongly decreased with temperature.

Data information: All data presented are mean ± SEM. n = 98–137 *Otof*+/+ cells and n = 90–97 *Otof*515T/515T cells in (K–N), 4–5 experiments. t-test (A–G) or Kruskal–Wallis test (K–N). *P < 0.05; **P < 0.01; ***P < 0.001.
Otoferlin is localized at the plasma membrane and at endosomal vesicles, and synaptic vesicles are enlarged in OtofI515T/IHCs

Next, we analysed the subcellular distribution of otoferlin at the ultrastructural level by performing pre-embedding immunogold labelling and electron microscopy (EM) of P15-P16 IHCs (Fig 7 and Appendix Fig S5). The near absence of gold clusters in OtofI+/− tissue confirmed specificity of immunogold labelling (Fig 7C). In both OtofI+/− and OtofI515T/IHCs, otoferlin immunogold signal was present at the plasma membrane including the active zone membrane (Fig 7A, B and D–G, and Appendix Fig S5), and at vesicular structures, some of which exhibited clathrin-coated vesicle budding (Fig 7I and J) indicating endosomal nature. The diameter of labelled intracellular membranous structures was, on average, larger in OtofI515T/IHCs (210 ± 13 nm; smallest diameter: 51 nm; n = 86) than in OtofI+/− IHCs (151 ± 8 nm, smallest 52 nm; n = 82, P < 0.001; Fig 7K) and thus clearly exceeded the typical synaptic vesicle diameter in IHCs (Chapochnikov et al., 2014). Hardly any otoferlin labelling was visible in < 100 nm distance to the ribbons where a halo of synaptic vesicles resides, neither in OtofI+/− nor in OtofI515T/IHCs, using two different pre-embedding protocols (Fig 7D–G). This discrepancy to earlier immunogold studies reporting a synaptic vesicle localization of otoferlin (Roux et al., 2006) might be due to pre- versus postembedding protocols used and/or different primary antibodies and a potential masking of our antibody epitope specifically at synaptic vesicles. In contrast, immunogold labelling for the vesicular glutamate transporter Vglut3 resulted in a strong signal around the ribbon (Fig 7H and Appendix Fig S5). Quantification of the gold particles revealed an overall reduction of otoferlin labelling in the cytoplasm and a strong trend towards lower levels at the membrane in OtofI515T/IHCs compared to OtofI+/− hair cells (Fig 7L and M), consistent with our immunofluorescence results. We conclude that the Ile515Thr mutation does not alter the subcellular distribution of otoferlin.

The larger average diameter of otoferlin-labelled structures in OtofI515T/IHCs might point towards impaired membrane turnover. We thus assessed the effects of the Ile515Thr mutation on synaptic ultrastructure and vesicle pool dynamics at 36 and 39°C by performing transmission EM of IHC ribbon synapses following inhibition or stimulation of exocytosis and subsequent chemical fixation. To inhibit IHC exocytosis, isolated organs of Corti were incubated in Ca2+-free saline, while stimulation employed extracellular saline with 1.3 mM Ca2+ and 40 mM K+ (according to the Nernst equation depolarizing IHCs to ~30 mV). We found the number of membrane-proximal and ribbon-associated vesicles quantified in random ultrathin sections to be similar in all four conditions (stimulatory and inhibitory at 36/39°C) and both genotypes (N = 2 mice/condition and genotype; Fig 7N and O, and Appendix Fig S6 and Appendix Table S1). The only significant effect was an increase in the number of membrane-proximal vesicles in OtofI515T as compared to OtofI+/− IHCs after stimulation at either temperature (Appendix Table S1).

Figure 7. The Ile515Thr mutation does not affect the subcellular localization of otoferlin but alters the size of endosomal and synaptic vesicles.

A–C Pre-embedding immunogold labelling for EM visualizes otoferlin localization in random ultrathin sections through the basal part of IHCs in OtofI+/− (A) and OtofI515T (B) but not in OtofI−/− (C) mice. IHCs are highlighted in beige. Scale bar, 200 nm.

D–G Magnified synaptic ribbons (R) and postsynaptic afferent boutons of SGNs (Aff), displaying otoferlin immunogold labelling at active zone membranes (pink arrowheads) but not around the synaptic ribbon, using saponin permeabilization (D–F), or Triton X-100 treatment (G). Scale bar, 100 nm.

H Vglut3 immunogold labelling adjacent to the ribbon as expected for a synaptic vesicle protein. Scale bar, 100 nm.

I, J Some otoferlin-labelled structures bear clathrin coats (red arrowheads), suggesting that they originate from bulk endocytosis. Scale bar, 100 nm.

K Otoferlin-labelled vesicles were on average larger in OtofI515T/IHCs (mean ± SEM; OtofI515T/IHCs, n = 86 vesicles, 18 images; OtofI+/−, n = 82 vesicles, 6 images; Wilcoxon rank-sum test; ***P < 0.001).

L Quantification of immunogold clusters at the plasma membrane revealed a strong trend towards reduced levels of otoferlin in OtofI515T/IHCs (six images each) (mean ± SEM; Wilcoxon rank-sum test).

M Distal from the plasma membrane, immunogold labels were reduced in OtofI515T/IHCs (mean ± SEM, Kruskal-Wallis test; *P < 0.05; **P < 0.01).

N Representative electron micrographs of IHC ribbon synapses in OtofI515T/IHCs and OtofI+/− (conventional embedding without immunogold) after pre-incubation for 10 min at the indicated temperature followed by high K+ stimulation or 0 Ca2+ inhibition for 1 min 45 s. Scale bars, 100 nm.

O Illustration depicting criteria for random section analysis (not drawn to scale). Ribbon-associated synaptic vesicles (SVs, green) in the first row ≤ 80 nm from ribbon (R) and membrane-proximal vesicles (yellow) in ≤ 25 nm membrane-to-membrane distance from the plasma membrane (blue) and ≤ 80 nm from each side of the presynaptic density.

P, Q Synaptic vesicles with > 45 nm diameter appeared at 39°C in random sections; fraction of large membrane-proximal (P, Kruskal-Wallis test followed by non-parametric multiple comparison test; *P < 0.05) and ribbon-associated (Q, one-way ANOVA followed by Tukey’s test; *P < 0.05; **P < 0.01) synaptic vesicles (mean ± SEM; OtofI+/− inhibitory, n = 24 synaptic ribbons, OtofI+/−stimulatory, n = 27 synaptic ribbons, OtofI515T/IHCs inhibitory, n = 36 synaptic ribbons, OtofI515T/IHCs stimulatory, n = 21 synaptic ribbons, see also Appendix Table S1).
Figure 7.
We next determined the vesicle diameter in all four conditions for both ribbon-associated and membrane-proximal vesicles. We found the majority of vesicle diameters to be in a 35-45 nm range, consistent with previous reports from IHC ribbon synapses (Neef et al., 2007; Appendix Fig S6). Remarkably, in the membrane-proximal pool at Otof\textsuperscript{I515T/I515T} synapses after stimulation at 39°C, vesicles with diameter > 45 nm were observed, that were less frequent after inhibition, and rarely observed in Otof\textsuperscript{+/+} (Fig 7P, Appendix Fig S6A and Appendix Table S1). In contrast, for the ribbon-associated pool, we found more large vesicles for inhibitory than for stimulatory condition at Otof\textsuperscript{+/+} (39°C) and, remarkably, even more for both conditions at Otof\textsuperscript{I515T/I515T} synapses (Fig 7Q, Appendix Fig S6B and Appendix Table S1).

We further analysed number and size of synaptic vesicles by performing electron tomography after stimulation at 36 and 39°C (Fig 8, n = 4 tomograms/genotype and condition; Table EV1). While the number of synaptic vesicles was unaltered in tomograms, we found the vesicles to be larger in Otof\textsuperscript{I515T/I515T} IHCs, both the membrane-proximal (Fig 8N and O) and the ribbon-associated ones (Fig 8P and Q). In all conditions tested, we also found some flattened vesicles (Table EV1), but their longest axis was not significantly different (Fig 8O and Q).

Together, the enlarged otoferlin-labelled endosomal vesicles and the enlarged synaptic vesicles point towards a deficit in vesicle reformation in Otof\textsuperscript{I515T/I515T} IHCs, which is more pronounced at febrile temperature.

Discussion

Here, we present a mouse model for auditory fatigue and otoferlin-related hearing impairment, allowing us to study the disease mechanism from molecular to systems level. Our analysis reveals that the remarkable IHC capability of indefinite fatigue sustained exocytosis requires otoferlin. This likely involves roles of otoferlin in reformation of synaptic vesicles from endocytosed membrane as demonstrated here, in addition to tethering synaptic vesicles to the plasma membrane (Vogl et al., 2015), and priming and/or active zone clearance (Pangrsic et al., 2010; Jung et al., 2015). Presynaptic function of IHCs and sound encoding correlate with the abundance of otoferlin at the plasma membrane. We postulate that upon an elevation of the body temperature in human patients with temperature-sensitive OTOF mutations, properly folded otoferlin at the plasma membrane drops below a level critical for promoting synaptic sound encoding.

Hearing impairment correlates with the amount of otoferlin at the IHC plasma membrane

Most human OTOF mutations lead to profound deafness, but some patients have residual hearing. While two mouse missense mutants, Otof\textsuperscript{I515T/I515T} and Otof\textsuperscript{Pga/Pga}, show a similar overall reduction of otoferlin protein levels, Otof\textsuperscript{Pga/Pga} IHCs, in addition, display a perturbed plasma membrane localization of otoferlin. This results in a reduction of absolute otoferlin membrane levels to only 3% of wild-type in Otof\textsuperscript{Pga/Pga} (Pangrsic et al., 2010 and this study), while Otof\textsuperscript{I515T/I515T} IHCs retain 34%. The comparison of the hearing phenotypes of Otof\textsuperscript{I515T/I515T}, Otof\textsuperscript{I515T/+}, Otof\textsuperscript{Pga/+} and Otof\textsuperscript{+/+} mice indicates that synaptic function and sound encoding scale with the amount of plasma membrane-bound otoferlin, which might generalize to human missense mutations. A further heat-induced reduction of the plasma membrane abundance of mouse Ile515Thr-otoferlin appeared rather slowly and the temperature-dependent hearing loss was relatively mild in Otof\textsuperscript{I515T/I515T} mice.

Direct comparison of mouse ABRs at febrile temperature with psychoacoustic testing of human patients is challenging. However, it seems that our mice are less susceptible to heat than human OTOF\textsuperscript{I515T/R1116*} subjects who exhibit a threshold elevation by ≥ 60 dB and loss of speech perception at 38.1°C body temperature. Our data indicate that this may be due to the RXR motif sequence presumably present in human otoferlin which reduces the plasma membrane abundance of Ile515Thr-otoferlin beyond what we found in Otof\textsuperscript{I515T/I515T} mice. The expression of this RXR motif depends on whether the first or the second splice acceptor site in exon 30 is used, which is currently unknown. Together, the lower cellular otoferlin protein levels due to the Ile515Thr mutation, the presumed presence of the RXR splice isofrom, which causes a weaker potency of the Ile515Thr-otoferlin for plasma membrane localization, and a likely heat-induced protein unfolding provide a candidate mechanism for temperature-sensitive hearing loss in humans.

High rates of synaptic vesicle exocytosis at physiological temperature challenge current models for release mechanisms at ribbon synapses

Reconciling IHC physiological and morphological data with \textit{in vitro} responses has remained difficult because of the clearly distinct experimental conditions (Buran et al., 2010; Pangrsic \textit{et al}, 2010). Here, we set out to remove one important variable by combining data sets obtained near PT. Similar to a previous report (Nouvian, 2007), membrane capacitance estimates showed a ~threefold increase of exocytosis at PT compared to RT. Considering the \textit{in vitro} patch clamp experiments at PT and assuming all exocytosis to occur at active zones, the calculated rate of sustained exocytosis amounts to ~2,300 vesicles/s/presynaptic active zone. This number appears very high when compared to EM counts of membrane- and ribbon-associated vesicles (on the order of 50/ribbon and with no obvious temperature dependence; Fig 8, Table EV1) and the average sustained SGN firing rate of 240 Hz in wild-type mice (Fig 4). Possible explanations for this discrepancy include more extrasynaptic exocytosis than described (Fuchs \textit{et al}, 2003), a higher failure rate than assumed from \textit{in vitro} and modelling studies (Siegel & Dallos, 1986; Rutherford \textit{et al}, 2012) and/or synchronized multivesicular release. The mechanism of release at the hair cell synapse is a topic of active research and both synchronized multivesicular release (e.g. Glowatzki \& Fuchs, 2002; Graydon \textit{et al}, 2011) and univesicular release through a dynamic fusion pore (Chapochnikov \textit{et al}, 2014) have been put forward to explain the heterogenous size and shape of EPSCs. Based on modelling Graydon \textit{et al} (2011) proposed synchronization of readily releasable vesicles by a common Ca\textsuperscript{2+} nanodomain at frog hair cell active zones, which, however, seemed insufficient to explain large and fast EPSCs in a model of the rat IHC active zone (Chapochnikov \textit{et al}, 2014). The comparable vesicle diameters in EM at RT and PT for Otof\textsuperscript{I515T+/+} mice seemingly argue against homotypic fusion of synaptic vesicles, although a rapid
Figure 8. EM tomography reveals enlargement of synaptic vesicles under continuous exocytosis in OtofI515T/I515T IHCs.

A–D Exemplary virtual sections through EM tomograms for stimulatory condition at 36 and 39°C. Scale bar, 100 nm.

E Illustration depicting the tomogram analysis (not drawn to scale). Ribbon-associated synaptic vesicles (SVs, green) in the first row ≤ 80 nm from ribbon (R) and membrane-proximal vesicles (yellow) in ≤ 50 nm membrane-to-membrane distance from the plasma membrane (blue) and ≤ 100 nm from each side of the presynaptic density.

F–M 3D reconstruction of the membrane-proximal pool (top view) (F–I) and the ribbon-associated pool of vesicles (front view) (J–M). Active zone membrane is depicted in blue, presynaptic density in pink and ribbons in red, scale bars, 100 nm.

N–Q Cumulative probability distribution of the diameter of round vesicles as well as the longest axis of flattened vesicles in the membrane-proximal pool (N, O) and ribbon-associated pool (P, Q). (N–P), one-way ANOVA followed by Tukey’s test; (Q), Kruskal–Wallis test followed by non-parametric multiple comparison test. Round vesicles tested in both pools appeared larger in OtofI515T/I515T IHC synapses but the long axis of flattened vesicles was comparable for both genotypes (see also Table EV1).
exocytosis of such enlarged vesicles might have prevented their detection so far.

Otoferlin plays a key role for vesicle reformation

Using immunogold labelling, we found otoferlin at the plasma membrane (also reported in Roux et al, 2006) and, specifically, at the membrane of the active zones, which is consistent with a recent immunofluorescence analysis (Vogl et al, 2015). We found otoferlin labelling also at endosomal structures partially exhibiting budding of clathrin-coated vesicles, which is in agreement with STED fluorescence imaging data (Revelo et al, 2014), but not on synaptic vesicles of < 50 nm diameter. Remarkably, in Otof<sup>I515T/I515T</sup> IHCs, we found enlarged otoferlin-labelled vesicular structures, potentially of endosomal origin, and on average larger synaptic vesicles after incubation both at 36 and 39°C. In contrast, deficiency of the otoferlin interacting molecule AP-2, adapter protein for clathrin-mediated endocytosis, caused ablation of ribbon-associated vesicles distal from the active zone membrane, and a reduction of clathrin-coated structures close to ribbon synapses (Jung et al, 2015), indicating that otoferlin and AP-2 interact on different steps of the vesicle reformation process.

In summary, we propose a new role for otoferlin in reformation of proper sized synaptic vesicles. The extent of the hearing loss in Otof<sup>I515T/I515T</sup> might be explained by such a synaptic vesicle reformation defect, given that the high vesicle turnover rates at the IHC ribbon synapse require an ultrafast vesicle reformation mechanism. However, the impaired sustained exocytosis in Otof<sup>I515T/I515T</sup> at RT points towards an additional deficit in priming (Pangrsic et al, 2010) and/or active zone clearance (Duncker et al, 2013; Jung et al, 2015).

Studying auditory fatigue at the level of single synapses in Otof<sup>I515T/I515T</sup> mice

The normal size of the RRP observed in Otof<sup>I515T/I515T</sup> IHCs in vitro seems in contrast to the SGN spike rate reduction in vivo. This discrepancy can be reconciled when considering that in the voltage clamp experiment, hyperpolarization allows for RRP recovery in the absence of Ca<sup>2+</sup> influx-driven exocytosis, while in vivo spontaneous release diminishes the occupancy of the release sites that compose the RRP, reducing the “standing” RRP. Moreover, the lower time resolution of the capacitance recordings obscures the finding of a combined reduction of rate and synchronicity of sound onset firing which is observed in single unit recordings and highlighted by the ABR amplitude reduction.

At physiological temperature, the phenotype of Otof<sup>I515T/I515T</sup> mice reproduces the clinical picture very well and single SGN response recordings in Otof<sup>I515T/I515T</sup> mice provide an explanation for the apparent discrepancy between the normal sound sensitivity and the poor speech perception and ABRs in human patients: while fewer spikes from individual SGNs with normal thresholds may suffice for tone detection, the combination of reduced spike rates and higher jitter of action potential firing at sound onset can cause a dramatic reduction in ABR wave I amplitude, as also observed in other mutants with impaired synaptic sound encoding (Buran et al, 2010). In response to constant or repetitive stimuli, Otof<sup>I515T/I515T</sup> SGNs display markedly enhanced adaptation and slowed recovery from adaptation. This is consistent with the finding that patients with otoferlin deficiency show auditory fatigue when challenged with continuous sounds (Wynne et al, 2013). In contrast, none of the patient reports mention tinnitus as an additional symptom. We thus consider it unlikely that impairment of the perception of silent gaps in noise in our mice is due to tinnitus (Turner et al, 2006). Instead, it is likely explained by the combination of the reduction of adapted spike rates and the delayed recovery of the sound onset response. The resulting deficit in the encoding of fast fluctuations in sound envelope structure seems well consistent with the speech comprehension difficulties experienced by human patients which obviously cannot be restored by sound amplification using traditional hearing aids. Our data suggests that a more promising approach would be to devise a new speech processing strategy that aims to compensate for the increased adaptation. Clinicians should expect ABR latencies to be close to normal (Fig EV1B) and late ABR waves to be better preserved than wave I, suggesting partial compensation in the auditory brainstem. Finally, we suggest that tests for sound adaptation and temporal coding (e.g. gap detection) should be more generally applied in clinical settings to detect deficits that are independent of sound sensitivity.

Materials and Methods

Mouse genetics

Otof<sup>I515T/I515T</sup> mice were generated by targeted mutagenesis of a genomic vector and homologous recombination in mouse embryonic stem cells (see Appendix Supplementary Methods for details).

Immunohistochemistry and quantification of fluorescence

Immunostaining and confocal fluorescence microscopy was performed essentially as described (Khimich et al, 2005) and detailed in Appendix Supplementary Methods.

For quantification of otoferlin and Vglut3 immunofluorescence, same experimental settings were used for each series. Using a Matlab routine implemented in Imaris, the cells were aligned in the xy- and yz-plane and the outer border of each cell was labelled manually in a 3D projection. Summed fluorescence intensities in voxels above or below the mid-nuclear plane were used for calculating the ratio of apical/basal fluorescence. Quantification of relative otoferlin membrane staining was performed after normalization of total cellular fluorescence levels and is illustrated in Fig 1D–F and was normalized to Otof<sup>+/+</sup> values (at 37°C if applicable).

Pre-embedding immunogold labelling

Anti-otoferlin (Abcam, 1:300) and anti-Vglut3 (Synaptic Systems, 1:300) antibodies were applied after fixation and permeabilization with either saponin (Fig 7A–F, I and J) or Triton X-100 (Fig 7G and H). Silver enhancement was used prior to sample embedding, as described in Appendix Supplementary Methods.

Patch clamp recordings

IHCs from the apical coils of freshly dissected organs of Corti were patch-clamped in the perforated-patch configuration as described.
(Moser & Beutner, 2000). Experiments at elevated temperature and flash photolysis were performed as described (Beutner et al., 2001; Nouvian, 2007; respectively). For details, see Appendix Supplementary Methods.

**Systems physiology and behaviour**

ABRs, DPOAE and recordings from individual SGNs were performed as described (Jing et al., 2013), except for offline action potential detection which was based on spike waveform and amplitude using a custom-written Matlab routine (see Appendix Supplementary Methods for details). For ABR recordings with local temperature changes, ABRs in response to click stimuli at 60, 80 and 100 dB were measured using a custom-written Matlab routine controlling TDT system III (Tucker Davis Technologies) and a JBL 2402 speaker. A custom-designed heat probe (Appendix Fig S2A) was placed on the bony wall of the mouse bulla. The local temperature of the heat probe was constantly monitored, same for two miniaturized thermo-probes (H*MT, Newport Omega, Deckenpfonn) placed on the promontory wall within the bulla and in the mouth or in the cerebellum (accessed through a small hole in the occipital bone). Operant conditioning in the “Audiobox” was carried out essentially as described (de Hoz & Nelken, 2014), and startle responses were recorded and quantified as detailed in Appendix Supplementary Methods.

**Electron microscopy and electron tomography**

Organs of Corti (P14) were explanted in HEPES-Hanks solution and incubated for 1 min 45 s in pre-warmed Ca^2+ free saline (5 mM K^+; 0 Ca^2+ and 5 mM EGTA; inhibitory condition) or in a solution containing 40 mM K^+ and 1.3 mM Ca^2+ (stimulatory condition). Samples were fixed and prepared as described (Wong et al., 2014) and detailed in Appendix Supplementary Methods. For random section analysis, electron micrographs were acquired with transmisión electron microscope (JEM-1011, 80 kV). For the 3D analysis, the semi-thin sections (250 nm) were imaged with JEM-2100 transmission electron microscope at 200 kV. Tomograms were reconstructed and analysed using IMOD package (bio3d.colorado.edu/ imod/) and ImageJ. For details, see Appendix Supplementary Methods.

**Molecular biology**

Quantitative PCR, subcloning of full-length otoferlin cDNA from mouse organs of Corti and mutagenesis thereafter are described in Appendix Supplementary Methods, as transfection of IHCs or HEK cells and mass spectrometric quantification of otoferlin protein levels.

**Statistics**

The data were analysed using Matlab (Mathworks), Microsoft Excel and Sigma Plot software. Averaged data are expressed as mean ± SEM unless otherwise specified. Normality was assessed using Jarque–Bera test for sample size of n ≥ 7 and by Kolmogorov–Smirnov test for n < 7. For normally distributed samples, statistic was tested using the unpaired, two-tailed Student’s t-test, others with Mann–Whitney U-test, unless stated otherwise. Statistical significance of fluence immunohistochemistry and EM analysis was calculated by one-way ANOVA followed by Tukey–Newman–Keuls test for parametric and Kruskal-Wallis test followed by non-parametric multiple comparisons (NPNC).

**Study approval**

Animal handling and experiments complied with national animal care guidelines and were approved by the University of Goettingen Board for animal welfare and the animal welfare office of the state of Lower Saxony.

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**Author contributions**

ER, NS and CW designed research; GH developed hard- and software; NS, RC, HA-M, ER, AM, TP, KTP and CL analysed data; NS, ER, RC, HA-M, AM, GY, CL, EA, TP and CW performed experiments; ER, NS, CW, RL, AM, HA-M, TP and NB wrote and edited the manuscript; NS, ER, HA-M and RC prepared figures; ER, CW, NS, HU, NB and RG-F supervised research; ER and NS managed and coordinated the study; and NS, ER, CW, HU, RG-F and NB acquired funding.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


Figure EV1. Partial central compensation of ABR wave I amplitude reduction, otoferlin protein levels during ageing and intact cochlear amplification.

A Amplitude growth of ABR waves II-IV with rising stimulus intensity to click stimulation in Otof\(^{+/+}\) (red, n = 5), Otof\(^{+/15T/15T}\) (blue, n = 6) and Otof\(^{+/+}\) (black, n = 5) animals tested at an age of 3–4 weeks, indicating a severe deficit of synchronous spiral ganglion neuron activation in Otof mutants but partial compensation in the auditory brainstem (mean ± SEM).

B Latencies of ABR waves I–IV from the same data set.

C Left: Interpolated DPOAE thresholds determined as the F1 intensity at which DPOAE intensity reached 10 dB SPL are comparable between Otof\(^{+/+}\) (red) and Otof\(^{+/+}\) (black) mice. Right: Amplitude growth of DPOAE in response to pairs of sine waves (frequencies indicated above each panel) at rising intensities. F1 intensity was always 10 dB above F2 intensity.

D Otoferlin protein levels were even further reduced during ageing in Otof\(^{+/+}\)/I\(^{15T/15T}\) IHCs, explaining the age-dependent decrease in ABR amplitudes. Numbers indicate number of IHCs analysed (Tukey–Newman–Keuls test for parametric multiple comparisons; ***P < 0.001).

E Representative images used for synapse counting in Otof\(^{+/+}\) (top) and Otof\(^{+/+}\) (bottom) IHCs. For 2- to 3-week-old mice (left), z-projections of confocal stacks stained for ribbons (CtBP2, red) and postsynaptic glutamate receptors (GluR2/3, green) are shown. For 9- to 16-week-old mice (right), single sections of IHCs imaged in z-stacks, stained for ribbons (CtBP2, red), the cytoplasmatic calcium buffer parvalbumin (green) and SGNs stained for Na/K-ATPase (blue) are shown.

F Quantification of synapses; numbers indicate the number of IHCs analysed; mean ± SEM.
Figure EV2. Single SGN responses show normal frequency tuning and thresholds, but abnormalities of latencies and spike rates.

A Representative examples of SGN tuning curves from Otof*515T/515T (pink/red) and Otof+/+ (grey/black) mice.

B Thresholds at CF were comparable in Otof*515T/515T (red) and Otof+/+ (black) SGNs.

C The median first spike latency (FSL) in response to 200 repetitions of tone bursts at CF, 30 dB above threshold at a stimulus rate of 5 Hz was significantly prolonged in Otof*515T/515T (red closed circles) and Otof+/+ (black closed circles) \( (P = 0.0003) \), t-test). However, when the expected median FSL for the respective onset spike rate was subtracted (open symbols), FSLs were normal. The expected median FSL was calculated by the following formula which was derived from a large population of wild-type SGNs: \(-2.5 \times \log(\text{onset rate}) + 20.23\). All FSL estimates were first corrected for the system delay (3.6 ms), for the time of the 4 ms tone burst ramp to reach fibre threshold and for the travelling wave delay (up to 0.8 ms according to our wild-type data set).

D Spike rate increases in individual Otof*515T/515T (red) SGNs in response to tone burst stimulation at CF and varying intensities were shallower than in Otof+/+ (black) SGNs. The sound intensity refers to the threshold of each SGN, defined by a spike rate increase of 20 Hz over spontaneous rate.

E Their dynamic range (the range of intensities over which the spike rate increased from 10% to 90% of the evoked rate) was normal.

F Spike rate increases in individual Otof*515T/515T (red) SGNs in response to continuous presentation of amplitude-modulated sounds (CF, varying intensities, referenced to threshold like in D, modulation frequency 500 Hz) were much shallower than in Otof+/+ (black) SGNs.
Figure EV3. Hearing impairment was assessed by startle responses and operant conditioning of mice.

A, B Acoustic startle responses to stimulation with 10 ms noise (A) or 12 kHz tone (B) bursts in Otof$^{I515T/I515T}$ (10 individuals pink, mean ± SEM red) and Otof$^{+/+}$ (9 individuals grey, mean ± SEM black) animals backcrossed to a CBA/J strain background.

C Prepulse inhibition of the startle response induced by silent gaps of varying duration was stronger in wild-type (eight individuals grey, mean ± SEM black) than in Otof$^{I515T/I515T}$ mice (eight individuals pink, mean ± SEM red). Gap detection thresholds were determined as the minimal duration of silent gaps in 70 dB background noise that were effective in significantly attenuating the amplitude of startle response elicited by 115 dB 12 kHz tone bursts (Mann–Whitney U-test followed by Bonferroni correction). Gap thresholds (squares) were elevated in Otof$^{I515T/I515T}$ mice (P = 0.04, Mann–Whitney U-test). In the control condition (no gap), the mean median startle amplitude for Otof$^{I515T/I515T}$ (0.07 ± 0.02) was identical to Otof$^{+/+}$ (0.07 ± 0.02; P = 0.99, t-test).

D Two Otof$^{I515T/I515T}$ mice (red) and one Otof$^{+/+}$ mouse (black) learned to avoid drinking when they heard 12 kHz tone burst stimuli (400 ms, 3 Hz stimulus rate, 80 dB) during their visits to the soundproof corner of the "IntelliCage" operant conditioning system and drank water only when they did not perceive sounds. All three mice reacted to stimuli at 20 and 30 dB like for silence and for stimuli above 30 dB like for 80 dB, indicating comparable hearing thresholds near 35 dB for all three mice at the age of 14–21 weeks.

E Subjective hearing thresholds in the same mice from (D) increased by approximately 5–15 dB when the mice were retested at an age of 42–53 weeks.

F Behavioural gap detection did not show clear age-related changes in a wild-type mouse tested at 15–23 and 40–55 weeks.
**Figure EV4. Sequence variations in mouse and human otoferlin and comparison with other species.**

A CLUSTAL Omega multiple sequence alignment for otoferlin homologues performed with the following sequences: Human otoferlin isoform e, NP_001274418.1; *Bos Taurus*, NP_001137579.1; *Rattus norvegicus*, NP_001263649; *Mus musculus* transcript variant 4, NM_001313767.1 (cDNA used in our experiments); *Mus musculus* transcript variant 1, NP_001093865.1; *Mus musculus* transcript variant 2, NP_114081.2; *Xenopus tropicalis*, XP_012826771.1. The presence of the 15 amino acid stretch SKG...GEH was tested in a PCR in (C).

B Sequence alignment, also including human otoferlin isoform b, NP_004793. Focus on the amino acid region between C2D and C2E bearing an arginine-rich sequence in human otoferlin isoform e, including an RXR motif (in red). Labelled in yellow is the human sequence that was introduced by site-directed mutagenesis into the mouse variant 4 for experiments in Fig 5.

C A PCR on mouse organ of Corti cDNA from P14 animals was performed to test for the expression of the mouse sequence variations. Left lane: using the primers AAGGACAGCCAGGAGACAGA and ATCTTGTCTTTGGGGCTCCT that bind before and after amino acid 168 in mouse variant 4 was used to distinguish between splice variants. For transcript variants 2 and 4, an amplicon of 155 bp was expected, whereas variant 1 should give rise to a 200-bp amplicon. The PCR indicates that almost exclusively the short variant is transcribed. Right lane: using primers TCATCTACCGACCTCCAGACC and CACATCCACCTTGACCACAGC binding before and after amino acid 1242 in mouse variant 4 expected to lead to an amplicon size of 148 bp for variants 1 and 4 or 208 bp for variant 2. The strong band at 148 bp indicates that the vast majority of cDNA molecules confirmed the expression of the short variant. In conclusion, our data indicate that transcript variant 4 seems to be the predominantly transcribed otoferlin isoform in mouse organs of Corti.

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