Topographic map refinement and synaptic strengthening of a sound localization circuit require spontaneous peripheral activity

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Key points

- Loss of the calcium sensor otoferlin disrupts neurotransmission from inner hair cells. Central auditory nuclei are functionally denervated in otoferlin knockout mice (Otof KOs) via gene ablation confined to the periphery.
- We employed juvenile and young adult Otof KO mice (postnatal days (P)10–12 and P27–49) as a model for lacking spontaneous activity and deafness, respectively. We studied the impact of peripheral activity on synaptic refinement in the sound localization circuit from the medial nucleus of the trapezoid body (MNTB) to the lateral superior olive (LSO).
- MNTB in vivo recordings demonstrated drastically reduced spontaneous spiking and deafness in Otof KOs.
- Juvenile KOs showed impaired synapse elimination and strengthening, manifested by broader MNTB–LSO inputs, imprecise MNTB–LSO topography and weaker MNTB–LSO fibres. The impairments persisted into young adulthood. Further functional refinement after hearing onset was undetected in young adult wild-types.
- Collectively, activity deprivation confined to peripheral protein loss impairs functional MNTB–LSO refinement during a critical prehearing period.

Abstract

Circuit refinement is critical for the developing sound localization pathways in the auditory brainstem. In prehearing mice (hearing onset around postnatal day (P) 12), spontaneous activity propagates from the periphery to central auditory nuclei. At the glycinergic projection from the medial nucleus of the trapezoid body (MNTB) to the lateral superior olive (LSO) of neonatal mice, super-numerous MNTB fibres innervate a given LSO neuron. Between P4 and P9, MNTB fibres are functionally eliminated, whereas the remaining fibres are strengthened. Little is

Nicolas Müller received an MSc in Cell and Neurobiology and recently finished his PhD thesis in the lab of Eckhard Friauf (University of Kaiserslautern). In his PhD thesis, he studied the developmental circuit refinement and synaptic performance of the MNTB–LSO projection upon peripheral and central manipulations of the auditory system. By doing so, he gained mechanistic insight into the maturation of inhibitory synapses. Nicolas’s next project will be to study the auditory cortex using in vivo two-photon calcium imaging in the lab of Jan Hirtz (University of Kaiserslautern)
known about MNTB–LSO circuit refinement after P20. Moreover, MNTB–LSO refinement upon activity deprivation confined to the periphery is largely unexplored. This leaves a considerable knowledge gap, as deprivation often occurs in patients with congenital deafness, e.g. upon mutations in the otoferlin gene (OTOF). Here, we analysed juvenile (P10–12) and young adult (P27–49) otoferlin knockout (Otof KO) mice with respect to MNTB–LSO refinement. MNTB in vivo recordings revealed drastically reduced spontaneous activity and deafness in knockouts (KOs), confirming deprivation. As RNA sequencing revealed Otof absence in the MNTB and LSO of wild-types, Otof loss in KOs is specific to the periphery. Functional denervation impaired MNTB–LSO synapse elimination and strengthening, which was assessed by glutamate uncaging and electrical stimulation. Impaired elimination led to imprecise MNTB–LSO topography. Impaired strengthening was associated with lower quantal content per MNTB fibre. In young adult KOs, the MNTB–LSO circuit remained unrefined. Further functional refinement after P12 appeared absent in wild-types. Collectively, we provide novel insights into functional MNTB–LSO circuit maturation governed by a cochlea-specific protein. The central malfunctions in Otof KOs may have implications for patients with sensorineural hearing loss.

Introduction

Hereditary hearing impairment (HI) is the most frequent sensorineural deficit in humans, with an incidence of ∼1 in 2000 newborns (Vohr, 2018). HI is linked to mutations in >120 genes (http://hereditaryhearingloss.org). HI due to mutations in the otoferlin-encoding gene OTOF is over-represented with an occurrence of ∼3–7% (Choi et al. 2009; Duman et al. 2011; Iwasa et al. 2013). Therefore, OTOF mutations (causative for non-syndromic autosomal recessive hearing loss DFNB9; Yasunaga et al. 1999) are of high clinical relevance. Otoferlin occurs sparsely in the brain, yet it is highly abundant in the inner ear, e.g. in cochlear inner hair cells (IHCs; Schug et al. 2006; Pangrscic et al. 2012; Ranum et al. 2019). Instead of synaptotagmin I and II, which mediate transmitter release from CNS synapses, IHCs employ otoferlin as a Ca$^{2+}$ sensor, which is crucial for vesicle exocytosis from P4 onwards (Roux et al. 2006; Beurg et al. 2009; Heidrych et al. 2009; Michalski et al. 2017). Otoferlin is also involved in vesicle replenishment and re-formation (Pangrscic et al. 2010; Duncker et al. 2013; Vogl et al. 2015, 2016; Michalski et al. 2017) and interacts with key synaptic proteins (Ramakrishnan et al. 2009, 2014; Hams et al. 2017). Collectively, otoferlin plays pivotal roles in transmitter release from IHCs. Thus, humans with OTOF mutations (Petersen & Willems, 2006; Mahdieh et al. 2012), as well as mutant Otof mice (Roux et al. 2006; Longo-Guess et al. 2007), generally suffer from defective transmission at IHC–auditory nerve fibre synapses (auditory synaptopathy; Starr & Rance, 2015; Moser & Starr, 2016). Auditory synaptopathy occurs in Otof$^{deaf5/deaf5}$ mice, which are profoundly deaf as they lack auditory brainstem responses up to 100 dB sound pressure level (SPL) (Wilson et al. 2005; Longo-Guess et al. 2007; Wright et al. 2014). The non-functionality of otoferlin is due to a missense point mutation in the gene part encoding the C2B domain, one of six C2 domains in the 230 kDa protein. Synaptic vesicle release from IHCs is reduced by ∼90% (Heidrych et al. 2009). Similar OTOF point mutations in regions encoding for the C2 domains of otoferlin occur in humans (Migliosi et al. 2002; Mirghomizadeh et al. 2002; Tekin et al. 2005).

Mice are sensory altricial and physiologically deaf until ~P12, when hearing onset occurs ( Mikaelian & Ruben, 1965; Ehret, 1976, 1983). Remarkably, spontaneous activity propagates through the auditory system in pre-hearing animals (Tritsch et al. 2007; Sonntag et al. 2009; Babola et al. 2018), and the feature is highly conserved across species (Wang & Bergles, 2015). The spontaneous activity arises from immature IHCs, which fire Ca$^{2+}$ action potentials and release glutamate (Kros et al. 1998; Tritsch et al. 2010; Johnson et al. 2012; Sendin et al. 2014; Eckrich et al. 2018). This results in action potential activity in spiral ganglion neurons and in excitation of auditory brainstem nuclei. The excitation occurs in a conserved temporal pattern (Tritsch et al. 2010), implying that IHCs generate pace-making activity. Spatially restricted and synchronous spontaneous activity provided by IHCs may be an instructive cue for the development of precise topographic maps in the auditory system (Friauf & Lohmann, 1999; Kandler et al. 2009; Leighton & Lohmann, 2016). Auditory synaptopathy in Otof$^{deaf5/deaf5}$ mice likely drastically reduces, or even abolishes, spontaneous activity and hence activity propagation from the periphery to central targets.

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In the mammalian auditory brainstem, a glycinergic projection from the MNTB to the LSO (Fischer et al. 2019) is involved in sound source localization by detecting interaural level differences (ILDs) upon precise integration of cochlear nucleus-mediated excitation and MNTB-mediated inhibition (Tollin, 2003; Yin & Kuwada, 2010; Beiderbeck et al. 2018; Friauf et al. 2019). The MNTB–LSO circuit is functionally refined during the first two postnatal weeks in rats and mice (Kim & Kandler, 2003; Hirtz et al. 2012; Clause et al. 2014), and refinement coincides with the presence of spontaneous prehearing activity. Initially, MNTB axons form exuberant and topographically imprecise synapses in the developing LSO (Sanes & Siverls, 1991). Between P4 and P8, exuberant synapses are eliminated, thereby increasing topographic precision (Sanes & Friauf, 2000; Kim & Kandler, 2003; Hirtz et al. 2012). In parallel, the remaining axons are strengthened between P5 and P9 (Kim & Kandler, 2003; Hirtz et al. 2012; Clause et al. 2014). We know very little about the impact of disturbed peripheral activity on central auditory circuit maturation, in particular in Otof mutations. This leaves us with a considerable knowledge gap of clinical relevance, as OTOF mutations frequently cause deafness in humans. Furthermore, virtually nothing is known about functional MNTB–LSO refinement after P20 (Walcher et al. 2011) both in normal development and upon deafness. Thus, by employing Otof\textsuperscript{deaf5/deaf5} mice, the aims of this study were twofold: (1) to examine the impact of peripheral activity on central MNTB–LSO circuit refinement during the prehearing period (P10–12), and (2) to investigate functional circuit refinement after hearing onset at a hitherto unexplored young adult stage (P27–49).

Via extracellular MNTB in vivo recordings, we verified drastically reduced spontaneous and acoustically evoked spike rates in Otof KOs. RNA sequencing demonstrated an absence of Otof transcripts in the MNTB and the LSO. Thus, Otof KOos are an elegant model to study the confined impact of disrupted spontaneous and acoustically evoked activity from the periphery on central circuit refinement without on-site protein loss, which is often a drawback in deafness models (Michalski & Petit, 2019). Reduced peripheral activity as a result of cochlea-specific Otof loss led to an imprecise MNTB–LSO topography due to impaired synaptic elimination in the MNTB–LSO circuit. MNTB single fibre strength was also weaker. Remarkably, P10–12 and P27–49 Otof KOos had a similar number of converging MNTB–LSO fibres and similar synaptic strength, arguing against delayed refinement in Otof KOos. Further functional MNTB–LSO refinement appeared absent in wild-types (WTs) after hearing onset. Our results provide novel insight into functional MNTB–LSO circuit maturation and are of potential medical relevance for the therapy of patients with non-functional ototoerin or other mutations leading to sensorineural HI.

### Methods

#### Animals and ethical approval

Animal breeding and experiments were approved by the regional councils of Rhineland-Palatinate and Saxony (TVV 66/13) according to the German Animal Protection Law (TschG §4/3) and followed the guidelines for the welfare of laboratory animals. Moreover, the authors understand and conform to the principles and regulations described in The Journal of Physiology (Grundy, 2015). Experiments were performed on Otof\textsuperscript{deaf5/deaf5} mice (Longo-Guess et al. 2007) and age-matched controls (Otof\textsuperscript{+/+} mice) of both sexes. Otof\textsuperscript{deaf5/deaf5} mice are designated Otof KOos in the text body and KOos in figures. Otof\textsuperscript{+/+} mice are designated WTos. Mice were genotyped as described (Longo-Guess et al. 2007).

#### In vitro electrophysiology

Coronal brainstem slices of P10–12 (labelled P11) mice were prepared as described (Hirtz et al. 2011; Kramer et al. 2014). To prepare viable brainstem slices from young adult (P31–49, labelled P38) animals, we adopted a protocol from Ting and colleagues (2014). Mice were injected with a lethal dose of ketamine hydrochloride (10 mg per animal), decapitated, and brains rapidly dissected in ice-cold preparation solution containing (in mM): 93 N-methyl-DL-glucamine, 93 HCl, 30 NaHCO\textsubscript{3}, 25 glucose, 20 HEPE\textsubscript{S}, 10 MgCl\textsubscript{2}, 3 L-ascorbic acid, 3 myo-inositol, 3 sodium pyruvate, 2.5 KCl, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 0.5 CaCl\textsubscript{2} (pH 7.4 when bubbled with carbogen; 300 ± 10 mOsm l\textsuperscript{-1}). Coronal brainstem slices, 270 µm thick, were cut in ice-cold preparation solution with a VT1200S vibratome (Leica Microsystems, Wetzlar, Germany) and transferred into artificial cerebral spinal fluid (ACSF) at room temperature containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl\textsubscript{2}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 2 sodium pyruvate, 3 myo-inositol, 0.44 l-ascorbic acid, 25 NaHCO\textsubscript{3}, 10 D-glucose, 2 CaCl\textsubscript{2} (pH 7.4 when bubbled with carbogen; 295 ± 5 mOsm l\textsuperscript{-1}). After 0.5–5 h, slices were transferred to the recording chamber, which was mounted on an upright microscope (Eclipse E600N, Nikon, Tokyo, Japan) equipped with infrared-differential interference contrast (DIC) optics (Nikon 4× CFI Achromat, 0.1×; 60× CFI Fluor W, 1.00 W ×) and a VX 44 CCD camera (PCO computer optics; PCO AG, Kelheim, Germany). Patch pipettes were pulled from glass capillaries (GB150(F)-8P, Science Products, Hofheim am Taunus, Germany) with a P-87 horizontal puller (Sutter Instruments, Novato, CA, USA). The resistance ranged from 2 to 6 MΩ when filled with internal solution containing (in mM):

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Gradual recruitment of MNTB fibres by electrical stimulation

A stimulation electrode (glass capillary, ~10 μm tip diameter) was connected to a stimulus isolator (STG 4002, Multi-Channel Systems, Reutlingen, Germany) and placed at the lateral edge of the MNTB. To stimulate MNTB fibres in a stepwise manner, the stimulation intensity was increased in small increments. Between 5 and 300 μA, increments were 5 μA, and 10 stimuli (100 μs monopolar pulses) were applied at each intensity. Above 300 μA, increments were 10 μA and five stimuli were applied. Synaptic depression was avoided by a low stimulation frequency of 0.5 Hz.

Paired-pulse experiments

Paired-pulse experiments were performed by stimulating MNTB fibres at several interstimulus intervals (ISIs; 10, 20, 50, 100, 500, 1000 ms). At each ISI, 10 repetitions (0.2 Hz) were performed and the paired-pulse ratio was calculated by dividing the peak amplitude of the second evoked inhibitory postsynaptic current (eIPSC) by the peak amplitude of the first eIPSC. In order to test for GABA\(_B\) receptor contribution in paired-pulse ratio experiments, 3 μM CGP55845 (CGRP; (2S)-3-[[((1S)-1-((3,4-dichlorophenyl)ethyl)amino-2-hydroxypropyl][phenyl methyl]phosphinic acid hydrochloride; Abcam, Cambridge, UK) was bath-applied in some P11 experiments.

Analysis of in vitro data

To allow reconstruction of the MNTB and LSO contours after mapping experiments, DIC images were taken at low magnification (4× objective). Contour reconstruction and determination of mediolateral and dorsoventral input widths were performed with CorelDraw X6 (Corel GmbH, München, Germany). The number of effective spots was determined by counting the number of sites in the fine raster from which a postsynaptic current could be elicited. An input cluster was classified as a continuum of effective spots (see Fig. 3De).

Peak amplitudes of eIPSCs were analysed with custom-written routines (Dr Alexander Fischer, Univ. Kaiserslautern) for IGOR Pro (WaveMetrics Inc., Lake Oswego, OR, USA) running Patcher’s Power Tools (Max Planck-Institute for Membrane Biophysics). Amplitude data were fed into a custom-written MatLab (MathWorks, Natick, MA, USA) routine (Dr Dennis Weingarten, Univ. Kaiserslautern) employing a k-Means-based cluster analysis and the determination of the silhouette value for each amplitude data point for the respective cluster. k-Means analysis assigns centroids to the dataset and applies an iterative optimization algorithm to minimize the error between centroids and data points. A higher cluster number thus reduces the error, but this alone does not warrant determination of the correct cluster number. A range of likely clusters is indicated when the error flattens as a function of cluster (see Fig. 5Ac). Those clusters at which errors flattened were further investigated using silhouette values. The silhouette value assigns a value to each amplitude data point, depending on how likely the data point fits into the determined cluster. Based on the mean silhouette values, we estimated the most likely cluster number (± number of converging MNTB fibres). For a detailed explanation of the approach and the algorithm, see https://de.mathworks.com/help/stats/k-means-clustering.html. Single fibre strength was calculated as the difference between subsequent centroids (centroid\(_{n+1}\) − centroid\(_n\)). Maximal amplitudes were determined by averaging eIPSCs in the plateau phase, i.e. their amplitude at which further increases of the stimulation intensity did not recruit further MNTB fibres. The fibre fraction was calculated by dividing the single fibre strength by the maximal amplitude. Amplitudes and kinetics (10–90% rise time, 100–37% decay time) of spontaneous inhibitory postsynaptic currents (sIPSCs) were analysed using Mini Analysis (Synaptosoft Inc., Decatur, GA, USA). To determine the quantal size \(q\), we performed a Gaussian fit on the binned amplitude distribution of sIPSCs \((n = 100\) sIPSCs, bin size 5 pA) ranging from the 0 pA bin to the bin after the first maximum (Krächan et al. 2017). By doing so, we avoided errors in calculating \(q\) when multiple quanta were spontaneously released (see Fig. 6Ab). Secondary peaks
that are not quantal multiples of the first peak are most likely due to the difference in synapse location with respect to the recording electrode (Silver, 2003). Gaussian fits were incorporated in Excel spreadsheets (Microsoft, Redmond, WA, USA) and goodness of fits were determined by least-squares fitting (Kemmer & Keller, 2010). The quantal content of every fibre (vesicles/fibre) was calculated by dividing the individual single fibre strength (pA/fibre) by \( q \) (pA/vesicle).

The number of release sites (\( N \)) was estimated as described (Wen et al. 2016a). Briefly, we employed a multinomial model in which we inserted the mean fibre amplitude (\( I_{\text{mean}} \)), the coefficient of variance of the mean fibre amplitude (\( CV_q \)), \( q \), and the coefficient of variance of \( q \) (\( CV_q \)), assuming uniform release probability across release sites:

\[
N = \frac{1}{\frac{q}{I_{\text{mean}}} - CV_q} \quad (1)
\]

In some cases (10.4% \( \pm \) 15/143 at P11; 9.5% \( \pm \) 15/157 at P38), the release site estimation yielded a negative value; such cases were excluded from further analysis. The release site release probability (\( p_r \)) was calculated by insertion of the estimated \( N \) in a binomial model:

\[
I_{\text{mean}} = \frac{N \times q}{p_r} \quad (2)
\]

**In vitro** electrophysiology and analysis were performed blind to the genotype.

**In vivo** electrophysiology and analysis

Experiments were performed on three WT and four KO mice at P27–32 (labelled P30) and on three KO mice at P11, P12 and P15 (labelled P11). Only the P11 mouse showed spike activity. To minimize the usage of laboratory mice according to the 3Rs guidelines, we reused a previously recorded dataset of P10–12 WT mice (C57BL/6 strain) from the same experimenter (Sonntag et al. 2015). KO mice were generated on a similar background (mixed C57BL/6, CH3 strain; Longo-Guess et al. 2017), allowing comparison of both datasets. Mice were deeply anaesthetized by intraperitoneal injections of ketamine hydrochloride (0.1 mg g\(^{-1}\); Ratiopharm, Ulm, Germany) and xylazine hydrochloride (0.005 mg g\(^{-1}\); Bayer, Leverkusen, Germany). They were fixed in a 

**RNA sequencing**

Coronal brainstem cryosections (30 \( \mu \)m thick) from P7, P11–12 (labelled P11) and P60 mice were placed on PEN membrane glass slides (Leica Microsystems) and dehydrated twice in ascending EtOH (75%, 95%, 100%, 1 min each). LSO and MNTB tissues were collected using a LMD6500/DM6000B laser microdissection system (Leica Microsystems). RNA was extracted with an Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality control and cDNA synthesis were done as described (Picelli et al. 2013). Libraries were prepared with Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced 1 \( \times \) 100 bp on a HiSeq 2500 (Illumina). Reads were trimmed for adapter sequences and low quality (phred score <20) with Trim Galore! (v0.4.2; http://www.bioinformatics.babraham.ac.uk/projects/trim_galore),
aligned to the mm10 mouse reference genome using two-step STAR \((v)2.5.2a\) alignment (Dobin & Gingeras, 2015) and marked for PCR duplicates with Picard tools (MarkDuplicate v1.115; http://broadinstitute.github.io/picard/). Gene-wise read counts were summarized with featureCounts (Liao et al. 2014) using Gencode annotation vM2 and normalized as counts per million (CPM). CPM \(\geq 0.5\) was used as a threshold for expressed genes. Normalization as reads per kilobase per million (RPKM) allowed comparison of expression levels. Gene, transcript and protein nomenclature is based on the Mouse Genome Informatics (MGI) guidelines.

### Statistics

Sample data are presented as mean ± standard error of the mean (SEM) or as box plots showing the median, 25–75% interquartile range, 0–25% and 75–100% whisker range. Statistical analysis was performed with Origin Pro 8.6 (OriginLab, Northampton, MA, USA). Normally distributed samples (Kolmogorov–Smirnov test) were compared in paired or unpaired two-tailed \(t\) tests. A homo- or heteroskedastic \(t\) test was performed based on the equality of variances determined by an equality of variances determined by an \(F\) test. If data were not normally distributed, a Mann–Whitney \(U\) test was applied. Significance levels are: \(* P < 0.05\), \(** P < 0.01\), \(*** P < 0.001\); n.s. is \(P \geq 0.05\). In the case of genotype (WT vs. KO) and developmental comparisons (P11 vs. P38), critical \(\alpha\) values were post hoc Sidák corrected (Abdi, 2007). Significance levels were then \(* P < 0.025\), \(** P < 0.005\) and \(*** P < 0.0005\). No statistical analysis of the spontaneous \(vivo\) MNTB spike rate was done at P11 due to a low \(n\) of 3 in the KO group (see Fig. 1C).

### Results

#### Reduced spontaneous MNTB spike rates in \(Otof\) KOs \(vivo\)

In prehearing rodents, spontaneous activity originates in the cochlea from where it is conveyed downstream auditory nuclei in a temporally conserved manner (Trisch et al. 2010; Babola et al. 2018). Because transmitter release from IHCs is decreased by \(\sim 90\%\) in \(Otof\) KOs (Heidrych et al. 2009), we expected strongly reduced spontaneous activity in auditory brainstem nuclei (Fig. 1A). To test the hypothesis, we performed single unit \(\text{in vivo}\) recordings in the MNTB at P11 and P30 (Fig. 1B). The recording probability (see Methods) was very low in P11 KOs (WT: 55 ± 2\%, \(n = 3\) animals; KO: 14 ± 11\%, \(n = 3\) animals), potentially because large parts of the MNTB were silent, as observed in cochlea-ablated rats and mice (Trisch et al. 2010; Babola et al. 2018). Spontaneous spike activity occurred only in one of three P11 KOs. In this KO, the spontaneous MNTB spike rate was reduced (Fig. 1C; WT: 1.3–107 spikes s\(^{-1}\); median: 18.9 spikes s\(^{-1}\), \(n = 34\) units/3 animals; KO: 0.7–2.1 spikes s\(^{-1}\), median: 1.4 spikes s\(^{-1}\), \(n = 3\) units/1 animal). At P30, the spontaneous spike rate was again reduced in the KOs (Fig. 1C; WT: 0.5–81 spikes s\(^{-1}\), median 25.4 spikes s\(^{-1}\), \(n = 26\) units/3 animals; KO: 0.5–11.6 spikes s\(^{-1}\), median: 7.4 spikes s\(^{-1}\), \(n = 16\) units/4 animals, \(P = 1.9 \times 10^{-3}\), unpaired \(t\) test). Similar to P11, the recording probability was lower for P30 KOs than for P30 WTs (WT: 84 ± 4\%, \(n = 3\) animals; KO: 36 ± 6\%, \(n = 4\) animals), again presumably due to parts of the MNTB being silent. Taken together, MNTB units of \(Otof\) KOs showed a strongly reduced spontaneous spike rate at both prehearing and posthearing age.

Next, we combined acoustic stimulation with \(\text{in vivo}\) MNTB recordings in P30 WTs and \(Otof\) KOs to assess sound-driven activity. Acoustic stimulation of an exemplary WT unit yielded a frequency tuning curve with a sharp peak at \(\sim 19\) kHz and an absolute threshold of \(\sim 0\) dB\(\text{SPL}\) (Fig. 1Da). A maximal spike rate of \(\sim 300\) spikes s\(^{-1}\) was reached \(\sim 10\) dB above threshold.

At higher intensities, maximal spiking was observed over a broader frequency range, for example between \(\sim 8\) and \(20\) kHz at 80 dB\(\text{SPL}\) (Fig. 1Da). Overall, a typical frequency tuning curve (Sonntag et al. 2009) was present, consistent with recordings from other WT MNTB units. In contrast, three \(Otof\) KO examples showed no tuning to specific frequencies even at 90 dB\(\text{SPL}\) and a lack of sound-evoked spiking (Fig. 1Db–d). This observation was made in all recordings from MNTB units in \(Otof\) KOs. Consequently, the maximal sound-evoked spike rate was reduced by \(\sim 95\%\) in KOs (Fig. 1E; WT median: 318 spikes s\(^{-1}\), \(n = 23\) units/3 animals; KO median: 25 spikes s\(^{-1}\), \(n = 12\) units/4 animals, \(P = 2.6 \times 10^{-18}\), unpaired \(t\) test). Taken together, the MNTB \(\text{in vivo}\) recordings demonstrate deafness in \(Otof\) KOs and corroborate previous findings of deafness in \(Otof\) KOs obtained from auditory brainstem responses (Longo-Guess et al. 2007). This clearly establishes \(Otof\) KOs as a genetic deafness model.

### No \(Otof\) transcripts in MNTB and LSO

Vglut3 and Ca\(_{2+}\)1.3 are two key players mediating vesicle release from cochlear IHCs (Platzer et al. 2000; Seal et al. 2008). Both proteins are also present in the auditory brainstem, including the MNTB–LSO circuit (Noh et al. 2010; Hirtz et al. 2012; Michalski & Petit, 2019). The Noh and Hirtz studies have employed systemic knockout mice to analyse MNTB–LSO circuit refinement. A dilemma of these studies is that they cannot pinpoint the defects to peripheral protein loss, or to central (on-site) protein loss in the MNTB–LSO circuit, or to a combination of both. To assess whether otoferlin is restricted to the cochlea, we checked for gene expression in MNTB and LSO and sequenced the mRNA of laser-dissected MNTB and LSO tissue (Fig. 2A). In P7, P11 and P60 WTs, RPKM values for \(Otof\) transcripts were below detection threshold (Fig. 2B).
Figure 1. Drastically reduced in vivo MNTB spike rate in Otof KOs

A, schematic overview of auditory circuitry in WT (black) and KO (red). Crosses indicate putative functionally denervated circuitry. HF, high frequency; LF, low frequency. B, original traces from extracellular MNTB in vivo recordings from a P11 WT (Ba), a P11 KO (Bb), a P30 WT (Bc) and a P30 KO mouse (Bd). Grey boxes indicate time-magnified close-ups depicted on the right of the respective trace. Note complex waveform consisting of a prepotential (pre, generated by the calyx of Held) and a postpotential (post, generated by the MNTB neuron).

C, quantification of the spontaneous spike rate. D, exemplary frequency tuning curves upon acoustic stimulation of a P30 WT (Da) and three units from P30 Otof KOs (Db–d). Spike rates are colour-coded, warmer colours indicate higher rates. Note virtual absence of sound-evoked spike activity in KOs. E, quantification of maximal spike rate. SPL, sound pressure level. [Colour figure can be viewed at wileyonlinelibrary.com]
and C) in both nuclei (MNTB: P7, 0.05 ± 0.04, n = 3; P11, 0 ± 0, n = 4; P60, 0.05 ± 0.03, n = 4; LSO: P7, 0.07 ± 0.06, n = 2; P11, 0.21 ± 0.2, n = 2; P60, 0.02 ± 0.01, n = 4).

For positive control and proof of concept, we determined the abundance of typical MNTB and LSO transcripts (Gapdh, GlyT2, Syt2, Pvalb, Kcnal1; cf. Ehmann et al. 2013; Bouhours et al. 2017). These transcripts showed moderate to robust levels, with RPKM values ranging from 21 ± 3 to 2451 ± 31 (Fig. 2B and C). To validate the quantification sensitivity, we checked for the developmental upregulation of Pvalb mRNA in auditory brainstem nuclei (Lohmann & Friauf, 1996). Indeed, Pvalb transcript levels were higher in the MNTB and LSO of adult mice (Fig. 2B and C). Taken together, the results imply that otoferlin is not present in the MNTB and LSO. Thus, Otof KOs are very suitable for studying the impact of strongly reduced spontaneous peripheral activity in isolation, without accompanying on-site protein loss in the auditory brainstem.

**Broader synaptic input width in Otof KO s**

To test the impact of reduced spontaneous activity on circuit refinement of the MNTB–LSO projection, we next determined MNTB input maps at P11 using glutamate uncaging. At this age, functional elimination in the MNTB–LSO projection has ceased for at least 2 days (Kim & Kandler, 2003). Initially, we determined the laser power required to elicit spikes in MNTB neurons. After

![Figure 2. No Otof transcripts in MNTB and LSO](image-url)

**Figure 2. No Otof transcripts in MNTB and LSO**

Aa, scheme of the experimental setup. Red dotted lines indicate laser-dissected MNTB and LSO tissue used for transcriptome analysis (see Methods). Ab, coronal Nissl-stained slice after laser-microdissection of the MNTB and LSO. B and C, RPKM values (reads per kilobase per million) of otoferlin transcripts and transcripts of five positive controls in the MNTB (B) and the LSO (C) at P7, P11 and P60. Note that RPKM values of otoferlin transcripts are below the 0.5 detection threshold in all biological replica (close-up diagram on the right). [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 3. Broader synaptic input width in P11 KOs
A, DIC image showing a patch-clamped MNTB neuron before (Aa) and during uncaging of 4-methoxy-7-nitroindolinyl (MNI) glutamate (Ab). Note similar size of laser spot and soma. Evoked potentials at 1.2 mW (light grey, subthreshold) and at 1.35 mW (dark grey, suprathreshold) laser power (Ac). The violet bar illustrates the laser activation (10 ms). Ba, DIC image of a slice containing MNTB and LSO, which are marked by white dotted lines. Red cross marks patch-clamped LSO neuron. d = dorsal, m = medial. Bb, MNTB reconstruction from the image in Ba. In the coarse raster, glutamate was uncaged in 20 µm steps over the whole extent of the MNTB, while synaptic connectivity to the LSO neuron was assessed for each uncaging spot. A laser pulse (10 ms, violet bar over original traces) evoked an eIPSC (red spot), confirming connectivity, or it failed to evoke a postsynaptic current (open circles). Synaptic currents are inward currents due to a high chloride concentration in the patch pipette (132 mM). Bc, same as Bb, but with a fine raster (10 µm) around the hot spot determined in the coarse raster (Bb). Ca, exemplary MNTB input maps of a WT and a KO. Cd, quantification of the normalized mediolateral and dorsoventral input width. Da and c, MNTB input maps depicting the number of effective spots (Da) and input clusters (Dc). Db and d, quantification of effective spots (Db) and input clusters (Dd). [Colour figure can be viewed at wileyonlinelibrary.com]
patching an MNTB neuron (Fig. 3Aa), light was focused directly onto the soma, and the laser power was gradually increased (Fig. 3Ab, note similar spot and soma size). This yielded a spike threshold of 1.35 mW (Fig. 3Ac). The sample threshold was 1.30 ± 0.1 mW (range 1.2–1.5 mW, n = 4 neurons/2 slices/2 mice, data not shown). Next, we determined the synaptic input width of the MNTB to a patch-clamped LSO neuron (Fig. 3Ba). We first uncaged glutamate in the whole MNTB with a coarse raster to identify hotspots and then applied a fine raster around these hotspots (Fig. 3Bb and c). Synaptic connectivity was confirmed by eIPSCs of short latency (Fig. 3Bb and c). Exemplary MNTB input maps for a WT and an Otof/KO are shown in Fig. 3Ca, demonstrating broader mediolateral and dorsoventral input widths in the KO. Sample data revealed a ~3-fold broader mediolateral input width (WT: 7.5 ± 1.5%, n = 16; KO: 20.5 ± 2.4%, n = 11, P = 9.6 × 10^{-5}, unpaired t test) and a ~2-fold broader dorsoventral input (Fig. 3Cb, WT: 19.4 ± 3.8%, n = 16; KO: 51.4 ± 6.9%, n = 11, P = 2.9 × 10^{-4}, unpaired t test). Accordingly, the number of effective spots was ~2.5-fold higher in KOs (Fig. 3Da and b; WT: 11.9 ± 2.3, n = 16; KO: 29.2 ± 6.4; n = 11, P = 0.03, unpaired t test), and ~60% more input clusters occurred (Fig. 3Dc and d; WT: 1.6 ± 0.2, n = 16; KO: 2.6 ± 0.2; n = 11, P = 0.04, unpaired t test). In a subset of mapping experiments, we assessed the reproducibility and repeated the mapping procedure at the same cell. Both runs revealed similar mediolateral input widths, dorsoventral input widths, and numbers of effective spots (data not shown; n = 8–9; P = 0.59; P = 0.28; P = 0.44; paired t tests), confirming reproducibility of the mapping experiments. Together, the findings from the mapping experiments strongly suggest that more MNTB neurons converge on a given LSO neuron in Otof KOs, implying impaired synaptic elimination.

**Imprecise topography of the MNTB–LSO projection in Otof KOs**

At P11, the MNTB–LSO projection is highly topographic (Sanes & Siverls, 1991; Kim & Kandler, 2003; Hirtz et al. 2012), thus providing the structural basis for precise sound frequency perception (Kandler et al. 2009). We reasoned that the impaired synaptic elimination (Fig. 3) reduces the precision of the MNTB–LSO map in Otof KOs. To address this hypothesis, we assigned 0% to the medial and 100% to the lateral border of reconstructed MNTBs and LSOs. This allowed us to determine the mediolateral percentile of the MNTB input centre and the position of the postsynaptic LSO cell (Fig. 4Aa and b). Plotting the location of the LSO somata against the MNTB input centre revealed a high linear relationship in WTs (Fig. 4Ba; Coefficient of determination R^{2} = 0.94, n = 13). KOs also showed a linear relationship, but data points were more scattered around the linear fit (Fig. 4Bb; R^{2} = 0.78, n = 10). To statistically quantify the scattering across our samples, we determined the residuals of the data points to the linear fits and found larger residuals in KOs (Fig. 4C; WT: 2.7 ± 0.4, n = 13; KO: 6.0 ± 1.1, n = 10, P = 0.02, unpaired t test). MNTB input locations did not differ (Fig. 4C; WT: 47.3 ± 2.8%, n = 13; KO: 50.6 ± 4.9%, n = 10, P = 0.56, unpaired t test), showing no sampling bias towards particular MNTB regions. The dataset thus supports the hypothesis that the impaired synaptic elimination in Otof KOs results in an imprecise topographic map.

**More MNTB fibres converge onto a given LSO neuron in Otof KOs at P11 and P38, yet have weaker strength**

Synaptic strengthening is a further hallmark of neuronal circuit development. Strengthening in the MNTB–LSO projection occurs between P5 and P9 (Kim & Kandler, 2003; Hirtz et al. 2012; Clause et al. 2014). To assess single fibre strength, we recorded from LSO neurons while activating MNTB fibres at gradually increasing stimulus intensity, allowing recruitment of individual fibres in a stepwise manner (Fig. 5Aa). We performed these experiments at P11 and P38 to investigate a potentially delayed circuit refinement in KOs. In addition, we addressed potential functional refinement in WTs after hearing onset. For this purpose, we recorded from LSO neurons up to P49, an advanced age at which MNTB–LSO refinement had not been investigated previously. Recruiting MNTB fibres with increasing stimulus intensity resulted in increasing eIPSC amplitudes (Fig. 5Ab). To determine the number of fibres and the single fibre strength, we employed a k-Means cluster analysis algorithm combined with a silhouette plot (Fig. 5Ac and d, see Methods). In P11 and P38 WTs, discrete eIPSC amplitude ‘jumps’ were the rule (Fig. 5Ba and b, Ca and b), whereas amplitude increases in strikingly smaller steps prevailed in age-matched KOs (Fig. 5Bc and d, Cc and d). Accordingly, we observed a ~1.4-fold higher number of converging MNTB fibres in P11 KOs (Fig. 5Da; WT: 3.9 ± 0.2, n = 16; KO: 5.5 ± 0.2, n = 15, P = 3.4 × 10^{-4}, unpaired t test), confirming the impaired elimination demonstrated in the mapping experiments. P38 KOs also displayed a higher number of converging MNTB fibres (~1.4-fold; WT: 3.9 ± 0.2, n = 20; KO: 5.4 ± 0.2, n = 15, P = 2.2 × 10^{-4}, unpaired t test). There was no age-related change in the MNTB fibre number between P11 and P38 in WTs and KOs (Fig. 5Da; P = 0.70, U test; P = 0.76, unpaired t test). These findings are interesting in two aspects. First, they indicate that synapse elimination in Otof KOs is not delayed. Second, they show that the MNTB–LSO circuit persists in the unrefined state following cochlea-specific Otoflloss. In addition, they show no further functional elimination from hearing onset to young adulthood in WTs.
The maximal eIPSC amplitudes did not differ significantly between KOs and WTs (Fig. 5Db; P11: WT, 1283 ± 140 pA, n = 16; KO, 1114 ± 123 pA, n = 15, P = 0.39; P38: WT, 1079 ± 115 pA, n = 16; KO 942 ± 130 pA, n = 15, P = 0.45, unpaired t tests). Furthermore, the maximal eIPSC amplitudes were unchanged throughout development for both WTs and KOs (WT: P = 0.27, KO: P = 0.36, unpaired t tests). With similar maximal strength, yet a higher MNTB fibre number in KOs, the single fibre strength is weaker in KOs. To verify this, we calculated the fibre fraction by dividing the individual fibre amplitude by the maximal amplitude. Indeed, the fibre fraction was reduced by ~30% (Fig. 5Dc and d) in P11 KOs (WT median: 24.2%; n = 60; KO median: 16.9%, n = 83, P = 6.5 × 10^{-4}, unpaired t test) and P38 KOs (WT median: 24.1%, n = 77; KO median: 16.7%, n = 80, P = 1.1 × 10^{-3}, U test). During development, the fibre fraction remained constant in WTs and KOs (P = 0.48, unpaired t test; P = 0.67, U test). Taken together, these results confirm the impaired synaptic elimination seen in the mapping experiments and demonstrate weaker single MNTB fibres in Otof KOs. Moreover, they show that further synaptic refinement is absent after hearing onset in WTs and that circuit refinement is not delayed in KOs.

**Lower quantal content accounts for weaker fibre strength in KOs**

In a further set of experiments, we addressed the synaptic mechanisms underlying reduced MNTB fibre strength. The reduced strength in Otof KOs may be due to a lower quantal content, a reduced release probability, and/or asynchrony in vesicle release. To assess the quantal content per fibre, we divided the single fibre strength by the quantal size, which we derived from a Gaussian fit over the sIPSC amplitude distribution (Fig. 6A; see Methods). At either age, q was not different between WTs and KOs (Fig. 6Ba; P11: WT, 21.6 ± 0.6 pA, n = 16; KO, 21.9 ± 0.5 pA, n = 15, P = 0.92; P38: WT, 21.7 ± 0.7 pA, n = 20; KO, 21.1 ± 1.0 pA, n = 15, P = 0.49, unpaired t tests). Furthermore, q remained constant throughout development in each genotype (P = 0.74, P = 0.65, unpaired t tests).

The sIPSC frequency did not differ between WT and KO, either at P11 or at P38 (Fig. 6Bb, Table 1). Moreover, it was unchanged during development (WT: P = 0.17, unpaired t test; KO: P = 0.07, unpaired t test). The rise and decay times of sIPSCs did not differ between genotypes at either age (Fig. 6Bc and d; Table 1). In each genotype, the rise time decreased with age (WT: P = 6.2 × 10^{-6}; KO: P = 2.1 × 10^{-3}, unpaired t tests) and the decay time became shorter (WT: P = 3.6 × 10^{-5}; KO: P = 6.0 × 10^{-5}, unpaired t tests).

In a next step, we compared single fibre eIPSC kinetics (Fig. 6C). Like sIPSCs, the kinetics did not differ between WT and KO at P11 and P38 (Table 1). They accelerated between P11 and P38 (Fig. 6Cc and d; rise time WT: P = 0.01; KO: P = 0.02; decay time WT: P = 6.8 × 10^{-6}; KO: P = 1.5 × 10^{-7}, unpaired t tests). Developmental acceleration of sIPSC as well as eIPSC kinetics after P11

![Figure 4. Imprecise topography of the MNTB–LSO circuit in P11 KOs](image-url)
Figure 5. Increased MNTB fibre number and weaker single fibre strength in KOs

Aa, scheme of experimental setup depicting stepwise recruitment of MNTB fibres. Ab, exemplary inhibitory postsynaptic currents (eIPSCs) evoked with increasing stimulation intensities. Note discrete amplitude jumps of eIPSCs, representing recruitment of an additional MNTB fibre. Ac, normalized error of k-Means cluster analysis for peak amplitudes of traces shown in Ab as a function of cluster number (1–15). Ad, silhouette plot reveals four clusters as the most likely cluster number for traces shown in Ab. Ba and c, exemplary eIPSCs of gradually recruited fibres from a P11 WT (black) and a KO cell (red). Stimulation artifacts were blanked; arrows indicate stimulus event. Bb and d, quantification of eIPSC peak amplitude as a function of stimulation intensity; same experiments as in Ba and c. Dotted line represent the strength of individual fibres. C, same as in B, but for P38 recordings. Da and b, quantification of the fibre number (Da) and the maximal amplitude (Db) in WTs and KOs at P11 and P38. Dc and d, quantification of fibre fraction. [Colour figure can be viewed at wileyonlinelibrary.com]
was prominent in both WTs and KOs, suggesting that the parameters are not shaped by acoustic experience but are rather genetically determined. Taken together, our analysis showed no difference in basic sIPSC and eIPSC properties between WT and KO, allowing a comparison of both datasets. Thus, we quantified the single fibre strength of gradually recruited MNTB fibres in terms of the absolute current amplitude and the quantal content (the number of released vesicles per stimulus). MNTB single fibre current amplitudes were 35% lower in P11 KOs compared to WTs (WT median: 264 pA, \( n = 60 \); KO median: 174 pA, \( n = 83 \), \( P = 0.001 \), U test). At P38, they were reduced by 38% (Fig. 6Aa and b; WT median: 245 pA, \( n = 77 \); KO median: 151 pA, \( n = 80 \); \( P = 1.1 \times 10^{-5} \), U test). Likewise, the quantal content of a single fibre was reduced in KOs at both ages (Fig. 6Ac and d; P11: WT median, 11.4 vesicles, \( n = 60 \); KO median, 8.2 vesicles, \( n = 83 \), \( P = 0.002 \), U test; P38: WT median, 11.5 vesicles, \( n = 77 \); KO median, 7.2 vesicles, \( n = 80 \), \( P = 0.001 \), U test).

**Figure 6.** Weaker single fibre strength due to lower quantal content in KOs

A, original traces of sIPSCs in P11 (Aa and c) and P38 (Ae and g) WT and KO and respective quantification of the quantal size (Ab, d, f and h) determined by a Gaussian fit. B, quantification of quantal size (Ba), sIPSC frequency (Bb), and sIPSC kinetics (Bc and d). Ca and b, exemplary single fibre eIPSCs from WT and KO at P11 and P38 (top). Bottom part depicts the same eIPSCs, but peak scaled to facilitate kinetics comparison. Stimulation artifacts were blanked. Cc and d, quantification of single fibre eIPSC kinetics. D, quantification of single fibre strength in absolute current (Da and b) and quantal content (Dc and d). [Colour figure can be viewed at wileyonlinelibrary.com]
Table 1. Basic parameters of sIPSCs and single fibre eIPSCs

| Parameter                        | P11 WT (n = 16) | KO (n = 15) | P   | P38 WT (n = 20) | KO (n = 15) | P   |
|---------------------------------|-----------------|-------------|-----|-----------------|-------------|-----|
| sIPSC rise time (ms)            | 0.54 ± 0.03     | 0.60 ± 0.03 | 0.17| 0.35 ± 0.01     | 0.33 ± 0.02 | 0.32|
| sIPSC decay time (ms)           | 1.65 ± 0.07     | 1.82 ± 0.09 | 0.15| 1.21 ± 0.06     | 1.18 ± 0.10 | 0.82|
| sIPSC frequency (1/s)           | 4.7 ± 1.2       | 4.0 ± 0.7   | 0.60| 7.9 ± 1.7       | 8.4 ± 2.1   | 0.85|
| Single fibre eIPSC rise time (ms)| 0.55 ± 0.05     | 0.63 ± 0.05 | 0.39| 0.40 ± 0.02     | 0.42 ± 0.02 | 0.50|
| Single fibre eIPSC decay time (ms)| 2.77 ± 0.20     | 2.76 ± 0.21 | 0.95| 1.41 ± 0.05     | 1.50 ± 0.06 | 0.57|

Values are means ± SEM (see Fig. 6). Probability values were determined by a t test.

vesicles, \( n = 80, P = 1.6 \times 10^{-5} \), unpaired t test). The single fibre strength remained unchanged during development in WTs and KOs in terms of current amplitude (WT: \( P = 0.63 \), unpaired t test; KO: \( P = 0.09 \), U test) and quantal content (WT: \( P = 0.64 \), unpaired t test; KO: \( P = 0.17 \), U test).

Collectively, the data show weaker single MNTB fibres in Otof KOs due to a lower quantal content.

To investigate whether a decreased release probability underlies the reduced quantal content, we performed paired-pulse ratio experiments with various interstimulus intervals.
intervals (ISIs, Fig. 7A). At every ISI, the paired-pulse ratio did not differ between WTs and KOs, either at P11 or at P38 (Fig. 7B, Table 2). During development, the paired-pulse ratio stayed constant for virtually all ISIs, implying no change of the release probability. To assess whether presynaptic GABA<sub>B</sub> receptors may have influenced the release probability (Magnusson et al. 2008; Fischer et al. 2019), we performed paired-pulse ratio experiments at P11 and applied the GABA<sub>B</sub> receptor blocker CGP into the bath (3 μM; Fig. 7C). In the presence of CGP, paired-pulse ratios did not differ between WTs and KOs at every ISI tested (Fig. 7Da, unpaired t tests). Furthermore, subtracting the mean paired-pulse ratios in control (without CGP application; Fig. 7Ba) from the mean paired-pulse ratios in the presence of CGP revealed closely matching values in P11 WTs and KOs (Fig. 7Db). Collectively, we conclude that GABA<sub>B</sub> receptors do not modulate the release probability in paired-pulse ratio experiments.

A further factor that may affect synaptic strength is synchrony of vesicular release. To test this possibility, we estimated the release synchrony by comparing decay kinetics of sIPSCs with single fibre eIPSCs from the same LSO neuron. Single MNTB fibres typically release ~10 vesicles per stimulus when stimulated at 0.5 Hz (Fig. 6Dd). In the case of asynchronous release, eIPSC decay times should be longer than sIPSC decay times, the latter reflecting gating mechanisms in response to transmitter from a single vesicle. To check this, averaged sIPSCs and averaged single fibre eIPSCs from the same neuron were peak scaled and peak aligned (Fig. 8Aa and b, Ba and b). As expected from a skewed temporal distribution of vesicle release (Minneci et al. 2012), eIPSC decay times were longer than those of sIPSCs (Fig. 8Ac and Bc). In P11 WTs, sIPSC decay times were 1.65 ± 0.07 ms, whereas eIPSC decay times were ~70% longer, amounting to 2.77 ± 0.20 ms (Fig. 8Ac; n = 16, P = 4.8 × 10<sup>−6</sup>, paired t test). In P11 KOs, the difference was ~60% (Fig. 8Ac; 1.74 ± 0.08 ms vs. 2.76 ± 0.09 ms, n = 15, P = 5.2 × 10<sup>−12</sup>, paired t test). In P38 WTs, eIPSC decay times were ~20% longer than sIPSC decay times (Fig. 8Bc; 1.21 ± 0.06 ms vs. 1.41 ± 0.05 ms, n = 20, P = 8.7 × 10<sup>−4</sup>, paired t test). In P38 KOs, the difference was ~30% (Fig. 8Bc; 1.18 ± 0.10 ms vs. 1.50 ± 0.15 ms, n = 15, P = 0.007, paired t test). Because kinetics accelerated between P11 and P38, we normalized the sIPSC decay time of every neuron to 100% to analyse eIPSC release synchrony over development. Release synchrony was similar between WTs and KOs at both ages (Fig. 8C; P11: WT: 167.0 ± 7.4%, n = 16; KO: 159.8 ± 8.4%, n = 15, P = 0.41; P38: WT: 119.8 ± 4.7%, n = 20; KO: 129.0 ± 8.4%, n = 15, P = 0.33, unpaired t tests). Notably, vesicle release became more synchronized during development in both WT (P = 4.9 × 10<sup>−6</sup>, unpaired t test) and KO (P = 0.004, unpaired t test), similar to the developmental increase in release synchrony in the adjacent medial superior olive and excitatory inputs to the LSO (Magnusson et al. 2005; Felix & Magnusson, 2016). This result suggests that maturation of vesicle release synchrony at MNTB–LSO synapses does not depend on acoustic experience. Taken together, the release probability and synchrony apparently do not contribute to reduced single fibre strength in Otof KOs.

**Fewer release sites per fibre cause a lower single fibre quantal content in Otof KOs**

Based on normal release probability and synchrony in Otof KOs, the reduced quantal content might be due to fewer release sites per MNTB fibre. To test this hypothesis, we estimated the number of release sites by employing a multinomial model of release that describes the relationship between the mean and the variance of postsynaptic currents, the quantal size, and the variance of the quantal size (Wen et al. 2016a, see Methods). The mean and variance of single fibre eIPSCs (Fig. 9A) were derived from MNTB fibre stimulation experiments (see Fig. 5), the quantal size, and the variance of the quantal size were determined from sIPSC amplitude distributions (see Fig. 6). Inserting the values into a multinomial model revealed ~35% fewer release sites per MNTB fibre in P11 KOs compared to WTs (WT median: 15.4, n = 57; KO median: 10.9, n = 71, P = 0.02, U test). It also revealed

| ISI (ms) | WT (n = 15) | KO (n = 12) | P     | WT (n = 9) | KO (n = 9) | P     |
|---------|------------|------------|-------|------------|------------|-------|
| 10      | 0.87 ± 0.02 | 0.82 ± 0.02 | 0.09  | 0.82 ± 0.03 | 0.86 ± 0.03 | 0.33  |
| 20      | 0.90 ± 0.01 | 0.90 ± 0.01 | 0.61  | 0.83 ± 0.02 | 0.86 ± 0.02 | 0.27  |
| 50      | 0.90 ± 0.01 | 0.90 ± 0.01 | 0.84  | 0.83 ± 0.03 | 0.84 ± 0.03 | 0.69  |
| 100     | 0.91 ± 0.01 | 0.90 ± 0.01 | 0.33  | 0.85 ± 0.02 | 0.87 ± 0.02 | 0.48  |
| 500     | 0.93 ± 0.01 | 0.90 ± 0.01 | 0.25  | 0.88 ± 0.02 | 0.89 ± 0.02 | 0.60  |
| 1000    | 0.93 ± 0.01 | 0.93 ± 0.01 | 0.64  | 0.91 ± 0.01 | 0.92 ± 0.01 | 0.35  |

Values are means ± SEM of paired-pulse ratios (see Fig. 7). Probability values were determined by a t test.
~40% fewer release sites in P38 KOs (Fig. 9Ba and b; WT median: 14.1, n = 71; KO median: 8.6, n = 71, P = 6.8 × 10−4, U test). In each genotype, the number of release sites remained constant throughout development (WT: P = 0.53, unpaired t test; KO: P = 0.07, U test). We also determined \(p_r\) by inserting the estimated number of release sites into a binomial model (Wen et al. 2016a, b). At neither age did \(p_r\) differ between genotypes (Fig. 9Bc and d; P11: WT median, 0.86; KO median, 0.85, n = 57, 71; P = 0.78; P38: WT median, 0.85; KO median, 0.88, n = 71, 71; P = 0.07, unpaired t tests). Taken together, impaired synaptic strengthening in Otof KOs can be explained by a lower number of release sites per MNTB fibre, which results in a lower quantal content. We summarized our findings in a schematic diagram (Fig. 10). We conclude that functional MNTB–LSO circuit refinement occurs

![Figure 8. Normal vesicular release synchrony in KOs](image)

**Figure 8. Normal vesicular release synchrony in KOs**

Aa and b, peak-scaled and peak-aligned sIPSCs (full line) and single fibre eIPSCs (dotted line) from a P11 WT (Aa, black) and a P11 KO (Ab, red). Ac, decay time quantification of sIPSCs and eIPSCs at P11. B, same as in A, but for P38. C, comparison of normalized increase in decay time (sIPSC − eIPSC in %) as a means for vesicular release synchrony. [Colour figure can be viewed at wileyonlinelibrary.com]

![Figure 9. Lower quantal content is due to fewer release sites per fibre in KOs](image)

**Figure 9. Lower quantal content is due to fewer release sites per fibre in KOs**

A, jitter of single fibre eIPSCs (grey) from WTs and KOs and respective mean (black: WT; red: KO) at P11 (Aa and b) and P38 (Ac and d). B, estimation of the number of release sites per fibre (Ba and b) derived from a multinomial model and the release site release probability \(p_r\) (Bc and d) derived from a binomial model. For details, see Methods. [Colour figure can be viewed at wileyonlinelibrary.com]
predominantly before P11. Lacking peripheral activity in Otof KOs affects the maturation of MNTB–LSO fibres. After P11, acoustic experience in WTs appears to have no impact on the investigated parameters.

Discussion

We have investigated functional and structural aspects of the MNTB–LSO circuit in WT and Otof KO mice. The latter are an elegant model for confined disruption of peripheral activity due to cochlea-specific protein loss. The main results are: (1) drastically reduced peripheral activity during the prehearing period impairs synapse elimination and strengthening, (2) impaired elimination leads to imprecise topography of the MNTB–LSO input map, (3) single fibre strength is reduced due a lower quantal content caused by fewer release sites, (4) functional refinement after hearing onset appears absent in WTs, and (5) refinement is not delayed in KOs, rather the circuit remains immature (see Fig. 10). Our study provides novel insight into MNTB–LSO circuit refinement upon functional denervation confined to peripheral protein loss and is the first to explore the functional circuitry as late as P49.

Impact of spontaneous prehearing activity on circuit refinement

Newborn mice are physiologically deaf until ~P12 (Ehret, 1983). Prior to P12, the periphery (cochlear IHCs) initiates spontaneous spike activity of bursting nature (Tritsch et al. 2007; Johnson et al. 2012; Sendin et al. 2014; Eckrich et al. 2018). The spike activity propagates to central auditory nuclei with a conserved temporal pattern (Sonntag et al. 2009; Tritsch et al. 2010; Babola et al. 2018). Therefore, IHCs appear to be peripheral pacemakers for central targets during prehearing development (Blankenship & Feller, 2010).

At P4, IHCs switch from synaptotagmin-mediated exocytosis of synaptic vesicles to otoferlin-mediated exocytosis (Beurg et al. 2010). Exocytosis is nearly

Figure 10. Schematic summary of MNTB–LSO circuit in normal and Otof KO mice

A, MNTB–LSO circuit in WT (black) and KO (red). KOs show impaired synapse elimination and strengthening as well as imprecise MNTB–LSO map. Bifurcation of MNTB fibres in the LSO is omitted for clarity. HF, high frequency; LF, low frequency. B, close-up depicting presynaptic MNTB bouton and part of postsynaptic LSO neuron. Reduced synaptic strength is due to fewer vesicles released from fewer release sites. Release probability, release synchrony and quantal size are normal. PSD, postsynaptic density; GlyR, glycine receptor. [Colour figure can be viewed at wileyonlinelibrary.com]
absent in IHCs of Otof KOs (Heidrych et al. 2009), suggesting that disrupted pacemaker activity in the periphery causes strongly reduced MNTB activity seen in our in vivo recordings (see Fig. 1). Thus, central auditory nuclei of Otof KOs are largely deprived of spontaneous prehearing activity from P4 onwards. The MNTB–LSO circuit undergoes synapse elimination and synaptic strengthening between P4 and P9 (Kim & Kandler, 2003; Hirtz et al. 2012; Clause et al. 2014). Therefore, spontaneous activity in Otof KOs is specifically reduced when MNTB–LSO refinement is normally initiated. At P11, we found impaired elimination and strengthening of MNTB fibres in Otof KOs, which was manifested by more, yet weaker MNTB fibres converging onto a given LSO neuron (see Figs 3–6). Temporally selective manipulation of peripheral spontaneous activity and absence of Otof mRNA (see Fig. 2 and http://developingmouse.brain-map.org/gene/show/58004) led us conclude that the impaired topographic refinement in the MNTB–LSO circuit is due to a lack of spontaneous activity originating in the cochlea (for an activity-independent increase of topographic precision, see Leake et al. 2002).

Animal models of absent cochlea-driven activity

Prior to the availability of genetic manipulations, abolishment of auditory activity has mostly been achieved by surgical cochlea ablation (Trune, 1982; Nordeen et al. 1983; Born & Rubel, 1985; Lippe, 1994). Such harsh ablation experiments have severe side effects and often unwanted pathological changes (Kandler et al. 2009; Wang & Bergles, 2015). Furthermore, surgical ablations are prone to error and larger variance in the cohort of ablated animals. Thus, each animal, as well as each cochlea, is likely manipulated to a different degree. This is especially true for bilateral ablations, in which asymmetries can occur between both ears, causing unwanted imbalances. In contrast, genetic models almost certainly provide standardized and relatively homogeneous cohorts. Furthermore, cochlea removal usually ablates spiral ganglion neurons (Rubel & Fritzsch, 2002), directly resulting in peripheral and central damage. These features draw caution towards surgical cochlea ablation studies. Nevertheless, when performed with great care and implementing appropriate controls (e.g. auditory brainstem recordings to assess deafness, cf. Hruskova et al. 2019), cochlear ablations can be valuable tools for manipulating activity in a temporally controlled manner. Historically, they have allowed critical periods to be identified (Hashisaki & Rubel, 1989; Moore, 1990; Mostafapour et al. 2000). Another advantage of cochlear ablations is that they can be made unilaterally. Such experiments have revealed impressive ectopic projections, such as an innervation of the ipsilateral MNTB from the cochlear nucleus (CN) on the unblasted side (Kitzes et al. 1995; Russell & Moore, 1995). Future studies could compare the central auditory circuitry in bilaterally cochlea-ablated animals with the results of genetic deafness models (as used in this study), providing insight into similarities and differences. For example, antipodal differences were revealed for MNTB membrane properties (cochlear ablation: Hassfurth et al. 2009; genetic deafness model: Leao et al. 2005).

dn/dn mice are a genetic model that lack cochlea-generated activity (Steel & Bock, 1980; Drury & Keats, 2003). The congenital deafness appeared in a breeding colony due to a spontaneous mutation in the 1950s (Deol, 1956; Deol & Kocher, 1958). Much later, the mutation was associated with the Tmcl gene (Kurima et al. 2002; Vreugde et al. 2002). The encoded TMC1 protein is part of the mechanotransduction channel complex, where it acts as the major pore-forming component of these channels. Similar to OTOF mutations (Migliosi et al. 2002; Mirghomizadeh et al. 2002; Tekin et al. 2005), TMC1 mutations cause deafness in humans (DFNB7/DFNB11; Kurima et al. 2002). Spontaneous prehearing activity is absent in dn/dn mice, as shown by in vivo auditory nerve recordings (Leao et al. 2006). Surprisingly, resurgence of spontaneous activity was discovered during in vivo recordings in the anteroventral CN (AVCN; Youssoufian et al. 2008). The basis for activity resurgence is unclear, but resurgence implies central compensation mechanisms. Such central activity compensations are unlikely in Otof KOs, as our MNTB in vivo recordings show strongly reduced spike rates at P11 and P30 (see Fig. 1). Extensive work on primary auditory circuits in dn/dn mice was performed by the Walmsley group. The activity-deprived AVCN in dn/dn mice shows larger evoked excitatory post-synaptic currents (eEPSCs) due to an increased release probability at endbulb–bushy cell synapses (Oleskevich & Walmsley, 2002; Oleskevich et al. 2004), similar to the observation in the AVCN of Otof KOs (Wright et al. 2014). In contrast, eEPSCs at the calyx–MNTB synapse are normal in dn/dn mice (Oleskevich et al. 2004), potentially due to the resurgence of compensatory spontaneous activity in the AVCN. However, reduced miniature IPSC (mIPSC) amplitudes in the MNTB (Leao et al. 2004b), altered MNTB cell excitability (Leao et al. 2004a), and loss of mediolateral protein gradients in the MNTB (Walmsley et al. 2006) are difficult to explain with activity resurgence. mIPSC amplitudes and kinetics in the LSO of P17 and P30 dn/dn mice are normal (Couchman et al. 2011), a finding similar to our observations (see Fig. 5). Functional MNTB–LSO connectivity in terms of input number and single fibre strength has not been investigated in dn/dn mice. Taken together, impairments in dn/dn mice are of a mosaic phenotype in primary auditory nuclei, likely due to the resurgence of spontaneous activity in the AVCN. Unfortunately, the basis and the temporal onset of activity...
resurgence are unknown, which hampers mechanistic conclusions towards the impairments. Such limitations are overcome in the Otof KO model, but the evidence obtained in the above-mentioned pioneer dn/dn and cochlear ablation studies provided a valuable basis for the present study.

Studies on two other genetic deafness models, namely mice lacking Vglut3 or Ca,1.3 (encoded by Slc17a8 or Cacna1d), have also revealed impaired MNTB–LSO circuit refinement (Noh et al. 2010; Hirtz et al. 2012). However, each of the two molecules is not only of uttermost importance for glutamate release from IHCs (Platzer et al. 2000; Seal et al. 2008), but also of functional relevance for synaptic transmission in the MNTB–LSO circuit (Gillespie et al. 2005; Noh et al. 2010; Hirtz et al. 2012; Jurkovicova-Tarabova et al. 2012). Consequently, it was not possible to distinguish between peripheral and central effects in systemic Slc17a8 and Cacna1d KOs. Furthermore, the frequency of spontaneous activity in Slc17a8 KOs is compensated by hyperexcitable spiral ganglion neurons that are directly depolarized and forced to generate spikes by K+ release from inner supporting cells, therefore bypassing abolished glutamate release from IHCs (Babolka et al. 2018; Sun et al. 2018). As explained above, such a compensatory mechanism is unlikely in Otof KOs, due to reduced spontaneous activity rates at P11 and P30. Therefore, we feel confident to provide a novel and confined view on the impact of peripheral spontaneous activity on MNTB–LSO circuit refinement, particularly when compared to previous studies.

Noh and coworkers (2010) employed another Otof KO line in which they stimulated MNTB fibres to analyse synapse refinement, as we did in the present study. In contrast to our study, they found normal MNTB–LSO development. The Otof line used in the Noh study features deleted exons 14 and 15 (OtofΔ14–15), and exocytosis from IHCs was reduced to an amount similar to our KO line (Roux et al. 2006; Heidrych et al. 2009). In OtofΔ14–15 mice, one would therefore expect impaired activity propagation from the periphery to central targets, similar to our study. Thus, we exclude that spontaneous prehearing activity differs substantially between both Otof KO lines. A potential explanation for the discrepancy are different approaches concerning the stimulation of MNTB fibres. To calculate the total number of converging fibres, the Noh group divided the maximal eIPSC amplitude from all MNTB fibres by the amplitude of a single fibre. A disadvantage of this method is that it assumes uniform strength across all MNTB fibres, which, however, is not warranted (see Figs 5 and 6). MNTB fibre strength is log normally distributed, and single fibre strength can vary up to ~60-fold within a given LSO neuron (Gjoni et al. 2018). Therefore, this stimulation method appears to be biased because it strongly depends on a largely deviating amplitude obtained from minimal stimulation. Collectively, it may not be able to resolve the ~40% higher input number present in our data (see Figs 5 and 6).

Our results are well in line with a recent study that investigated mice lacking the α9 subunit of the nicotinic acetylcholine receptor (Clause et al. 2014). In these α9 KOs, the cholinergic feedback from the brainstem to the inner ear via olivo-cochlear efferents is functionally denervated (Simmons et al. 1996; Glowatzki & Fuchs, 2000). This results in subtle modifications of the spontaneous activity pattern, without changing the overall frequency (Clause et al. 2014). The change is sufficient to impair MNTB–LSO refinement. The elegant Clause study provides strong evidence that the spontaneous activity pattern carries information for proper MNTB–LSO circuit refinement. Moreover, our study is in line with results from other developing neural circuits in the neocortex, the spinal cord, the hippocampus and the cerebellum (Blankenship & Feller, 2010; Leighton & Lohmann, 2016; Luhmann et al. 2016). Therefore, our results of impaired MNTB–LSO circuit refinement upon strong spontaneous activity reduction are in good agreement with impairments upon changed activity patterns.

The reduction in single fibre strength was more severe in Slc17a8 and Cacna1d KOs (~70–75%) than in Otof KOs (~40%). The stronger reduction may be due to the on-site abundance of Vglut3 and Ca,1.3. Indeed, the quantal size at MNTB–LSO synapses was reduced in systemic Slc17a8 and Cacna1d KOs (Noh et al. 2010; Hirtz et al. 2012), whereas it was normal in Otof KOs (see Fig. 6). The quantal size is mainly associated with the number of postsynaptic receptors (Edwards, 2007). Weaker MNTB fibres in systemic Slc17a8 and Cacna1d KOs are therefore likely the result of combined presynaptic and postsynaptic deficits. This might argue for different strengthening mechanisms at pre- and postsynaptic loci, in which different molecules are involved. For example, signalling by L-type voltage-gated calcium channels, such as Ca,1.3, is necessary for glycine receptor clustering (Kirsch & Betz, 1998) and thus potentially involved in the quantal size increase occurring between P4 and P9 at the MNTB–LSO projection (Kim & Kandler, 2010).

Collectively, spontaneous prehearing activity appears to govern the refinement of the MNTB–LSO circuit before hearing onset. However, as discussed above, on-site molecules likely act in cooperativity. This raises the question of whether the refinement of the MNTB–LSO circuit may be retarded in Otof KOs and may develop normally with time in a delayed manner due to potential compensation by on-site molecules. We found no evidence for a delayed circuit refinement in P38 Otof KOs as the MNTB–LSO circuit persisted in an immature and unrefined state (see Figs 5–9). The results imply a critical period for MNTB–LSO refinement between P4 and P9 during which spontaneous activity is necessary. If this
activity is absent, the defects cannot be compensated later on and appear to be irreversible in the context of persisting lack of activity.

Impact of acoustic experience on circuit refinement

Prior to our study, very little was known about the functional MNTB–LSO circuit refinement after P20. Our study is the first to investigate functional MNTB–LSO connectivity in young adults (up to P49). Previously, the oldest age had been P21 (Walcher et al. 2011). We found mainly three to four converging fibres in young adult WTs, similar to the Walcher study. This indicates the absence of further functional refinement after hearing onset, at least until P49. Although further functional MNTB–LSO refinement is absent in WTs, single MNTB fibres refine structurally after hearing onset by reducing the number of boutons (Sanes & Takacs, 1993; Clause et al. 2014). Unilateral cochlea ablation at P7 causes P19–23 MNTB axonal boutons to spread a greater distance along the LSO’s topographic axis (Sanes & Takacs, 1993). Whether this is due to a lack of spontaneous prehearing activity and/or acoustic experience cannot be concluded from this classic study, but the imprecise topography in our data closely resembles these results (see Fig. 4). Our analysis shows that the number of release sites is similar between P11 and P38. Fewer boutons per MNTB fibre, yet the same number of release sites per fibre, might argue in favour of a developmental increase of release sites per bouton.

To our surprise, none of the investigated parameters in the present study appears to depend on acoustic experience. For example, acceleration of glycinergic currents after P11 (Pilati et al. 2016) is normal, as well as the increase in vesicular release synchrony (Figs 6 and 8). They may be genetically hard-wired and may thus also develop normally in deaf humans. Nevertheless, our observations should not be interpreted as conclusive evidence that acoustic experience is not involved in refining other parameters of the MNTB–LSO circuitry.

Clinical implications

Among the >120 genes involved in HI (http://hereditaryhearingloss.org), OTOF mutations occur with a frequency of ~3–7%, much higher than average (Rodriguez-Ballesteros et al. 2008; Choi et al. 2009; Duman et al. 2011; Iwasa et al. 2013). Are there any implications of our study for the relatively large cohort of patients carrying OTOF mutations? We found imprecise inputs and weaker MNTB–LSO fibres in Otof KO mice, and the deficits in synaptic refinement become manifested prior to hearing onset. In stark contrast to humans, mice are already able to hear in utero, at 26–28 weeks of gestation (Starr et al. 1977; Fawer & Dubowitz, 1982; Lary et al. 1985). Therefore, spontaneous prehearing activity likely occurs during the first half of gestation. Hence, we reason that the MNTB–LSO circuit is deficient at birth in humans with non-functional otoferlin.

Would OTOF patients be able to detect interaural level differences (ILDs) after receiving a cochlear implant? Even though inhibition of a single MNTB fibre was weaker in Otof KOs (see Fig. 6Db), maximal inhibition was normal (see Fig. 5Db) and the MNTB–LSO circuit was generally functional. Therefore, OTOF patients with cochlear implants might be able to process ILDs efficiently. Indeed, deaf patients wearing bilateral cochlear implants can detect ILDs (Nopp et al. 2004; van Hoesel, 2004; Seeber & Fastl, 2008; Potts & Litovsky, 2014).

Might circuit deficiencies in OTOF patients be restored upon receiving cochlear implants? To render such an approach successful, the central auditory system must be plastic. In humans, central auditory plasticity is present until the age of 3.5 years (Sharma et al. 2002a,b), indicating most effective circuit restoration if children are implanted as early as possible. The frequency representation might be disturbed due to imprecise topography (see Fig. 4). However, currently available cochlea implants introduce frequency imprecision due to unspecific current spread and crosstalk between stimulation electrodes (Dombrowski et al. 2019). Therefore, technical flaws associated with the current technology might dominate the deficits in MNTB–LSO topography. To obtain a more complete understanding of the ILD circuitry in deafness models, further studies should investigate CN–LSO refinement in models such as the Otof KO model. Different molecular pathways are likely to regulate the refinement of the MNTB–LSO and the CN–LSO circuits, because each circuit can refine independently of each other (Noh et al. 2010; Garcia-Pino et al. 2017).

Conclusions and outlook

Using Otof KO mice as a model for cochlea-specific protein loss, we demonstrate that spontaneous peripheral prehearing activity governs central auditory circuit refinement. In particular, we show that reduced spontaneous activity impairs synapse elimination and synaptic strengthening in the MNTB–LSO circuit. Moreover, acoustic experience during posthearing development plays a minor role in MNTB–LSO refinement. We employed the Otof KO model to decipher maturation processes of the MNTB–LSO circuit that depend on peripheral activity. Although we argue that Otof KOs are an elegant model, we admit that there are some drawbacks. For example, spontaneous activity is unaffected until P4, the time point when otoferlin becomes indispensable for vesicle release from IHCs (Beurg et al. 2010). While the temporally restricted manipulation turned out to be advantageous for our study (functional MNTB–LSO refinement is initiated...
at P4), the model would cause problems for studies aimed at activity-dependent mechanisms before P4. To overcome such limitations, future studies could employ time- and tissue-specific KOs using the cre/lox system in combination with tamoxifen (Feil et al. 2009).

Our deafness-related findings on central auditory circuit maturation during distinct developmental periods may have implications for deafness treatment strategies. It will be important to investigate whether cochlear implants can diminish, or even rescue, the central deficits present in several deafness animal models.

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