SOUND ENCODING
AT THE FIRST AUDITORY SYNAPSE

PhD Thesis

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Herewith I declare, that I prepared the PhD Thesis "Sound encoding at the first auditory synapse" on my own and with no other sources and aids than quoted.

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Göttingen, 30.06.2019
Abstract

The inner hair cell (IHC) synapse, the first relay station of the auditory system, is able to encode sound with sub-millisecond precision and without any signs of fatigue (Matthews and Fuchs, 2010; Pangršić et al., 2012; Safieddine et al., 2012). Consequently, the IHC synapse manages a high rate of neurotransmitter release per active zone (AZ), which requires a fine balance between fast exocytosis and subsequent endocytosis (Pangrsic et al., 2010; Pangrsic and Vogl, 2018). The molecular physiology of this synapse is unconventional and far from understood. Furthermore, for a given sound frequency, single-AZ driven spiral ganglion neurons (SGNs) can collectively cover the whole audible spectrum of sound pressures, ranging within six orders of magnitude. How individual AZs of an IHC, which is believed to be isopotential, can drive activity of SGNs with different firing properties is also largely unknown. The presynaptic heterogeneity is one of the candidate mechanisms (Frank et al., 2009; Meyer et al., 2009; Ohn et al., 2016). This thesis provides further insight on the endocytic machinery of the IHC synapse, and the mechanisms contributing to encoding of sound pressures over a wide dynamic range. First, we combined electron tomography, membrane capacitance ($C_m$) measurements, systems physiology and confocal microscopy to decipher the role of AP180 in IHC synaptic transmission. Based on our results, we propose that AP180 is involved in release site clearance, clathrin assembly for clathrin-mediated endocytosis and synaptic vesicle (SV) reformation. Secondly, we explored whether the planar polarity mechanisms establishing the hair bundle orientation at the apical part of the IHC could contribute to the spatial heterogeneity of AZs at the basal part of the IHC, and thereby contribute to diverse SGN firing properties. Using patch-clamp combined with Ca$^{2+}$ imaging, $C_m$ measurements and high and superresolution light microscopy, we analyzed PTXa-expressing IHCs, in which the G$\alpha_i$ signaling was blocked. We propose that G$\alpha_i$ signaling involved in planar polarity mechanisms contributes to setting up diverse SGN firing properties (Jean et al., 2019). Lastly, we studied the IHC synaptic transfer function to understand how the heterogeneous Ca$^{2+}$ signaling translates into neurotransmitter release. By dual-color imaging of synaptic Ca$^{2+}$ influx and glutamate release, we discovered a heterogeneity of the Ca$^{2+}$ dependence of release among the synapses, even within an individual IHC. We propose that the IHC partitions the sound information contained in the receptor potential via heterogeneous presynaptic control of release, and thereby contributes to the diverse SGN firing properties that are thought to underlie encoding over a wide dynamic range of sound pressures.
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1 General Introduction

1.1 Sound

Sensory processing requires the ability to transduce, encode and consequently perceive the information of the stimuli from different sources. Even though the basic senses—vision, audition, olfaction, somatic and vestibular sensation—deal with different modalities, they share a common path: the receptor cells convert the energy from different sources into neural signals that deliver the stimulus information to the brain. In the case of the auditory system, the external stimuli are sound waves, and the auditory system is able to detect the acoustical cues with high sensitivity and can respond rapidly. This has a critical importance not only for communication but also for initial orientation of the body to novel stimuli.

Sound is a pressure wave caused by vibrating air molecules that consists of alternating compressions and rarefactions. Sound waves have four components: waveform, phase, amplitude and frequency. While the amplitude of the sound roughly corresponds to the loudness, the frequency is perceived as the pitch of the sound. Mammalian species have sensitivity to different frequency and sound pressure ranges. While humans can detect frequency range from 20 Hz to 20 kHz, mice hearing spans the range of 1 to 100 kHz (Ashmore, 2008). Mammals are able to encode sound pressures in a range of six orders of magnitude.

1.2 The external and middle ear

The external and middle ear are responsible for collecting sound waves and transmitting to the inner ear with amplification. The external ear comprises auricle and ear canal, which together direct sound energy to the eardrum, so called tympanic membrane (Figure 1.1). The auricle detects the elevation of the sound source by its asymmetrical nature. This corrugated surface allows us to localize sounds from different locations with respect to the head, especially in the vertical axis. The anatomy of ear canal is important to boost the sound pressure around 3 kHz, where the elementary human speech sounds are concentrated (Purves et al., 2004). The airborne sound is collected by the external ear, reaching the tympanic membrane, a
diaphragm, which is connected to the three tiny bones in the middle ear: the malleus, incus and stapes. The major role of middle ear is to ensure the transmission of the airborne sound vibrations to the fluid-filled inner ear, cochlea. The middle ear overcomes the problem of transition from a low impedance medium like air to a high impedance medium like water by increasing the pressure by 200-fold (Purves et al., 2004). The middle ear is able to boost the pressure both by the lever function of the ossicles and by the small diameter of oval window, linking the middle ear to the inner ear, compared to the tympanic membrane diameter.

![Figure 1.1 – The structure of the human ear.](image)

The human ear has three functional parts: external, middle and inner ear (right bottom). The external ear collects and focuses the sound into the ear canal (external auditory meatus). The tympanic membrane, connecting the external ear and the middle ear, receives the air pressure changes caused by sound waves. These vibrations are conveyed through the three middle bones: the malleus, the incus and the stapes (right top). Vibration of the stapes stimulates the cochlea. (Modified from (Kandel et al., 2012)).

1.3 The inner ear

The cochlea (from cochlos in Greek, meaning snail) of the inner ear is where the sound pressure is translated into the neural signals. The cochlea not only amplifies and converts the mechanical signals into the neural signals, but also acts as a frequency decomposer, mostly thanks to its structure. The cochlea is a tube-like structure and turns around the conical bone, modiolus. The cross-section of this tube-like structure reveals three fluid-filled compartments called scalae (Figure 1.2B). The upper compartment is called scala vestibuli; it is connected
1.4. The organ of Corti

The organ of Corti is the receptor organ of the inner ear. It is an epithelial tissue that sits atop the basilar membrane. It consists of four rows of hair cells and different types of supporting cells such as inner phalangeal cells, Deiter's cells, Hensen's cells, inner and outer pillar cells. The supporting cells are important for the homeostasis and provide structural support to the organ of Corti as it vibrates in response to the sound. The hair cells, the receptor cells of the organ of Corti, are innervated by the afferent nerve fibers - spiral ganglion neurons (SGNs) - which carry the information to the brain. There are two types of hair cells in the organ of Corti: inner hair cells (IHCs) forming the inner row and outer hair cells (OHCs) organized in three outer rows. The hair cells are columnar cells. They are named for the hair-like microvilli, called stereocilia that form ‘hair bundles’ at the apical part of the cells facing scala media. The innermost epithelium in the scala media forms the tectorial membrane and it is in contact with the OHC stereocilia. Since the basilar membrane and the overlying tectorial membrane have different pivot points, the traveling wave creates the shearing movement for the hair cells that are sandwiched between those two structures. This movement directly reflects the
mechanosensitive stereocilia of OHCs, while the resulting radial fluid stream displaces the stereocilia of IHCs that are not connected to the tectorial membrane.

Figure 1.2 – Schematic of the cochlea and the organ of Corti.
A. Schematic of the inner ear showing the tube-like structure of the cochlea, the vestibular organ, and the corresponding vestibular and cochlear nerve. B. Cross section of the cochlea shows the three liquid filled ducts (scalae). The scala vestibuli is closed by the oval window and the scala tympani is closed by the round window. The organ of Corti, the receptor organ of the cochlea, is located on the basilar membrane in the middle duct - the scala media. C. The organ of Corti consists of three rows of outer hair cells and one row of inner hair cells, innervated by the auditory nerve. Hair cells are equipped with mechanosensitive hair bundles. (Modified from (Kandel et al., 2012)).
Figure 1.3 – Basilar membrane motion and tonotopy.

A. The schematic of the uncoiled cochlea shows the flow of the sound energy. Sound waves vibrate the tympanic membrane, and this vibration is transferred to the middle ear bones. The piston-like movement of stapes, partially integrated to the oval window, causes the oscillations propagate along the scala vestibuli and scala tympani. Both scalae are connected via the helicotrema in the apex. These oscillatory pressure moves the basilar membrane. (Modified from (Kandel et al., 2012))

B. As the basilar membrane has different mechanical properties along its length, each frequency of stimulation cause a maximal displacement at a particular position. This frequency dependent displacement of the basilar membrane gives rise to a topographical frequency mapping, called tonotopy. The movement of the basilar membrane activates the IHCs at that position, and consequently excites the SGNs. Mapping the SGN response thresholds over the audible spectrum constitutes the tuning curve of the fibers, where they exhibit the most sensitivity at the characteristic frequency. (Modified from (Purves et al., 2004)).

1.4.1 Hair cell transduction

Hair cells transduce the vibrational energy into an electrical signal. They can faithfully detect movements of atomic dimensions with sub-millisecond precision. The sound-driven displacement of the basilar membrane almost instantaneously deflects the stereocilia of the hair cells, equipped with the mechanoelectrical transducer (MET) channels (Figure 1.4A). Stereocilia are tightly regulated and organized in a bundle of 50-100 in IHCs and OHCs (Barr-Gillespie, 2015). They are reinforced by an array of parallel actin filaments, that are cross-linked via several actin-binding proteins. The OHC stereocilia are organized in V-shaped rows, and their distal tip is embedded in the tectorial membrane (Andrade et al., 2016). Such an attachment is important for a tight mechanical coupling. Several rows of stereocilia form a staircase structure that increases in height, the tallest one lacking MET channels (Beurg et al., 2009). The staircase organization is related to a functional polarization: the deflections towards the taller edge of the hair bundle are excitatory, as they open MET channels, and the deflections towards the shorter edge are inhibitory, as they cause the closing of MET channels (Fettiplace and Kim,
2014). Extracellular filaments called tip links join the tip of a stereocilium to the lateral wall of its taller neighbor (Figure 2.4A). The tip link is composed of cadherin-23 at the upper end and of protocadherin-15 at the bottom end (Kazmierczak et al., 2007), where it is connected to the putative MET channel candidate TMC1 (transmembrane channel-like protein) (Kurima et al., 2015). Excitatory deflection of the tip links conveys force to MET channels and their ensuing opening causes inward MET current carrying Na\(^+\), K\(^+\) and Ca\(^{2+}\), among other small cations (Beurg et al., 2006). Due to high K\(^+\) concentration of endolymph (see above) and the positive endocochlear potential of approximately 100 mV, K\(^+\) is the main cation carrying the MET current, which generates a depolarizing receptor potential. The disruption of the tip links abolishes transduction (Assad et al., 1991; Furness et al., 2008).

1.4.2 Outer hair cells

OHCs are one of the two types of hair cells present in the mammalian cochlea. They are thought to amplify sound mainly by their somatic ‘electromotility’, with some contribution of the hair bundle motility (reviewed in (Fettiplace, 2017)). The somatic electromotility, contractions and elongations of the cell body, is determined by voltage-dependent changes in the piezoelectric membrane protein prestin on the lateral membrane of the OHC (Zheng et al., 2000). Prestin is a member of solute transporter family SLC26A5 and the electromotility of mammalian prestin is dependent on intracellular Cl\(^-\) (Oliver et al., 2001). The genetic deletion of prestin in mice resulted in the loss of electromotility in isolated OHCs and 50 dB reduction in cochlear sensitivity \textit{in vivo} (Liberman et al., 2002). Since prestin is very densely packed on the lateral membrane of OHCs, the loss of stiffness in OHCs could cause this decreased sensitivity. Nonetheless, further evidence came from a mutant mouse with nonfunctional prestin: while the stiffness of OHCs was preserved, 60 dB loss of acoustic sensitivity \textit{in vivo} was observed, further supporting the role of prestin and prestin-driven somatic electromotility in cochlear amplification (Dallos, 2008).

OHCs show different channel properties and cell size along the tonotopic axis. For instance, the macroscopic MET currents in OHCs show an increasing gradient in amplitude from the apex to the base of the cochlea. This might enhance the sensitivity of high-frequency hair cells where the basilar membrane is rigid (Beurg et al., 2006). In addition to providing the high acoustic sensitivity of hearing, cochlear amplification by OHCs enhance frequency tuning beyond what is achieved by the passive properties of the basilar membrane (tonotopic frequency decomposition, see above). OHCs receive inhibitory synaptic transmission from medial olivocochlear neurons that controls the extent of electromotility and, hence, cochlear amplification. Afferent synaptic transmission of OHCs to non-myelinated type II SGNs employs glutamatergic ribbon synapse (discussed below) and seems to contribute little or none to sound encoding (for review; see (Fuchs and Glowatzki, 2015)).
1.4. The organ of Corti

Figure 1.4 – IHC mechanotransduction and receptor potential.

A. The sound-driven vertical movement of the basilar membrane deflects the stereocilia. Upon stereocilia deflection towards the tallest one, tip links apply force to the mechanoelectrical transducer (MET) channels. This force opens the MET channels, carrying inward current of mainly $\text{K}^+$, but also $\text{Ca}^{2+}$ and $\text{Na}^+$. Increase and decrease in inward MET current depolarizes and hyperpolarizes the IHC, respectively. B. Receptor potentials of an IHC recorded from an anesthetized guinea pig for different sound frequencies. At low frequencies, the IHC receptor potential is solely sinusoidal, following the waveform the sound stimulus. At frequencies above 1 kHz, the receptor potential transforms into a sustained depolarization, similar to a step depolarization. The periodic (AC) component is filtered by the membrane time constant, leaving the DC component. (Data taken from (Palmer and Russell, 1986)).

1.4.3 Inner hair cells

IHCs, the genuine receptor cells of the cochlea, encode most of the acoustic signal. The myelinated type I SGNs, composing 95% of the auditory afferent neurons, innervate IHCs. The resting potential of IHCs is mainly set by MET channels open at rest and slow delayed rectifier $\text{K}^+$ channels (KCNQ4) (Kharkovets et al., 2006; Marcotti et al., 2003b; Oliver et al., 2003; Johnson et al., 2011). Upon deflection of stereocilia by radial fluid displacement, more MET channels of IHCs open and generate a depolarizing receptor potential. At low frequency sounds, the IHC receptor potential follow the waveform of the sound stimulus (Figure 1.4B). On the other hand, frequencies above a few kilohertz, the receptor potential converts into a sustained depolarization, similar to a step depolarization, as the periodic component is filtered by the membrane time constant (Palmer and Russell, 1986). Once IHC is depolarized, it activates voltage-gated L-type calcium channels: $\text{Ca}_{\text{V}}1.3$. $\text{Ca}^{2+}$ influx-triggered exocytosis causes release of glutamate onto SGNs.

IHCs make overall 5-20 synapses with SGNs depending on the tonotopic position, where
single synapse drives fiber activity (Merchan-Perez and Liberman, 1996). IHC synapse, the first synapse in the auditory pathway, is able to encode sound with sub-millisecond precision and without any signs of fatigue (Moser and Beutner, 2000; Pangrsic et al., 2010). In response to a constant stimulus, however, SGNs show a fast adaptation (Westerman and Smith, 1984), which is believed to stem from the presynaptic depression related to RRP depletion (Moser and Beutner, 2000). The IHC synapse has one of the highest rates of neurotransmitter release per active zone, requiring mechanisms of fast exocytosis, and tight coupling to subsequent endocytosis. Given the peculiar and largely unknown molecular composition of the IHC synapse, synaptic transmission in this synapse is not fully understood. Furthermore, for a given frequency, single-active zone (AZ) driven SGNs can collectively cover the whole audible spectrum of sound pressures, ranging within six orders of magnitude. How individual AZs of an IHC, believed to be isopotential, can drive activity of SGNs with different properties is also largely unknown.

1.4.3.1 Ribbon synapses

IHC synapses are different from conventional glutamatergic synapses. They comprise an electron-dense structure, called synaptic ribbon. In vertebrates, ribbon synapses are found solely in sensory cells. Besides IHCs, these cells include OHCs, vestibular and lateral-line hair cells, photoreceptors and retinal bipolar neurons, the sensory cells of the fish electrical organs and pineal gland cells (for review, see (Matthews and Fuchs, 2010). All exhibit sustained release of neurotransmitter upon graded changes in membrane potential, even though this feature is more prominent in IHCs.

The ribbon is an electron-dense mostly ovoid structure, around which the SVs are tethered like a halo (for review; see (Matthews and Fuchs, 2010; Pangršić et al., 2012; Safieddine et al., 2012)). The main component of the ribbon is the RIBEYE protein, which comprises A and B domain (Schmitz et al., 2000). The A domain, unique to the ribbons, forms the structural core by mediating self-assembly (Magupalli et al., 2008; Schmitz et al., 2000) and the B domain is very similar to the transcription factor COOH-terminal binding protein 2 (CtBP2; (Schmitz et al., 2000). Furthermore, due to its resemblance to NADH dehydrogenase, the B domain might be enzymatically active (Schwarz et al., 2011).

The function of the ribbon has remained enigmatic for a long time. In zebrafish hair cells, RIBEYE was shown to be required for proper trafficking of the Ca\(^{2+}\) channels to the AZs (Sheets et al., 2011; Sheets et al., 2012). At mammalian hair cell ribbon synapses, however, it has been a challenge to target RIBEYE specifically due to its high resemblance to CtBP2, disruption of which is lethal (Hildebrand and Soriano, 2002). The first approaches were by genetic targeting of the main scaffold protein bassoon. Bassoon binds to RIBEYE and anchors the ribbon to the presynaptic density (Dick et al., 2003) and concomitantly its disruption causes the loss of the ribbon from the AZs (Khimich et al., 2005). In bassoon KO, the lack of AZ-anchored ribbons in IHCs reduced fast exocytosis and disrupted the synchronous auditory signaling (Buran et al., 2010; Khimich et al., 2005). Furthermore, disruption of bassoon resulted in reduced
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Ca\(^{2+}\) influx due to the reduced abundance of Ca\(^{2+}\) channels and disrupted the Ca\(^{2+}\) channel organization (Frank et al., 2010). Recently, specific KO of the Ribeye A domain achieved complete loss of ribbons in the mouse retina and organ of Corti (Becker et al., 2018; Jean et al., 2018; Maxeiner et al., 2016). Surprisingly, the Ribeye KO IHCs had normal Ca\(^{2+}\) influx and abundance of Ca\(^{2+}\) channels, suggesting bassoon itself, rather than the ribbon, may contribute to Ca\(^{2+}\) channel tethering and stabilization at the AZ (for review; see (Pangrsic et al., 2018)). On the other hand, the Ribeye KO IHCs show a depolarized shift in the activation of Ca\(^{2+}\) channels, which possibly explains the reduced spontaneous and evoked firing rates of Ribeye KO SGNs (Jean et al., 2018). The reduced replenishment rate observed in Ribeye KO IHCs supports the long-lasting hypothesis of the ribbon operating as a conveyor belt. The ribbon replenishes release sites potentially by facilitating the diffusion of SVs on the ribbon surface towards the AZ, and SV priming (Becker et al., 2018; Jean et al., 2018; Frank et al., 2010; Grayon et al., 2014; Mehta et al., 2013; Zenisek and Matthews, 2000).

IHC ribbon synapses can maintain high and sustained SV release rates and they utilize an unconventional fusion machinery that is not fully understood. IHCs express the unconventional vesicular glutamate transporter Vglut3 for loading of SVs with glutamate and genetic disruption of Vglut3 causes deafness in mice (Ruel et al., 2008; Seal et al., 2008) and potentially in humans (Greene et al., 2001). Furthermore, they lack the major SV proteins, such as synaptophysin, complexin and synapsin (Safieddine and Wenthold, 1999; Strenzke et al., 2009). Surprisingly, Nouvian et al. (2011) found that exocytosis in mouse IHCs operate without the neuronal soluble NSF attachment protein receptor (SNARE) proteins, which mediate otherwise membrane fusion in conventional synapses (Nouvian et al., 2011). Mouse IHC exocytosis, probed by membrane capacitance (C\(_m\)) measurements, was insensitive to genetic ablation (SNAP-25, synaptobrevin-1 or 2 and 3) or neurotoxin-mediated cleavage of neuronal SNAREs.

In addition, mRNA, as previously reported (Safieddine and Wenthold, 1999), was present, but synaptically located protein was not detected by immunohistochemistry in IHCs (Nouvian et al., 2011). Moreover, SV priming factors of the CAPS and Munc13 families are also missing from IHCs (Vogl et al., 2015). Different from the conventional synapses, Ca\(^{2+}\) influx is mainly mediated via Ca\(_V\) 1.3 channels in IHCs – described in detail in the next chapter.

In this atypical synapse, a lot of research was done to identify the Ca\(^{2+}\) sensor for exocytosis, as the neuronal vesicular Ca\(^{2+}\) sensors, synaptotagmin 1 and 2, are only expressed during early development (Beurg et al., 2010; Reisinger et al., 2011). The multi-C\(_2\) domain protein otoferlin has been suggested as the Ca\(^{2+}\) sensor for exocytosis in IHCs (Roux et al., 2006; Johnson and Chapman, 2010; Michalski et al., 2017). However, final proof of the Ca\(^{2+}\) sensor hypothesis remains to be delivered. Alternatively, synaptotagmin 4 has been suggested as a candidate Ca\(^{2+}\) sensor (Johnson et al., 2010), but its expression in mature IHCs is controversial.

Overall, the IHC ribbon synapse is characterized by the ribbon, a halo of SVs around, scaffold proteins and a large Ca\(^{2+}\) channel cluster underneath. Besides bassoon, the other main scaffold proteins are RIM proteins and a short splice variant of piccolo called piccolino (Jung et al., 2015a; Regus-Leidig et al., 2013). SV pools in IHC AZs can be identified in different
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ways. Morphologically, they are divided into two main pools: the membrane-proximal and the ribbon-associated SV pools (Chakrabarti et al., 2018; Lenzi et al., 1999). On average, an IHC ribbon tethers 70 SVs: approximately 14 of them constitute the membrane-proximal SV pool (Jung et al., 2015a; Wong et al., 2014). Physiologically, the fast component of the release, recruited by brief depolarizations (<20ms), is thought to reflect the fusion of the readily releasable pool of SVs (RRP), and their potential morphological correlate is the membrane-proximal pool of SVs (Moser and Beutner, 2000). Moreover, the sustained component of exocytosis or the slowly releasable SV pool likely reflects the ribbon-associated SV pool (Moser and Beutner, 2000).

1.4.3.2 Voltage-gated calcium channels

Since each step of presynaptic activity, such as SV fusion, endocytosis, and SV replenishment to the RRP may be dependent on Ca$^{2+}$ in some way, it is important to understand the Ca$^{2+}$ dynamics at the active zone. The voltage-gated Ca$^{2+}$ (Ca$_V$) channels (VGCC) transduce membrane potential change into intracellular Ca$^{2+}$ transients in order to mediate neurotransmission. They are grouped based on the characteristic currents they mediate: the Ca$_V$1 family give rise to L-type currents, the Ca$_V$2 family is responsible for P/Q, N and R-type currents, and the Ca$_V$3 family mediates T-type currents (for review, see (Catterall, 2011)).

VGCCs are complexes made of different subunit proteins; pore-forming α1 subunit, auxiliary α2δ and β subunits (for review, see (Catterall, 2011); Figure 1.5). The functional diversity of VGCCs, clustered to three Ca$_V$ families -as mentioned above-, come from the existence of different subunit isoforms; ten α1, four α2δ and four β subunit isoforms are present. The α1 subunit defines the kinetics and the voltage dependence of VGCCs together with their pharmacology. The auxiliary subunits can modulate these properties and define the abundance and the location of VGCCs (for review, see (Dolphin, 2012)). The α1 subunit contains four homologous domains, each comprising six transmembrane α helices (S1-S6). The S4 segments of each domain act as the voltage sensors for activation, and initiate the conformational change that opens the pore.

In contrast to conventional synapses where exocytosis depends largely on channels of the Ca$_V$2 family, in IHC ribbon synapses, Ca$_V$1.3 channels mediate 90% of the Ca$_V$ current (Dou et al., 2004; Platzer et al., 2000; Brandt et al., 2003). Indeed, the Ca$_V$1.3 KO IHCs show minimal exocytosis upon depolarization, while the exocytosis evoked by Ca$^{2+}$ uncaging seemed normal (Brandt et al., 2003). The remaining Ca$^{2+}$ current in Ca$_V$1.3 KO mice – which are congenitally deaf – suggests involvement of other Ca$_V$ channels (Brandt et al., 2003). The candidates are Ca$_V$1.4 and Ca$_V$2.3, based on the sensitivity of the residual current to Ca$^{2+}$ channel modulators (Brandt et al., 2003). Furthermore, Ca$_V$1.3 KO IHCs lack the Ca$^{2+}$ action potentials during development, showing its importance for normal hair cell development (Brandt et al., 2003). Ca$_V$3.1 was also shown to be transiently present during IHC development, possibly contributing to Ca$^{2+}$ action potentials with their low-voltage activation (Levic and Dulon, 2012; Nie et al., 2008).
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Figure 1.5 – Voltage-gated calcium channel subunits. The VGCCs are made of the pore-forming α1 subunit, the auxiliary α2δ and the β subunits. The α1 subunit has four homologous domains, each containing six transmembrane helices (S1-S6). The S4 segments of each domain serve as the voltage sensors for the activation. The CaV1.3 channel C-terminus contains a C-terminal modulatory domain (CTM). The EF-motif together with the preIQ and IQ domains comprise the CDI machinery. The α2δ subunit contains an extracellular α2 subunit and membrane associated δ subunit, while the β subunit is intracellular. (Modified after (Dolphin, 2012)).

The biophysical properties of CaV1.3 channels make them suitable to mediate neurotransmission evoked by graded membrane potential changes. Single-channel recordings in IHCs showed that CaV1.3 channels have fast activation kinetics (less than 1 ms) and slow inactivation, and are active at relatively negative potentials (~60 mV) (Zampini et al., 2010; Zampini et al., 2013). Furthermore, the pharmacology of the CaV1.3 channels is well-understood: the CaV1 family is sensitive to dihydropyridines (DHPs) such as isradipine or nifedipine (Ca2+-channel agonist) and BayK8644 (Ca2+-channel antagonist), which act by changing the open probability (Brown et al., 1984; Hess et al., 1984). Nonstationary fluctuation analysis on Ca2+-tail currents suggested single-channel current of -0.63 pA (at -62 mV, in the presence of 10 mM Ca2+ and 1 mM Ba2+) with open probability of 0.82 (in the presence of BayK 8644) (Brandt et al., 2005). The single-channel recordings from immature mouse IHCs (P5-10) showed single-channel currents of -1.1 pA at the resting potential of the cell (from -50 mV to -70 mV, (Marcotti et al., 2003a); in the presence of 5 mM Ca2+ and BayK 8644) with maximum open probability of 0.15 (Zampini et al., 2010). Combining several data obtained from mouse apical IHCs, Pangrsic et al. (2018) estimated a single-channel current of approximately -0.14 pA and a maximal open probability of 0.2-0.4 (without BayK8644) (Pangrsic et al., 2018). The single-channel current decreases linearly with membrane depolarization, while the open probability increases, following a Boltzmann equation (Zampini et al., 2010; Zampini et al., 2013). These changes in the single-channel current and open probability in relation to voltage give rise to the I-V (current-voltage) curve on the macroscopic level.

In mature IHCs (after the onset of hearing), CaV1.3 channels are mainly clustered at the AZs, beneath the ribbon, showing mostly linear arrangement, while extrasynaptic channels also exist (Brandt et al., 2005; Neef et al., 2018; Zenisek et al., 2003). Several studies have aimed
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at estimating the number of CaV channel per active zone. Based on the fluctuation analysis on Ca²⁺-tail currents, the presence of ~1700 Ca²⁺ channels were approximated on the whole IHC level (Brandt et al., 2005). With the assumption of 500 extrasynaptic Ca²⁺ channels (at a density of one channel per μm²), and average number of 14 synapses per IHC led to the number of ~80 Ca²⁺ channels per AZ (Brandt et al., 2005). Recently, Ca²⁺ imaging upon the microiontophoresis of Ca²⁺ chelator EGTA and the optical fluctuation analysis estimated the average number of Ca²⁺ channels to 125 and 78 per AZ, respectively (Neef et al., 2018). However, the counts varied drastically between 20-330 per AZ, showing a major heterogeneity of synapses (Neef et al., 2018).

The roles of the auxiliary subunits in regulating CaV channels have been also studied in IHCs. Of the β subunits IHCs predominantly express CaVβ2. Hearing in CaVβ2 KO animals was severely impaired (Neef et al., 2009). CaVβ2 KO IHCs showed strongly decreased Ca²⁺ influx and exocytosis, attributable to decreased number of CaV channels on the membrane (Neef et al., 2009). Together, CaVβ2 controls the abundance of the CaV1.3 channels in IHCs (Neef et al., 2009). Recent evidence shows that IHCs express α2δ2 subunit, and IHCs with non-functional α2δ2 have decreased Ca²⁺ influx and exocytosis (Fell et al., 2016). In addition, the spatial coupling between the presynaptic CaV1.3 channels and postsynaptic PSD-95 clusters was altered in the absence of functional α2δ2, suggesting a trans-synaptic role of it in IHCs (Fell et al., 2016).

VGCCs exhibit two types of inactivation: voltage-dependent inactivation (VDI) and Ca²⁺-dependent inactivation (CDI). CaV1 channels differ from the other CaVs, as they have slow VDI (Tsien et al., 1988). CDI is mediated by Ca²⁺ binding to the Ca²⁺ sensor calmodulin, which, otherwise, is in the apo-state and associated with the C-terminal isoleucine-glutamine (IQ) domain of CaVα1 (Figure 1.5). Upon Ca²⁺ binding to its EF-hand motif, calmodulin undergoes conformational change promoting CDI (Ben Johny et al., 2013). The EF-hand motif is found in many calcium-binding proteins (Moncrief et al., 1990). The C-terminal regulatory domain (CTM) of CaVα1 was shown to compete with calmodulin for binding the IQ domain of the channels, thus weakening calmodulin-mediated CDI and also shift the voltage dependence of activation (Bock et al., 2011; Liu et al., 2010; Singh et al., 2008). Alternative splicing of the C-terminus of CaVα1 could therefore change the biophysical properties of CaV channels. IHCs have both short and long CaV1.3 splice variants (Vincent et al., 2017), likely contributing to the functional heterogeneity of the channels.

In addition to calmodulin, calcium binding proteins (CaBPs), too, modulate CaV channel properties. CaBPs are EF-hand Ca²⁺ binding proteins that show high homology with calmodulin, and can antagonize calmodulin-mediated CDI (Tsien et al., 1988). In the cochlea, multiple CaBP members (CaBP1, 2, 4 and 5) are expressed (Cui et al., 2007). So far, CaBP2 is the only member, whose disruption was found to cause significant hearing impairment in humans (Schrauwen et al., 2012). Patch-clamp recordings from mutant mouse IHCs lacking CaBP2 showed enhanced Ca²⁺-channel inactivation (Picher et al., 2017a). On the other hand, CaBP4 is a weak modulator of CDI (Cui et al., 2007). Besides the protein-protein interactions,
H$^+$ released during SV exocytosis was shown to transiently reduce Ca$^{2+}$ current in hair cells and suggested as a mechanism of short-term plasticity (Cho and Gersdorff, 2014).

### 1.4.3.3 Calcium domains

The abundance and the distribution of VGCCs determine their physical distance to SVs and consequently, they have a key importance in stimulus-secretion coupling. There are two limiting scenarios; Ca$^{2+}$ nanodomain and Ca$^{2+}$ microdomain control of exocytosis. The term Ca$^{2+}$ nanodomain control of release is used when the mean coupling distance between Ca$^{2+}$ source and Ca$^{2+}$ sensor is less than 30 nm, while the distance is typically more than 100 nm for Ca$^{2+}$ microdomain control of release (Neher, 1998). In the case of a tight coupling, opening of a single (Ca$^{2+}$ nanodomain) or a few (Ca$^{2+}$ nanodomain-like) Ca$^{2+}$ channels matter for the release of a readily-releasable SV and therefore, the exocytosis of the RRP is near-linearly dependent on the number of open Ca$^{2+}$ channels (Figure 1.6).

Both coupling types offer different advantages. Ca$^{2+}$ nanodomain control of release provides increased efficacy and speed of transmission, as it reduces the synaptic delay (Schneggenburger and Neher, 2005). Furthermore, linear relationship between Ca$^{2+}$ and release leads to a greater dynamic range and sensitivity (for review; see (Eggermann et al., 2012)). It can, however, increase the ‘jitter’ of the evoked and spontaneous neurotransmitter release, as release is triggered by the stochastic opening of a single or few Ca$^{2+}$ channels. In contrast, in Ca$^{2+}$ microdomain control of release, there is high specificity and all-or-none response, although this loose coupling causes higher metabolic cost for the synapse (for review; see (Matveev et al., 2011)).

There are several indirect ways to probe the coupling distance between Ca$^{2+}$ channels and Ca$^{2+}$ sensors of exocytosis. One way is by intracellular application of two exogenous Ca$^{2+}$ chelators that have different binding rates but comparable affinities (Adler et al., 1991; Naraghi and Neher, 1997). The principle behind is the following: if the coupling distance is short, only the fast Ca$^{2+}$ chelator BAPTA (1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid) binds quickly enough to buffer out Ca$^{2+}$ before it reaches the Ca$^{2+}$ sensor of exocytosis from the Ca$^{2+}$ source. On the other hand, the slowly acting Ca$^{2+}$ chelator EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) will not have an effect on the exocytosis, unless exocytosis is under control of a Ca$^{2+}$ microdomain.

For instance, in the squid giant synapse and the mature calyx of Held synapse, neurotransmitter release is blocked by intracellular BAPTA, but not EGTA, suggesting a nanodomain coupling (Adler et al., 1991; Fedchyshyn and Wang, 2005); further reviewed in (Eggermann et al., 2012; Wang and Augustine, 2014)). Similarly, in mature mouse IHCs, there is a differential effect of BAPTA and EGTA on the release of the RRP on the whole-cell level, consistent with nanodomain coupling (Moser and Beutner, 2000; Beutner and Moser, 2001). It is important to note that in both studies exocytosis was studied by $C_{m0}$ measurements, which sum a dozen of synapses. Moreover, the differential effect of BAPTA and EGTA was used to separate different
pools of IHC SVs: the fast component of release, thought to reflect RRP, was insensitive to EGTA, while the slow component of release, interpreted as sustained release, was sensitive to 5 mM EGTA. Postsynaptic recordings from rat IHCs revealed consistently that the amplitude of the SGN current did not change in response to 5 mM intracellular EGTA, however, the onset and rise time were reduced (Goutman and Glowatzki, 2007). Indeed, disruption of endogenous Ca\(^{2+}\) buffers and substitution with BAPTA and EGTA together with modelling shed light on the properties of the physiological Ca\(^{2+}\) buffers in IHCs, and the effective coupling distance (Pangršić et al., 2015). The KO mice lacking endogenously expressed EF-hand Ca\(^{2+}\) binding proteins, parvalbumin-α, calbindin-D28k, and calretinin showed unaltered RRP suggesting a tight coupling between Ca\(^{2+}\) channels and release sites (Pangršić et al., 2015). In addition, overall sound encoding was largely unaffected while sustained exocytosis was reduced, suggesting excess exocytosis occurs extrasynaptically (Pangršić et al., 2015). Mathematical modelling suggested an effective coupling distance of 17 nm, supporting a nanodomain coupling in IHCs (Pangršić et al., 2015). Furthermore, substitution experiments with EGTA and BAPTA in ruptured-patch experiments indicated the presence of endogenous Ca\(^{2+}\) buffers equivalent to 1 mM synthetic Ca\(^{2+}\) binding sites with nearly half of them having kinetics as fast as BAPTA (Pangršić et al., 2015).

**Figure 1.6 – Ca\(^{2+}\) nanodomain versus microdomain control of release.**

A. IHCs make 5-30 synapses with SGNs. Upon the IHC depolarization, Ca\(_{V}\) 1.3 channels open. Ca\(^{2+}\) influx through the channels triggers the fusion of vesicles filled with glutamate. The distance between the Ca\(^{2+}\) sensor of the fusion machinery and the Ca\(^{2+}\) source –Ca\(_{V}\) 1.3 channels– defines the stimulus-release coupling. There are two limiting scenarios. B. The term “Ca\(^{2+}\) nanodomain” control of release is used when the distance is smaller than 30 nm, and a few Ca\(^{2+}\) channels can trigger release of a SV. In this scenario, the release of a vesicle is linearly dependent on the number of open Ca\(^{2+}\) channels, and consequently have a quasi-linear Ca\(^{2+}\) dependence (\(m = 1\)). C. “Ca\(^{2+}\) microdomain” control of release is defined when the distance is larger than 100 nm. Several Ca\(^{2+}\) channels has to open to trigger release of a vesicle, which gives rise to a supra-linear dependence of release (\(m > 1\)).
Ca\textsuperscript{2+} domain manipulations can be used to probe the spatial organization of presynaptic Ca\textsuperscript{2+} signaling. To interpret the effects of Ca\textsuperscript{2+} domain manipulations, it is critical to understand the biochemical (intrinsic) Ca\textsuperscript{2+} cooperativity. The classic work of Dodge and Rahamimoff (1967) suggested multiple Ca\textsuperscript{2+} ions act cooperatively, as they observed a power function relationship between neurotransmitter release and \([\text{Ca}\textsuperscript{2+}]_e\) (Dodge and Rahamimoff, 1967).

A more direct approach to assess the intrinsic Ca\textsuperscript{2+} cooperativity is by Ca\textsuperscript{2+} uncaging. This technique uses caged-Ca\textsuperscript{2+} compounds to elevate intracellular Ca\textsuperscript{2+} concentrations in a spatially homogenous manner, and simultaneous Ca\textsuperscript{2+} imaging allows to determine the Ca\textsuperscript{2+} concentration across the cell (Neher and Zucker, 1993). In IHCs, combination of Ca\textsuperscript{2+} uncaging and \(C_m\) measurements revealed a power of five. This means that binding of five Ca\textsuperscript{2+} ions to the Ca\textsuperscript{2+} sensor is required for vesicle fusion to happen (Beutner et al., 2001).

The exponent defining the power function relation of neurotransmitter release on Ca\textsuperscript{2+} influx is referred to as the "apparent" Ca\textsuperscript{2+} cooperativity \(m\). The Ca\textsuperscript{2+} domain manipulations to probe apparent Ca\textsuperscript{2+} cooperativity include varying the single channel current and number of open Ca\textsuperscript{2+} channels in several ways. First, the single-channel current on a time scale relevant for exocytosis (>500 \(\mu\text{s}\)) can be varied either by changing \([\text{Ca}\textsuperscript{2+}]_e\) or by application of a Ca\textsuperscript{2+} channel flicker blocker, such as inorganic blocker Zn\textsuperscript{2+}. Changing the single-channel current is expected to reflect the high intrinsic Ca\textsuperscript{2+} cooperativity of exocytosis, since the Ca\textsuperscript{2+} domain amplitude driving fusion would gradually change at the site of the vesicle, independent of the topography of the active zone. Secondly, the number of open channels can be changed by depolarizations of different amplitude, by tail current protocols recruiting different numbers of channels or by application of DHP antagonists (see the previous chapter). It is important to note that changing voltage amplitude, if the voltage range is broad, would also vary the single-channel current by the change in the driving force. On the other hand, tail current protocol could possibly help avoiding this complication. In this case, a pre-depolarization around the Ca\textsuperscript{2+} reversal potential, which activates all the Ca\textsuperscript{2+} channels, is applied and followed by a hyperpolarized voltage to allow Ca\textsuperscript{2+} influx by increase in the driving force. In such a situation, varying the duration of the non-permissive pre-depolarization pulse allows to control the number of open channels. Changing the number of open Ca\textsuperscript{2+} channels by any of the means described above, could cause a linear or supralinear rise of exocytosis depending on the precise topography of the active zone. In the case of a Ca\textsuperscript{2+} nanodomain control of release, varying the open Ca\textsuperscript{2+} channel number would linearly correlate with the release, since single-channel opening is sufficient to trigger exocytosis. On the other hand, Ca\textsuperscript{2+} microdomain control of release would require several Ca\textsuperscript{2+} channels to be open to trigger exocytosis.

In IHCs, so far, the apparent Ca\textsuperscript{2+} cooperativity was assessed by relating whole-cell Ca\textsuperscript{2+} influx either to whole-cell exocytosis via \(C_m\) measurements or to postsynaptic recordings. Brandt \textit{et al.} (2005) studied apparent Ca\textsuperscript{2+} cooperativity in mouse IHCs on the whole-cell level by capacitance measurements of RRP exocytosis elicited by 20-ms-long depolarizations (Brandt \textit{et al.}, 2005). Initially, they have varied single channel current by varying \([\text{Ca}\textsuperscript{2+}]_e\), which resulted in a supralinear Ca\textsuperscript{2+} dependence of exocytosis as expected from the high intrinsic Ca\textsuperscript{2+}
cooperativity found with Ca\(^{2+}\) uncaging. To avoid the potential differences in open channel number at different \([\text{Ca}^{2+}]_e\), unstable leaks at low \([\text{Ca}^{2+}]_e\) and differences in SV priming, they next changed the Ca\(^{2+}\) current by application of Zn\(^{2+}\). Zn\(^{2+}\) causes a flicker block of L-type Ca\(^{2+}\) channels, which does not result in true reduction of Ca\(^{2+}\) current (Winegar and Lansman, 1990). However, in the presence of Zn\(^{2+}\), an apparent reduction in the Ca\(^{2+}\) domain “seen” by the Ca\(^{2+}\) sensor of release would occur, as the kinetics of the fusion machinery cannot follow microsecond flickering of the Ca\(^{2+}\) ions. Similar to the effects of varying \([\text{Ca}^{2+}]_e\), Zn\(^{2+}\) manipulation revealed a nonlinear relationship between Ca\(^{2+}\) influx and exocytosis, further supporting the high intrinsic Ca\(^{2+}\) cooperativity. The same study further probed the Ca\(^{2+}\) cooperativity by varying the number of open channel through slow application of DHP antagonists, as the DHP antagonists decrease the open probability rather than the single-channel current (Brandt et al., 2005; Hess et al., 1984). A near-linear relationship was observed during DHP block, supporting the notion of Ca\(^{2+}\) nanodomain-like control of exocytosis in IHCs. Another study used postsynaptic recordings of SGNs to interpret the Ca\(^{2+}\) cooperativity of release in rat IHCs, where they have manipulated the depolarization voltage (Goutman and Glowatzki, 2007). They found a supralinear relationship between Ca\(^{2+}\) influx and EPSCs (excitatory postsynaptic currents) in the positive voltage range (>0mV), where the open probability of Ca\(^{2+}\) channels is constant, while the single-channel current changes. This relation was attributed to the high intrinsic Ca\(^{2+}\) cooperativity of release. On the other hand, in the physiological range for receptor potentials (-50 mV to -30 mV), a linear relation was found in agreement with the previous observations, hence supporting the notion of a Ca\(^{2+}\) nanodomain-like control of exocytosis.

A summation model was proposed to explain the observed linear relation between Ca\(^{2+}\) influx and neurotransmitter release in receptor cells (Heil and Neubauer, 2010). They proposed that the near-linear relation arises from the sum of several supralinear, but saturating, dependencies with different sensitivities at individual AZs of a cell. This hypothesis is still to be tested on a single AZ level, since so far Ca\(^{2+}\) cooperativity was studied either relating whole-cell Ca\(^{2+}\) influx to whole-cell exocytosis or to postsynaptic recordings.

Overall, the current state of knowledge supports the notion of Ca\(^{2+}\) nanodomain-like control of exocytosis in mature mammalian IHCs. They, however, go through changes in their coupling efficiency during development. Before the onset of hearing, exocytosis is evoked by Ca\(^{2+}\) action potentials with low coupling efficiency. Upon changes of the open channel number, a supralinear Ca\(^{2+}\) dependence turned into a near-linear one after the onset of hearing (Wong et al., 2014). Wong et al. (2014) proposed that the observed changes are due to the confinement of Ca\(^{2+}\) influx to the AZs and establishment of a nanodomain coupling between Ca\(^{2+}\) channels and release sites. The authors found it unlikely that there was a change in the intrinsic Ca\(^{2+}\) dependence of release machinery, as the Ca\(^{2+}\) uncaging experiments showed a high intrinsic Ca\(^{2+}\) cooperativity for both immature and mature mouse IHCs. Moreover, biophysical modeling of exocytosis in immature and mature IHCs suggested a scheme of molecular coupling of the release sites and the Ca\(^{2+}\) channels, where the Ca\(^{2+}\) influx through the coupled channel dominates the Ca\(^{2+}\) signal at the Ca\(^{2+}\) sensor (Wong et al., 2014). Such a transition
1.5. Spiral Ganglion Neurons

from an apparent high Ca$^{2+}$ cooperativity to a low one is also observed in calyx of Held synapse (Fedchyshyn and Wang, 2005). This tightening of Ca$^{2+}$ channel-sensor coupling in calyx of Held was suggested to be due to the spatial reorganization of GTP/GDP binding protein, septin 5 (Yang et al., 2010). This protein is thought to form a physical barrier between Ca$^{2+}$ channels and SVs, and maintain a microdomain coupling in immature calyx of Held synapses (Yang et al., 2010). In IHCs, however, no protein with similar function has been identified so far. On the other hand, Johnson et al. (2010) proposed that the differential expression of synaptotagmin IV is responsible for the apparent linearization of the Ca$^{2+}$ cooperativity but its expression in mature IHCs is controversial (Johnson et al., 2010).

1.5 Spiral Ganglion Neurons

In the organ of Corti, type I SGNs transmit the sensory information from an individual IHC, where they make 1:1 connection (Spoendlin, 1969). A type I SGN sends out a single unbranched, myelinated dendrite (except for the short initial segment near the synapse) to an IHC. Type I SGNs constitute 95% of the total afferent innervation in the organ of Corti. The remaining 5% of the innervation is by type II SGNs, which have a branched pattern and connect to several OHCs. The efferent axons from the superior olivary nuclei innervate OHCs directly, and also regulate the activity of type I SGNs (Figure 1.7).

Upon IHC depolarization by sound-driven vibrations, presynaptic Ca$^{2+}$ channels activate. The following Ca$^{2+}$ influx triggers exocytosis of vesicles filled with glutamate onto SGNs. SGNs employ glutamate receptors of the \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type, which forms a ring structure across the presynaptic density (Khimich et al., 2005; Meyer et al., 2009; Liberman et al., 2011). This ring structure of AMPA receptors is thought to optimize detection of glutamate released from the vesicles more uniformly (Chapochnikov et al., 2014). The presence of NMDA receptors is not clear, although metabotropic receptors were implicated (Peng et al. 2004). Upon glutamate release, glutamate receptors get activated, and this results in excitatory postsynaptic potentials (EPSPs) in SGNs. In type I SGNs, spikes are likely generated in the initial segment close to the synaptic ribbon (Hossain et al., 2005). In vivo and ex vivo studies show that almost every IHC release event triggers a spike (Rutherford et al., 2012; Siegel, 1992). This suggests the EPSCs are large enough to exceed the spike threshold. There is a heterogeneity in size and amplitude of EPSCs, which was interpreted as multivesicular release from IHCs (Glowatzki and Fuchs, 2002). On the other hand, recently, univesicular release mechanism was proposed to explain the heterogeneity of the EPSCs, where a single vesicle can undergo a full collapse or a pore flickering fusion (Chapochnikov et al., 2014; Grabner and Moser, 2018; Huang and Moser, 2018).

Single unit recordings of SGNs give insight about the SGN properties: the most prominent feature of SGNs is the frequency selectivity. SGNs show increased discharge rates above the spontaneous rates (SRs) when acoustically stimulated with a characteristic frequency (Figure 1.3B). Furthermore, SGN has a lower response threshold at a characteristic frequency, com-
Chapter 1. General Introduction

Figure 1.7 – Innervation pattern in the organ of Corti.
The type I and II SGNs constitute 95% and 5% of the afferent innervation in the organ of Corti. The type I SGNs (depicted in purple) make 1:1 connection with IHCs, while the type II SGNs (depicted in orange) have a branched pattern and connect to several OHCs. The efferent axons (green) directly connect with OHCs or modulate the type I SGNs. (Modified from (Kandel et al., 2012)).

pared to the high threshold displayed at other frequencies. The response thresholds over the audible spectrum give rise to the tuning curve of a SGN, with a sharp minimum at the characteristic frequency (Taberner and Liberman, 2005; Kiang et al., 1965; Liberman, 1978; Figure 1.3). This frequency selectivity comes from the tonotopic mapping of the cochlea as described above. Another feature of SGNs is the phase-locking: SGNs tend to fire action potentials at the same phases of a periodic stimulus. The quality of phase-locking depends on the frequency and intensity of the stimulus (Heil and Peterson, 2015). The level of phase-locking decreases with higher frequencies (up to 4 kHz). This limit coincides with the maximum frequency that can be encoded by the AC component of the IHC receptor potential (Figure 1.4B).

SGNs show variable spontaneous activity: individual fibers show different mean discharge rates between 0 to >120 spikes/s. Liberman (1982) traced individual cat SGNs after measuring their SRs and response properties back to the IHC synapse (Liberman, 1982). He found 3 classes of fibers in cat: low- and medium-SR fibers with high threshold preferentially contacting the modiolar side of IHC, and high-SR fibers with low threshold innervating the pillar side of IHC. In mice, the rate-level functions -discharge rate (spike/second) versus tone burst level (dB SPL)- showed the low SR-high threshold fibers have bigger dynamic range (>20 dB) than the high SR-low threshold fibers (Taberner and Liberman, 2005). These findings point
1.6 Candidate mechanisms driving diverse SGN firing properties

Out that SGNs originating from a single isopotential IHC vary considerably in their spiking behavior. While the mammalian cochlea encodes sound pressures spanning over six orders of magnitude, individual SGNs seem to cover only a fraction of it. This heterogeneous spiking behavior of SGNs contacting single IHC is thought to increase the dynamic range of sound intensity coding with an ensemble of fibers covering different fractions. This mechanism, however, might not be the only way to deal with the wide dynamic range intensity coding; the dynamic range adaptation of SGNs could also contribute (Dean et al., 2005; Wen et al., 2009). The exact mechanism driving the position-dependent firing properties of SGNs is not known.

1.6 Candidate mechanisms driving diverse SGN firing properties

As a single isopotential IHC can drive SGNs with diverse spiking behavior, several underlying mechanisms were proposed to explain this. These mechanisms include pre- and postsynaptic heterogeneity and efferent innervation (for review; see (Meyer and Moser, 2010)). The IHC synapses were indeed shown to have morphological and functional differences. A classical ultrastructural study in cats, based on a small sample of backtraced SGNs, showed the low SR-high threshold fibers contacting synapses with bigger ribbons and high SR-low threshold fibers contacting synapses with smaller ribbons (Merchan-Perez and Liberman, 1996). Although this difference is not as clear-cut in smaller rodents, studies showed similar gradient of AZ properties (Liberman et al., 2011; Ohn et al., 2016). As well, an opposing gradient of ribbon size and postsynaptic glutamate receptor patch was observed: bigger ribbons facing smaller glutamate receptor patches on the modiolar side of IHC, while the opposite is observed on the pillar side of IHC (Liberman et al., 2011). This is, however, controversial, as an opposite observation was made in guinea pig (Zhang et al., 2018). The modiolar-pillar gradient of AZ properties was disrupted when lateral olivocochlear efferents were damaged, showing the contribution of efferent innervation to the diversity of type I SGNs (Yin et al., 2014). Besides the observed differences in the anatomy of SGNs –larger diameter on the pillar side–, three studies employing single-cell RNA sequencing showed three molecularly distinct classes of SGNs (Petitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018).

In addition to the morphological differences observed in AZs, subcellular Ca\(^{2+}\) imaging showed the AZs from a single IHC are functionally different (Frank et al., 2009; Meyer et al., 2009; Ohn et al., 2016). Initially, Frank et al. (2009) observed a heterogeneity of Ca\(^{2+}\) signaling in a single IHC, in terms of amplitude and voltage dependence. They further investigated the putative mechanisms contributing to the heterogeneity: they exclude the contribution of mitochondrial Ca\(^{2+}\) uptake, Ca\(^{2+}\)-induced Ca\(^{2+}\) release, cytosolic buffering, and open probability of Ca\(^{2+}\) channels on the amplitude variability. Meyer et al. (2009) also showed high variability of Ca\(^{2+}\) signaling in a single IHC with modiolar synapses having larger Ca\(^{2+}\) microdomain amplitude compared to pillar synapses. Ohn et al. (2016) took a more advanced approach of 3D-live imaging of most, if not all, AZs of an IHC. By applying voltage ramps to IHC, they studied the voltage dependence of Ca\(^{2+}\) influx, and found that the pillar synapses activated at weaker depolarizations than the modiolar synapses. The more negative activation
of the pillar synapses were argued to relate to the high SR-low threshold fibers. All previous studies leave us with a complicated picture of how SGN spiking behaviour might be shaped to encode complementarily sound intensities over a wide dynamic range (Figure 1.8).

Figure 1.8 – Spatial heterogeneity of SGN firing properties and IHC synapses.
SGNs innervating a single IHC show different spiking behavior: fibers with high SR - low threshold preferentially contact the pillar (abneural) side of the IHC, and low SR - high threshold fibers contact the modiolar (neural) side of the IHC. Exemplary rate-level functions from the two different class of SGNs were shown. High SR – low threshold fibers exhibit smaller dynamic range compared to the low SR – high threshold fibers. Moreover, they exhibit a spatial heterogeneity of synapses: the pillar synapses exhibit smaller ribbon size, while the modiolar synapses have bigger ribbons. Consistent with the small ribbon size, pillar synapses exhibit smaller Ca$^{2+}$ domain amplitude with a more negative activation kinetics compared to the modiolar synapses in the same IHC. (modified after (Pangrsic et al., 2018))
1.7 Scope of the thesis

This thesis had three main goals:

First, we aimed at understanding the role of AP180 in IHC synaptic transmission. We used confocal microscopy to check the presence and cellular localization of AP180 in WT IHCs. We next checked the impact of the absence of AP180 on hearing by systems physiology. Moreover, we performed patch-clamp $C_m$ measurements to probe the replenishment rate of SVs and endocytotic membrane retrieval in IHCs lacking AP180. We, furthermore, used high-pressure freezing and electron tomography to assess the SV pools in the absence of AP180 compared to the WT control mice.

Secondly, we explored whether the planar polarity mechanisms establishing the hair bundle orientation at the apical part of the IHC could contribute to the spatial heterogeneity of IHC AZs at the basal part. The AZ heterogeneity is thought to contribute to diverse SGN firing properties, and thereby widen the dynamic range of sound encoding. We used a mouse line expressing PTXα in hair cells to block the $G_{αi}$ signaling. We checked the effects of disrupted $G_{αi}$ signaling, by patch-clamp $C_m$ measurements, patch-clamp combined with $Ca^{2+}$ imaging, high- and super-resolution microscopy (Jean et al., 2019).

Lastly, we studied the synaptic transfer function of IHCs and explored its contribution to sound encoding over a wide dynamic range. We performed imaging of synaptic $Ca^{2+}$ influx and glutamate release combined with patch-clamp recordings. For the detection of synaptic glutamate release, we used AAV-mediated expression of a glutamate sensor, iGluSnFR, on the postsynaptic membrane (Marvin et al., 2013). We initially probed the voltage dependence and intrinsic $Ca^{2+}$ dependence of release by relating whole-cell $Ca^{2+}$ influx to synaptic glutamate release. By dual-color imaging of $Ca^{2+}$ influx and glutamate release on a single-synapse level, we then assessed the “apparent” $Ca^{2+}$ dependencies of individual synapses.
2 AP180 promotes release site clearance and clathrin-dependent vesicle reforma-
tion in inner hair cells of the mouse cochlea

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the manuscript with help of S.J., T.Ma., and I.M.

Figure contributions of Jana Kroll*:
- Immunohistochemistry (Figure 2.1, 2.3, S1)
- High pressure freezing and electron tomography (Figure 2.4, 2.7)

Figure contributions of Özge Demet Özçete*:
- analysis of the ABR recordings and DPOAEs (Figure 2.2)
- patch-clamp recordings of Ca\(^{2+}\) currents and C\(_m\) (Figure 2.5, 2.6)
Chapter 2. AP180 in IHC synaptic transmission

2.1 Abstract

High-throughput neurotransmission at ribbon synapses of cochlear inner hair cells (IHCs) requires tight coupling of neurotransmitter release and balanced recycling of synaptic vesicles (SVs). Moreover, release sites need to be cleared from exocytosed material for a new SV to come in. Here, we examined the role of the adaptor protein AP180 for IHC synaptic transmission in AP180-KO mice using high-pressure freezing and electron tomography, confocal microscopy, patch-clamp membrane-capacitance measurements and systems physiology. AP180 was found predominantly at the synaptic pole of IHCs. AP180-deficient IHCs had drastically reduced SV numbers, slowed endocytic membrane retrieval, and accumulated endocytic intermediates near ribbon synapse. Together, this indicates that AP180 is required for clathrin-dependent endocytosis and SV reformation in IHCs. Moreover, AP180 deletion led to a high prevalence of SVs in a multi-tethered docked state after stimulation, a reduced rate of SV replenishment, and a hearing impairment. We conclude that AP180 contributes to release site clearance in addition to its role in clathrin recruitment in IHCs and, thereby, is required for indefatigable hearing.

2.2 Significance Statement

Both, the ultrastructure and the molecular composition of inner hair cell (IHC) ribbon synapses are specialized to accomplish the very high synaptic vesicle (SV) throughput required for indefatigable hearing. We show here that the adaptor protein AP180 is not only essential for the recruitment of clathrin during slow endocytosis and during SV reformation following bulk retrieval. Moreover, our data indicate that AP180 contributes to efficient SV replenishment to the readily releasable pool of SVs by promoting the clearance of previously exocytosed material from the release sites.

Keywords: hearing, sound encoding, ribbon synapse, calcium, exocytosis, endocytosis, adaptor proteins, clathrin, high-pressure freezing, electron tomography, membrane capacitance

2.3 Introduction

Ribbon synapses, formed between cochlear inner hair cells (IHCs) and spiral ganglion neurons, feature one of the highest rates of continuous neurotransmitter release in the mammalian body (Pangršić et al., 2012). High-throughput synaptic vesicle (SV) exocytosis is a prerequisite for indefatigable sound encoding. It goes hand in hand with highly efficient SV replenishment, endocytosis and reformation of fusion-competent SVs. The specialized structure of the IHC active zone (AZ) - notably the electron-dense ribbon that is made of RIBEYE and tethers a halo of SVs (Jean et al., 2018; Lv et al., 2016; Maxeiner et al., 2016) - and a unique protein composition of release machinery and AZ cytomatrix are thought to accomplish this function (reviewed in: (Rutherford and Moser, 2016; Safieddine et al., 2012; Wichmann and Moser, 2015)).
Endocytic membrane retrieval in IHCs comprises a slow, linear component that likely reflects clathrin-mediated endocytosis (CME), and a fast, exponential component thought to represent bulk retrieval (Moser and Beutner, 2000; Neef et al., 2014). Morphological evidence for CME and bulk retrieval in hair cells has been reported previously (e.g. (Neef et al., 2014; Jung et al., 2015a; Lenzi et al., 2002; Siegel and Brownell, 1986)). Molecular mediators in SV recycling in hair cells include dynamin, endophilin-As, clathrin and synaptojanin-1 (Neef et al., 2014; Jung et al., 2015a; Boumil et al., 2010; Kroll et al., 2019; Trapani et al., 2009). Their disruption primarily affected the slow, clathrin-dependent component of IHC membrane retrieval. Surprisingly, the absence of the adaptor protein 2 (AP-2) did not significantly impair endocytic membrane retrieval (Jung et al., 2015a). Yet, like in neurons (Kononenko et al., 2014), absence of AP-2 from IHCs compromised the clathrin-dependent reformation of SVs from endosome-like vacuoles (ELVs). Being further required for the sorting of the multi-C2 domain protein otoferlin, AP-2 was shown to play a dual role in release site clearance and in SV reformation in IHCs (Jung et al., 2015a).

Similar to AP-2, the neuronal adaptor protein AP180 acts in clathrin recruitment and in the sorting of membrane-stranded SV cargo following exocytosis at conventional synapses (Koo et al., 2015). Therefore, it represents an interesting candidate regulator of SV recycling at IHC synapses. Via binding of clathrin, AP-2 and phospholipid PI(4,5)P2, AP180 was shown to be involved in the early steps of clathrin-coated pit (CCP) formation in neurons (Ford et al., 2001; Hao et al., 1999; Lindner and Ungewickell, 1992; Morris et al., 1993; Moshkanbaryans et al., 2016). In AP180 knock-out mice (AP180-KO), accumulations of ELVs and lower numbers of clathrin-coated structures have been observed, underlining the importance of AP180 for CME as well as for clathrin-dependent SV reformation from ELVs (Koo et al., 2015). Via its AP180 N-terminal homology (ANTH) domain, AP180 binds to the SNARE domain of VAMP2 and other members of the VAMP family (Koo et al., 2011; Maritzen et al., 2012). This way, AP180, as well as its ubiquitously expressed homolog CALM, organize the recycling of VAMP2 from the plasma membrane after SNARE complex disassembly following SV fusion and, thereby, contribute to the clearance of release sites at conventional synapses (Koo et al., 2015; Koo et al., 2011; Maritzen et al., 2012; Miller et al., 2011). AP180-dependent clearance of release sites seemed to be of greater importance in inhibitory synapses that show higher rates of transmitter release than excitatory synapses (Koo et al., 2015).

The relevance of AP180 for high-throughput synaptic transmission at IHC ribbon synapses is unclear, especially because IHCs seem to employ an unconventional molecular machinery. Notably, IHCs operate without AP180’s cargo protein VAMP2 and other neuronal SNARE proteins like SNAP25 and syntaxin-1 and seem to lack SNARE regulators such Munc-13 and Munc-18 (Nouvian et al., 2011; Vogl et al., 2015). To date, the only well-characterized key player in IHC exocytosis is otoferlin. Otoferlin is thought to be the Ca2+-sensor of exocytosis in IHCs and mediates efficient SV replenishment, possibly by advancing SV tethering and release site clearance (Jung et al., 2015a; Kroll et al., 2019; Vogl et al., 2015; Chakrabarti et al., 2018; Duncker et al., 2013; Johnson and Chapman, 2010; Michalski et al., 2017; Pangrsic et al., 2010; Roux et al., 2006; Strenzke et al., 2016; Vogl et al., 2016). Here, we studied the role of AP180 at
Chapter 2. AP180 in IHC synaptic transmission

IHC ribbon synapses from the molecular to the systems level. Combining confocal microscopy, chemical depolarization of IHCs, high-pressure freezing and electron tomography, perforated patch-clamp, and recordings of otoacoustic emissions and auditory brainstem responses, we discovered that AP180 has a role in release site clearance, clathrin assembly for CME and SV reformation following bulk retrieval in IHCs.

2.4 Results

2.4.1 AP180 is present at synapses of the murine cochlea and redistributes upon stimulation

First, we aimed to identify if the adaptor protein AP180 is present in the murine organ of Corti, and, more specifically, in IHCs. Therefore, we performed immunohistochemistry in acutely explanted apical cochlear turns (approximately 2-12 kHz) of wild-type mice (Wt) after the onset of hearing, as well as of age-matched AP180-KO mice (used as a control for antibody specificity). We labeled for AP180 in combination with markers for efferent synapses (synapsin1/2), the synaptic ribbon (RIBEYE/CtBP2), and the hair cells (otoferlin). Immunofluorescence (Fig. 2.1A left panel) revealed the presence of AP180 in IHCs and outer hair cells (OHCs), as well as in efferent fibers reaching to the hair cells and possibly in spiral ganglion neurons (see their somata in the inset to Fig. 2.1A). A lack of AP180 staining in organs of Corti from AP180-KO mice confirmed the high specificity of the AP180 antibody (Fig. S1A). Higher magnifications of maximum intensity z-projections (Fig. 2.1A right panel) revealed that AP180 is most prevalent in the basal half of IHCs where the ribbon synapses are located (‘synaptic pole’). Single xy-sections through the synaptic pole of immunolabeled IHCs indicate an intracellular as well as plasma membrane distribution of AP180. Line profiles through the longitudinal axis of IHCs (Fig. 2.1B) revealed the highest fluorescence intensity levels at the IHC plasma membrane, followed by IHC lumen and efferent presynaptic terminals.

The distribution of AP180 immunofluorescence was altered upon high-K+ depolarization (65 mM for 1 min, Fig. S1B). Compared to resting conditions, the intensity of AP180 fluorescence was significantly reduced in a distance of 2 µm from the plasma membrane (Fig. S1C; \( p = 0.0003 \), unpaired student’s t-test) after high-K+ stimulation, whereas the staining near the membrane was preserved (Fig. S1C; \( p = 0.5468 \), unpaired student’s t-test). These data indicate the stimulation-induced recruitment of AP180 to the plasma membrane, pointing towards a relevance of AP180 for IHC synaptic transmission.

2.4.2 AP180 is required for hearing

In order to probe for a function of AP180 in IHC synaptic transmission on the systems level, we next recorded auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAE). Wt and AP180-KO mice showed comparable ABR wave amplitudes (Fig. 2.2A) and ABR latencies (Fig. 2.2E), while the ABR thresholds were significantly increased
Figure 2.1 – AP180 is present in the murine organ of Corti.

(A) Maximum intensity z-projection of confocal sections of an apical cochlear turn (left) and higher magnification of IHCs and OHCs (right) from a P15 Wt animal immunolabeled for AP180, RIBEYE/CtBP2 (staining ribbons and nuclei) and synapsin-1/2. Scale bars: left image 200 µm; right image 10 µm. Inset in (A) represents a zoom into the spiral ganglion area and shows AP180 immunofluorescence in the somata of spiral ganglion neurons. (B) Single confocal sections from synaptic pole of an IHC (Wt, P15) using markers for AP180, otoferlin and synapsin1/2. Scale bar 5 µm. (Bi and Bi i) Average fluorescence intensity line profiles through the longitudinal axis of seven IHCs from apical to basal. AP180 fluorescence is strongest in proximity to the IHC basal membrane, but can also be detected in nerve terminals of lateral olivocochlear efferents (yellow arrows) and potentially in the postsynaptic boutons of spiral ganglion neurons.

for the frequencies 6 and 16 kHz (Fig. 2B; for 6 kHz, $p = 0.032$, for 16 kHz, $p = 0.024$, Mann-Whitney U test). DPOAEs, reflecting cochlear amplification, were intact in AP180-KO mice, which indicates normal mechanoelectrical transduction and electromotility of OHCs (Fig.
Therefore, we conclude that the elevation of ABR thresholds arises from a dysfunction downstream of mechanoelectrical transduction and cochlear amplification, i.e. in IHCs, their synapses or the auditory pathway.

![Image](image-url)

**Figure 2.2** – Auditory brainstem responses show elevated thresholds in AP180-KO animals. (A) ABR waveforms were recorded in response to 80 dB clicks (mean ± SEM, AP180-KO N (number of mice) = 9, Wt N = 9). (B) ABR thresholds were significantly elevated for AP180-KO animals compared to Wt animals (mean ± SEM, AP180-KO N = 9, Wt N = 9, for 6 kHz, \( p = 0.032 \), for 16 kHz, \( p = 0.024 \), Mann-Whitney U test). (C) DPOAE amplitudes (mean ± SEM) recorded in response to the pair of simultaneous sine waves \( f_1 = 13.3 \text{ kHz} \) and \( f_2 = 16 \text{ kHz} \) at increasing intensity \( f_2 \) intensity was 10 dB bigger than \( f_1 \) intensity) were unaltered. (D) Cumulative amplitude from wave 1 to 5 was comparable in AP180-KO and Wt animals. (E) ABR latencies did not show any difference in AP180 animals compared to Wt animals.

### 2.4.3 AP180 absence alters the levels of Vglut3, but not of otoferlin

In IHCs of AP-2\( \mu \) KO mice, otoferlin levels were shown to be strongly reduced, with the reduction being less pronounced at the plasma membrane suggesting impaired sorting of otoferlin (Jung and Schnitzer, 2003). Therefore, we tested by semi-quantitative analysis of otoferlin immunofluorescence if deletion of AP180 likewise changes otoferlin levels and/or distribution (Fig. 2.3A and A\(_i\)). Yet, neither otoferlin distribution, nor total fluorescence intensity, were altered in IHCs of AP180-KO mice compared to Wt \( (p = 0.17, \text{ unpaired student's t-test}) \). Yet, we noted that the immunofluorescence intensity of the vesicular glutamate transporter of IHCs, Vglut3, was reduced by 17.1 ± 3.2% throughout the IHC in AP180-KO mice compared to Wt (Fig. 2.3B and B\(_i\); \( p < 0.0001, \text{ unpaired student's t-test} \)). Notably, co-staining
Figure 2.3 – Reduced Vglut3 levels, but unaltered otoferlin levels in AP180-deficient IHCs.
(A) Maximum intensity z-projections of otoferlin-stained apical cochlear turns from AP180-KO and Wt littermate controls. Scale bars 5 nm. (A_i) Unaltered otoferlin levels in AP180-KO (n = 114 IHCs from N = 4 mice) compared to Wt (n = 107 IHCs from N = 4 mice). (B) Maximum intensity z-projections of confocal sections of Wt and AP180-KO IHCs stained for Vglut3. Scale bars 5 µm. (B_i) Intracellular Vglut3 levels were 17% reduced in AP180-KO IHCs (n = 114 IHCs from 4 animals) compared to Wt (n = 107 IHCs from N = 4 animals). (C) Exemplary confocal sections of RIBEYE/CtBP2 (magenta) labeling the synaptic ribbon and Homer (green) staining labeling the postsynaptic density. Scale bar 5 µm. (C_i) Equal numbers of ribbon synapses in Wt (6 images from 3 animals, approx. 10 cells per image) and in AP180-KO (6 images from 3 animals, approx. 10 cells per image). *** p < 0.001.

for RIBEYE/CtBP2 and homer (a marker for the postsynaptic density; Fig. 2.3C) indicated an unaltered ribbon anchorage to the AZ as well as a normal number of ribbon synapses (Fig. 2.3C_i; ribbon synapses per IHC for Wt: 14.5 ± 0.4, for AP180-KO: 14.5 ± 0. 5 synapses per IHC; p = 0.96, unpaired student’s t-test). In summary, deletion of AP-180 did not alter otoferlin levels, synapse number or ribbon anchorage, but it reduced the Vglut3 levels potentially via diminishing SV numbers.
2.4.4 Numbers of SVs and clathrin-coated structures are reduced in AP180-KO mice

Next, we performed high-pressure freezing followed by freeze substitution and electron tomography of ribbon synapses shortly after the onset of hearing (P15-P16). Exemplary virtual sections (Fig. 2.4A), obtained from semithin-sections via tomogram generation, as well as 3D models of reconstructed ribbon synapses from Wt and AP180-KO IHCs (Fig. 2.4B) revealed reduced numbers of SVs not only in direct vicinity to the ribbon, but also in the cytoplasm. We investigated the following three morphological pools of SVs, as defined previously (Kroll et al., 2019) and indicated in the schematic drawing (Fig. 2.4D): (i) ribbon-associated SVs (RA-SVs) in max. 80 nm distance to the ribbon, (ii) membrane-proximal SVs (MP-SVs) that are within a 50 nm distance from the plasma membrane and 100 nm laterally from the presynaptic density, and (iii) cytosolic SVs (operationally defined as all uncoated vesicles with a max. diameter of 70 nm and a max. distance of 500 nm to the ribbon, excluding the aforementioned SV pools). In all three pools, numbers of SVs were reduced in IHCs of AP180-KOs compared to Wt. The strongest reduction by almost 60% was observed for the pool of cytosolic SVs (Fig. 2.4I; \( p < 0.0001 \), unpaired student’s t-test). Numbers of RA-SVs (Fig. 2.4E; \( p = 0.0062 \), unpaired student’s t-test) and of MP-SVs (Fig. 2.4F; \( p = 0.0328 \), unpaired student’s t-test) were reduced by approximately 24% and 22%, respectively. The reduction of SVs, found in electron tomography, exceeded the expectation based on Vglut3 immunofluorescence (reduction by 17%). The outer diameter of the cytosolic SVs, on the other hand, was increased on average (Fig. 2.4J; \( p < 0.0001 \), Kolmogorov-Smirnov test), which may account for the modest reduction of Vglut3 fluorescence intensity. The observed increase of the SV diameter is consistent with results of previous studies on mice and invertebrates missing AP180 or its homolog UNC-11 (Koo et al., 2015; Zhang et al., 1998; Nonet et al., 1999; Vanlandingham et al., 2014). Interestingly, the average distance of MP-SVs to the AZ plasma membrane was slightly increased in IHCs from AP180-KO mice compared to Wt (Fig. 2.4G; \( p = 0.0288 \), Mann-Whitney U test).

We further quantified clathrin-coated structures in proximity to the ribbon, including coated vesicles and coated pits budding from the plasma membrane or from ELVs, as can be seen exemplarily in Fig. 2.4C. The numbers of coated structures in total (Fig. 4H; \( p = 0.0002 \), unpaired student’s t-test), and of coated vesicles in particular (\( p < 0.0001 \), Mann-Whitney U test, please also see Table S1 for further information on statistical analyses), were strongly reduced in AP180-KO compared to Wt. This suggests an involvement of AP180 in the early steps of clathrin-mediated membrane retrieval. While numbers of ELVs were not significantly increased in tomograms of AP180-KO mice (Fig. 2.4K; \( p = 0.7645 \), unpaired student’s t-test), we found a strong increase in their average volume (Fig. 2.4L; \( p = 0.0043 \), Mann-Whitney U test). Therefore, it is likely that the reduced numbers and increased size of SVs are a consequence of impaired clathrin- and AP180-dependent SV reformation from ELVs.
2.4. Results

Figure 2.4 – Ultrastructural changes of IHC ribbon synapses in AP180-KO mice.

(A) Representative virtual sections of tomograms from P15 Wt and AP180-KO ribbon synapses. Scale bars 100 nm. (B) Reconstructed models of the area around the ribbon from P15 Wt and AP180-KO IHCs. Scale bars 200 nm. (C) Exemplary coated structures observed at the IHC base. Scale bars 100 nm. (D) Schematic drawing illustrating parameters for the quantifications in (E-L; n for all quantifications = 10 tomograms from 2 different animals in each group). (E) Numbers of RA-SVs were 24% reduced in AP180-KO mice. (F) The number of MP-SVs was likewise reduced, and (G) the distance of MP-SVs to the AZ plasma membrane was increased. (H) Numbers of coated structures were decreased with the number of coated vesicles being most strongly affected. (I) In the cytosol, numbers of small uncoated vesicles were drastically (>60%) reduced in absence of AP180. (J) The average diameter of cytosolic SVs was increased. Generally, SV diameters were more heterogeneous in absence of AP180. (K) Numbers of ELVs were unaltered, (L) while the average volume per ELV was strongly increased in AP180-KO mice. * p < 0.05; ** p < 0.01; *** p < 0.001 for all quantifications.
2.4.5 IHCs from AP180-KO mice show normal Ca\(^{2+}\) influx, but impaired SV replenishment

In order to test for the physiological correlates of the morphological changes observed in the AP180-KO IHCs, we performed perforated patch-clamp experiments soon after the onset of hearing (5 mM Ca\(^{2+}\), P14-18) in acutely explanted apical cochlear coils (approximately 6-10 kHz). We recorded whole-cell CaV1.3-mediated Ca\(^{2+}\) influx in response to step depolarizations of 10 ms from -84 mV to +63 mV in 5 mV increments (Fig. 2.5A inset). Ca\(^{2+}\) influx of AP180-KO IHCs was statistically indistinguishable from that of Wt IHCs (Fig. 2.5A). Furthermore, we performed measurements of membrane capacitance increments (ΔC\(_m\)) reporting exocytosis evoked by the Ca\(^{2+}\) influx. SV pool dynamics was probed by a train of stimuli that consisted of 10 step depolarizations to -14 mV of 20 ms duration each, with 150 ms inter-stimuli intervals (Fig. 2.5B). No significant differences of ΔC\(_m\) in response to the first depolarization, which is thought to represent fusion of the readily releasable pool (RRP), or in response to the full train of stimuli, was observed (Fig. 2.5B). We further examined the replenishment rate of the SVs by using the 'SMN analysis' (Schneggenburger et al., 1999). We plotted the cumulative ΔC\(_m\) versus the stimulus number and fitted a line in the last 5 stimuli (Fig. 2.5C). The obtained apparent replenishment rate (fF/stimulus) was significantly lower in AP180-KO IHCs (with the outliers, marked with filled diamond shape and identified via IQR method, \(p = 0.037\), without the outliers, \(p = 0.01\), unpaired student’s t-test). The increased SV size leads to an overestimation of the true rate of SV replenishment. Assuming a spherical SV shape and a specific capacitance of 9.1 fF/µm\(^2\) (Albillos et al., 1997), we estimated the expected capacitance added by single SV fusion (SV diameter was obtained from the tomograms; Fig. 2.4J). The mean estimated outer SV diameter values were 44.0 ± 0.1 nm and 48.4 ± 0.3 nm, for Wt and AP180-KO IHCs, respectively. We subtracted the average membrane thickness (6.2 nm, (Neef et al., 2007) to obtain the mid-membrane diameter, which is relevant for calculating the capacitance increase upon single SV fusion. The estimated capacitance for Wt SVs (41.3 ± 0.3 aF) was close to the measured value of 40 aF (Grabner and Moser, 2018), while the SV capacitance predicted for AP180-KO IHCs amounted to 51.8 ± 0.7 aF. Based on these estimates, we converted the apparent SV replenishment rate (fF/stimulus, see above) into number of vesicles per stimulus. This revealed a strong impairment in the SV replenishment rate in AP180-KO IHCs (Fig. 2.5C, \(p = 0.0003\) unpaired student’s t-test).

Potential reasons for the observed reduction of the replenishment rate in AP180-KO IHCs include (i) deficits in SV docking and/or priming, (ii) disturbed release site clearance, (iii) impaired SV resupply including SV reformation and recruitment of SVs to the active zone, which we aimed to decipher in the following experiments.
2.4. Results

Figure 2.5 – Impaired SV replenishment, but normal Ca\(^{2+}\) influx and SV fusion in AP180-KO IHCs.

(A) In order to quantify whole-cell Ca\(^{2+}\) current IHCs were step depolarized for 10 ms from -84 mV to +63 mV in 5 mV increments. AP180-KO IHCs showed comparable Ca\(^{2+}\) currents (AP180-KO n = 11 IHCs, N = 9 mice; Wt n = 11 IHCs, N = 7 mice) (A\(_i\)). Inset shows example Ca\(^{2+}\) currents in response to the stimuli paradigm shown above. (B) IHCs were depolarized with a train of stimuli that consists of 10 step depolarizations with duration of 20 ms separated by 150 ms (mean ± SEM, AP180-KO n = 13 IHCs, N = 9 mice; Wt n = 12 IHCs, N = 8 mice). AP180-KO IHCs had comparable ∆C\(_m\) in response to the first stimulus (B\(_i\)), and a tendency towards lower total ∆C\(_m\) evoked by the train of stimuli (B\(_{ii}\)). (C) Cumulative ∆C\(_m\) versus stimulus number. A line was fitted to the last 5 points of the train of stimuli. The replenishment rate estimates (fF/stimulus) were calculated from the slope. (C\(_i\)) AP180-KO IHCs showed a significantly slower replenishment rate (fF/stimulus) compared to Wt IHCs (AP180-KO n = 13 IHCs, N = 9 mice; Wt n = 12 IHCs, N = 8 mice, with the outliers, marked with filled diamond shape (identified via IQR method), \(p = 0.037\), without the outliers, \(p = 0.01\), t-test). (C\(_{ii}\)) Further calculation was done by taking into consideration the diameter of the SVs obtained from the EM data (Fig. 2.4J). The replenishment rate of SVs (vesicles/stimulus) was significantly reduced in AP180-KO IHCs compared to Wt IHCs (AP180-KO n = 13 IHCs, N = 9 mice; Wt n = 12 IHCs, N = 8 mice, student’s t test, \(p = 0.0003\)). Box plots indicate 25-75 quartile with whiskers reaching from 10-90%. The outliers were detected via inter-quartile range (IQR) method and are depicted as filled diamonds.
Figure 2.6 – Reduced rate of the slow component of endocytic membrane retrieval, but intact fast endocytosis.

(A) Exemplary traces of Ca\(^{2+}\) current (top) and capacitance (bottom) upon 20 ms step depolarization to -14 mV from the holding potential of -84 mV. AP180-KO IHCs had comparable ΔC\(_m\) (A\(_i\)) and efficiency of Ca\(^{2+}\) current to drive exocytosis, probed by the ratio of ΔC\(_m\) and Q\(_{Ca}\) (A\(_{ii}\)), in response to 20 ms step depolarization, primarily eliciting RPP release (AP180-KO n = 15 IHCs, N = 12 mice; Wt n = 15 IHCs, N = 11 mice). (B) Exemplary traces of Ca\(^{2+}\) current (top) and C\(_m\) (bottom) upon 200 ms step depolarization. ΔC\(_m\) in AP180-KO IHCs were comparable with Wt IHCs in response to 200 ms depolarization, eliciting sustained release (B\(_i\)). The efficiency of Ca\(^{2+}\) current to drive exocytosis was comparable (B\(_{ii}\)). Average traces of C\(_m\) in response to 20 ms (C) and 200 ms (D) depolarization (mean ± SEM, AP180-KO n = 16 IHCs, N = 12 mice; Wt n = 17 IHCs, N = 14 mice). (E) Time of return to baseline after 20 ms depolarization was comparable between AP180-KO and Wt IHCs. (F) The slope of the slow linear component of endocytic membrane retrieval, most likely reflecting CME, was calculated by fitting a line to the first 20 s of the C\(_m\) recording after the depolarization. AP180-KO IHCs showed a shallower slope (without the outliers, p = 0.002, t-test, with the outliers, p = 0.043, Mann-Whitney U test). (G) The slope of the linear component following 200 ms depolarization was not statistically distinguishable. We did not observe significant differences in the amplitude (H) and the time constant (I) of the fast, exponential component of endocytosis, likely to represent the bulk endocytosis. Box plots indicate 25-75 quartile with whiskers reaching from 10-90%. The outliers were detected via inter-quartile range (IQR) method and were depicted as filled diamonds.
2.4. Results

2.4.6 Deletion of AP180 impairs CME and clathrin-dependent SV reformation in IHCs

Given the role of AP180 in CME and clathrin-dependent SV reformation at conventional synapses (Koo et al., 2015; Zhang et al., 1998), we investigated endocytic membrane retrieval in IHCs of AP180-KO mice. Using perforated-patch recordings, we determined the endocytic $C_m$ decline following exocytosis in response to step depolarizations to -14 mV for 20 ms and for 200 ms (Neef et al., 2014). As above, both AP180-KO and Wt IHCs showed comparable exocytic $\Delta C_m$ upon 20 ms step depolarizations, thought to primarily induce RRP release (Fig 2.6 A and A[i]). The slow and linear component of endocytosis, likely to reflect CME (Neef et al., 2014), dominated the endocytic $C_m$ decline following 20 ms depolarizations in IHCs of both genotypes (Fig 2.6 C, E, F). The slope of the linear component was significantly smaller in AP180-KO IHCs compared to Wt IHCs, indicating a deficit in CME (Fig. 2.6 F; $p = 0.043$, Mann-Whitney U test, when including the data points identified as outliers; $p = 0.002$, unpaired student’s t-test, when excluding the outliers). In contrast, AP180-deletion did not impair the fast, exponential component of the endocytosis elicited by exocytosis in response to 200 ms depolarization (Fig 2.6 D, H, I), which likely reflects bulk endocytosis (Neef et al., 2014).

In a next step, we stimulated freshly dissected P15-P16 organs of Corti for 5 min using high-K+ followed by high-pressure freezing, freeze substitution and electron tomography (Fig 2.7). Similar to under resting conditions, we observed a lower total number of coated structures in IHCs of AP180-KO mice (Fig 2.7 I; $p = 0.002$, Mann-Whitney U test). We detected several ELVs with multiple budding CCPs in proximity to ribbon synapses in Wt, indicative of active SV reformation following bulk endocytosis (exemplar shown in the virtual section and in the 3D model in Fig 2.7 C). Yet, in AP180-KO mice, we did not observe ELVs with more than one or two budding CCPs. Moreover, the number of ELVs with one or more budding CCPs was reduced in AP180-KO IHCs compared to Wt IHCs, indicating a deficit in CME (Fig. 2.6 F; $p = 0.043$, Mann-Whitney U test, also see Table S1). As at rest, the total number of ELVs was unaltered in AP180-KO IHCs compared to Wt ($p = 0.113$, student’s t-test, Table S1) whereas the average ELV volume was again increased (Fig. 2.7H; $p = 0.0355$, Mann-Whitney U test).

The number of cytosolic SVs was decreased by more than 40% in AP180-KO IHCs (Fig. 2.7F; $p = 0.0026$, Mann-Whitney U test), so not beyond the reduction found at resting conditions. Interestingly, numbers of SVs directly connected to the ribbon did not differ between IHCs of Wt and AP180-KO mice after stimulation. We did neither observe a difference in the number of RA-SVs (Fig. 2.7D; $p = 0.445$, Mann-Whitney U test) nor in the number of MP-SVs (Fig. 2.7E; $p = 0.721$, Mann-Whitney U test). These data indicate that AP180- and clathrin-dependent reformation of SVs is required for maintaining high numbers of cytosolic SVs in the synaptic pole of the IHC. Still, even in absence of AP180, endocytic SV recycling was sufficient to provide enough SVs for refilling the pools of RA-SVs and MP-SVs. Therefore, the role of AP180 in CME and SV reformation seems unlikely to fully account for the observed impairment in SV replenishment.
2.4.7 After stimulation, SVs remain in a multi-tethered state at the AZ of AP180-KO IHCs

In order to investigate potential mechanisms underlying impaired SV replenishment, we further examined the MP-SV pool by electron tomography (Fig. 2.7A and 2.7B). As mentioned
above (Fig. 2.4G), the distance of MP-SVs to the AZ membrane was increased in AP180-KO IHCs under resting conditions. After stimulation, we did not observe significant differences in the average membrane distance of MP-SVs between Wt and AP180-KO IHCs (Table S1; \( p = 0.451 \), unpaired student's t-test). Since SV tethering to the AZ membrane has been suggested to reduce the distance between MP-SVs and AZ membrane (e.g. (Chakrabarti et al., 2018; Fernández-Busnadiego et al., 2013)), we quantified the numbers of tethers to the AZ membrane under resting conditions and after stimulation. For that purpose, we determined the following SV groups: (i) SVs without such tethers, (ii) SVs with a single tether, (iii) SVs with multiple tethers, and (iv) docked SVs, i.e. that the distance between SV outer membrane and AZ membrane was 0-2 nm in electron tomography virtual sections, as previously described for IHCs and neurons (Chakrabarti et al., 2018; Imig et al., 2014).

In resting conditions, we did not observe any significant differences in the tethering of MP-SVs in AP180-KO IHCs (Fig. 2.7G, one-way ANOVA followed by Tukey's post-hoc test; for individual \( p \)-values see Table S1). In line with a previous study of IHCs, high-K\(^+\) stimulation resulted in more tethered and less untethered SVs (Chakrabarti et al., 2018). This was the case in both, Wt and AP180-KO IHCs (Fig. 2.7G; for the fraction of untethered SVs, resting Wt vs. stimulated Wt \( p = 0.001 \), resting Wt vs. stimulated AP180-KO \( p = 0.001 \), one-way ANOVA followed by Tukey's post-hoc test). Yet, we noticed a shift towards multi-tethered SVs in AP180-KO IHCs after stimulation, whereas most SVs in stimulated Wt IHCs remained connected via a single tether (\( p = 0.008 \) for the fraction of multi-tethered MP-SVs in stimulated Wt vs. stimulated AP180-KO IHCs). Furthermore, we observed significantly more docked SVs in stimulated AP180-KO IHCs compared to resting conditions (Wt resting condition vs. AP180-KO stimulated condition: \( p = 0.004 \), one-way ANOVA followed by Tukey's post-hoc test; AP180-KO rest. vs. AP180-KO stim: \( p = 0.002 \), one-way ANOVA followed by Tukey's post-hoc test), as well as compared to stimulated Wt (\( p = 0.037 \), one-way ANOVA followed by Tukey's post-hoc test). Together, these observations indicate that MP-SVs in IHCs of AP180-KO mice remained in the multi-tethered or docked state prior to fusion, which has previously been indicated as a morphological correlate of impaired release site clearance in IHCs (Chakrabarti et al., 2018).

### 2.5 Discussion

In the present study, we addressed the role of the endocytic adaptor protein AP180 in synaptic sound encoding in the cochlea. There, IHC ribbon synapses must sustain high rates of SV release for reliable transmission of auditory information. When investigating IHC ribbon synapses of AP180-KO mice by a combination of functional patch-clamp recordings, electron tomography and systems physiology, we found evidence for a dual function of AP180. Firstly, AP180 is required for clathrin-dependent SV reformation from ELVs and aids in the early steps of classical CME. Secondly, AP180 plays a role in efficient replenishment of SVs to the release site, likely via promoting the clearance of the release site from proteins and lipids added by preceding SV fusion events.
2.5.1 **AP180 is required for clathrin-dependent endocytosis and SV reformation following bulk retrieval in IHCs**

The reduced rate of endocytic $C_m$ decline and the reduction of clathrin-coated SVs in resting and stimulated AP180-KO IHCs support a role of AP180 in CME. Beyond that, we observed less budding of CCPs from enlarged ELVs in AP180-KO IHCs after high-$K^+$ stimulation, suggesting that SV reformation from ELVs following bulk retrieval operates in an AP180-dependent manner. Clathrin-dependent SV reformation from ELVs was also impaired in AP-2 KO IHCs (Neef et al., 2018). Therefore, like in hippocampal neurons (Kononenko et al., 2014; Koo et al., 2015), both adaptor proteins seem to play an essential role in the recruitment of clathrin triskelia not only to the plasma membrane, but also to ELVs.

Notably, SV numbers were more drastically reduced in AP180-deficient IHCs (60%) than in AP180-deficient inhibitory CNS neurons (~33%; note that no SV reduction was found in excitatory neurons, (Vanlandinghing et al., 2014)). Nonetheless, CME still occurred, and bulk retrieval seemed unaffected in AP180-deficient IHCs. Moreover, prolonged stimulation by high-$K^+$ left the RA- and MP-SVs pools unchanged and did not further reduce the number of cytosolic SVs in AP180-KO IHCs. Therefore, AP180-independent forms of membrane retrieval must exist in IHCs, and other adaptor proteins like the AP180 homolog CALM, AP-2 or AP-3 may contribute to the recruitment of clathrin. The precise interplay of adapter proteins and membrane retrieval mechanisms in IHCs requires further investigation. Also, future studies might address the question whether accumulation of exocytosed proteins and lipids at the plasma membrane, or increased membrane tension, shift membrane retrieval towards clathrin-independent forms (Maritzen and Haucke, 2018).

2.5.2 **A role of AP180 in release site clearance at IHC active zones**

Notwithstanding the SV reformation deficit, the number of MP-SVs at the AZ was not significantly reduced in AP180 KO IHCs during the strong stimulation. Still, the measured rate of SV replenishment during trains of brief depolarizations was reduced in AP180-KO IHCs, while $Ca^{2+}$ triggered fusion of RRP-SVs seemed intact. These findings suggest that slowed SV replenishment of the release sites was primarily due to impaired AZ clearance rather than to insufficient SV resupply to the AZ. Further support for this hypothesis comes from the observation of an increased number of multi-tethered and docked SVs in the AP180-KO IHCs. Similarly, in IHCs of the otoferlin mutant *pachanga*, which was shown to be profoundly deaf with a reduced rate of SV replenishment to the RRP (Pangrsic et al., 2010), the number of multi-tethered SVs was significantly higher than in Wt after stimulation (Chakrabarti et al., 2018). Multi-tethered SVs in neurons were also shown to be closer to the AZ membrane compared to single-tethered or untethered SVs and probably display a prerequisite for SV docking and priming (Fernández-Busnadiageto et al., 2013). As SV fusion was intact in AP180 KO IHCs, we take the higher prevalence of multi-tethered SVs as a potential morphological correlate of unproductive release sites due to impaired clearance.
In a previous study, absence of RIM-binding protein 2 (RIM-BP2) likewise resulted in an increased distance of MP-SVs to the AZ-membrane in IHCs at resting conditions, that has been attributed to disturbances in the composition of the cytomatrix of the AZ (Krinner et al., 2017). Changes in the cytomatrix or in the AZ plasma membrane itself may also serve as an explanation for the increased distance of MP-SVs in absence of AP180. More precisely, absence of AP180 could lead to accumulation of proteins or lipids at the membrane of the release sites, which possibly causes an energetically unfavorable environment, ultimately slowing down exocytic processes after prolonged stimulation.

These findings are remarkable, given that AP180 has previously been identified as a key sorting factor for VAMPs (Maritzen and Haucke, 2018; Miller et al., 2011; Sahlender et al., 2013). Thereby, in contrast to many other interactions between cargo proteins and their adaptors, the conserved SNARE motif of VAMPs itself binds to the ANTH domain of AP180 (Koo et al., 2015; Miller et al., 2011). Consequently, AP180 deficiency led to accumulations of VAMP2 at the plasma membrane of neurons (Koo et al., 2015). However, the architecture and molecular composition of the IHC active zone are unique - IHCs seem to operate without VAMP1-3 and other neuronal exocytic proteins like Munc-13, Munc-18 and complexin (Nouvian et al., 2011; Vogl et al., 2015; Strenzke et al., 2009). Moreover, the levels of the key protein of the IHC exocytosis, otoferlin, were unaltered in IHCs in absence of AP180 suggesting that otoferlin sorting for endocytosis does not require AP180. Our data, therefore, indicate that hair cells require at least one other, yet to be identified protein for SV exocytosis that might also harbor a SNARE domain. This unknown exocytic IHC protein probably interacts with AP180 and accumulates at the plasma membrane in absence of AP180. Of note, the unaltered otoferlin levels suggest that otoferlin sorting does not depend on AP180 and allow to attribute the IHC phenotype to AP180 deficiency itself rather than to concomitant loss of otoferlin.

2.6 Materials and Methods

2.6.1 Animals

Constitutive knockout mice for AP180 as well as their wild-type, previously described in (Koo et al., 2015), were used in this study. For all experiments, mice of either sex were examined shortly after the onset of hearing (P14-P18). For the analysis of AP180 localization and distribution, C57BL6/J mice were used. All experiments complied with national animal care guidelines and were approved by the University Medical Center Göttingen board for animal welfare and the animal welfare office of the state of Lower Saxony.

2.6.2 Immunohistochemistry and Confocal Microscopy

Freshly dissected apical turns of organs of Corti from two-weeks old mice were (i) chemically stimulated for 1 min using high-\(K^+\) stimulation solution (65.36 mM KCl, 79.7 mM NaCl, 2 mM CaCl\(\text{}_2\), 1 mM MgCl\(\text{}_2\), 0.5 mM MgSO\(\text{}_4\), 10 mM HEPES, 3.4 mM L-glutamine, and 6.9 mM
D-glucose, pH 7.4) or incubated in a high Ca\(^{2+}\), low K\(^+\) solution for control (5.36 mM KCl, 139.7 mM NaCl, 2 mM CaCl\(^2\), 1 mM MgCl\(_2\), 0.5 mM MgSO\(_4\), 10 mM HEPES, 3.4 mM L-glutamine, and 6.9 mM D-glucose, pH 7.4; also see (49) at room temperature followed by fixation using 4% formaldehyde (FA) in phosphate buffered saline (PBS) on ice, or (ii) directly fixed with 4% FA in PBS for 10 min (CtBP2/Homer) or for 60 min (Otoferlin/Vglut3, AP180) on ice. After 3x10 min washing in PBS, blocking solution (goat serum dilution buffer (GSDB); 16% normal goat serum, 450 mM NaCl, 0.3% Triton X-100, 20 mM phosphate buffer, pH 7.4) was applied for 1 h in a wet chamber at room temperature. Primary antibodies were diluted in GSDB and applied overnight at 4°C in a wet chamber. After washing 3x10 min (wash buffer: 450 mM NaCl, 20 mM phosphate buffer, 0.3% Triton X-100), secondary antibodies diluted in GSDB were applied in a light-protected wet chamber for 1 h at room temperature. Then, the specimens were washed thrice in wash buffer and finally in 5 mM phosphate buffer and mounted onto glass microscope slides with mounting medium (Mowiol 4-88, Sigma). The following primary antibodies were used: rabbit anti-AP180 (1:300, Synaptic Systems, cat. No. 155 003), mouse anti-CtBP2 (also recognizing the ribbon protein ribeye, 1:200, BD Biosciences, cat. No. 612044), mouse anti-otoferlin (1:300, Abcam, cat. No. ab53233), rabbit anti-Vglut3 (1:300, Synaptic Systems, cat. No. 135 203), rabbit anti-Homer1 (1:200, Synaptic Systems, cat. No. 160 002) and guinea pig anti-synapsin1/2 (Synaptic Systems, cat. No. 106 002). The secondary antibodies used for confocal microscopy were goat anti-rabbit AlexaFluor488 (1:200, Invitrogen, cat. No. A 11008), goat anti-mouse AlexaFluor568 (1:200, Invitrogen, cat. No. A 11004), goat anti-mouse AlexaFluor633 (1:200, Invitrogen, cat. No. A 21136), and goat anti-guinea pig AlexaFluor568 (1:200, Invitrogen, cat. No. A 11075). Confocal images were acquired using a laser scanning confocal microscope (Zeiss LSM800, Carl Zeiss AG, Oberkochen, Germany) with 488 nm (Ar) and 561 nm (He-Ne) lasers for excitation and 1.4 NA 63x oil immersion objectives. Z-axis stacks of 2D images were generated with a step size of 0.6 μm from comparable tonotopic regions. Images were processed using ImageJ (http://imagej.net/) and assembled for display in Adobe Illustrator software. For semiquantitative analysis of immunofluorescence, samples of AP180-KO and control were processed identically and in parallel throughout immunohistochemistry, confocal imaging, and analysis.

### 2.6.3 High-Pressure Freezing and Freeze Substitution

HPF was essentially performed as described previously (Chakrabarti et al., 2018; Jung et al., 2015b; Wong et al., 2014). Briefly, the apical cochlear turns from P15-P16 Wt and AP180-KO mice were dissected in Ca\(^{2+}\)-free Hanks’ balanced salt solution (HBSS) and carefully placed on aluminium specimen carriers in the 0.2 mm cavity (type A, 0.1 and 0.2 mm cavity, Leica Microsystems, Wetzlar, Germany) filled with HBSS solution. A second specimen carrier (0.3 mm cavity, type B, Leica Microsystems) was dipped in hexadecene and placed onto the first specimen carrier with the cavity upwards. An EM HPM100 (Leica Microsystems) was used for sample freezing. Immediately afterwards, samples were transferred into liquid nitrogen and stored until freeze substitution was performed. For stimulation, organs of Corti were dissected in Ca\(^{2+}\)-free HBSS solution and transferred into stimulation solution (identical with
2.6. Materials and Methods

the solution used for immunohistochemistry experiments). Within the stimulation solution, samples were placed on specimen carriers and frozen exactly 5 min after the transfer.

Freeze substitution was performed with an EM AFS2 (Leica Microsystems). Organs of Corti were transferred into the AFS2 precooled to -90°C. After 4 d incubation in 0.1% tannic acid in acetone, samples were washed 3x1 h in acetone before 2% (w/v) osmium tetroxide in acetone was applied at -90°C. The temperature was increased from -90°C to -20°C (5°C/h), stayed at -20°C for 17 h and was further increased from -20°C to +4°C (10°C/h). Osmium tetroxide was removed at 4°C and samples were washed 3x1h with acetone. Subsequently, samples were slowly warmed to room temperature. Finally, organs of Corti were infiltrated with Epon resin (acetone/Epon 1:1 (v/v) for 2h, 100% Epon overnight), placed into embedding molds and polymerized for 48 h at 70°C.

2.6.4 Electron Tomography

Electron tomography of ribbon synapses was performed as described previously (Strenzke et al., 2009; Jung et al., 2015b). 250 nm sections of the embedded samples were obtained approaching from the anterior edge using an ultramicrotome (UC6, Leica Microsystems, Wetzlar, Germany) with a 35° diamond knife (Diatome, Nidau, Switzerland) and applied to formvar-coated copper 75-mesh grids. Sections were post-stained with uranyl acetate replacement solution (Science services, Munich, Germany) for 40 min and lead citrate for 1 min following standard protocols. To both sides of the grids, 10 nm gold beads (British Bio Cell, Crumlin, UK) were applied as fiducial markers. Using Serial-EM software, single tilt series from -60° to +60° (increment 1°) were acquired with a JEM2100 (JEOL, Freising, Germany) at 200 kV and 10,000x magnification. For tomogram generation, the IMOD package etomo was used, models were generated with 3dmod (bio3d.colorado.edu/imod/). Auditory Brainstem Recordings and Distortion Product Otoacoustic Emissions ABRs and DPOAE were performed on 6-weeks-old mice as previously described (Jing et al., 2013). Briefly, mice were anesthetized with a combination of ketamine (125 mg/kg) and xylazine (2.5 mg/ kg) i.p. The core temperature was maintained constant at 37°C using a heat blanket (Hugo Sachs Elektronik–Harvard Apparatus). The TDT II system run by BioSig software was used for stimulus generation, presentation, and data acquisition (Tucker Davis Technologies) (MathWorks). Tone bursts (4/6/8/12/16/24/32 kHz, 10 ms plateau, 1 ms cos2 rise/fall) or clicks of 0.03 ms were presented at 40 Hz (tone bursts) or 20 Hz (clicks) in the free field ipsilaterally using a JBL 2402 speaker.

2.6.5 Patch-clamp recordings from IHCs

The apical turn of the organs of Corti were acutely dissected from P14 to P18 animals in HEPES Hank’s solution containing (in mM): 5.36 KCl, 141.7 NaCl, 10 HEPES, 0.5 MgSO4·7H2O, 1 MgCl2·6H2O, 5.6 D-glucose, and 3.4 L-glutamine (pH 7.2, 300 mOsm/l). The IHC basolateral membranes were exposed by cleaning of nearby cells with a suction pipette. IHCs were
patch clamped at room temperature (20-25°C) in perforated-patch configuration as described previously (Moser and Beutner, 2000). For Ca\(^{2+}\) current and capacitance (\(C_m\)) measurements, the extracellular solution contained the following (in mM): 110 NaCl, 35 TEA-Cl, 2.8 KCl, 1 MgCl\(_2\), 1 CsCl, 10 HEPES, 5 CaCl\(_2\), and 11.1 D-glucose (pH 7.2, 305 mOsm/l) and was introduced into the recording chamber via a perfusion system. The pipette solution contained (in mM): 130 Cs-gluconate, 10 TEA-Cl, 10 4-AP, 10 HEPES, 1 MgCl\(_2\), as well as 300 mg/ml amphotericin B (pH 7.17, 290 mOsm/l). The measurements were done via EPC-10 amplifiers controlled by Patchmaster software (HEKA Elektronik, Germany). IHCs were held at -84 mV. All voltages were corrected for liquid junction potential offline (14 mV). Currents were leak corrected using a p/10 protocol. Recordings were used only if the leak current was lower than 30 pA and the series resistance (Rs) was lower than 30 mOhm. Recordings were discarded when the Ca\(^{2+}\) current rundown exceeded 25%.

For IV recordings, the IHCs were step depolarized for 10 ms to -14 mV from the holding potential of -84 mV to +63 mV in 5 mV increments. For \(C_m\) measurements, IHCs were stimulated by step depolarizations to -14 mV for durations of 20 or 200 ms at intervals of 60s-100s. The Lindau-Neher technique was used to measure the capacitance changes (Lindau and Neher, 1988). Exocytosis and endocytosis were quantified from \(\Delta C_m\) as described previously (Neef et al., 2014).

### 2.6.6 Data Analysis

**Immunohistochemistry.** Intensities of AP180, otoferlin and synapsin1/2 fluorescence signals along the longitudinal axis of IHCs (Fig. 2.1) were measured via line profiles using ImageJ software. Igor Pro 6.3 software was used to generate average intensity profiles. AP180 distribution in membrane proximity (Fig. S1) was assessed from intensity line scans reaching from the basal plasma membrane towards the cytosol along the longitudinal axis of IHCs. Using ImageJ software, middle planes of IHCs were selected and cell borders were defined via otoferlin co-staining. In the otoferlin channel, lines from the cell membrane towards the nucleus were drawn and fluorescence intensity was measured in the AP180 channel (pixel intensity values ranging from 0 to 255) to avoid bias. Igor Pro 6.3 software was used to generate average intensity profiles of the individual cells. IHC ribbon synapses were manually counted in z-projections of confocal sections from CtBP2/Homer-immunolabeled organs of Corti using ImageJ software. Otoferlin and Vglut3 levels of IHCs were semi-quantitatively assessed as immunofluorescence intensity values and analyzed using Imaris (Bitplane) and Matlab (Mathworks), as described previously (Strenzke et al., 2009). Intensities of each cell are normalized to the average intensity of the Wt group for otoferlin or for Vglut3.

**Electron tomography.** Only tomograms with a single ribbon synapse clearly anchored to the active zone were selected for quantification using the IMOD package 3Dmod (bio3d.colorado.edu/imod/) and as described previously (Kroll et al., 2019; Chakrabarti et al., 2018). Small, clear vesicles were classified as synaptic vesicles (SVs) if they appeared round and...
if their outer diameter (defined by fitting a circle to the borders at the maximum projection of
the respective SV) was smaller than 70 nm. SVs were categorized in three groups: (i) ribbon-
associated SVs (RA-SVs), if they were located in the first row around the synaptic ribbon and
with a max. distance of 80 nm to the ribbon, (ii) membrane-proximal SVs (MP-SVs) if the
distance between SV and plasma membrane was less than 50 nm and if the distance between
SV and presynaptic density was less than 100 nm, and (iii) cytosolic SVs if the distance between
SV and ribbon was less than 500 nm thereby excluding RA-SVs and MP-SVs (all criteria being
valid at maximum projection of the respective SV). MP-SVs were further subdivided into four
groups depending on their connection to the AZ plasma membrane as described in a previous
study (Chakrabarti et al., 2018): SVs were counted as docked, if the distance between SV outer
membrane and AZ membrane was 0-2 nm. Single-tethered SVs were all SVs with only one
tether reaching from the SV to the AZ membrane, multi-tethered SVs were all SVs with more
than one tether from SV to the AZ. SVs were categorized as untethered if no filament was
observed between SV and plasma membrane (thereby ignoring possible filamentous connec-
tions between SVs, as well as between SVs and presynaptic density or between SV and ribbon).
Furthermore, all structures containing a clathrin-coat were counted as coated structures if the
minimal distance between organelle and ribbon was less than 500 nm. This quantification
includes structures with a coat around the entire clathrin-coated SV (independent of its size),
coated pits budding from the plasma membrane, and endosome-like vacuoles (ELVs) with
at least one budding coated pit (in this case, the ELV with pit was only counted once and not
included in the additional ELV quantification). All membranous organelles were defined as
ELVs and counted, if their max. diameter was larger than 70 nm and/or if they were tubular,
and if the minimal distance between ELV and ribbon was less than 500 nm. The volume of
each ELV was assessed from a modelled mesh calculated from contours drawn in every virtual
section of the ELV in 3Dmod.

Patch-clamp recordings. Electrophysiological data was analyzed with custom-written pro-
grams in Igor Pro 6.3 and Python. For the analysis of the IV curves, last 5 ms of the evoked
Ca\(^{2+}\) currents were averaged after subtracting the mean of the first 5 ms of the recording. For
the analysis of the stimuli train, 70 ms of C\(_m\) was averaged to calculate ΔC\(_m\), skipping first
50 ms after each depolarization to avoid impact of non-exocytic C\(_m\) transients. In order to
estimate the replenishment rate of SVs, we used the “SMN analysis” (Schneggenburger et al.,
1999). We plotted cumulative ΔC\(_m\), versus the stimulus number. A line was fitted to the last 5
points, and the slope of the fit was taken as the replenishment rate. We furthermore calculated
the replenishment rate in terms of vesicles per stimulus. We have used the diameter values
obtained from the tomograms of Wt and AP180-KO IHCs, substracting the mean membrane
thickness (Neef et al., 2007) to obtain the midmembrane diameter. For the conversion of SV
diameter to capacitance values, a specific capacitance value of 9.1 fF / µm\(^2\) (Neef et al., 2007)
was used and spherical shape was assumed.

For calculating the ΔC\(_m\), the difference between the mean 400 ms C\(_m\) before and 200 ms
after the depolarization was taken. The Ca\(^{2+}\) charge (Q\(_{Ca}\)) was calculated by taking the
integral of the leak-subtracted current during depolarization. The endocytosis recordings
were analyzed as described previously (Neef et al., 2014). The overall observed decrease in $C_m$ during perforated patch-clamp recordings were corrected by the slope of the global fit to $C_m$ values taken from each recording. We have calculated the slow component of the endocytosis by a linear fit of post-depolarization $C_m$, skipping 200 ms. The recordings did not return to baseline within 80 s were excluded from the analysis. The amplitude of the exponential component of $C_m$ decline after 200 ms depolarization was calculated by substraction of linear fit to last 15 s of $C_m$. An exponential function was fit to the residual $C_m$ using a genetic curve-fitting algorithm (Sanchez del Rio and Pareschi, 2001). IHCs that did not exhibit exponential component were excluded from the analysis.

Statistical analysis. Sample sizes were chosen according to typical observation numbers used in each respective field and can be found in the respective figures and/or corresponding figure legends. For quantification, the following software was used: Matlab (Mathworks), Excel (Microsoft), Igor Pro 6 (Wavemetrics), Origin 9.0 (Microcal Software), GraphPad Prism (GraphPad Software) and Python. Averages are expressed as mean ± SEM, box plots indicate 25-75 quartile with whiskers reaching from 10-90%. Data sets were tested for normal distribution (Jarque-Bera test, D’Agostino and Pearson omnibus normality test or the Shapiro-Wilk test) and equality of variances. Statistical significance was calculated using unpaired, two-tailed student’s t-test for normally distributed data, Mann-Whitney U test for non-normally distributed data and Kolmogorov–Smirnov test to compare data distribution. For Fig. 2.7G, one-way ANOVA followed by Tukey’s post-hoc test was used to compare multiple groups. Significant differences are reported as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. 

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2.7 Supplementary information

Figure S1 – Subcellular distribution of AP180 changes after stimulation.
(A) AP180 immunofluorescence in maximum intensity z-projections of confocal sections of apical cochlear turns from Wt and AP180-KO (both P15). Scale bar 5 µm. (B) Exemplary single confocal sections from synaptic pole of IHCs from Wt P15 mice after 1 min high K⁺ stimulation compared to IHCs after 1 min in low K⁺ control solution. Scale bars 5 µm. (C) Average AP180 intensity line scans from confocal sections measured from the basal IHC membrane towards the nucleus indicate unchanged max. intensities in membrane proximity \( (C_1) \) and reduced AP180 intracellular levels in 2 µm distance from the membrane \( (C_{ii}) \) after stimulation \( (n = 93 \text{ IHCs from } N = 3 \text{ mice}) \) compared to resting condition \( (n = 73 \text{ IHCs from } N = 3 \text{ mice}) \). Otoferlin co-staining was used to determine IHC intracellular regions. *** \( p < 0.001 \).
### Table 2.1 – Auditory brainstem responses show elevated thresholds in AP180-KO animals (Fig 2.2)

<table>
<thead>
<tr>
<th>Data</th>
<th>Avg. Wt</th>
<th>Avg. KO</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B: ABR threshold at 4 kHz [dB]</td>
<td>61.25 ± 5.57</td>
<td>67.5 ± 3.27</td>
<td>Student's t</td>
<td>t = -0.96</td>
<td>0.349</td>
</tr>
<tr>
<td>2B: ABR threshold at 6 kHz [dB]</td>
<td>43.1 ± 6.0</td>
<td>53.3 ± 3.7</td>
<td>Mann-Whitney U</td>
<td>U = 17.0</td>
<td>0.0325 (*)</td>
</tr>
<tr>
<td>2B: ABR threshold at 8 kHz [dB]</td>
<td>35.0 ± 4.85</td>
<td>46.11 ± 3.2</td>
<td>Student's t</td>
<td>t = -1.909</td>
<td>0.0743</td>
</tr>
<tr>
<td>2B: ABR threshold at 12 kHz [dB]</td>
<td>33.75 ± 5.72</td>
<td>43.33 ± 2.88</td>
<td>Student's t</td>
<td>t = -1.54</td>
<td>0.1426</td>
</tr>
<tr>
<td>2B: ABR threshold at 16 kHz [dB]</td>
<td>32.7 ± 5.4</td>
<td>45.0 ± 3.6</td>
<td>Mann-Whitney U</td>
<td>U = 18.0</td>
<td>0.0240 (*)</td>
</tr>
<tr>
<td>2B: ABR threshold at 24 kHz [dB]</td>
<td>39.44 ± 5.67</td>
<td>47.77 ± 4.64</td>
<td>Mann-Whitney U</td>
<td>U = -1.135</td>
<td>0.064</td>
</tr>
<tr>
<td>2B: ABR threshold at 32 kHz [dB]</td>
<td>57.77 ± 6.82</td>
<td>60.55 ± 4.74</td>
<td>Student's t</td>
<td>t = -0.33</td>
<td>0.742</td>
</tr>
<tr>
<td>2B: ABR threshold for 20 Hz click [dB]</td>
<td>41.11 ± 1.11</td>
<td>41.11 ± 1.11</td>
<td>Mann-Whitney U</td>
<td>U = 40.5</td>
<td>0.467</td>
</tr>
</tbody>
</table>

### Table 2.2 – Reduced Vglut3 levels but unaltered otoferlin levels in AP180-deficient IHCs (Fig 2.3)

<table>
<thead>
<tr>
<th>Data</th>
<th>Avg. Wt</th>
<th>Avg. KO</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A: Norm. otoferlin fluorescence [%]</td>
<td>100 ± 4.6</td>
<td>95.7 ± 6.0</td>
<td>Student's t</td>
<td>t = 1.37</td>
<td>0.17</td>
</tr>
<tr>
<td>3B: Norm. Vglut3 fluorescence [%]</td>
<td>100 ± 6.6</td>
<td>82.5 ± 6.1</td>
<td>Student's t</td>
<td>t = 5.34</td>
<td>p &lt; 0.0001 (***p value)</td>
</tr>
<tr>
<td>3C: N of ribbon synapses</td>
<td>14.5 ± 0.4</td>
<td>14.5 ± 0.4</td>
<td>Student's t</td>
<td>t = 0.054</td>
<td>0.96</td>
</tr>
</tbody>
</table>
### Table 2.3 – Ultrastructural Changes of IHC ribbon synapses in AP180-KO mice (Fig 2.4)

<table>
<thead>
<tr>
<th>Data</th>
<th>Avg. Wt</th>
<th>Avg. KO</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E: N of RA-SVs</td>
<td>47.7 ± 1.7</td>
<td>36.4 ± 3.2</td>
<td>Student’s t</td>
<td>t = 3.1</td>
<td>p = 0.0062 (**)</td>
</tr>
<tr>
<td>4F: N of MP-SVs</td>
<td>18.5 ± 1.5</td>
<td>14.4 ± 0.9</td>
<td>Student’s t</td>
<td>t = 2.31</td>
<td>p = 0.0328 (*)</td>
</tr>
<tr>
<td>4G: Distance to membrane (MP-SVs) [nm]</td>
<td>-</td>
<td>-</td>
<td>Kolmogorov-Smirnov</td>
<td>D = 0.239</td>
<td>p = 0.0002 (***)</td>
</tr>
<tr>
<td>4G: Avg. # distance to membrane (MP-SVs)</td>
<td>20.1 ± 0.7</td>
<td>26.7 ± 2.1</td>
<td>Mann-Whitney U</td>
<td>U = 21</td>
<td>p = 0.0288 (*)</td>
</tr>
<tr>
<td>4H: Total N of coated structures</td>
<td>8 ± 0.7</td>
<td>3.6 ± 0.5</td>
<td>Student’s t</td>
<td>t = 4.71</td>
<td>p = 0.0002 (***)</td>
</tr>
<tr>
<td>4H: N of coated vesicles</td>
<td>6.9 ± 0.8</td>
<td>1.8 ± 0.3</td>
<td>Mann-Whitney U</td>
<td>U = 1</td>
<td>p &lt; 0.0001 (***)</td>
</tr>
<tr>
<td>4H: N of coated pits at ELVs</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>Mann-Whitney U</td>
<td>U = 40.5</td>
<td>p = 0.4699</td>
</tr>
<tr>
<td>4H: N of coated pits</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>Mann-Whitney U</td>
<td>U = 34</td>
<td>p = 0.1626</td>
</tr>
<tr>
<td>4I: N of cytosolic SVs</td>
<td>90.2 ± 7.9</td>
<td>37 ± 4.6</td>
<td>Student’s t</td>
<td>t = 5.82</td>
<td>p &lt; 0.0001 (***)</td>
</tr>
<tr>
<td>4J: Diameter of cytosolic SVs [nm]</td>
<td>-</td>
<td>-</td>
<td>Kolmogorov-Smirnov</td>
<td>D = 0.44</td>
<td>p &lt; 0.0001 (***)</td>
</tr>
<tr>
<td>4J: Avg. # diameter of cytosolic SVs [nm]</td>
<td>44.1 ± 0.4</td>
<td>48.1 ± 0.6</td>
<td>Student’s t</td>
<td>t = 5.56</td>
<td>p &lt; 0.0001 (***)</td>
</tr>
<tr>
<td>4K: N of ELVs</td>
<td>21.3 ± 2.3</td>
<td>22.2 ± 1.8</td>
<td>Student’s t</td>
<td>t = 0.304</td>
<td>p = 0.7645</td>
</tr>
<tr>
<td>4L: Avg. # ELV volume [nm³]</td>
<td>569,333 ± 28,301</td>
<td>1,210,064 ± 225,248</td>
<td>Mann-Whitney U</td>
<td>U = 1</td>
<td>p = 0.0043 (**)</td>
</tr>
</tbody>
</table>

### Table 2.4 – Impaired SV replenishment, but normal Ca2+ influx and SV fusion in AP180-KO IHCs (Fig 2.5)

<table>
<thead>
<tr>
<th>Data</th>
<th>Avg. Wt</th>
<th>Avg. KO</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A: I_{max} [pA]</td>
<td>258.84 ± 9.68</td>
<td>257.88 ± 14.74</td>
<td>Mann-Whitney U</td>
<td>U = 60.0</td>
<td>0.5</td>
</tr>
<tr>
<td>5C: ΔC_{m} after first pulse [fF]</td>
<td>25.3 ± 1.7</td>
<td>25.5 ± 1.9</td>
<td>Student’s t</td>
<td>t = 0.08</td>
<td>0.936</td>
</tr>
<tr>
<td>5C_{ii}: ΔC_{m} after train [fF]</td>
<td>157.91 ± 10.47</td>
<td>131.45 ± 11.5</td>
<td>Student’s t</td>
<td>t = 1.68</td>
<td>0.106</td>
</tr>
<tr>
<td>5D: Replenishment rate [fF/stimulus]</td>
<td>10.91 ± 0.98</td>
<td>7.98 ± 0.89</td>
<td>Student’s t</td>
<td>t = 2.83</td>
<td>0.0103 (*)</td>
</tr>
<tr>
<td>5D_{ii}: Replenishment rate [SVs/stimulus]</td>
<td>264.14 ± 23.74</td>
<td>153.99 ± 17.22</td>
<td>Student’s t</td>
<td>t = 4.35</td>
<td>0.00031 (***)</td>
</tr>
</tbody>
</table>
Table 2.5 – Reduced rate of the slow component of endocytic membrane retrieval, intact fast endocytosis (Fig 2.6)

<table>
<thead>
<tr>
<th>Data</th>
<th>Avg. Wt</th>
<th>Avg. KO</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
<td>20 ms - ΔC&lt;sub&gt;m&lt;/sub&gt; [fF]</td>
<td>27.8 ± 1.6</td>
<td>26.32 ± 1.83</td>
<td>Student's t</td>
<td>t = 0.609</td>
</tr>
<tr>
<td>6A</td>
<td>20 ms - ΔC&lt;sub&gt;m&lt;/sub&gt;/Q&lt;sub&gt;Ca&lt;/sub&gt; [fF/pA]</td>
<td>6.52 ± 0.28</td>
<td>6.9 ± 0.45</td>
<td>Student's t</td>
<td>t = 0.715</td>
</tr>
<tr>
<td>6B</td>
<td>200 ms - ΔC&lt;sub&gt;m&lt;/sub&gt; [fF]</td>
<td>209.54 ± 27.51</td>
<td>167.59 ± 16.82</td>
<td>Student's t</td>
<td>t = 0.1974</td>
</tr>
<tr>
<td>6B</td>
<td>200 ms - ΔC&lt;sub&gt;m&lt;/sub&gt;/Q&lt;sub&gt;Ca&lt;/sub&gt; [fF/pA]</td>
<td>5.7 ± 0.8</td>
<td>5.0 ± 0.4</td>
<td>Mann-Whitney U</td>
<td>U = 1.3213</td>
</tr>
<tr>
<td>6E</td>
<td>20 ms - Time of return to baseline [s]</td>
<td>38.32 ± 2.05</td>
<td>41.83 ± 2.67</td>
<td>Student's t</td>
<td>t = 0.3021</td>
</tr>
<tr>
<td>6F</td>
<td>20 ms - Slope of the linear component [fF/s]</td>
<td>-0.85 ± 0.052</td>
<td>-0.79 ± 0.06</td>
<td>Mann-Whitney U</td>
<td>U = 88.0</td>
</tr>
<tr>
<td>6F</td>
<td>without outliers</td>
<td>-0.91 ± 0.04</td>
<td>-0.73 ± 0.03</td>
<td>Student's t</td>
<td>t = -3.22</td>
</tr>
<tr>
<td>6G</td>
<td>200 ms - Slope of the linear component [fF/s]</td>
<td>-1.54 ± 0.25</td>
<td>-1.23 ± 0.12</td>
<td>Student's t</td>
<td>t = 1.093</td>
</tr>
<tr>
<td>6H</td>
<td>200 ms - Amplitude of the exp. component [fF]</td>
<td>103.84 ± 18.12</td>
<td>78.64 ± 9.88</td>
<td>Student's t</td>
<td>t = 0.7492</td>
</tr>
<tr>
<td>6I</td>
<td>200 ms – time constant of the exp. component [s]</td>
<td>6.01 ± 0.6</td>
<td>6.34 ± 0.9</td>
<td>Mann-Whitney U</td>
<td>U = 94.0</td>
</tr>
</tbody>
</table>
### Table 2.6 – SV-tethering to the AZ and SV reformation are altered in stimulated IHCs from AP180-KO mice (Fig 2.7)

<table>
<thead>
<tr>
<th>Data</th>
<th>Avg. Wt stim</th>
<th>Avg. KO stim</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>7D: N of RA-SVs</td>
<td>38.6 ± 3.6</td>
<td>34.1 ± 1.6</td>
<td>Mann-Whitney U</td>
<td>U = 39.5</td>
<td>p = 0.445</td>
</tr>
<tr>
<td>7E: N of MP-SVs</td>
<td>16.1 ± 1.9</td>
<td>16.1 ± 1.3</td>
<td>Mann-Whitney U</td>
<td>U = 45</td>
<td>p = 0.7207</td>
</tr>
<tr>
<td>Avg. distance to membrane (MP-SVs) [nm]</td>
<td>22.3 ± 2.3</td>
<td>24.4 ± 1.4</td>
<td>Student's t</td>
<td>t = 0.77</td>
<td>p = 0.4505</td>
</tr>
<tr>
<td>7F: N of cytosolic Svs</td>
<td>58.9 ± 6.1</td>
<td>34.4 ± 2.8</td>
<td>Mann-Whitney U</td>
<td>U = 12</td>
<td>p = 0.0026 (**)</td>
</tr>
<tr>
<td>N of ELVs</td>
<td>17.7 ± 3.0</td>
<td>24.0 ± 2.5</td>
<td>Student's t</td>
<td>t = 1.62</td>
<td>p = 0.1127</td>
</tr>
<tr>
<td>7H: Avg.# ELV volume [nm³]</td>
<td>770,287 ± 110,267</td>
<td>1,418,517 ± 288,188</td>
<td>Mann-Whitney U</td>
<td>U = 11.5</td>
<td>p = 0.0022 (**)</td>
</tr>
<tr>
<td>7I: Total N of coated structures</td>
<td>5.3 ± 0.4</td>
<td>2.3 ± 0.7</td>
<td>Mann-Whitney U</td>
<td>U = 12</td>
<td>p = 0.002 (**)</td>
</tr>
<tr>
<td>7J: N of coated vesicles</td>
<td>3.7 ± 0.4</td>
<td>1.6 ± 0.5</td>
<td>Mann-Whitney U</td>
<td>U = 12</td>
<td>p = 0.002 (**)</td>
</tr>
<tr>
<td>7K: N of coated pits</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>Mann-Whitney U</td>
<td>U = 45</td>
<td>p = &gt; 0.999</td>
</tr>
<tr>
<td>7L: N of coated pits at ELVs</td>
<td>1.2 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>Mann-Whitney U</td>
<td>U = 32</td>
<td>p = 0.006 (**)</td>
</tr>
</tbody>
</table>

### Table 2.7 – SV-tethering to the AZ and SV reformation are altered in stimulated IHCs from AP180-KO mice (Fig 2.7G)

<table>
<thead>
<tr>
<th>7G:</th>
<th>Untethered SV</th>
<th>Single-tethered SV</th>
<th>Multi-tethered SV</th>
<th>Docked SV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg. Wt rest</td>
<td>48.0 ± 3.1 %</td>
<td>40.4 ± 1.7 %</td>
<td>10.1 ± 2.1 %</td>
<td>1.6 ± 1.1 %</td>
</tr>
<tr>
<td>Avg. KO rest</td>
<td>46.4 ± 3.9 %</td>
<td>38.0 ± 2.6 %</td>
<td>14.8 ± 3.5 %</td>
<td>0.8 ± 0.8 %</td>
</tr>
<tr>
<td>Avg. Wt stim</td>
<td>22.6 ± 3.6 %</td>
<td>54.1 ± 2.6 %</td>
<td>19.9 ± 3.0 %</td>
<td>3.4 ± 1.9 %</td>
</tr>
<tr>
<td>Avg. KO stim</td>
<td>20.0 ± 5.6 %</td>
<td>30.1 ± 4.3 %</td>
<td>39.6 ± 6.4 %</td>
<td>10.3 ± 2.6 %</td>
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### Table 2.8 – Untethered Svs

<table>
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<tr>
<th>Data</th>
<th>Statistical test</th>
<th>t/ U/ F</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Untethered SV</td>
<td>one-way ANOVA</td>
<td>F = 13.2296</td>
<td>p &lt; 0.0001 (***), 0.8999947</td>
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</table>

<table>
<thead>
<tr>
<th>Post-hoc Tukey</th>
<th>Wt rest</th>
<th>KO rest</th>
<th>Wt stim</th>
<th>KO stim</th>
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<tbody>
<tr>
<td>Wt rest</td>
<td>-</td>
<td>0.8999947</td>
<td>0.0010053</td>
<td>0.0010053</td>
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<tr>
<td>KO rest</td>
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<td>-</td>
<td>0.0017751</td>
<td>0.0010053</td>
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<td>Wt stim</td>
<td>-</td>
<td>-</td>
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<td>0.8999947</td>
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Table 2.9 – Single-tethered SVs

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<th>Data</th>
<th>Statistical test</th>
<th>t/ U/ F</th>
<th>p-value</th>
</tr>
</thead>
</table>
| Single-tethered SV | one-way ANOVA    | F = 11.5683 | \( p < 0.0001 \) (***)

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<th>Wt stim</th>
<th>KO stim</th>
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</thead>
<tbody>
<tr>
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<td>0.0093707</td>
<td>0.0724638</td>
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<tr>
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Table 2.10 – Multi-tethered SVs

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<th>Data</th>
<th>Statistical test</th>
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<th>p-value</th>
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</thead>
</table>
| Multi-tethered SV | one-way ANOVA    | F = 10.5239 | \( p < 0.0001 \) (***)

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<tbody>
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<td>Wt stim</td>
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Table 2.11 – Docked SVs

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<th>t/ U/ F</th>
<th>p-value</th>
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| Docked SV       | one-way ANOVA    | F = 6.3848 | \( p = 0.0016 \) (**)

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<th>Wt stim</th>
<th>KO stim</th>
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<tr>
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<td>0.8603228</td>
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<td>Wt stim</td>
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Table 2.12 – Subcellular distribution of AP180 changes after stimulation (Fig S1)

<table>
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<tr>
<th>Data</th>
<th>Avg. Wt rst</th>
<th>Avg. Wt stim</th>
<th>Statistical test</th>
<th>t/ U</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>S1B\textsubscript{1}i: Membrane fluorescence gray value (0-255)</td>
<td>103 ± 5.7</td>
<td>107 ± 4.8</td>
<td>Student's t-test</td>
<td>t = 0.604</td>
<td>( p = 0.5468 )</td>
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</table>
| S1B\textsubscript{1}i: Cytosolic fluorescence at 2 µm from membrane | 53.3 ± 3.6 | 37 ± 2.7 | Student's t-test | t = 3.69 | \( p = 0.0003 \) (***)

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References


References


Krinner, S., Butola, T., Jung, S., Wichmann, C. and Moser, T. (2017). RIM-binding protein 2 promotes a large number of CaV1.3 Ca2+-channels and contributes to fast synaptic vesicle replenishment at hair cell active zones. Frontiers in Cellular Neuroscience 11.
References


3 Intrinsic planar polarity mechanisms influence the position-dependent regulation of synapse properties in inner hair cells

Published article:

Philippe Jean, Özge Demet Özçete, Basile Tarchini, and Tobias Moser

Author contributions: PJ., B.T., and T.M. designed research; PJ., Ö.D.Ö., and B.T. performed research; PJ. and Ö.D.Ö. analyzed data; and PJ., Ö.D.Ö., B.T., and T.M. wrote the paper.

Figure contribution of Özge Demet Özçete:
· patch-clamp recordings of Ca^{2+} current and C_m (Figure 4C-F)
Intrinsic planar polarity mechanisms influence the position-dependent regulation of synapse properties in inner hair cells

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Edited by Robert H. Edwards, University of California, San Francisco, CA, and approved March 19, 2019 (received for review November 2, 2018)

Encoding the wide range of audible sounds in the mammalian cochlea is collectively achieved by functionally diverse type I spiral ganglion neurons (SGNs) at each tonotopic position. The firing of each SGN is thought to be driven by an individual active zone (AZ) of a given inner hair cell (IHC). These AZs present distinct properties according to their position within the IHC, to some extent forming a gradient between the modiolar and the pillar IHC side. In this study, we investigated whether signaling involved in planar polarity at the apical surface can influence position-dependent AZ properties at the IHC base. Specifically, we tested the role of Gu\(i\) proteins and their binding partner LGN/Gpsm2 implicated in cytoskeleton polarization and hair cell (HC) orientation along the epithelial plane. Using high and superresolution immunofluorescence microscopy as well as patch-clamp combined with confocal Ca\(^{2+}\) imaging we analyzed IHCs in which Gu signaling was blocked by Cre-induced expression of the pertussis toxin catalytic subunit (PTX\(\alpha\)). PTX\(\alpha\)-expressing IHCs exhibited larger Ca\(^{2+}\) channel clusters and consequently greater Ca\(^{2+}\) influx at the whole-cell and single-synapse levels, which also showed a hyperpolarized shift of activation. Moreover, PTX\(\alpha\) expression collapsed the modiolar–pillar gradient in azimutal ribbon size and maximal synaptic Ca\(^{2+}\) influx. Finally, genetic deletion of Gu\(i2\) and LGN/Gpsm2 also disrupted the modiolar–pillar gradient of ribbon size. We propose a role for Gu proteins and LGN in regulating the position-dependent AZ properties in IHCs and suggest that this signaling pathway contributes to setting up the diverse firing properties of SGNs.

\textbf{Significance}

The wide dynamic range of sound encoding enables perception of sounds varying over six orders of magnitude, thought to be collectively achieved by distinct classes of type I spiral ganglion neurons (SGNs) contacting each inner hair cell (IHC). These synaptic contacts have been shown to vary according to the SGN firing properties along the modiolar–pillar axis. It has also been shown that the IHCs spatially segregate active zones (AZs) with different Ca\(^{2+}\)-influx properties, proposing an attractive candidate mechanism for the diverse sound encoding properties among SGNs. Our study, combining confocal/STED immunofluorescence microscopy, patch-clamp, and Ca\(^{2+}\) imaging, proposes that Gu and LGN, involved in planar polarity mechanisms at the IHC apex, contribute to establish the spatial gradient of IHC AZ properties.

The sense of hearing relies on vivid, temporally precise, and tireless encoding of sounds ranging in pressure over six orders of magnitude. Active amplification of cochlear vibrations for soft sounds and compression at strong ones enable the inner hair cell (IHC) receptor potential to represent the broad range of audible sound pressures. Each IHC forms ribbon synapses with several type I spiral ganglion neurons (SGNs) that relay the auditory information to the brain. Each of the functionally diverse SGNs only encodes a fraction of the audible range of sound pressures. Collectively, they cover the entire dynamic range: high-spontaneous rate, low-sound-threshold SGNs (high SR) encode soft sounds, and low-spontaneous rate, high-threshold SGNs (low SR) encode loud sounds (2–4). Since such functionally diverse SGNs can exhibit comparable frequency tuning, they are thought to receive input from neighboring or even the same IHC at a given tonotopic position (2, 5, 6). Based on back-tracing experiments it was proposed that low-SR SGNs preferentially contact the modiolar side of the IHCs, facing the spiral ganglion, while high-SR SGNs are more likely to target the pillar side of the IHCs, facing the outer hair cells (OHCs) (6).

Scientists and funders have a critical interest in the potential for hearing restoration. PNAS is a forum for the dissemination of findings on hearing research. The success of this journal depends on the originality and impact of the contributions that it receives. PNAS has a long tradition of providing a venue for emerging research fields. Experiments and data should be clear and robust. Reporting data in a timely and efficient manner facilitates the progress of science.

Several mechanisms have been proposed to explain the different SGN firing patterns. Postsynaptic morphology shows different fiber caliber and mitochondrial content (6) as well as different amounts of AMPA receptors (7–9). The lateral olivocochlear system could provide differential efferent modulation (7, 10). Finally, presynaptic mechanisms could establish the different firing properties of SGNs (6, 11–14), where larger ribbons associated with more synaptic vesicles and Ca\(^{2+}\) channels are found at modiolar active zones (AZs) likely facing low-SR SGNs (6, 11–14). How larger AZs, likely having greater maximal synaptic strength, could drive low-SR SGNs remained a mystery until it was discovered that their Ca\(^{2+}\) influx seems to operate at more depolarized potentials than that of AZs of the pillar side (14). This could readily explain the higher spontaneous rate and lower sound threshold of the SGNs contacting the pillar AZs, where substantial release is expected at the resting potential of IHCs and small receptor potentials might suffice to increase the ribbons of AZs to decompose the full auditory information contained in the receptor potential into different neural channels remains largely unclear. Nonmutually exclusive candidate mechanisms for the
observed spatial gradients of AZ properties include (i) a cell autonomous signaling mechanism in IHCs or an instructive influence by (ii) SGNs or (iii) effector innervation.

Here we explored the exciting possibility that planar polarity mechanisms responsible for establishing the proper architecture of the hair bundles and their uniform orientation across HCs (15–17) also influence the observed basolateral gradients of IHC synapse properties. During early development, the apical HC compartment located between the tallest stereocilia and the abneural junctions of the cell (the bare zone) lacks protrusions and hosts the polarized localization of Ins, LGN/Gmp2, and Gai (18). This protein complex was first shown to control mitotic spindle orientation and thereby asymmetric cell division in Drosophila, a function broadly conserved in mammals (19). Gai/InsLGN exclude aPKC, and the resulting compartmentalization of the HC apex influences the position of the kinocilium and the stereocilia, shaping cytoskeleton intrinsic asymmetry and possibly aligning it with tissue-wide polarity cues residing at cell-cell junctions, including core planar cell polarity (PCP) proteins (18, 20, 21). Moreover, Gai and Gai are also enriched at the tips of the tallest stereocilia (22–24), making the Incis-LGN-Gai complex a promising candidate for conveying information across compartments (23). Pericentrin toxin (PTXx) is well known to ADP ribosylate and block Gai signaling, and interestingly, PTX application led to PCP defects and a deficit of kinocilium migration in HCs in coculture controls (20). Moreover, transgenic or Cre-induced expression of PTX catalytic subunit (PTXx) in HC in vivo induced PCP defects, stereocilia staining, and severe hair bundle disorganization associated with profound deafness (18, 23).

LGN mutants shared stereocilia stunting and profound deafness (22–25), while milder hair bundle defects and hearing loss were reported in mutant mice lacking Gai (22), and these phenotypes were accentuated in conditional double Gai2/Gai3 mutants (25).

Neckless LGN mutants mouse showed that the defective AZs vs. pillar, 0.95 ± 0.03 (SD = 0.03), n = 192 AZs vs. pillar, 0.95 ± 0.03 (SD = 0.03), n = 148 AZs in 22 HCs, N (number of animals) = 5, P < 0.00001, Mann–Whitney–Wilcoxon test] (Fig. 1B and C, in black). Initially, we considered using PTXα-expressing, noninverted HCs as internal controls. Interestingly, however, we observed a complete collapse of the ribbon size gradient upon PTXα expression regardless of apical IHC orientation [PTXα mutant noninverted, in blue, modiolar, 1.00 ± 0.03 (SD = 0.35), n = 190 AZs vs. pillar, 0.99 ± 0.03 (SD = 0.31), n = 173 AZs in 22 HCs, N = 12, P = 0.88; PTXα mutant 180° inverted, in red, modiolar, 1.00 ± 0.02 (SD = 0.31), n = 192 AZs vs. pillar, 0.95 ± 0.02 (SD = 0.31), n = 174 AZs in 22 HCs, N = 12, P = 0.75, Mann–Whitney–Wilcoxon test for both conditions] (Fig. 1B and C).

To enhance the specificity of our manipulation, we turned to mutants constitutively lacking either Gai2 or Gai3. Both littermate controls and homozygous mutants lacking Gai2 exhibited a modiolar–pillar gradient of RIBEYE-immunofluorescence [Gai2KO/−, modiolar, 1.00 ± 0.03 (SD = 0.38), n = 220 AZs vs. pillar, 0.64 ± 0.02 (SD = 0.26), n = 107 AZs vs. Gai2KO/−] or Gai3KO/−, modiolar, 1.00 ± 0.03 (SD = 0.35), n = 43 AZs vs. pillar, 0.77 ± 0.03 (SD = 0.31), n = 114 AZs; in 18 HCs for N = 4 for both conditions, P < 0.00001, Mann–Whitney–Wilcoxon test] (SF Appendix, Fig. S1A). Here we combined morphological and physiological analysis of mouse HCs expressing PTXα (23) and found that the disruption of Gai signaling affects the properties and spatial heterogeneity of the AZs in HCs. Confocal and stimulated emission depletion (STED) microscopy showed that the mutant synapses were unchanged in number but were reorganized with larger and more complex Ca2+ channel clusters and a loss of the modiolar–pillar gradient of ribbon size. Performing patch-clamp and live confocal Ca2+ imaging, we showed that the mutant HCs exhibited an increased amplitude and hyperpolarized activation of Ca2+ influx at the whole-cell and single-synapse levels. Moreover, the modiolar–pillar gradient for synaptic Ca2+ influx strength observed in control condition was collapsed in PTXα-expressing HCs. The gradient of ribbon size was also disrupted in Gai3 and LGN KOi, suggesting that HC-intrinsic mechanisms influencing planar polarity at the apical membrane might also regulate the modiolar–pillar distribution of ribbon synapse properties.

Next, we combined F-actin labeling to record IHC orientation with immunostaining against Otoferlin or Vglut3 to outline the IHC shape and C9BP2/RIBEYE to estimate the localization of the ribbons and approximate their size. Thereby, we investigated whether the altered apical orientation might go along with a change of the previously reported modiolar–pillar gradient of ribbon size in HCs (14, 27). For analysis, we reconstructed the HCs in cylindrical coordinates to overlay multiple cells and study the position dependence of ribbon size [SF Appendix, SI Materials and Methods]. As expected, in HCs of PTXα control littermates, the modiolar–pillar gradient of RIBEYE-immunofluorescence intensity [proxy of ribbon size (28), normalized to the mean modiolar RIBEYE immunofluorescence intensity] was observed [modiolar, 1.00 ± 0.03 (SD = 0.40), n (number of replicates) = 211 AZs vs. pillar, 0.82 ± 0.03 (SD = 0.34), n = 148 AZs in 22 HCs, N (number of animals) = 5, P < 0.00001, Mann–Whitney–Wilcoxon test] (Fig. 1B and C).
transduction resulting from stunted stereocilia is not the primary cause for the lost gradient of ribbon size observed in IHCs lacking G\(\alpha\)i-LGN function.

**Inactivating G\(\alpha\)i Reorganizes and Potentiates Ca\(^{2+}\) Influx at IHC AZs.**

Next, we performed a detailed immunofluorescence analysis of ribbon synapse morphology in PTXa-expressing IHCs. We stained against CtBP2/RIBEYE and found no significant differences between PTXa control and mutant conditions for the average number of ribbons per IHC [PTXa mutant, 12.41 \(\pm\) 0.43 (SD = 2.12) vs. PTXa control, 12.54 \(\pm\) 0.29 (SD = 1.44); \(n = 24\) IHCs, \(N = 6\), \(P = 0.81\), t test] or their mean RIBEYE immunofluorescence intensity [PTXa mutant, 6.51 \(\pm\) 0.13 a.u. (SD = 2.40 a.u.); \(n = 360\) AZs, \(N = 9\) vs. PTXa control, 6.62 \(\pm\) 0.13 a.u. (SD = 2.26 a.u.); \(n = 327\) AZs, \(N = 9\), \(P = 0.28\), Mann–Whitney–Wilcoxon test for both conditions].

Using confocal microscopy, presynaptic Ca\(^{2+}\)-channel clusters were identified as spots of Ca\(_{\text{V1.3}}\) immunofluorescence juxtaposed to the postsynaptic density (PSD) detected as PSD-95 immunofluorescent spots. Ca\(_{\text{V1.3}}\) channels remained clustered at mutant AZs and were further quantified by fitting Ca\(_{\text{V1.3}}\) immunofluorescent spots with a 2D Gaussian function. The amplitudes of the fits in PTXa-expressing IHCs were significantly greater for Ca\(_{\text{V1.3}}\) [PTXa mutant, 0.74 \(\pm\) 0.03 a.u. vs. PTXa control, 0.64 \(\pm\) 0.02 a.u.].
Inactivating Glu2 or LGN collapses the modiolar-pillar gradient for ribbon size. (A) The polar charts display locations and intensities of immunofluorescently labeled CBP2 marking the ribbons. Modiolar and pillar refer to facing toward or away from the ganglion in the modiolar, apical and basal refer to the tonotopic axis of the organ of Corti. The fluorescence intensity of each CBP2 signal point is reflected by its color, with warmer, yellow tones indicating higher intensity and cooler, darker tones indicating lower intensity. The spatial gradient for ribbon strength was lost upon Glu3 inactivation (modiolar, \( n = 218 \) AZs vs. pillar, \( n = 145 \) AZs for 20 IHCs, \( P < 0.00001 \), Mann–Whitney–Wilcoxon test), whereas it was preserved in the heterozygote littermate controls (modiolar, \( n = 225 \) AZs vs. pillar, \( n = 143 \) AZs in 20 IHCs, \( P < 0.00001 \), Mann–Whitney–Wilcoxon test). Box plots show 10th, 25th, 50th, 75th, and 90th percentiles with individual data points overlaid; each radial circle is 2.5 \( \mu m \), and means are shown as crosses as for B. (B) The inactivation of LGN collapsed the gradient of ribbon size [LGN KO, modiolar, \( n = 231 \) AZs vs. pillar, \( n = 184 \) AZs, \( P = 0.11 \) vs. LGN control (heterozygotes), \( n = 298 \) AZs vs. pillar, \( n = 128 \) AZs, \( P < 0.00001 \); 24 hICs, \( n = 4 \), Mann–Whitney–Wilcoxon test for both conditions].
Inactivating Gsi Causes an Increased Amplitude and Hyperpolarized Activation of IHC Ca\(^{2+}\) Influx

To physiologically characterize the effect of inactivating Gsi on Ca\(^{1.3}\) Ca\(^{2+}\) channels, we first recorded the whole-cell Ca\(^{2+}\) current of PTXa-expressing IHCs in the ruptured-patch configuration (5 mM [Ca\(^{2+}\)]; Fig. 4A). The amplitude of the Ca\(^{2+}\) influx [PTXa mutant, \(-28.16 \pm 0.54\) mV (SD = 3.41 mV), n = 40 IHCs; 13 vs. PTXa control, \(-23.76 \pm 8\) pA (SD = 50 pA), n = 40 IHCs, N = 13 vs. PTXa control, \(-19.1 \pm 5\) pA, SD = 38 pA, n = 40 IHCs, N = 14; P < 0.00001, t test] was significantly increased compared to the more numerous Ca\(^{1.3}\) channels expressed by immunohistochemistry. Next, we analyzed the voltage dependence of Ca\(^{2+}\)-channel activation (Fig. 4B) and found a significant hyperpolarizing shift of \(-4\) mV of the voltage of half-maximal Ca\(^{2+}\)-channel activation, V\(_{1/2}\) [Fig. 4, B; PTXa mutant, \(-28.16 \pm 0.54\) mV (SD = 3.41 mV), n = 40 IHCs; 13 vs. PTXa control, \(-23.76 \pm 8\) pA (SD = 50 pA), n = 40 IHCs, N = 13 vs. PTXa control, \(-19.1 \pm 5\) pA, SD = 38 pA, n = 40 IHCs, N = 14; P < 0.00001, t test] compared to the control. Moreover, we found a subtle but significant increase of the voltage sensitivity of activation (apparent as a decrease of the slope factor k) in mutant IHCs [PTXa mutant, \(7.33 \pm 0.06\) mV (SD = 0.41 mV), n = 40 IHCs, N = 13 vs. PTXa control, \(7.72 \pm 0.07\) mV (SD = 0.42 mV), n = 40 IHCs, N = 14; P < 0.00001, t test; Fig. 4, B]).

To test the consequences of the Ca\(^{1.3}\) Ca\(^{2+}\) channel rearrangement in the PTXa mutant IHCs on exocytosis, we performed membrane capacitance measurements (C\(_{m}\)) in the perforated patch-clamp configuration (1.3 mM [Ca\(^{2+}\); Fig. 4 C-H]). IHCs were step depolarized to \(-17\) mV for different durations (2 to 50 ms) to probe the vesicle pool dynamics. Consistent with the above results, the integrated Ca\(^{2+}\) influx (Ca\(^{2+}\) charge Q(T)) of the PTXa mutant IHCs was significantly increased for every pulse duration tested (Q(T) at 2 ms, P = 0.002; Q(T) at 5 ms, P = 0.004; Q(T) at 10 ms, P = 0.004; Q(T) at 20 ms, P = 0.01; Q(T) at 50 ms, P = 0.01, n = 10 IHCs, N = 7 in the PTXa mutant; n = 10 IHCs, N = 6 in the PTXa control; t test; Fig. 4D). Surprisingly, despite the significantly increased Ca\(^{2+}\) influx, the PTXa mutant IHCs tended to have smaller exocytic events, which we observed using the automated tracking software (Fig. 3 D and E). However, we cannot exclude a contribution of other mechanisms such as a potentiation of the exocytosis.
PTXa-expressing IHCs show an increased and hyperpolarized Ca\textsuperscript{2+} influx at the whole-cell level. (A) IV relationship of the whole-cell Ca\textsuperscript{2+} current (ruptured-patch configuration, 5 mM [Ca\textsuperscript{2+}]) shows a significantly increased current amplitude in the PTXa mutant IHC (n = 40 IHCs, N = 13 in the PTXa mutant; n = 40 IHCs, N = 14 in the PTXa control; P < 0.0001, t test) (A). The protocol, consisting of 20 ms steps of 5 mV from −82 to +63 mV, as well as the resulting currents of a representative control IHC, are shown in the Bottom Right. Mean (line) ± SEM (shaded area) are displayed: the box plots show 10th, 25th, 50th, 75th, and 90th percentiles with individual data points overlaid, and means are shown as crosses, as for B. (B) Fractional activation of the whole-cell Ca\textsuperscript{2+} current derived from the IV relationships (A) was fitted to a Boltzmann function. (B) Box plots of the voltage for half-maximal activation V\textsubscript{1/2} and V\textsubscript{h} of individual IHCs show a significant hyperpolarized shift of the fractional activation (h) of the Ca\textsuperscript{2+} channels in the PTXa mutant condition (n = 40 IHCs, N = 13 in the PTXa mutant; n = 40 IHCs, N = 14 in the PTXa control; P < 0.0001, t test) (B). Box plots of the voltage sensitivity or slope factor k and k estimates of individual IHCs illustrate increased voltage sensitivity in the PTXa mutant condition (n = 40 IHCs, N = 13 in the mutant; n = 40 IHCs, N = 14 in the PTXa control; P < 0.0001, t test). (C) Representative Ca\textsuperscript{2+} currents (Top) and corresponding low-pass filtered capacitance (Bottom) traces recorded from PTXa mutant and control IHCs in response to 20-ms step depolarization to −17 mV from the holding potential of −87 mV (ruptured-patch configuration, 1.3 mM [Ca\textsuperscript{2+}]). The PTXa mutant IHCs showed bigger Ca\textsuperscript{2+} currents than the control IHCs, while the capacitance jumps (ΔC) were comparable. (D) Mean exocytic ΔC\textsubscript{m} (Top) and Ca\textsuperscript{2+} current integrals (Q\textsubscript{m}) (Bottom) as a function of depolarization duration (mean ± SEM, n = 10 IHCs, N = 7 in the PTXa mutant; n = 10 IHCs, N = 6 in the PTXa control; for Q\textsubscript{m}, at 2 ms, P = 0.002; for Q\textsubscript{m} at 5 ms, P = 0.005; for Q\textsubscript{m} at 10 ms, P = 0.003; for Q\textsubscript{m} at 20 ms, P = 0.007; for Q\textsubscript{m} at 50 ms, P = 0.007, t test; for ΔC\textsubscript{m} at 2 ms, P = 0.02, t test; at 10 ms, P = 0.03, Mann-Whitney-Wilcoxon test) (D). (E) Relation between exocytic ΔC\textsubscript{m} and Q\textsubscript{m} from PTXa mutant and control IHCs (mean ± SEM; fill color of the mean points darkens with increasing depolarization duration). PTXa mutant IHCs showed significantly lower efficiency of Ca\textsuperscript{2+} influx to drive exocytosis for every depolarization duration. (F) Ratio of ΔC\textsubscript{m} and Q\textsubscript{m} from PTXa mutant and control IHCs upon 20 ms step depolarization (n = 10 IHCs, N = 7 in the PTXa mutant; n = 10 IHCs, N = 6 in the PTXa control; for ratio at 2 ms, P = 0.02; at 5 ms, P = 0.01; at 10 ms, P = 0.006; at 20 ms, P = 0.001; Q\textsubscript{m} at 50 ms, P = 0.005, t test). (E, F) Representative Ca\textsuperscript{2+} currents (Middle) and corresponding low-pass filtered capacitance (Bottom) traces recorded from PTXa mutant and control IHCs upon 100 ms step depolarization to −41 mV from the holding potential (perforated-patch configuration, 1.3 mM [Ca\textsuperscript{2+}]). Stimulus template (Top) illustrates the 2-ms steps starting from −53 to −37 mV. (G) Mean exocytic ΔC\textsubscript{m} (Top) and Ca\textsuperscript{2+} current integrals (Q\textsubscript{m}) (Bottom) as a function of depolarization voltage (mean ± SEM, n = 8 IHCs, N = 4 in the PTXa mutant; n = 9 IHCs, N = 5 in the PTXa control; P < 0.0001; for Q\textsubscript{m} to −37 mV, P = 0.002, for Q\textsubscript{m} at −39 mV, P = 0.005; for ΔC\textsubscript{m} at −41 mV, P = 0.01, t test; for Q\textsubscript{m} at −43 mV, P = 0.02; for Q\textsubscript{m} at −45 mV, P = 0.016; for Q\textsubscript{m} at −47 mV, P = 0.05; for ΔC\textsubscript{m} at −49 mV, P = 0.046, Mann-Whitney-Wilcoxon test) (G). (H) Relation between exocytic ΔC\textsubscript{m} and Q\textsubscript{m} from PTXa mutant and control IHCs (mean ± SEM; fill color of the mean points darkens with increasing depolarization voltage). The ratio between the ΔC\textsubscript{m} and Q\textsubscript{m} was comparable throughout the different voltage range (−53 to −37 mV, with 2-mV increments). (H) Ratio of ΔC\textsubscript{m} and Q\textsubscript{m} from PTXa mutant and control IHCs upon 100 ms step depolarization to −39 mV.
Ca²⁺ sensor of exocytosis. Furthermore, we have probed the voltage dependence of exocytosis given the hyperpolarized shift (~5 mV) in the activation of Ca²⁺ influx in the PTXa mutant HICS. HICS were step depolarized for 100 ms in the physiologically relevant voltage range, starting from ~53 to ~37 mV with 2-mV increments (Fig. 4F). PTXa mutant HICS exhibit significantly bigger Q_{hot} for most voltage steps, whereas the tendency toward larger ∆C_{m} did not reach statistical significance (n = 8 HICS, N = 4 in the PTXa mutant; n = 9 HICS, N = 5 in the PTXa control) (Fig. 4G). Interestingly, the ratio of ∆C_{m} over Q_{hot} was comparable between PTXa mutant and control HICS in the physiologically relevant voltage range (Fig. 4 H and H1).

Next, we studied presynaptic Ca²⁺ signaling at individual AZs using spinning-disk confocal microscopy of HICS loaded with the low-affinity Ca²⁺ indicator Fluo-4FF (500 μM), the nonfluorescent chelator EGTA (10 mM), and a TAMRA (tetramethylrhodamine)-conjugated CBP2-binding peptide. Under these conditions the Ca²⁺ indicator fluorescence serves as a proxy of synaptic Ca²⁺ influx (12, 14, 33). AZs were identified by spots of the CBP2-binding peptide fluorescence where Ca²⁺ signals (Fluo-4FF hotspots) commenced upon depolarization. We found an increased maximal rise of Fluo-4FF fluorescence, i.e., synaptic Ca²⁺ influx, in PTXa expressing HICS (PTXa mutant, ΔF/F_{max} = 0.05 ± 0.10 (SD = 1.34), n = 175 AZs vs. PTXa control, 1.59 ± 0.08 (SD = 1.10), n = 175 AZs; in 20 HICS for N = 12 for both conditions, P = 0.0002, Mann–Whitney–Wilcoxon test; Fig. 5i). This gain of synaptic Ca²⁺ influx strength agrees with the analysis of Ca²⁺ immunofluorescence and with the enhanced Ca²⁺ influx at the whole-cell level. Moreover, as at the whole-cell level, we found a significant hyperpolarized shift (approximately ~4 mV) of activation of the synaptic Ca²⁺ influx [PTXa mutant, V_{h} = −28.77 ± 0.71 mV (SD = 8.42 mV), n = 142 AZs in 20 HICS, N = 12 vs. PTXa control, V_{h} = −24.11 ± 0.57 mV (SD = 6.77 mV), n = 141 AZs in 20 HICS, N = 12, P < 0.0001, Mann–Whitney–Wilcoxon test; Fig. 5.b)].

Finally, we studied the spatial extent of the synaptic Ca²⁺ signals by measuring the FWHM of 2D Gaussian function fits to the hotspots of Ca²⁺-indicator fluorescence. We found a greater spread of synaptic Ca²⁺ signals in PTXa-expressing HICS [PTXa mutant, long axis (L.A.) = 1.061 ± 0.21 nm (SD = 266 nm), short axis (S.A.) = 0.779 ± 0.18 nm (SD = 228 nm); n = 162 AZs in 20 HICS, N = 12, P = 0.55, Mann–Whitney–Wilcoxon test; Fig. 5.b)].

Characteristically, we found a greater spread of 2D Gaussian fits to the Ca²⁺ fluorescence hotspots in PTXa-expressing HICS likely due to higher variability at the single synapse level [PTXa mutant, k = 6.74 ± 0.17 mV (SD = 1.99 mV), n = 142 AZs in 20 HICS, N = 12 vs. PTXa control, 6.83 ± 0.16 mV (SD = 2.13 mV), n = 141 AZs in 20 HICS, N = 12, P = 0.17 mV (SD = 252 nm); S.A. = 672 ± 17 nm (SD = 218 nm), n = 160 AZs in 20

Fig. 5. PTXa-expressing HICS possess an increased, more spread-out and hyperpolarized Ca²⁺ influx at the single-synapse level. (A) FV relationship (ΔF/F vs. depolarization level in ramp), approximating the voltage dependence of synaptic Ca²⁺ influx. Voltage ramps from −87 to +63 mV during 150 ms were used to trigger synaptic hotspots of Fluo-4FF fluorescence and HIC Ca²⁺ influx (in the middle at the bottom, 10 AZs in one exemplary HIC). (A) ΔF_{max}/F_{min} was calculated by averaging five values at the FV peak and was significantly increased in the PTXa mutant condition (n = 175 AZs in 20 HICS, N = 12 in the PTXa mutant; n = 175 AZs in 20 HICS, N = 12 in the PTXa control; P = 0.0002, Mann–Whitney–Wilcoxon test). Mean (line) ± SEM (shaded area) are displayed; the box plots show 10th, 25th, 50th, 75th, and 90th percentiles with individual data points overlaid, and means are shown as crosses, as for B and D. (B) Fractional activation curves derived from fits to the FV relationships (C) were fitted to a Boltzmann function. (E) The voltage for half-maximal activation V_{h} was significantly hyperpolarized in the mutant condition (n = 142 AZs in 20 HICS, N = 12 in the PTXa mutant; n = 141 AZs in 20 HICS, N = 12 in the PTXa control; P = 0.0001, Mann–Whitney–Wilcoxon test), while the voltage sensitivity (B) did not differ significantly between both conditions (n = 142 AZs in 20 HICS, N = 12 in the PTXa mutant; n = 141 AZs in 20 HICS, N = 12 in the PTXa control; P = 0.55, Mann–Whitney–Wilcoxon test). (C) Exemplary ΔF pictures of Fluo-4FF hotspots at PTXa control (Left) and mutant (Right) synapses fitted and overlaid by 2D Gaussian functions to estimate spatial extent as FWHM for the S.A. and the L.A. (D) AZs of PTXa mutant HICS showed a greater spatial spread of the Fluo-4FF fluorescence changes. FWHM calculated from the Gaussian fitting to the Fluo-4FF fluorescence hotspot was larger for both short and long axes in PTXa mutant HICS (n = 162 AZs for 20 HICS, N = 12) compared with PTXa control ones (n = 160 AZs for 20 HICS, N = 12) (P < 0.0001, Mann–Whitney–Wilcoxon test for both axes).
IHCs, N = 12, P < 0.0001, Mann–Whitney–Wilcoxon test for both axes] (Fig. 5 C and D). This larger spread of the presynaptic Ca2+ signals is in agreement with the higher prevalence of AZs with multiple Ca2+-channel clusters in PTXa-expressing IHCs.

The Modiolar–Pillar Gradient of Maximal Synaptic Ca2+ Influx Is Lost upon PTXa Expression, but the Pillar–Modiolar Gradient of Its Voltage-Dependent Activation Is Maintained. By reconstructing the imaged IHCs as cylindrical models, we then studied the position dependence of AZ properties in live imaging experiments as previously described (14). At P14–18 a tendency for a stronger maximal Ca2+ influx was found for modiolar AZs, which also showed a significantly more depolarized activation of the Ca2+ channels. Performing this analysis on the synaptic Ca2+ influx of PTXa control and mutant IHCS at P21-26, as expected, we found a stronger ΔF/Fmax for modiolar than for pillar AZs in PTXa control IHCS [modiolar, 1.68 ± 0.11 (SD = 1.17), n = 104 AZs vs. pillar, 1.39 ± 0.11 (SD = 0.91), n = 65 AZs in 19 IHCS, P = 0.033; Mann–Whitney–Wilcoxon test]. PTXa expression completely collapsed this gradient [modiolar, 1.96 ± 0.13 (SD = 1.32), n = 98 AZs vs. pillar, 1.94 ± 0.17 (SD = 1.51), n = 57 AZs in 18 IHCS, N = 10, P = 0.91, Mann–Whitney–Wilcoxon test] (Fig. 6 A). The “winning” AZs, defined for each cell as the synapse exhibiting the strongest Ca2+ influx (highlighted in blue in the box plot and polar charts), were in great majority positioned on the modiolar side in PTXa control IHCS, as previously described (14). In contrast, their localization seemed more random in PTXa-expressing IHCS. However, there was no significant difference in their average positions along the modiolar–pillar axis (P = 0.10; t test) (M Appendix; Fig. S4) and their respective contributions (ratio between mean ΔF/Fmax of winner/mean ΔF/Fmax of the rest of the AZs) for a given cell [PTXa mutant, 2.49 ± 0.18 (SD = 0.78) vs. PTXa control, 2.27 ± 0.24 (SD = 1.08), P = 0.46, t test]. In contrast to the ΔF/Fmax gradient, the pillar–modiolar gradient for the V0 of Ca2+-channel activation was maintained in PTXa expressing IHCS [PTXa mutant, pillar, −26.35 ± 0.83 mV (SD = 7.50 mV), n = 80 AZs vs. pillar, −30.64 ± 1.47 mV (SD = 9.57 mV), n = 42 AZs in 18 IHCS, N = 10; P = 0.013 vs. PTXa control, pillar, −23.19 ± 0.74 mV (SD = 6.69 mV), n = 81 AZs vs. pillar, −25.70 ± 0.96 mV (SD = 6.96 mV), n = 53 AZs in 19 IHCS, N = 11; P = 0.039, t test for both conditions] (Fig. 6 B).

Discussion

The auditory system processes sound pressures ranging over six orders of magnitude. SGNs with different and complementary firing properties work together to encode this wide dynamic range of audible sounds. Despite progress in defining mechanisms, the functional diversity of SGNs remains largely enigmatic. Defining where the primary mechanism resides and how it is established and maintained are active fields of research. Here we investigated whether Gxi-LGN function, known to influence cell-intrinsic planar polarity at the apex of the HCs, also affects the

Fig. 6. The modiolar–pillar gradient for synaptic Ca2+ influx strength is collapsed upon PTXa expression, but the gradient for voltage-dependent activation is preserved. (A) The polar charts display intensities of maximal AZ Ca2+ influx (ΔF/Fmax) as a function of AZ positions in live-imaging experiments. Modiolar and pillar refer to facing toward or away from the ganglion in the modiolus; apical and basal refer to the tonotopic axis of the organ of Corti, as for B. Box plots of the ΔF/Fmax estimates of individual IHCS show that the PTXa control conditions exhibit significantly stronger Ca2+ hotspots in the modiolar side compared with the pillar side (PTXa control, modiolar, n = 104 AZs; pillar, n = 65 AZs, P = 0.033, Mann–Whitney–Wilcoxon test), whereas this gradient is completely collapsed in the PTXa mutant (PTXa mutant, modiolar, n = 98 AZs; pillar, n = 57 AZs, P = 0.91, Mann–Whitney–Wilcoxon test). The strongest AZs from each cell are highlighted in blue in both polar charts and box plots. Data were pooled from 19 and 18 IHCS in PTXa control and mutant conditions, respectively; box plots show 10th, 25th, 50th, 75th, and 90th percentiles with individual data points overlaid; each radial circle is 2 μm, and means are shown as crosses, as for B. (B) The polar charts display voltages for half-maximal activation V1/2 as a function of AZ positions in live-imaging experiments. Box plots of the voltage for half-maximal activation V1/2 and V0 estimates of individual IHCS show a significant hyperpolarized shift of the fractional activation of the Ca2+,1.3 channels in the pillar side compared with the modiolar side in both control and mutant conditions (PTXa mutant, modiolar, n = 80 AZs; pillar, n = 42 AZs, P = 0.013; PTXa control, modiolar, n = 81 AZs; pillar, n = 53 AZs, P = 0.039; t test for both conditions).
presynaptic heterogeneity at the base of IHCs. Interestingly, disrupting Gnr activity via PTXa expression in IHCs collapsed the modiolar–pillar gradient of ribbon size and maximal synaptic Ca\textsuperscript{2+} influx, while reorganizing and enlarging the synapses. Indeed, we found larger and more complex Ca\textsuperscript{2+}-channel clusters by immunofluorescence microscopy, which was corroborated by the observation of increased Ca\textsuperscript{2+} influx at the whole-cell and single-synapse levels. Importantly, PTXa consistently disrupted the modiolar–pillar gradient of ribbon size, suggesting that reliance on Gnr function differs to polarize apical and basal IHC features. This could potentially be explained by a strict requirement for Gnr3 to establish the ribbon gradient, while the apical cytoskeleton might be redundantly oriented by multiple Gnr isoforms. Overall, our results suggest that Gnr3–LGN participates in establishing the position-dependent properties of IHC AZs.

A Role for Gnr in Setting Up the Spatial Gradient of AZ Properties in IHCs. Although it is well established that PTXa specifically ADP-ribosylates and impairs Gnr function (35), PTXa might affect IHC development in more ways than strictly disrupting planar polarity mechanisms. While the enlarged synaptic Ca\textsuperscript{2+} influx observed upon PTXa argues against decreased IHC fitness or impaired IHC activity, simultaneous disruption of Gnr2 and Gnr3 was reported to impair IHC maturation (26). This conclusion was based on the absence of large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channels at the IHC neck and the persistence of small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (SK2) channels present before hearing onset and juxtaposed to efferent boutons. PTXa-expressing as well as LGN KO IHCs showed variable BK signals from IHC to IHC ranging from normal clustering to no signal (SI Appendix, Fig. S5 B and C). Agreement with this modulatory effect was observed in PTXa-expressing IHCs at P22 but were completely absent in littermate controls (SI Appendix, Fig. S6 A–C). However, several observations argue against a leading role of delayed IHC maturation on the observed PTXa mutant synaptic phenotype. Gnr3 mutant IHCs lost their modiolar–pillar gradient for ribbon size but exhibited a clustering of BK channels at their neck (SI Appendix, Fig. S5A). Supportive evidence also includes the report of a significant gradient in immature IHCs before hearing onset (27). Moreover, targeted inactivation of the Gnr3–binding partner LGN also collapsed the gradient of the size of synaptic ribbons, suggesting that the similar collapse upon PTXa likely reflects defective Gnr3–LGN cell polarization. While the increased whole-cell Ca\textsuperscript{2+} current per se could be consistent with impaired IHC maturation (28), further arguments against a major maturational deficit include (i) a qualitatively similar amount of extrasynaptic Ca\textsuperscript{1.3}Ca\textsuperscript{2+} channels at the confocal level as well as a comparable maximal Ca\textsuperscript{2+} influx potentiation in PTXa mutants at the whole-cell and synapse levels (30 and 23%, respectively), (ii) well-defined PSDs at the confocal and STED levels, in contrast to the several PSDs per contact found at earlier stages (28); (iii) same ribbon intensity and number, while more and smaller ribbons were found in immature IHCs; and finally, (iv) stronger synaptic Ca\textsuperscript{2+} influx, while weaker Ca\textsuperscript{2+} influx per AZ was found before hearing onset (28). Altogether, Gnr signaling could thus be required for aspects of IHC maturation independently from its role in diversifying ribbon synapse properties.

In conclusion, our results strongly suggest that Gnr–LGN signaling directly regulates the modiolar–pillar gradient of synaptic properties. Considering other possible confounding mechanisms, it is worth noting that stunted and disorganized stereocilia affecting hair bundle function and thus sound encoding per se are unlikely to alter synapses, as we did not find obvious changes in position-dependent morphological synapse properties in Myo15\textsuperscript{p20} mutants. Nonetheless, some aspects of the synaptic phenotype observed in the PTXa mutant IHCs are reminiscent of theUSH1C deaf-circular mutant mice, where absence of the harmonin protein induces severe hair bundle defects. There, too, an increased amplitude and hyperpolarized activation of synaptic Ca\textsuperscript{2+} channels was observed, with no evidence for an immaturity phenotype was found (14). A gain of synaptic function through increased number and more hyperpolarized activation of Ca\textsuperscript{2+} channels could be a mechanism to increase spontaneous SGN firing to compensate for the lack of sound-evoked firing. Hence, the potentiated Ca\textsuperscript{2+} influx could be secondary to a deficit of mechanotransduction.

Candidate Mechanisms for Defining Position-Dependent AZ Ca\textsuperscript{2+} Influx Properties. Using single-cell RNA sequencing, recent studies (37–39) characterized three distinct subpopulations of type I SGNs in the mouse. Interestingly, these transcriptome-based subpopulations expressed distinct complements of transcription factors but also ion channels, receptors, and synaptic proteins. Moreover, as these different subpopulations were shown to differentially target the IHC basolateral periphery, their profiling offered potential postsynaptic determinants that could account for heterogeneous firing properties of the SGNs. Moreover, these SGN subpopulations might exert differential instructive influence on the properties of presynaptic IHC AZs. By reconstructing the patch-clamped cells we found that blocking Gnr signaling abolishes the modiolar–pillar gradient for the maximal strength of synaptic Ca\textsuperscript{2+} influx but preserves the pillar-modiolar gradient for the voltage dependence of Ca\textsuperscript{2+} channel activation. This finding might indicate that distinct mechanisms govern the spatial distribution of the number of Ca\textsuperscript{2+} channels per AZ and their voltage dependence of activation. This observation of unchanged spatial gradient of voltage dependence of activation would be consistent with observations in IHCs of Gipc3 KO mice, where the gradient for the strength of synaptic Ca\textsuperscript{2+} influx was reversed, while the pillar–modiolar one for voltage dependence of activity was preserved. In the absence of large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, including modified expression of RIBEYE (48, 49) and frame shift \textit{rhb} mutations (50), as well as Crg-deletion of RIBEYE in the mouse cochlea and retina (39, 40), several studies in the neuromast HCs of larval zebrafish, including modified expression of RIBEYE (48, 49) and frame shift \textit{rhb} mutations (50), as well as Crg-deletion of RIBEYE in the mouse cochlea and retina (39, 40), have shown a relationship between the ribbon and the Ca\textsuperscript{2+}-channel cluster organization. A depolarized shift of the voltage dependence activation of Ca\textsuperscript{2+} influx was found in RIBEYE-deficient IHCs (54), but a direct interaction of RIBEYE and the Ca\textsuperscript{2+}-channel complex has not yet been reported. Aside from RIBEYE, Bassoon has been shown to promote Ca\textsuperscript{2+}-channel tethering at the AZ (53) likely via interaction with RIM-binding protein (54). RIM-binding protein was reported to interact with Ca\textsuperscript{1.3}Ca\textsuperscript{2+} channels (55) and is necessary to establish a normal Ca\textsuperscript{2+}-channel complement at the IHC AZ (56). Moreover, RIM2Δz and β have been reported to promote the clustering of these channels at the synapse (47), and RIM2 and RIM3 were shown to directly interact with the pore-forming subunit of Ca\textsuperscript{1.3}Ca\textsuperscript{2+} channel (57). It will be of interest to explore possible interactions determining whether the trafficking of these different AZ proteins are governed by the Gnr3–LGN complex. Future experiments are required to investigate the importance of LGN for synaptic physiology and identify potential alternative interacting partners for Gnr besides LGN, including membrane receptors or other planar polarity proteins. In particular, it will be interesting to ask whether tissue-level mechanisms regulating IHC planar orientation at cell–cell junctions (e.g., core PCP proteins)
also influence the spatial gradient of ribbon size and/or synaptic physiology. In addition, emphasis should be put on ultrastructural studies by electron microscopy to further characterize the spatial reorganization of the synapses, as well as recordings from auditory nerve fibers to assess the effect on the SGN firing rate diversity.

Materials and Methods

All experiments complied with national animal care guidelines and were approved by the Göttingen University’s Committee for Animal Welfare, the Animal Welfare Office of the State of Lower Saxony, and the Animal Care and Use Committee of The Jackson Laboratory. For details of patch-clamp and confocal Ca2+ imaging, immunohistochemistry and confocal/STED imaging, and data analysis, see Supplemental Methods and Materials.

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Supplementary information for

“Intrinsic planar polarity mechanisms influence the position-dependent regulation of synapse properties in inner hair cells”

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This PDF file includes:

Supplementary text: Materials and Methods
Figs. S1 to S6
References for SI reference citations

Supplementary Information Text

Materials and Methods.

Animals

The Cre-inducible PTXa allele at the Rosa26 locus and the LGN KO mice have been previously described (1, 2). Myo15<sup>sh2</sup> (JR#109) and Atoh1-Cre (JR#11104) (3) were obtained from The Jackson Laboratory. The Gai3 mutant strain was derived by breeding from the Gai1; Gai3 double mutant strain obtained from The Jackson Laboratory (JR#24525) (4). The Gai2 mutant strain was generated with CRISPR/Cas9 and carries a deletion of Gai2 exons 2-4.

Patch-clamp recordings

The apical 2/3 turns of organs of Corti from P21-26 old mice were freshly dissected in HEPES Hank’s solution containing (in mM): 5.36 KCl, 141.7 NaCl, 10 HEPES, 0.5 MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 MgCl<sub>2</sub>·6H<sub>2</sub>O, 1 mg/ml D-glucose, and 0.5 mg/ml L-glutamine (pH 7.2, ~300 mOsm). The native morphologies and positions of the IHCs within the organ of Corti were maintained as much as possible by accessing them from the modiolus. Patch pipettes were made from GB150F-8P or GB150-8P borosilicate glass capillaries for whole cell and perforated patch-clamp recordings, respectively (Science Products, Hofheim, Germany). All experiments were conducted at room temperature (20-25°C). For whole cell recordings, the patch pipette solution contained (in mM): 111 L-glutamate, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 EGTA, 13 TEA-Cl, 20 HEPES, 4 Mg-ATP, 0.3 Na-GTP and 1 L-Glutathione (pH 7.3, ~290 mOsm). Perforated patch-clamp experiments were done as
described previously (5). The patch pipette solution contained (in mM): 130 Cs-gluconate, 10 TEA-Cl, 10 4-AP, 10 HEPES, 1 MgCl₂, as well as 300 mg/ml amphotericin B (pH 7.17, ~290 mOsm). The extracellular solution contained the following (in mM): 2.8 KCl, 102 NaCl, 10 HEPES, 1 CsCl₂, 1 MgCl₂, 35 TEA-Cl, 2 mg/ml D-Glucose and either 5 CaCl₂ or 1.3 CaCl₂ for whole cell and perforated patch-clamp configuration, respectively (pH 7.2, ~300 mOsm). Membrane capacitance measurements in Fig. 4. F-H were done with 100 nM apamin in the extracellular solution. An EPC-10 USB amplifier (HEKA, Lambrecht/Pfalz, Germany) controlled by PatchMaster software (HEKA) was used for the acquisition. IHCs were held at -87 mV after liquid junction potential correction. All voltages were corrected offline for liquid junction potential (-17 mV) and voltage-drops across the series resistance (Rₛ). For whole-cell recordings, recordings were discarded when the leak current exceeded -50 pA at -87 mV, Rₛ exceeded 15 MΩ within 4 min after break-in, or Ca²⁺-current rundown exceeded 25%. The membrane capacitance were performed using the Lindau-Neher technique (6). For capacitance measurements, only traces with the leak current lower than -40 pA and Rₛ lower than 30 MΩ were used for further analysis. In one data set, IHCs were depolarized from the holding potential of -87 mV to -17 mV for durations of 2 ms to 50 ms and in another one to different voltage range from -53 mV to -37 mV with 2 mV increments for 100 ms, in order to probe weak depolarizations, with 20 s interval, in each case.

**Spinning disk microscopy and live confocal Ca²⁺ imaging**

Ca²⁺-imaging was performed with a spinning disk confocal scanner (CSU22, Yokogawa, Germany) mounted on an upright microscope (Axio Examiner, Zeiss, Germany) with 63x, 1.0 NA objective (W Plan-Apochromat, Zeiss). The spinning disk was set to 2000 rpm to synchronize with the 100 Hz acquisition rate of the camera to avoid the uneven illumination. Images were acquired by a scientific CMOS camera (Neo, Andor, Northern Ireland), with a pixel size of 103 nm. To visualize the Ca²⁺-hotspots and the ribbons, the low affinity Ca²⁺-indicator Fluo-4FF penta-K⁺ salt (0.8 mM, Life Technologies, Germany) and the TAMRA-conjugated CtBP2/RIBEYE-binding dimer peptide (10 µM, Biosynthan, Germany) were added to the
intracellular solution just before experiment. The Ca\(^{2+}\)-indicator Fluo-4FF was excited by the 491 nm diode-
pump solid-state (DPSS) laser (Calypso, Cobolt AB, Solna, Sweden), and the red fluorescence from TAMRA 
was excited by the 561 nm DPSS laser (Jive, Cobolt AB). Using a piezo positioner for the objective 
(Piezosystem, Germany), a scan of the entire cell was performed 4 min after breaking into the cell, taking 
sections each 0.5 µm at an exposure time of 0.5 s in the red (TAMRA-peptide) channel from the bottom to 
the top of the cell. In order to study the voltage-dependence of Ca\(^{2+}\)-indicator fluorescence increments at 
the synapses, the confocal scans were acquired every 0.5 µm from the bottom-most to the top-most 
ribbon. Ca\(^{2+}\)-currents were evoked by applying a voltage ramp stimulus from -87 to +63 mV during 150 ms 
(1 mV/ms) in each focal plane. Simultaneously, fluorescence measurements were made in the green 
channel (Fluo-4FF) with a frame rate of 100 Hz. In order to overcome the limitations of the frame rate and 
increase the voltage resolution of the fluorescent signal acquired, the voltage ramp protocol was applied 
twice, once shifted by 5 ms such that for any given frame during the second ramp the voltage was shifted 
by 5 mV compared to the first stimulus. The interval between 2 sequential sections was 2 s to avoid Ca\(^{2+}\) 
dependent inactivation of Ca\(^{2+}\)-channels. Alternating planes were acquired to avoid photobleaching 
encountered with the consecutive plane acquisitions.

**Immunohistochemistry, confocal and high resolution STED imaging**

The samples were fixed in formaldehyde (4%, 10 min to 1h on ice depending on the antibodies). 
Afterwards, the following primary antibodies were used: mouse anti-CtBP2 (1:200, BD Biosciences, 
612044), mouse anti-PSD-95 (1:200, Sigma Aldrich, P246-100ul), rabbit anti-Cav1.3 (1:100, Alomone Labs, 
ACC 005), rabbit anti Vglut3 (1/300, Synaptic Systems, Germany, 135 203), guinea-pig anti Vglut 3 (1/300, 
Synaptic Systems, Germany, 13 204), rabbit anti- otoferlin (1:100, Cedarlane/Synaptic Systems 
178003[SY]), rabbit anti-KCNMA1 (BK) (1:200, Alomone Labs, APC 021). Secondary antibodies used were 
Alexa Fluor 488 conjugated anti-rabbit, Alexa Fluor 488 conjugated anti-guinea-pig, Alexa Fluor 568 
conjugated anti-mouse, and Alexa Fluor 647 conjugated anti-rabbit (1:200, Invitrogen, A11008, A11073.
A11004, A31573 respectively). For high resolution STED microscopy, STAR580 and STAR635p conjugated anti-mouse and rabbit respectively (1:200, Abberior, Germany, 2-0002-005-1 and 2-0012-007-2 respectively) have been used as secondary antibodies. Images were acquired using a Zeiss LSM800 or an Abberior Instruments Expert Line STED microscope, with excitation lasers at 488, 561, and 633 nm and STED lasers at 775 nm, 1.2 W, using a 1.4 NA 100x oil immersion objective, either in confocal or in 2D-STED mode. Images were adjusted for brightness and contrast using Image J.

Data analysis

Live-imaging and IHC-patch-clamp

The data were analyzed using custom programs in Igor Pro 6.3 (Wavemetrics, Portland, OR, USA). For analysis of IV-curves, the evoked Ca\(^{2+}\)-currents were averaged from 5 to 10 ms after the start of the depolarization. \(\Delta F\) images were created by subtracting the fluorescence intensities inside the cell at resting state (\(F_0\), average of 10 frames) from the ones at the depolarized state (average of 6 frames during voltage ramp protocol). \(\Delta F\) for each hotspot was calculated as the average of a 9 pixel square placed in the region exhibiting the greatest intensity increase within the fluorescent hotspot. The fluorescence-voltage relationship (\(FV\)) was sorted by the corresponding voltage of each fluorescence intensity value. Maximal \(\Delta F\) \((\Delta F_{\text{max}})\) was the average of 5 \(\Delta F\) values obtained during the voltage ramp protocol (at the peak of Ca\(^{2+}\) influx). Only fluorescent increments presenting a \(\Delta F_{\text{max}}\) greater than the average of the fluorescence intensity plus 2 standard deviations at rest were defined as synaptic Ca\(^{2+}\) signals and considered for further analysis. Due to their variance, analysis of their voltage dependence was performed on fits to the raw \(FV\) traces using the following function:
\[ F(V) = F_0 + f_v \cdot \left( \frac{V - V_r}{1 + e^{(V_h - V)/k}} \right) \]

where \( V \) stands for the voltage command. The fitting parameters were determined by Igor Pro automatically, and their initial guess resulted from the estimations of \( F_0 \), the signal at rest, \( V_h \) for the voltage value of half-maximal activation and \( k \) for the voltage sensitivity obtained from a sigmoid fitting. The slope factor \( f_v \) was obtained by linear fitting of the FV-trace in the range of 3 to 23 mV, where the decrease of fluorescence at positive voltages results from the declining driving force despite full activation of the Ca\(^{2+}\) channels. The resulting fitting trace was forced to reach the reversal potential \( V_r \), obtained from the corresponding whole-cell Ca\(^{2+}\)-currents. The FV fit was then divided by the \( f_v \) line extrapolated to all the corresponding voltages, to estimate the hotspot fractional activation curves. The fractional activation curves were then fitted by the Boltzmann function to obtain the voltage for half activation (\( V_h \)) and slope-factor (\( k \)).

Synaptic ribbon fluorescence (visualized with the TAMRA-conjugated CtBP2/RIBEYE-binding dimer peptide) was estimated by measuring the ratio of the strongest fluorescence pixel intensity to that of the pixel nearby the hotspot (8-9 pixels away inside the cell) \( (F_{\text{ribbon}}/F_{\text{nearby}}) \). The spatial extent of the synaptic Ca\(^{2+}\)-signals was estimated by fitting of a 2D Gaussian function to the fluorescent hotspot using a genetic fit algorithm (7) to obtain the full width at half maximum in the long and short axis. For each spot, the calculations were made at those confocal sections where the intensity of the spot was strongest.

In order to combine live-imaging data from multiple cells, we reconstructed the morphology of individual IHCs and the positions of their synapses based on the fluorescence of the TAMRA-conjugated RIBEYE-binding peptide and then transformed the Cartesian coordinates into cell-aligned cylindrical coordinates (for more details see (8)). In brief, for each cell we identified the plane of symmetry orthogonal to the tonotopic axis. Then we sectioned the IHC orthogonally to a straight line fitting the pillar edge of
the cell in the plane of symmetry. We calculated the center of mass for each section and connected those of the bottom-most and of the largest section to define the central axis for our cylindrical coordinate system. We projected the AZ coordinates of multiple cells along their central axis for the polar charts, with the 4 sides annotated as modiolar or pillar (facing toward or away from the ganglion), and apical or basal (toward the cochlear tonotopic apex or base).

**Immunostaining**

Confocal and STED immunofluorescence images were analyzed and z-projected with Fiji software and further analyzed using Igor Pro. The spatial extents of the $\text{Ca}^{2+}$ channel clusters and PSD-95 was estimated in 2D STED images by assessing the full width at half maximum in the long and short axis by fitting a 2D Gaussian function (7). The intensities of the spots were estimated by taking the amplitude of the Gaussian fits at the confocal level. The position-dependent intensity of the ribbons was analyzed by a customized algorithm in MATLAB software and used as a plug-in in Imaris. The positions of the ribbons were defined as the centers of mass of CtBP2-immunofluorescent spots. The spots were subjectively selected by thresholding the quality of a 3D Gaussian fitting, including or excluding spots. Immunofluorescence intensities were measured as the sum of the voxel values within a defined region (3 voxels in X, Y, and 2 in Z) with the center of mass of the spot as origin. The cytosolic staining allowed to assign each ribbon to an IHC and to properly place the vectors. After marking the center of each IHC nucleus, a vector passing by this point and defining the central axis of the cylindrical model of the cell was adjusted to the relative orientation of each IHC cell in the XY and YZ axis. The Cartesian coordinates of the ribbons were transformed to cell-centric cylindrical coordinates in order to adjust differences in cellular orientation relative to the XYZ axes of the microscope. Multiple cells were then plotted by overlaying their central axes with alignment to the center of each nucleus.
Statistical analysis:

The data were analyzed using Matlab (Mathworks), Igor Pro 6 (Wavemetrics), Imaris 7.6.5 (Bitplane) and Python. Averages were expressed as mean ± standard error of the mean (S.E.M.). For every dataset, the standard deviation (S.D.), number of replicates (n) and animals (N) were indicated. In order to compare two samples, data sets were tested for normal distribution (Jarque-Bera test) and equality of variances (F-test), followed by two-tailed unpaired Student’s t-test, or, when data were not normally distributed and/or variance was unequal between samples, the unpaired two-tailed Mann-Whitney-Wilcoxon test was used. The non-significant difference between samples is reported as n.s., significant differences are reported as * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \).
Supplemental Figures

Fig. S1: Myo15<sup>sh2</sup> and Gαi2<sup>KO</sup> mice retain a modiolar-pillar gradient for ribbon size

(A) The polar charts display locations and intensities of immunofluorescently labelled CtBP2 marking the ribbons. Modiolar and pillar refer to facing towards or away from the ganglion in the modiolus, apical and basal refer to the tonotopic axis of the organ of Corti. The fluorescence intensity of each CtBP2 signal point is reflected by its color, with warmer, yellow tones indicating higher intensity, and cooler, darker tones indicating lower intensity. Each radial circle is 2.5 µm. The IHCs from both Gαi controls (Gαi2<sup>+/+</sup> or Gαi2<sup>KO/+</sup>) and mutant (Gαi2<sup>KO/KO</sup>) conditions display a significant modiolar (M)–pillar (P) gradient of ribbon strength (Gαi2 control (black): modiolar: n = 213 AZs vs. pillar: n = 114 AZs, Gαi2<sup>KO/KO</sup> (red): modiolar: n = 220 AZs vs. pillar: n = 107 AZs, p < 0.00001, Mann-Whitney-Wilcoxon test for both conditions) (B) The IHCs from both Myo15 control (Myo15<sup>+/+</sup>) and Myo15 mutant conditions (Myo15<sup>sh2/sh2</sup>) display a significant gradient of stronger ribbons in the modiolus (M) as compared to the pillar side (P) (Myo15 control (black): modiolar:
Fig. S2: Disruption of Gαi signaling does not change ribbon size and number

(A) Maximal projection of confocal sections from PTXa control and mutant organs of Corti co-immunolabeled for the IHC marker Vglut3 (green) and the ribbon marker CtBP2 (magenta). Scale bar = 5 µm. (B) The average number of ribbons per cell is comparable between PTXa control and mutant conditions (n = 24, N = 6 for both conditions, p = 0.81, t.test). Box plots show 10, 25, 50, 75 and 90th percentiles with individual data points overlaid, means are shown as crosses as for (C). (C) The average intensity of the ribbons is comparable between PTXa control and mutant conditions (PTXa control: n = 327, PTXa mutant: n = 360; N = 9 in both conditions, p = 0.28, Mann-Whitney-Wilcoxon test).
Fig. S3: PTXα expressing IHCs exhibit enlarged AMPAR clusters with preserved ring-like shape

(A) Maximum intensity projections of confocal sections from organs of Corti immunolabeled for GluA3 (magenta) and ribbon marker CtBP2 (green). Scale bar = 5 µm. (B) Mean intensities of the synaptic ribbons were unchanged but intensities of the postsynaptic GluA3 receptors were significantly increased in the PTXα mutant IHCs (red circles) as compared to PTXα control IHCs (black circles) (n = 120 synapses, N = 4 for both conditions, p < 0.00001, Mann-Whitney-Wilcoxon test for both conditions).
Maximal Ca\textsuperscript{2+} influx of the winning AZs (PTXa mutant in red, n = 18 winners vs. PTXa control in black, n = 19 winners) as a function of position along the pillar–modiolar axis. Mean (dashed line) ± S.E.M. (shaded areas) of their position did not differ significantly (p = 0.10, t.test)

Fig. S4: Winning AZ Ca\textsuperscript{2+} influx strength along the pillar–modiolar axis

Chapter 3. Supplementary information
Fig. S5: Immunostaining study of BK channel clustering in Gαi3, PTXα and LGN mutant inner hair cells

(A) Maximal projection of confocal sections from organs of Corti co-immunolabeled for the large-conductance Ca²⁺-activated K⁺ (BK) channels (green as for B, C) and the HC marker Myo7 (magenta as for B and C). The BK positive spots are still present in the Gαi3 KO IHCs, but in smaller amount. Scale bar = 5 µm as for (B) and (C).

(B) The BK channels are co-stained with the IHC marker Vglut3 and exhibit a mosaic expression in the PTXα mutant IHCs, showing normal clustering or being absent (no signal).

(C) The BK channels are co-stained with the IHC marker Vglut3 and exhibit a mosaic BK-channel expression in the LGN mutant IHCs, showing normal clustering in some IHCs while no signal was detected in other IHCs.

(A) Maximal projection of confocal sections from organs of Corti co-immunolabeled for the large-conductance Ca²⁺-activated K⁺ (BK) channels (green as for B, C) and the HC marker Myo7 (magenta as for B and C). The BK positive spots are still present in the Gαi3 KO IHCs, but in smaller amount. Scale bar = 5 µm as for (B) and (C).

(B) The BK channels are co-stained with the IHC marker Vglut3 and exhibit a mosaic expression in the PTXα mutant IHCs, showing normal clustering or being absent (no signal).

(C) The BK channels are co-stained with the IHC marker Vglut3 and exhibit a mosaic BK-channel expression in the LGN mutant IHCs, showing normal clustering in some IHCs while no signal was detected in other IHCs.
Fig. S6: SK2 channel clustering in PTXa mutant IHCs shows a mosaic phenotype

(A) Maximal projection of confocal sections from organs of Corti co-immunolabeled for the small-conductance Ca\(^{2+}\) activated K\(^+\) (SK2) channels (green) and the efferent marker Synapsin 1/2 (magenta as for B, C): SK2 positive spots (likely clusters of SK2) are present in every immature P7 control IHC before hearing onset. The IHCs are delineated in white from bottom (letter B) to top (letter T). Scale bar = 10 µm as for (B, C). (B) The SK2 channels are almost completely absent in mature P22 littermate PTXa controls. (C) PTXa mutant P22 IHCs exhibit a mosaic expression of the SK2 channels with either normal clustering or no signal observed in a given IHC.
REFERENCES


Studying synaptic sound encoding by fluorescence imaging of single synapses

4.1 Introduction

The auditory system is able to encode sound pressures varying over six orders of magnitude. The IHC receptor potential represents the full range (Palmer and Russell, 1986), while each SGN cover only a fraction of it (Liberman and Kiang, 1978; Sachs and Abbas, 1974; Taberner and Liberman, 2005; Zagaeski et al., 1994). For a given sound frequency, SGNs innervating the same IHC show a spatial heterogeneity: low- and medium-SR fibers with high threshold innervate preferentially the modiolar side of the IHC, while high-SR fibers with low threshold preferentially innervate the pillar side of the IHC (Liberman, 1982). These findings point out that SGNs possibly innervating a single isopotential IHC vary considerably in their firing properties. The functional diversity of SGNs is thought to increase the dynamic range of sound intensity coding with an ensemble of fibers covering different fractions. The mechanisms driving the diverse SGN firing properties, however, are not fully understood.

Several candidate mechanisms were proposed to explain how the functional diversity of SGNs is achieved: pre- and postsynaptic heterogeneity, and efferent modulation. On the presynaptic level, IHC synapses vary drastically in their morphological and functional properties even within individual IHCs. Morphologically, the ribbons on the modiolar side of the IHCs are bigger than the pillar ones (Merchan-Perez and Liberman, 1996). Functionally, the AZs differ in the number and voltage dependence of their Ca\(^{2+}\) channels, independent of the tonotopic position (Frank et al., 2009; Meyer et al., 2009). Furthermore, they exhibit a spatial heterogeneity with pillar synapses having a more negative activation voltage (Ohn et al., 2016). It was proposed that pillar synapses with more negative activation voltage could increase the glutamate release, which consequently leads to high SR (Ohn et al., 2016). On the postsynaptic level, recent evidence shows that the SGNs are molecularly different in their cohort of ion channels, receptors, synaptic proteins, and adhesion molecules, which in turn could affect their excitability (Shrestha et al., 2018; Sun et al., 2018; Petitpré et al., 2018). In addition, the
innervation of the lateral olivocochlear efferent fibers can modulate the SGN postsynaptic excitability (Ruel et al., 2001), and contribute to the establishment of the spatial heterogeneity of the IHC AZs (Yin et al., 2014).

The sound encoding properties of individual AZs in IHCs might be different based on the presynaptic control of release, including the previously reported differences in Ca\(^{2+}\) signaling and potentially in Ca\(^{2+}\) channel-exocytosis coupling. In IHC ribbon synapses, a tight functional coupling between Ca\(^{2+}\) channels and SVs has been suggested so far (Brandt et al., 2005; Goutman and Glowatzki, 2007; Wong et al., 2014; Pangršić et al., 2015). In these studies, the apparent Ca\(^{2+}\) cooperativity was assessed by relating whole-cell Ca\(^{2+}\) influx either to whole-cell exocytosis (C\(_m\) measurements; (Brandt et al., 2005; Pangršić et al., 2015; Wong et al., 2014)) or to postsynaptic recordings (Goutman and Glowatzki, 2007). The current state of knowledge supports the notion of Ca\(^{2+}\)-nanodomain-like control of exocytosis in mature mammalian IHCs. A summation model, however, was proposed to explain the observed linear Ca\(^{2+}\) dependencies (Heil and Neubauer, 2010). According to this model, the near-linear relation between Ca\(^{2+}\) influx and exocytosis stem from the sum of several supralinear, but saturating, dependencies with different sensitivities at individual AZs of a receptor cell. This model is yet to be tested on a single synapse level.

Here, I studied the presynaptic heterogeneity of AZs in IHCs, by sequential dual-color imaging of synaptic Ca\(^{2+}\) influx and glutamate release. I established the optical detection of glutamate release at individual IHC synapses using AAV-mediated expression of intensity-based glutamate reporter, iGluSnFR (Marvin et al., 2013). When relating whole-cell Ca\(^{2+}\) influx to synaptic glutamate release, I obtained a near-linear relation, consistent with the previous studies. The dual-color imaging of synaptic Ca\(^{2+}\) influx and glutamate release, however, revealed a heterogeneity of the Ca\(^{2+}\) cooperativities at individual synapses, in addition to the heterogeneity of voltage dependence of Ca\(^{2+}\) influx and glutamate release. I further showed different modes of Ca\(^{2+}\) domain control (Ca\(^{2+}\) nano- or microdomain-like) co-existed within a single IHC. We propose that different Ca\(^{2+}\) dependencies of release help the IHC partition the wide dynamic range of sound intensities by contributing to the diverse SGN firing properties.

### 4.2 Results

#### 4.2.1 Early attempts to optimize an exocytosis reporter in inner hair cells

As the IHC synapse is the first relay station in the auditory pathway, deciphering its transfer function is of crucial importance. In conventional synapses, fluorescence imaging methods have been developed to monitor presynaptic activity such as vesicle fusion and endocytosis (for review; see (Kavalali and Jorgensen, 2014)). In IHCs, however, it has been a long journey and remained a challenge to optically monitor exocytosis with genetically encoded sensors. Although pHluorin imaging—a pH sensitive GFP variant—is well established in other systems to monitor synaptic exo- and endocytosis, Vglut1-pHluorin had a very low signal-to-noise
4.2. Results

ratio (SNR) in IHCs (Neef et al., 2014). The low SNR was attributed to less acidic SV pH in IHCs, estimated to be \( \sim 6.5 \), compared to the intravesicular pH of 5.5 in conventional synapses (Miesenböck et al., 1998; Neef et al., 2014). This low SNR can as well stem from the high membrane expression of Vglut1-pHluorin and/or the expression of Vglut1, which is not the endogenous vesicular transporter in IHCs (Ruel et al., 2008; Seal et al., 2008). In the early stages of my PhD, I worked to optimize the pH-sensitive proteins in the pursuit of having a reliable, functional exocytosis reporter in IHCs. I would like to summarize this work in a short paragraph, since it will not be further mentioned: I have used two red-shifted pH sensitive proteins; mOrange2 (Li et al., 2011) and pHtomato (Li and Tsien, 2012), and cloned them into the first luminal loop of the IHC endogenous vesicular transporter, Vglut3. I have characterized the two exocytosis reporters, Vglut3-mOrange2 and Vglut3-pHtomato, in cultured HEK293 cells and hippocampal neurons. Based on the pH-calibration curve in hippocampal neurons, we selected Vglut3-mOrange2 to clone into pAAV1/2 virus backbone under human \( \beta \)-actin promoter/CMV enhancer for characterization of the sensor in IHCs. Prenatal otocyst injections at E18 and postnatal injections at P6 to wild-type (WT) mice showed successful but variable transduction efficiencies. The pH-calibration curve showed a similar pKa compared to literature (Vglut1-mOrange2; (Li et al., 2011)), but the sensor failed to dim sufficiently at pH 5.5 in IHCs. IHCs were patch-clamped in order to stimulate exocytosis, but no promising fluorescence signal was obtained. I tried strong step depolarizations (200-ms-long) to stimulate IHCs, in the presence of high \([Ca^{2+}]_e\) (5 mM), and increased the extracellular pH from 7.2 to 7.3 to improve SNR of Vglut3-mOrange2 in IHCs. Due to the lack of promising functional signal, we concluded that pH-sensitive exocytosis reporters are not suitable to monitor synaptic activity in IHCs.

4.2.2 Establishing optical detection of glutamate release in inner hair cells

As the pH-sensitive sensors failed to report exocytosis in IHCs, we next tried the intensity-based glutamate sensing fluorescent reporter (iGluSnFR; (Marvin et al., 2013)). iGluSnFR is a glutamate sensor that is constructed from *E.coli* GltI, encoding the periplasmic component of the ABC transporter complex for glutamate and aspartate, and circularly permuted GFP (Marvin et al., 2013). It can be positioned to the synaptic cleft, by targeting either the presynaptic or postsynaptic membrane, as it has a single transmembrane domain. We have targeted iGluSnFR to the postsynaptic membrane of SGNs, via postnatal injections of pAAV9.hSyn.iGluSnFR to the round window of WT mice at P5-7. Immunohistochemistry of the organ of Corti from the AAV-injected ear showed high transduction levels, demonstrating several iGluSnFR-expressing boutons per IHC (Figure 4.1). After the onset of hearing (P15-P19), IHCs from acutely dissected organ of Corti were patch-clamped and imaged simultaneously. The functional signal of iGluSnFR was detected in conditions that would “boost” the glutamate release: IHCs were stimulated with 200-ms-long step depolarizations in ruptured-patch configuration with intracellular solution containing 1 mM EGTA in the presence of high \([Ca^{2+}]_e\) (5 mM). After confirming the presence of the functional response of iGluSnFR in IHCs, I used physiological buffering to assess the dynamic range of the sensor in terms of sensitivity and
Figure 4.1 – Transduction of SGNs with AAV9.hSyn.iGluSnFR.

The maximal intensity projection of A. immunolabeled iGluSnFR-expressing SGNs (GFP) and B. IHCs, OHCs and all SGNs (parvalbumin) of the organ of Corti dissected from the right ear of P25 mouse postnatally injected (at P6) with AAV9.hSyn.iGluSnFR virus. C. Overlay of A and B shows high transduction efficiency of AAV9.hSyn.iGluSnFR virus. The membrane iGluSnFR expression (green) can be seen from the SGN somas, located lower right, as parvalbumin (magenta) is an Ca$$^{2+}$$ binding protein present mostly in the cytoplasm (Scale bar: 50 µm) D. Close-up of C shows single IHCs innervated by several iGluSnFR-expressing boutons. (Scale bar: 10 µm)

saturation (perforated patch-clamp, 1.3 mM [Ca$$^{2+}$$]e: reported perilymphatic Ca$$^{2+}$$ concentration; (Wangemann and Schacht, 1996)). For the acquisition of the fluorescence signal, I switched to using spinning disc confocal microscopy, which allows fast acquisition with spatial information, instead of laser-scanning confocal microscopy. An acquisition rate of 50 Hz was fast enough to detect reliable signal given the kinetics of iGluSnFR. Moreover, I used CtBP2-binding peptide to localize the presynaptic ribbons corresponding to the postsynaptic boutons: CtBP2-binding peptide was introduced to IHC after the perforated-patch recordings by rupturing the IHC membrane patch (Figure 4.2). The localization of corresponding presynaptic ribbons was used for separating individual synapses. Thereby, I have established a successful way of detecting iGluSnFR response at a single IHC synapse.
4.2. Results

Figure 4.2 – Optical detection of glutamate release at individual IHC synapses.
A. SGNs were transduced with AAV9.hSyn.iGluSnFR, and IHCs were patch-clamped from the pillar side. Two exemplary images of IHCs, showing iGluSnFR expressing boutons from both modiolar (cell 1) and pillar (cell 2) side. B. Perforated patch-clamp was performed to evoke glutamate release in IHCs, which can be detected via iGluSnFR expressed on the membrane of SGNs. ΔF of iGluSnFR was obtained upon 10 ms step depolarization to -22 mV from the holding potential of -87 mV. The normalized color map reflects synaptic glutamate release. C. IHCs were ruptured following the perforated patch-clamp recordings to load TAMRA-conjugated CtBP2-binding peptide for ribbon localization. (*:transduced boutons, >:ribbons) (Scale bar: 5 µm)

4.2.3 Hearing was unaffected in mice expressing iGluSnFR in SGNs

To assess whether AAV-mediated iGluSnFR expression in SGNs affects hearing, recordings of auditory brainstem responses (ABR) were performed on WT mice 2 weeks after postnatal injection of AAV9.hSyn.iGluSnFR virus (Figure 4.3). ABR waveforms and thresholds were comparable between the injected and non-injected ear which was used as a control. After the ABR recordings, the expression levels were checked by immunohistochemistry to confirm successful transduction of the virus. As the hearing was unaffected, iGluSnFR expression in SGNs does not seem to affect sound encoding and signal propagation along auditory pathway and is a good candidate to study IHC synaptic physiology.
Figure 4.3 – Hearing is normal in mice expressing iGluSnFR in SGNs.

A. ABR waveforms were recorded in response to 80 dB clicks (mean ± SEM, 3 animals). The non-injected ear was used as a control. B. ABR thresholds were comparable between AAV9.hSyn.iGluSnFR injected ear and the non-injected control. A statistical test was not applied, as it is a small sample size. The transduction levels were checked after ABR recordings to confirm the presence of iGluSnFR expression. C. The maximal intensity projection of immunolabeled iGluSnFR-expressing SGNs (GFP) and synaptic ribbons (CtBP2), showing the iGluSnFR expression in the injected ear. (Scale bar: 5 µm)

4.2.4 Glutamate release increases with stimulus duration

To assess whether iGluSnFR is adequate to detect glutamate release at IHC synapses, I combined iGluSnFR imaging with patch-clamp membrane capacitance (Cm) measurements, which are well-established to detect IHC exocytosis on a whole-cell level (Moser and Beutner, 2000). As Cm measurements, however, sum over approximately a dozen of synapses from an IHC, it lacks the resolution of a single synapse. Figure 4.4A shows an example iGluSnFR response and corresponding Cm measurement in response to a 50-ms-long step depolarization. Glutamate release at the single synapse level could be detected for weak stimuli compared to those eliciting detectable signal for the synapse population of an IHC.

Stimuli of varying lengths can be applied to resolve the time course of release as it can not be resolved using single sinewave Cm measurements (see Schnee et al., 2005) for dual sinewave or due to the kinetics of iGluSnFR. I applied step depolarizations of varying length (2 ms to 100 ms) from the holding potential of -87 mV to -23 mV. I checked the correlation between the exocytic change in Cm (ΔCm) and the peak (max(ΔF/Φ0)) or the area under the curve (AUC(ΔF/Φ0)) of the iGluSnFR signal. Both max(ΔF/Φ0) and AUC(ΔF/Φ0) of iGluSnFR showed significant positive correlation with ΔCm (Pearson’s correlation coefficient ρ = 0.63, p < 0.0001 and ρ = 0.66, p < 0.0001, respectively; Figure 4.4D). Moreover, iGluSnFR signal was sensitive enough to detect glutamate release in response to 2-ms-long step depolarization: the peak of iGluSnFR was significantly higher from the preceding baseline (average of 4 frames during and 10 frames before stimulation; Mann-Whitney-U test, p < 0.0001). Given together, both
max(ΔF/F₀) and AUC(ΔF/F₀) of iGluSnFR reliably report glutamate release for brief depolarizations, while release in response to longer depolarizations might be better represented by the AUC(ΔF/F₀) of iGluSnFR. Due to this reason, we used the AUC(ΔF/F₀) of iGluSnFR as the measure of glutamate release in the following experiments.

The time course of release can be reconstructed by the “Bookman” plot, which plots the increments of the responses during stimuli against the stimulus length (Horrigan and Bookman, 1994). In IHCs, the “Bookman” plot was used to probe the SV pool dynamics by Cₘ measurements (Moser and Beutner, 2000). Cₘ measurements show a fast component of exocytosis, thought to represent RRP release which tends to saturate near 20 ms of stimulation, and a subsequent near linear sustained component of exocytosis (Moser and Beutner, 2000; Figure 4.4C - middle graph). I fitted the mean ΔCₘ and AUC(ΔF/F₀) values acquired for different stimulus durations using sum of an exponential and linear function to assess how fast the RRP was depleted in both cases (equation is taken from (Pangršić et al., 2010)):

\[
\Delta C_m(t) = RRP \text{size} \cdot (1 - e^{\frac{t}{\tau}})^n + \text{slope} \cdot t
\]

The time constant of RRP depletion was comparable for ΔCₘ (RRP size: 4.9 fF; \(\tau_{\text{depletion}} = 7.94\) ms) and AUC(ΔF/F₀) (RRP size: 2.4 arbitrary unit (a.u) of AUC(ΔF/F₀), \(\tau_{\text{depletion}} = 11.39\) ms) (perforated patch-clamp, 1.3 mM \([Ca^{2+}]_e\)). In conclusion, the iGluSnFR signal as a read-out of glutamate release increases with stimulus duration along with IHC’s ΔCₘ and follows the exocytosis dynamics observed on the whole-cell level.

### 4.2.5 Voltage dependence of release in inner hair cells

Next, I checked the voltage dependence of release in IHCs. I applied 10-ms-long step depolarizations to monitor RRP exocytosis from the holding potential of -87 mV to a voltage within the physiologically relevant range of receptor potentials (Palmer and Russell, 1986); from -62 mV to -22 mV in 5 mV increments (Figure 4.5A). Glutamate release was already detected from -42 mV (Figure 4.5B-C). Postsynaptic recordings from rat IHCs showed similar results: release was detected at -49 mV for longer step depolarizations (100 ms; (Goutman and Glowatzki, 2007)). I used stimuli of varying voltage to probe the “apparent” Ca²⁺ cooperativity of release, as varying the voltage in the negative range results mainly in changes in the open Ca²⁺ channel number. By fitting a power function to all the data points collected from 33 boutons, I found a near-linear relation between whole-cell Ca²⁺ influx and AUC(ΔF/F₀) of iGluSnFR as a read-out of synaptic glutamate release (\(m = 1.53\), Figure 4.5D). Consistently, the mean power of 1.71 was calculated from the individual synapse fits (Figure 4.5E). The near-linear Ca²⁺ dependence of release supports the previous findings of Ca²⁺ nanodomain-like control of release in IHCs (Brandt et al., 2005; Goutman and Glowatzki, 2007; Pangršić et al., 2015; Wong et al., 2014).
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Figure 4.4 – Glutamate release increases with stimulus duration.
A. A representative whole cell Ca\textsuperscript{2+}-current, corresponding C\textsubscript{m} and single synapse iGluSnFR fluorescence trace upon a 50-ms-long step depolarization to -23 mV. Recordings were from WT mice (P15-P19) injected with AAV9.hSyn.iGluSnFR virus at P5-7 (perforated patch-clamp, 1.3 mM [Ca\textsuperscript{2+}]\textsubscript{e}). B\textsubscript{i}. Average iGluSnFR response upon step depolarizations to -23 mV from a holding potential of -87 mV with increasing durations of 2 ms to 100 ms (color coded) (n = 31 boutons). B\textsubscript{ii}. Corresponding average C\textsubscript{m} traces upon step depolarizations with increasing duration (N = 10 IHCs from 8 mice). C. The AUC of the iGluSnFR response with respect to whole cell \Delta C\textsubscript{m} and Ca\textsuperscript{2+} charge (Q\textsubscript{Ca}) (mean ± SD). D. The relation of the peak (pearson’s correlation coefficient (\rho) = 0.63, p<0.0001) or the AUC (\rho = 0.66, p<0.0001) of the iGluSnFR signal and whole cell \Delta C\textsubscript{m} (mean ± SD). Both the peak and the AUC of iGluSnFR response positively correlate with the whole cell \Delta C\textsubscript{m}. Depolarization duration is color coded, and the black circles indicating the means darken with depolarization duration.

4.2.6 Calcium domain manipulation by Zn\textsuperscript{2+} perfusion

To probe the intrinsic Ca\textsuperscript{2+} cooperativity of release in IHCs, I manipulated single Ca\textsuperscript{2+}-channel current by application of 1 mM Zn\textsuperscript{2+}. Zn\textsuperscript{2+} causes a rapid flicker block of L-type Ca\textsuperscript{2+} channels (Winegar and Lansman, 1990). This flicker block results in an apparent decrease of the Ca\textsuperscript{2+}
4.2. Results

A. Exemplary single synapse iGluSnFR signal in response to a 10-ms-long step depolarization from a holding potential of -87 mV to a voltage within the physiologically relevant range of receptor potentials: from -22 mV to -62 mV in 5 mV increments. The voltage range is color-coded: darker points indicate more negative potentials. B. The peak iGluSnFR fluorescence (top; max(ΔF/F(0)10ms) from A and corresponding whole-cell Ca\(^{2+}\) current (bottom; perforated patch-clamp, 1.3 mM [Ca\(^{2+}\)]\(_{c}\)). The glutamate release can already be detected at -42 mV. C. Average ΔF/F(0) iGluSnFR traces recorded from n = 33 boutons (N = 11 IHCs). Shaded areas show ± SD. D. Normalized AUC(ΔF/F(0)10ms) and Q_{Ca} is plotted (n = 33 boutons from N = 11 IHCs). A power function was fitted before an obvious saturation of the RRP release (normalized Q_{Ca} < 1), and a quasi-linear relation was observed (m = 1.53). E. A histogram showing the distribution of m when individual fits were made per synapse before an obvious saturation was observed for a given synapse (m_{average} = 1.71). Only the fits with R² value higher than 0.7 were used for further analysis (n = 33 boutons). The rug plot under the histogram displays the individual data points. Every depolarization step was repeated at least two times and average was taken per synapse.
domain “seen” by the Ca\(^{2+}\) sensor of release within the limits of exocytosis kinetics, as the fusion machinery cannot follow microsecond flickering of the single channel Ca\(^{2+}\) current. Varying the single Ca\(^{2+}\) channel current is expected to mirror the intrinsic Ca\(^{2+}\) cooperativity of release, as it is independent of the AZ topography. Previously, a cooperative binding of 4-5 Ca\(^{2+}\) ions to the fusion machinery was proposed to be required for vesicle fusion (Beutner et al., 2001; Wong et al., 2014). Here, I monitored the RRP exocytosis by repetitive 10-ms-long depolarizations while slowly perfusing 1 mM Zn\(^{2+}\) in and out of the recording chamber. Figure 4.6A-B shows a single synapse example: as the whole-cell Ca\(^{2+}\) current decreases, glutamate release almost abolishes. Washing out of Zn\(^{2+}\) from the recording chamber recovers both Ca\(^{2+}\) current and consequently the release. To exclude the possibilities that the observed decrease in the Ca\(^{2+}\) current and the iGluSnFR signal are not due to a dramatic Ca\(^{2+}\) rundown (more than 25%), or photobleaching of the sensor, the recordings were used only if both signals were recovered after washing out Zn\(^{2+}\). I found a supra-linear relation between Ca\(^{2+}\) influx and release, by power function fitting to all the data points recorded from 24 boutons \((m = 2.56)\) or from the mean of the individual fits (19 boutons, \(m = 2.46\); Figure 4.6C-D). The observed supralinearity supports the high intrinsic Ca\(^{2+}\) cooperativity of release in IHCs.

4.2.7 Paired optical recordings of synaptic Ca\(^{2+}\) influx and glutamate release in inner hair cells

IHCs make overall 5-20 synapses with SGNs, where single synapses drive fiber activity (Merchan-Perez and Liberman, 1996). For a given frequency, single-AZ driven SGNs can collectively cover the whole audible spectrum of sound pressures, ranging within 6 orders of magnitude. How individual AZs from an IHC, which is believed to be isopotential, can drive activity of SGNs with different properties is largely unknown. Previous work showed a heterogeneity of synapses in a single IHC (Ohn et al., 2016). Synapses differed in their voltage dependence of Ca\(^{2+}\) channel activation and amplitude of maximal synaptic Ca\(^{2+}\) influx (Frank et al., 2009; Meyer et al., 2009; Ohn et al., 2016). These differences in the voltage-dependence of synaptic Ca\(^{2+}\) influx could explain the greater spontaneous release of glutamate at the resting IHC potential, which leads to higher SR. Furthermore, the sound encoding properties of individual synapses might differ based on the differences in Ca\(^{2+}\) signaling and potentially in Ca\(^{2+}\) channel-exocytosis coupling. In addition, recently, three parallel studies showed molecular differences in SGN classes (Petitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018).

Taken together, understanding IHC synaptic input-output relation is of critical importance to understand sound encoding. In this part, I established and performed dual-color imaging of single-synapse Ca\(^{2+}\) influx and glutamate release, which can bridge the gap between the pre- and postsynapse. As the ribbon development is more complete and the heterogeneity of the synapses is more established after P21 (Liberman and Liberman, 2016), I performed the paired optical recordings of Ca\(^{2+}\) influx and glutamate release on P21-26 mice.
4.2. Results

Figure 4.6 – Effects of changes in single channel Ca\(^{2+}\) current on glutamate release.
A. Exemplary single-synapse iGluSnFR signal in response to repetitive 10-ms-long step depolarizations to -23 mV from a holding potential of -87 mV as 1 mM Zn\(^{2+}\) is perfused in and out from the recording chamber. The temporal sequence is encoded in color; darker colors indicate earlier time points. B. The time course of the peak iGluSnFR response (top; max(ΔF/ΔF\(_0\))\(_{10\text{ms}}\)) from A and corresponding whole-cell Ca\(^{2+}\) current (bottom; perforated patch-clamp, 1.3 mM [Ca\(^{2+}\)]\(_{e}\)). The whole-cell Ca\(^{2+}\) influx decreases with Zn\(^{2+}\) perfusion, since Zn\(^{2+}\) causes a rapid flicker block of L-type Ca\(^{2+}\) channels. C. Normalized AUC(ΔF/ΔF\(_0\))\(_{10\text{ms}}\) and Q\(_{Ca}\) is plotted (n = 24 boutons from N = 10 IHCs). A power function was fitted before an obvious saturation of the RRP release (normalized Q\(_{Ca} < 0.7\)), and a supralinear relation was observed (m = 2.54). D. A histogram showing distribution of m when individual fits were made per synapse before an obvious saturation was observed for the given synapse (m\(_{average} = 2.46\)). Only the fits with R\(^2\) value higher than 0.7 were used for further analysis (n = 19 boutons). The rug plot under the histogram displays the individual data points.

4.2.7.1 Establishing red-shifted Ca\(^{2+}\) imaging in IHCs

Ca\(^{2+}\) imaging in IHCs is well-established by the use of green-emitting low-affinity chemical Ca\(^{2+}\) indicators, such as Fluo-5N and Fluo-4FF (Frank et al., 2009; Meyer et al., 2009; Ohn et al., 2016). As I aimed to combine Ca\(^{2+}\) imaging with iGluSnFR imaging, optimization of red-shifted Ca\(^{2+}\) indicators was required. Overall, the availability and SNR of the red-shifted Ca\(^{2+}\) dyes are low compared to the green-emitting chemical Ca\(^{2+}\) dyes (for review; see (Oheim et al., 2014)).
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Figure 4.7 – Rhod-FF is suitable for subcellular Ca\textsuperscript{2+} imaging in IHCs.

A. ∆F images of before, during and after 100-ms-long step depolarization to -17 mV from a holding potential of -87 mV. The stimulus ∆F image shows single confined Ca\textsuperscript{2+} “hotspot” from an IHC, while the spot is not present before and after the stimulus. The right top image shows the fluorescence of the Abberior Star 488 conjugated CtBP2-binding peptide, co-localizing with the Ca\textsuperscript{2+} hotspot. A\textsubscript{i} depicts the color bars of the ∆F and F images in arbitrary units (a.u.).

B. The corresponding ∆F/F\textsubscript{0} trace of Rhod-FF signal.

After a careful search for an optimal red-shifted Ca\textsuperscript{2+} indicator, we decided to use the red-shifted Ca\textsuperscript{2+} indicator Rhod-FF; which has a K\textsubscript{d} of 19 µM (excitation peak at 552 nm, emission peak at 580 nm; commercially available at AAT Bioquest). As a proof of principle, I applied step depolarizations and imaged with 800 µM Rhod-FF in combination with an Abberior Star 488-conjugated CtBP2-binding peptide in high-buffering conditions (5 mM [Ca\textsuperscript{2+}]\textsubscript{e} and 10 mM EGTA; (Ohn et al., 2016)). Figure 4.7 shows a confined Ca\textsuperscript{2+} “hotspot”, co-localizing with the AbberiorStar488-conjugated CtBP2-binding peptide in response to a 100-ms-long step depolarization. We did not have any evidence for mitochondrial sequestration of Rhod-FF as it was previously indicated with some of the rhodamine-based Ca\textsuperscript{2+} indicators (for review; see (Oheim et al., 2014)). Furthermore, the observed Ca\textsuperscript{2+} hotspots consistently co-localized with the ribbon peptide. The fast kinetics of the Ca\textsuperscript{2+} signal also supports the detection of synaptic Ca\textsuperscript{2+} influx through Ca\textsubscript{V}1.3 channels. As changing buffering conditions, lower [Ca\textsuperscript{2+}]\textsubscript{e} and EGTA resulted in broader Ca\textsuperscript{2+} hotspots with low SNR, we used the high-buffering conditions established in Ohn et al., 2016. Taken together, I showed Rhod-FF can be used for single-synapse Ca\textsuperscript{2+} imaging in IHCs and suitable for combining with iGluSnFR imaging.

4.2.7.2 Experimental design of dual-sequential imaging of Ca\textsuperscript{2+} and glutamate release in IHCs

In the experimental design of the dual-color imaging, we aimed to capture the voltage dependence and amplitude of synaptic Ca\textsuperscript{2+} influx and glutamate release, as these properties are the key to understand synaptic input-output function. Since the spinning disc microscopy is
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Figure 4.8 – Dual-sequential imaging of synaptic Ca$^{2+}$ influx and glutamate release.

A. $\Delta F$ images of Rhod-FF and iGluSnFR in response to a voltage ramp and a 50-ms-long step depolarization, respectively. The analysis of the marked synapse on the overlaid image is further explained in the following panels. B and C. (upper) the voltage command, (middle) the corresponding whole-cell Ca$^{2+}$ influx and (bottom) the functional fluorescence responses from Rhod-FF (B) and iGluSnFR (C). A boltzman function was fitted either to an individual Rhod-FF fluorescence trace in response to a voltage ramp (B$_i$) or to AUC($\Delta F/F_0$) of iGluSnFR per depolarization voltage (C). D. The obtained fits from a Ca$^{2+}$ “hotspot” (B$_i$) and from glutamate release (C$_i$) were plotted against each other in a voltage range from -57 mV to -17 mV in 1 mV increments. This plot shows an example linear ($m = 1.01$) synaptic input-output relation in a physiologically relevant voltage range. (ruptured patch-clamp, 10 mM intracellular EGTA, 5 mM [Ca$^{2+}$]$_o$)
equipped with a single sCMOS camera and a dual-viewer optics was not available at that time, I have performed dual-sequential imaging of Ca\(^{2+}\) influx and glutamate release. I detected the imaging plane by the baseline fluorescence of iGluSnFR-transduced boutons. I applied a voltage-ramp depolarization from -87 mV to +63 mV to IHCs, while simultaneously recording Rhod-FF fluorescence (Figure 4.8B). The voltage ramps are useful to estimate voltage dependence of synaptic Ca\(^{2+}\) influx in a single depolarization (Ohn et al., 2016). To capture the presynaptic Ca\(^{2+}\) influx of the corresponding SGN bouton, I repeated the voltage ramps in 5 different planes in 0.5 \(\mu\)m alternating steps (Figure 4.19). The alternating steps were used to avoid additional photobleaching (Ohn et al., 2016). After recording the presynaptic Ca\(^{2+}\) influx in 5 planes, I focused on the initially determined middle plane, and applied 50-ms-long step depolarizations to a physiologically relevant voltage range to record the iGluSnFR signal (Figure 4.8C). 50-ms-long depolarizations were chosen to stimulate sufficient IHC release in the presence of 10 mM EGTA, which is expected to constrain the Ca\(^{2+}\) signal to the nanometer-proximity of the Ca\(^{2+}\) channels and to inhibit Ca\(^{2+}\)-dependent SV replenishment. I positioned the depolarization voltage to cover the dynamic range of the glutamate release (-57, -49, -45, -41, 37, -33, -25, -17 mV, applied in a pseudo-randomized manner). Boltzmann functions were fitted to the voltage-dependence of synaptic Ca\(^{2+}\) influx and glutamate release. These fit functions approximating the synaptic Ca\(^{2+}\) influx and glutamate release were then used to relate their behavior in the physiologically relevant voltage range (synaptic Ca\(^{2+}\) influx, Figure 4.8B; synaptic glutamate release, Figure 4.8C; example relation of both, Figure 4.8D; see Materials and Methods for details).

### 4.2.7.3 Voltage-dependence and apparent Ca\(^{2+}\) dependence of glutamate release

I assessed the voltage of half-maximal activation (V\(_{1/2}\)) of the whole-cell Ca\(^{2+}\) influx (Q\(_{Ca}\)) of 23 IHCs by fitting a Boltzmann function (V\(_{1/2}\) = -36.25 ± 2.77 mV, SD; Figure 4.9A). Similarly, I obtained the mean V\(_{1/2}\) of synaptic Ca\(^{2+}\) influx (V\(_{1/2}\) = -37.66 ± 5.6 mV, SD; Figure 4.9C) and synaptic glutamate release (V\(_{1/2}\) = -37.37 ± 3.81 mV, SD; Figure 4.9B) of 41 synapses. As expected, I found a wider V\(_{1/2}\) distribution for individual synapse Ca\(^{2+}\) influx and glutamate release, compared to that of the whole-cell Ca\(^{2+}\) influx. While, obviously, the presynaptic Ca\(^{2+}\) influx shapes the release of glutamate at a given AZ, the whole-cell Ca\(^{2+}\) influx remains relevant as its estimation is very precise and it has been used for studying Ca\(^{2+}\) cooperativity in past and present studies. I next related the glutamate release to either whole-cell Ca\(^{2+}\) influx or to synaptic Ca\(^{2+}\) influx, as it was described step by step in Figure 4.8 (Figure 4.9D-E). To estimate the coupling of Ca\(^{2+}\) influx and glutamate release, I fitted a power function until the 40% of the AUC(∆F/F\(_0\)) of iGluSnFR to capture the initial rise, where RRP depletion is unlikely to occur. I showed that Ca\(^{2+}\) cooperativity (m) of glutamate release obtained by relating it to single synapse Ca\(^{2+}\) influx was higher on average (m\(_{Rhod-FF}\) = 2.23), compared to the whole-cell Ca\(^{2+}\) influx (m\(_{QCa}\) = 1.48; Figure 4.9F). The majority of the synapses, however, seemed to operate near linearly, suggesting a Ca\(^{2+}\) nanodomain-like control of release (arbitrary cut-off for linearity; m < 1.6), while overlap of Ca\(^{2+}\) domains of several channels might occur at the remaining synapses with greater m (Ca\(^{2+}\) microdomain-like) control of release.
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Figure 4.9 – Voltage-dependence and apparent Ca$^{2+}$ dependence of glutamate release.
A. The normalized $Q_{Ca}$, calculated in response to 50-ms-long step depolarizations, is plotted against the depolarization voltage. A Boltzman function was fitted to estimate the voltage of half-maximal Ca$^{2+}$ influx and the slope-factor ($k$). Individual IHCs are color coded in the shades of blue (N = 23 IHCs). B. The normalized AUC($\Delta F/F_0$) of iGluSnFR, in response to 50-ms-long step depolarizations, is plotted against depolarization voltage, like in (A) (n = 41 synapses; the individual synapses are color coded). C. A voltage ramp was applied to obtain the Rhod-FF trace. The voltage of half-maximal synaptic Ca$^{2+}$ influx was calculated from the Boltzman function fitted to baseline-normalized traces ($\Delta F/F_0$). (mean ± SD) D. The relation of whole-cell Ca$^{2+}$ influx (A) and synaptic glutamate release (B). The bold line shows the mean. E. The relation of synaptic Ca$^{2+}$ influx and glutamate release. F. The histogram shows the Ca$^{2+}$ cooperativity ($m$) obtained by individual power function fitting until 40% of normalized iGluSnFR response from (D; gray) and from (E; green). The mean $m$ was found to be 1.48 and 2.23, respectively. The rug plot shows the individual data points. The dashed line shows the arbitrary cut-off for linearity ($m = 1.6$).

In IHCs, the Ca$^{2+}$ cooperativity of release has been studied only by relating the whole-cell Ca$^{2+}$ influx to whole-cell exocytosis or to postsynaptic recordings, always yielding $m_{QCa}$. I next checked the correlation between $m_{QCa}$ and $m_{Rhod–FF}$ to test how much $m_{QCa}$ can actually reflect about what is happening on a single synapse level: there is a positive correlation, yet they are not the same (Figure 4.10; $\rho = 0.58$, $p < 0.0001$). Since the whole-cell Ca$^{2+}$ influx sums over all the synapses in an IHC, I further focused on single synaptic properties.
4.2.7.4 Linear synapses exhibit wider dynamic range

I checked if there is a cellular preference to employ either type of Ca\(^{2+}\) domain control of release (Ca\(^{2+}\) nano- or microdomain), or if they can co-exist in a single IHC. Figure 4.11 shows an example IHC where 3 nearby modiolar synapses show different Ca\(^{2+}\) cooperativities (\(m = 7.18, 1.31, 2.35\)). Interestingly, the linear synapse (depicted in green) showed wider dynamic range (33.5 mV) of glutamate release, while the other two nonlinear synapses covered narrower voltage ranges (9.5 and 11.2 mV). Indeed, in this subpopulation of 41 synapses, the linear synapses, suggesting a Ca\(^{2+}\) nanodomain like control of release, had significantly wider dynamic range compared to non-linear synapses (Figure 4.12; student’s t-test; \(p < 0.0001\)).

Every synapse seems to contribute for the sound encoding with variable dynamic range width and \(V_{1/2}\) (Figure 4.13A). This shows how individual synapses could contribute to collective sound intensity encoding. Even though synapses have shown differences in their Ca\(^{2+}\) cooperativities with linear and non-linear input-output relation, there was a positive correlation between the \(V_{1/2}\) of synaptic Ca\(^{2+}\) influx and glutamate release (Figure 4.13B; \(r = 0.36, p = 0.021\)).

4.2.7.5 Pillar synapses seem to release at more negative potentials compared to modiolar ones in a single IHC

There is a spatial heterogeneity of SGN firing properties, where high SR-low threshold fibers innervate preferentially on the pillar side of the IHC, and low SR-high threshold fibers contact the
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Figure 4.11 – Single IHC shows different modes of Ca\(^{2+}\) domain control of glutamate release.

A. The overlaid ∆F image of Rhod-FF (red) and iGluSnFR (green) shows synaptic Ca\(^{2+}\) influx and glutamate release at three nearby modiolar synapses. Individual synapses are color-coded. B. The relation between Ca\(^{2+}\) influx and glutamate release on a single synapse level in the voltage range of -57 to -17 mV plotted in 1 mV increments. A power function was fitted until the 40% of normalized AUC(∆F/F_0)\(_{iGluSnFR}\). Synapses showed different Ca\(^{2+}\) cooperativities (m = 7.18, 1.31, 2.35; blue, green, yellow). C. Dynamic ranges of corresponding Ca\(^{2+}\) influx (gray) and glutamate release (color-coded) with the voltage of half-maximum (V\(_{1/2}\)) depicted. The green synapse with Ca\(^{2+}\) nanodomain-like control of release (m = 1.31) exhibit bigger dynamic range than the other synapses.

Modiolar side of the IHC (Merchan-Perez and Liberman, 1996). Previously, the heterogeneity of presynaptic Ca\(^{2+}\) influx was proposed to explain the differences in SRs: in this Ca\(^{2+}\) imaging study, pillar synapses showed activation of the Ca\(^{2+}\) influx at weaker depolarizations compared to the modiolar synapses (Ohn et al., 2016). A more hyperpolarized voltage-dependence of the synaptic Ca\(^{2+}\) influx could explain the greater spontaneous glutamate release at the resting IHC potential that consequently leads to higher SR. In support of this hypothesis, the present work shows a positive correlation between the V\(_{1/2}\) of synaptic Ca\(^{2+}\) influx and glutamate release. On the other hand, until now, to our knowledge, the single synaptic transfer function remained to be measured to fully comprehend stimulus-secretion coupling as well as the impact of heterogeneous Ca\(^{2+}\) signaling properties for spontaneous and evoked SGN firing. Here, I show an exemplary recording from iGluSnFR-expressing boutons at both modiolar and pillar sides of the IHC. Interestingly, the only pillar synapse in the given plane was active at weaker potentials compared to the modiolar synapses (Figure 4.14). It is, however, not possible to make general conclusion, as out of 41 boutons only 5 of them were recorded from the pillar side of the IHC. In this small set of pillar synapses, we did not observe a significant
tendency of glutamate release at more negative potentials compared to the modiolar ones.

### 4.2.7.6 Clustering of synapses based on their properties

With the paired optical recordings of \( \text{Ca}^{2+} \) influx and glutamate release, I acquired 19 properties per synapse. I created a correlation map, which uses Pearson correlation coefficient and allows individual comparisons of the synaptic properties (Figure 4.15). As this leaves us with a big number of variables, I next performed Principal Component Analysis (PCA) and K-means clustering to discover possible clusters in this dataset. Principal Component Analysis is a dimension-reduction tool that converts a set of potentially correlated variables into linearly uncorrelated variables called principal components (PCs) that can still account for most of the information of a given dataset. Figure 4.16 shows the first three PCs, on 2-D and 3-D level, color-coded based on the K-means clustering results. Figure 4.16D shows that the first three PCs are able to explain more than 60% of the variance observed in the data. Furthermore, I analyzed the glutamate release response properties based on the clustering results (Figure 4.17). The first cluster is composed of linear synapses with big dynamic range and low threshold, while the third cluster has non-linear synapses with smaller dynamic range and high threshold. Furthermore, the synapses in the cluster one seem to be active already around the IHC receptor potential (\( \sim -55 \text{ mV} \); Figure 4.17D). The second cluster falls in between those properties. Interestingly, no pillar synapses were found in the third cluster.

![Figure 4.12 – Linear synapses exhibit wider dynamic range.](image)

**Figure 4.12 – Linear synapses exhibit wider dynamic range.**

A. The histogram from Figure 4.9F, showing the distribution of \( m \) obtained by relating synaptic \( \text{Ca}^{2+} \) influx and glutamate release. The dashed line shows the arbitrary cut-off for linearity (\( m = 1.6 \)). B. The dynamic ranges of glutamate release are significantly wider for linear synapses compared to non-linear synapses (student’s t-test, \( p < 0.0001 \)).
4.3 Discussion

In the auditory system, single-AZ driven SGNs can collectively cover the whole audible spectrum of sound pressures, ranging within 6 orders of magnitude. The mechanisms underlying this dynamic range partition are not fully understood. Presynaptic AZ heterogeneity, in terms of maximal amplitude of synaptic Ca$^{2+}$ influx and voltage dependence of Ca$^{2+}$ channel activation, was previously suggested as a candidate mechanism (Frank et al., 2009; Meyer et al., 2009; Ohn et al., 2016). On the other hand, recent single-cell transcriptomic studies showed molecular differences between SGN subtypes that might affect their neuronal excitability (Pettitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018). It is, however, not clear how the heterogeneous Ca$^{2+}$ signaling translates into differences in the release and whether potential heterogeneity of other presynaptic mechanisms might be involved, such as the coupling of Ca$^{2+}$ influx and exocytosis. Here, we studied sound encoding at individual IHC synapses, by dual-color imaging of Ca$^{2+}$ influx and glutamate release, providing insight to its synaptic transfer function. We found that half of the synapses exhibited linear relation between Ca$^{2+}$ influx and glutamate release, suggesting nanodomain coupling (19 synapses out of 41; $m < 1.6$), while the other half was non-linear ($m > 1.6$; mean $m = 2.23$). Surprisingly, individual IHC synapses showed different Ca$^{2+}$ dependencies of release even within a single IHC. Moreover, linear synapses exhibited wider dynamic range compared to the nonlinear synapses. We propose that IHC partitions dynamic range through heterogeneous presynaptic control of

Figure 4.13 – Each synapse contributes with a fraction to the wide dynamic range of sound encoding.
A. Dynamic ranges of the synaptic Ca$^{2+}$ influx (magenta) and glutamate release (green) with their $V_{1/2}$ depicted. B. The $V_{1/2}$ of Ca$^{2+}$ influx shows a significant positive correlation with the $V_{1/2}$ of glutamate release ($r = 0.36$, $p = 0.021$). The marginal histograms show the distribution of each axis, with a rug plot overlaid.
Previously, the apparent \( \text{Ca}^{2+} \) dependence of release was studied by relating the whole-cell \( \text{Ca}^{2+} \) influx either to whole-cell exocytosis (\( \Delta C_{m} \)) or to single-synapse release (EPSCs), always yielding \( m_{QCa} \) and summing information from many synapses (Brandt et al., 2005; Goutman and Glowatzki, 2007; Pangrižić et al., 2015; Wong et al., 2014). These studies obtained a quasi-linear apparent relation between \( \text{Ca}^{2+} \) influx and glutamate release, supporting the notion of \( \text{Ca}^{2+} \) nanodomain-like control of release in IHCs. Indeed, when relating whole-cell \( \text{Ca}^{2+} \) influx and single-synapse glutamate release, I obtained similar results in two age group and buffering conditions (the first dataset: P15-19, perforated-patch, 1.3 mM [\( \text{Ca}^{2+} \)]\(_{e} \), Figure 4.5E: \( m_{QCa} = 1.7 \); the second dataset: P21-26, ruptured-patch, 5 mM [\( \text{Ca}^{2+} \)]\(_{e} \), Figure 4.9F: \( m_{QCa} = 1.48 \)). Interestingly, a summation model was proposed to explain the linear apparent \( \text{Ca}^{2+} \) dependence of release in receptor cells (Heil and Neubauer, 2010). This model suggested that the near-linear relations result from the sum of supralinear and saturating dependencies with different sensitivities at individual synapses of the cell. In IHCs, studying the synaptic input-output relation has critical importance to understand its function, since a substantial AZ heterogeneity was found in the voltage dependence of synaptic \( \text{Ca}^{2+} \) influx, deviating from
Figure 4.15 – Correlation map of synaptic properties.
This correlation map shows the Pearson correlation coefficients calculated between the given synaptic properties. The degree of correlation is color-coded; positive correlations are depicted in red, and the negative correlations are depicted in blue. The colors darken with the strength of correlation.

the whole-cell Ca\(^{2+}\) influx (Ohn et al., 2016). By single-synapse dual imaging of Ca\(^{2+}\) influx and glutamate release, I showed that indeed half of the synapses had a supralinear relation with saturation in the physiologically relevant range of receptor potentials, while the other half, which was not predicted by this model, was linear, employing Ca\(^{2+}\) nanodomain-like control of release. Moreover, I showed that these different modes of release control, i.e. Ca\(^{2+}\) nanodomain and microdomain-like, could co-exist in a single IHC, excluding possible cellular preference of either mode.

Both coupling types offer different advantages and could have different functional implications for the IHC (for review; see Eggermann et al., 2012; Moser et al., 2006)). Ca\(^{2+}\) nanodomain control of release provides increased efficiency and speed, as it decreases the synaptic delay. This feature of Ca\(^{2+}\) nanodomain control of release would be relevant for the temporal precision of auditory signaling. On the other hand, Ca\(^{2+}\) microdomain control of release shows high specificity and all-or-none response, which consequently decreases the “jitter” of the evoked transmitter release and spontaneous release at rest. Interestingly, IHCs were found
to drive SGNs with diverse spiking behavior: high SR – low threshold fibers preferentially connecting to the pillar side of the IHC and low SR – high threshold fibers innervating the modiolar side of the IHC (Merchan-Perez and Liberman, 1996). In this study, the non-linear synapses, employing microdomain coupling, exhibited significantly higher release threshold compared to the linear synapses. Previously, the spatial differences in the SR was explained by the heterogeneity in the voltage dependence of synaptic Ca\(^{2+}\) influx, with the pillar synapses activating in slightly lower potentials compared to the modiolar synapses (Ohn et al., 2016). In addition to the AZ heterogeneity in voltage dependence of Ca\(^{2+}\) influx, different coupling mechanisms could contribute to the variable SR of SGNs innervating single IHC. Even though this study sampled limited amount of pillar synapses compared to the modiolar ones, no pillar synapse was found in the cluster 3 (identified by K-means clustering), where synapses employed Ca\(^{2+}\) microdomain-like control of release with high threshold (Figure 4.17).

The Ca\(^{2+}\) dependence of release could affect the ratio of the synchronous to asynchronous release. As the precision of the synchronous release highly depends on the tight coupling between the Ca\(^{2+}\) source and the Ca\(^{2+}\) sensor at the AZ, heterogeneous coupling in a single IHC could give rise to different types of release. Interestingly, IHCs show two modes of release: monophasic EPSCs, with sharp rising phases and a single exponential decay, and multiphasic EPSCs, with nonuniform rise and decay phases. Both modes of release appear in every recording, while the percentage of monophasic EPSCs differs (30% to >75%; (Grant...
Two hypotheses were proposed to explain the modes of release in IHCs. First hypothesis is the multi-quantal release (MQR), where AZs employ coordinated fusion of 6 vesicles, and the multiphasic EPSCs occur when there is less synchronization (Glowatzki and Fuchs, 2002). Second is the uni-quantal release (UQR), where the spontaneous EPSCs result from either a full collapse of few vesicles (monophasic), or a flickering fusion pore of SVs (multiphasic) (Chapochnikov et al., 2014; Grabner and Moser, 2018; Huang and Moser, 2018). In the case of MQR, heterogeneous Ca\(^{2+}\) dependence of release could explain the appearance of two modes in different ratios. Interestingly, transmitter release increases its level of synchronization during postnatal development (Grant et al., 2010), which coincides with the transition from an apparent microdomain control of release to a nanodomain-like control of release in IHCs (Wong et al., 2014).

One of the hypothesis is that Ca\(^{2+}\) nanodomain control of release leads to a wider dynamic range of the response. In support, I found that linear synapses employing Ca\(^{2+}\) nanodomain-like control of release had significantly wider dynamic range compared to the nonlinear
synapses in IHCs. While the mammalian cochlea encodes sound pressures spanning six orders of magnitude, individual SGNs seem to cover only a fraction of it. The diverse spiking behavior of SGNs contacting single IHC was suggested to increase the dynamic range of sound intensity coding with an ensemble of fibers covering different fractions. Heterogeneity in Ca$^{2+}$ dependence of release within an IHC may help IHC to partition the wide dynamic range of sound pressures. It is puzzling though how high SR-low threshold fibers, if they are employing nanodomain coupling, could exhibit smaller dynamic range in rate-level functions (Taberner and Liberman, 2005). One possible explanation could be that high SRs at linear synapses may narrow the dynamic range of the evoked response, possibly by affecting the standing SV pool.

One example of a single cell employing both linear and nonlinear synapses comes from zebrafish bipolar cells (Odermatt et al., 2012). Retinal bipolar cells were found to display luminance (light intensity) sensitivity over four orders of magnitude through linear and nonlinear synapses, with nonlinear synapses showing more efficiency (Odermatt et al., 2012). While it is not known how the bipolar cells can achieve the linear and nonlinear synapses, heterogeneous control of release is one of the hypotheses. It is tempting to think this kind of heterogeneity of Ca$^{2+}$ dependencies is acquired where wide dynamic range of sensory coding is needed. How a single cell can establish different Ca$^{2+}$ dependencies of release is, however, not known. Future work to decipher the relevance of the Ca$^{2+}$ domain control of release on the diverse SGN spiking would be interesting. Single unit recordings from a mutant mouse that favors either type of Ca$^{2+}$ domain control of release would be a good candidate. One example comes from the postnatal development of the mice: a tighter coupling of Ca$^{2+}$ influx and exocytosis is established after the onset of hearing (Wong et al., 2014). This time window coincides with the appearance of high SR fibers (Wong et al., 2013).

4.4 Materials and Methods

4.4.1 Animals and postnatal injections

Postnatal AAV-injections were performed by Christiane Senger-Freitag, and initially by Dr. Vladan Rankovic. Postnatal AAV-injections, as described previously (Jung et al., 2015a), were made into scala tympani of the right ear through the round window. P5-7 wild-type (WT) C57Bl/6 mice was used for the injection of iGluSnFR virus under human synapsin (hSyn) promoter (pAAV9. hSyn.iGluSnFR, commercially available at Addgene, USA) to drive iGluSnFR expression in SGNs. Injected WT mice was used for experiments either 1 week (P15-19) or 2 weeks (P21-26) after the injection.

In brief, under general and local anesthesia (isoflurane and xylocaine, respectively), the right ear –due to the preference of the experimenter- was accessed through a dorsal incision. Once the round window membrane was located, a quartz capillary pipette was used to gently puncture and inject $\sim 1-1.5 \mu l$ of pAAV. hSyn.iGluSnFR (Titer $\geq 1 \times 10^{13}$ vg/ml). After the injection of the virus, the wound was sutured and buprenorphine (0.1 mg per kg) was applied.
4.4. Materials and Methods

as a painkiller. The recovery of the animals was monitored on a daily basis. All animals were kept prior and after the injection in a 12 hour light/dark cycle, with access to food and water *ad libitum* and with the mother until the end of the weaning period (~P21).

4.4.2 Auditory brainstem recordings

The auditory brainstem response (ABR) recordings were performed by Nadine Dietrich. They were done on P25 mice as previously described (Jing et al., 2013). Briefly, mice were anesthetized with a combination of ketamine (125 mg/kg) and xylazine (2.5 mg/ kg) i.p. The core temperature was maintained constant at 37°C using a heat blanket (Hugo Sachs Elektronik–Harvard Apparatus). The TDT II system run by BioSig software was used for stimulus generation, presentation, and data acquisition (Tucker Davis Technologies) (MathWorks). Tone bursts (6/12/24 kHz, 10 ms plateau, 1 ms cos2 rise/fall) or clicks of 0.03 ms were presented at 40 Hz (tone bursts) or 20 Hz (clicks) in the free field ipsilaterally using a JBL 2402 speaker.

4.4.3 Patch-clamp recordings

The apical 2/3 turn of organs of Corti were acutely dissected from P15 to P26 animals in HEPES Hank’s solution containing (in mM): 5.36 KCl, 141.7 NaCl, 10 HEPES, 0.5 MgSO$_4$·7H$_2$O, 1 MgCl$_2$·6H$_2$O, 5.6 D-glucose, and 3.4 L-glutamine (pH 7.2, ~300 mOsm/l). The IHC basolateral membranes were exposed by cleaning of nearby cells with a suction pipette by approaching from either pillar side or modiolar side. All experiments were conducted at room temperature (RT) (20-25°C). Patch pipettes were made from GB150-8P or GB150F-8P borosilicate glass capillaries for perforated and whole-cell patch-clamp recordings, respectively (Science Products, Hofheim, Germany). The pipettes were coated with Sylgard to decrease capacitive noise. Afterwards, the pipettes were polished by the custom-made microforge to smooth the tip. All patch-clamp recordings were done simultaneously with fluorescent imaging of iGluSnFR or of iGluSnFR and Ca$^{2+}$.

*Perforated-patch recordings.* Perforated-patch clamp was performed as described previously (Moser and Beutner, 2000). For Ca$^{2+}$ current and membrane capacitance ($C_m$) measurements, the extracellular solution contained the following (in mM): 110 NaCl, 35 TEA-Cl, 2.8 KCl, 1 MgCl$_2$, 1 CsCl, 10 HEPES, 1.3 CaCl$_2$, and 11.1 D-glucose (pH 7.2, ~305 mOsm/l) and was introduced into the recording chamber via a perfusion system. The pipette solution contained (in mM): 130 Cs-gluconate, 10 TEA-Cl, 10 4-AP, 10 HEPES, 1 MgCl$_2$, as well as 300 mg/ml amphotericin B (pH 7.17, ~290 mOsm/l). The intracellular solution also contained the TAMRA-conjugated CtBP2/RIBEYE-binding dimer peptide (10 µM, Biosynthan, Germany). After the perforated-patch recordings, each cell was ruptured to load the peptide in order to label the synaptic ribbons. All the measurements were done via EPC-10 amplifiers controlled by Patchmaster software (HEKA Elektronik, Germany). IHCs were held at ~87 mV. All voltages were corrected for liquid junction potential offline (17 mV). Currents were leak corrected...
using a p/10 protocol. Recordings were used only if the leak current was lower than 30 pA and the series resistance (Rs) was lower than 30 mOhm (in the perforated-patch configuration). The Lindau–Neher technique was used to measure the $C_m$ changes (Lindau and Neher, 1988). Exocytosis was quantified from $C_m$ changes ($\Delta C_m$) as described previously (Neef et al., 2014). In one dataset, IHCs were stimulated by step depolarizations of different durations (2 to 100 ms, in a randomized manner) to -23 mV at intervals of 60s-100s. In order to probe voltage-dependence of release, in another dataset, IHCs were step-depolarized for 10 ms from -62 mV to -22 mV in 5 mV increments in a randomized order. For the Zn$^{2+}$ perfusion experiments, 1 mM Zn$^{2+}$, added to the extracellular solution, was slowly perfused in and out of the recording chamber, while IHCs were step depolarized for 10 ms to -23 mV simultaneously up to 20 times.

**Ruptured-patch recordings.** Ruptured-patch experiments were performed in extracellular solution containing (in mM): 2.8 KCl, 102 NaCl, 10 HEPES, 1 CsCl$_2$, 1 MgCl$_2$, 35 TEA-Cl, 2 mg/ml D-Glucose and 5 CaCl$_2$ (pH 7.2, 300 mOsm). The patch pipette solution contained (in mM): 111 L-glutamate, 1 MgCl$_2$, 1 CaCl$_2$, 10 EGTA, 13 TEA-Cl, 20 HEPES, 4 Mg-ATP, 0.3 Na-GTP and 1 L-Glutathione (pH 7.3, ~290 mOsm). The intracellular solution also contained 800 µM of the low affinifity (19 µM) chemical Ca$^{2+}$ indicator Rhod-FF tripotassium salt (AAT Bioquest, USA) for fluorescent imaging. The recordings were discarded when the leak current exceeded -50 pA at -87 mV or RS was greater than 15 MΩ within 4 min after break-in. For Ca$^{2+}$ imaging experiments, a voltage ramp stimulus (from -87 to +63 mV during 150 ms; 1 mV/ms) was applied to evoke Ca$^{2+}$ currents were evoked by applying a voltage ramp stimulus.

For all IV recordings, the IHCs were step depolarized for 20 ms from -87 mV to +63 mV in 5 mV increments. The IV recordings were used to assess the fitness of the cell, and recordings were discarded when the Ca$^{2+}$ current rundown exceeded 25%.

### 4.4.4 Spinning disk confocal imaging of Ca$^{2+}$ and iGluSnFR

Imaging experiments were performed with a spinning disk confocal scanner (CSU22, Yokogawa, Germany) mounted on an upright microscope (Axio Examiner, Zeiss, Germany) with 63x, 1.0 NA objective (W Plan-Apochromat, Zeiss). The spinning disk speed was set to 2000 rpm to avoid uneven illumination. A scientific CMOS camera (Neo, Andor, Northern Ireland) with a pixel size of 103 nm was used to acquire images. iGluSnFR and Ca$^{2+}$ indicator Rhod-FF or TAMRA-peptide were excited by diode- pumped solid-state lasers with 491 nm and 561 nm wavelength, respectively (Cobolt AB).

**iGluSnFR imaging.** iGluSnFR-expressing SGN boutons were detected via low intensity 491 nm excitation in order to avoid photobleaching. Generally, the baseline expression levels of iGluSnFR were high enough to be detected with this low laser power (sixfold lower laser power than that typically used for recording, with 200 ms exposure time). The imaging plane for the target IHC was selected when several transduced boutons were visible, preferentially in the mid-basal section of the cell, to avoid the “synaptically crowded” basal pole. A brief step depolarization was applied to the cell to check for the functional signal in the given plane. If
needed the laser intensity was changed to improve the signal (between 0.0100 to 0.0304). Once this was set, iGluSnFR fluorescence was acquired at 50 Hz (20ms/frame) simultaneously with patch-clamp recordings. The iGluSnFR signal was evoked by step depolarizations of different durations to different voltage values, as it is specified in every dataset. 50 Hz acquisition rate was sufficient and fast enough to detect reliable signal given the kinetics of iGluSnFR, compared to 20 Hz and 100 Hz acquisition rates tried.

**Sequential imaging of Ca$^{2+}$ and iGluSnFR.** For the dual-color imaging of Ca$^{2+}$ and glutamate release approach, as described above for iGluSnFR imaging, the imaging plane was selected based on the baseline fluorescence of iGluSnFR. Once the middle plane was set, the red fluorescence of the Ca$^{2+}$ indicator Rhod-FF, excited with 561 nm laser, was imaged at 100 Hz while Ca$^{2+}$ currents were triggered by applying a voltage ramp from -87 to +63 mV during 150 ms (1 mV/ms) in 5 planes separated by 0.5 µm. A piezo positioner for the objective (Piezosystem, Germany) was used to precisely control the Z-plane. Once the Ca$^{2+}$ imaging was done, the iGluSnFR signal was acquired in the green channel at 50 Hz by applying step depolarizations of 50 ms from the holding potential of -87 to different voltage values in the middle plane. The depolarization values were picked in a range from -57 mV to -17 mV (-57, -49, -45, -41, 37, -33, -25, -17 mV, applied in a pseudo-randomized manner) to maximize the points in the dynamic range of the signal rather than evenly distributed values.

### 4.4.5 Immunohistochemistry and confocal microscopy

Acutely dissected apical turns of organs of Corti were fixed in formaldehyde (4% in phosphate buffered saline (PBS), 1h on ice). After 3x5 min PBS washing step, the samples were blocked with a donkey serum dilution buffer (DSDB; 16% normal donkey serum, 450 mM NaCl, 0.3% Triton X-100, 20 mM phosphate buffer, pH 7.4) or with a goat serum dilution buffer -depending on the secondary antibody- for 1 hour at room temperature in a wet chamber. The blocking was followed by an overnight incubation with the primary antibodies at 4°C. After 3x5 min PBS washing step, the samples were incubated with the secondary antibodies for 1 hour at RT. Following the final 4x5 min PBS washing step, the samples were mounted in mounting medium (Mowiol 4-88, Sigma). The primary antibodies used were the following: mouse anti-CtBP2 (1:200, BD Biosciences, 612044) –to detect ribbons–, chicken anti-GFP (1:200, Abcam, 13970) –to detect iGluSnFR expression– and guinea pig anti-parvalbumin (1:200, SYSY, 195004) –to detect SGNs, OHCs and IHCs. Secondary antibodies were used with 1:200 dilution: Alexa Fluor 488 conjugated anti-chicken (Dianova, 703-45-155), Alexa Fluor 633 conjugated anti-mouse (Invitrogen, A31571), Alexa Fluor 488 anti-chicken (Invitrogen, A11039) and Alexa Fluor 568 anti-guinea pig (Invitrogen, A11075). Images were acquired using an Abberior Instruments Expert Line STED microscope, with excitation lasers at 488, 568 and 633 nm using a 1.4 NA 100x or 20x oil immersion objective, in confocal mode. Images were adjusted for brightness and contrast using Image J for illustration purposes.
Chapter 4. Fluorescence imaging of single IHC synapses

4.4.6 Data Analysis

4.4.6.1 Patch-clamp recordings

Electrophysiological recordings were analyzed using custom written programs in Igor Pro 6.3. The Ca\textsuperscript{2+} charge was calculated by the time-integral of the leak-subtracted current during the depolarization step. $\Delta C_m$ was calculated as the difference between the average $C_m$ 400 ms before and after the depolarization (the initial 100 ms after the depolarization was skipped.)

4.4.6.2 Imaging of iGluSnFR

I implemented all the image analysis routines in Python.

Region of interest (ROI) picking. The $\Delta F$ image was created by subtracting baseline fluorescence ($F_0$, an average of 15 frames before stimulus) from the fluorescence images acquired during/after stimulation ($F$, an average of 5 frames). The $\Delta F$ image was median-filtered with the size of 4-6 depending on the signal amplitude. Maximum entropy thresholding was then applied to the median-filtered $\Delta F$ image in order to create a mask for ROI picking. After the generation of the mask, a watershed segmentation algorithm was used to label the individual ROIs and separate them. A single mask was generated per cell, using the recording with strongest stimulation, and applied for all images. The individual ROIs, corresponding to postsynaptic SGN boutons, were further confirmed by the presence of presynaptic ribbon peptide (CtBP2-binding peptide). The fluorescence of every pixel contributing to the defined ROI was averaged from the raw time series for further analysis. The background fluorescence was calculated by averaging 60 x 60 pixels in the pillar region of the image, where no iGluSnFR fluorescence is expected: by the anatomy, SGNs innervate IHCs and leave the cochlea towards the modiolus.

Analysis of fluorescence traces. The average background value was subtracted from the raw fluorescence traces ($F$). Following background subtraction, $\Delta F$ traces were generated by subtraction of mean baseline value ($F_0$). $\Delta F$ was normalized to $F_0$ to create $\Delta F/F_0$ traces. For peak detection, $\Delta F/F_0$ were smoothened using a Hanning window function with the window size of 7 to avoid false peak detection (Figure 4.18A). The area under the curve (AUC) was calculated by fitting a single exponential to $\Delta F/F_0$ traces and subtracting the sum of this fit from the sum of the $\Delta F/F_0$ trace in an interval of 40 frames from the beginning of the stimulus (Figure 4.18B).

4.4.6.3 Sequential imaging of Ca\textsuperscript{2+} and iGluSnFR.

ROI picking-iGluSnFR. The ROIs were picked as described above. Differently, a Gaussian filter with sigma of 1-3 was applied to be consistent with the detection of Ca\textsuperscript{2+} hotspots. The ROIs were confirmed by the presence of a corresponding Ca\textsuperscript{2+} “hotspot”.

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Figure 4.18 – Peak detection and calculation of area under the curve of iGluSnFR signal. A. For peak detection, Hanning window smoothening with window size of 7 was applied to baseline-normalized ($\Delta F/F_0$) iGluSnFR signal (black) in response to 100-ms-long step depolarization. The outcome trace of smoothening algorithm (orange) is overlaid to the original trace for illustration. B. A single exponential function was fitted to $\Delta F/F_0$ trace to account for photobleaching, and the area between the two traces (blue shade) was calculated as area under the curve (AUC). The initial observed rise in the raw traces is a laser artifact.

ROI picking-Rhod-FF. Similarly, a $\Delta F$ image was created from the mean time series created, in this case, by averaging all the trials from all the planes. This $\Delta F$ image was treated the same way as described for iGluSnFR-ROI picking. The created mask was applied to all recording planes and the plane that had the maximum $\Delta F$ for a given ROI was used for further analysis. This way, it was made sure that the plane with the highest signal was used for every Ca$^{2+}$ "hotspot" (Figure 3.2).

Analysis of fluorescence traces. A bandstop filter at 33.3 Hz was applied to the raw traces in order to remove the noise caused by the spinning disc speed 2000 rpm. The obtained traces were background-subtracted and normalized to baseline fluorescence ($F_0$) as described above. Fluorescence-voltage (FV) relations for iGluSnFR were estimated from the step depolarizations to different voltage values. AUC of iGluSnFR for each depolarization was calculated as described above. Boltzman fit was used to estimate the two fitting parameters: voltage of half-maximum activation ($V_{1/2}$) and slope factor ($k$) of the glutamate release.

$$AUC_{iGluSnFR}(V) = \frac{1}{1 + e^{\frac{V-V_{1/2}}{k}}}$$

For Ca$^{2+}$ imaging with Rhod-FF, FV curves were estimated from ramp depolarization. The raw FV curves were affected by noise, such as readout or shot noise from the CCD camera. Therefore, the following equation was used to optimize the raw traces (Ohn et al., 2016):

$$F_{Rhod-FF}(V) = F_0 + \frac{g_{max}(V-V_r)}{1 + e^{\frac{V-V_{1/2}}{k}}}$$
Slope factor ($k$) was obtained with this equation. The resulting fit was used to estimate $V_{1/2}$ by minimizing the scalar at the mid-point. The reversal potential was fixed to the value of +47.6 mV after LJ potential correction. The peak of the Rhod-FF signal was obtained by averaging 3 frames corresponding the voltages values between -17 mV and 3 mV during ramp depolarization.

The FV fits for Rhod-FF or $Q_{Ca}$ and $AUC_{iGluSnFR}$ were plotted against each other in the voltage range of -57 to -17 mV. A power function was fitted in order to get single-synapse glutamate release-$Ca^{2+}$ signal/whole-cell $Ca^{2+}$ charge relationship:

$$AUC_{iGluSnFR}(V) = A(F_{Rhod−FF})^m$$ (4.4)

$$AUC_{iGluSnFR}(V) = A(Q_{Ca})^m$$ (4.5)

4.4.6.4 Statistical analysis

All the statistical tests were performed with custom-written Python code. Averages are expressed as mean ± SD, box plots indicate 25-75 quartile with whiskers reaching from 10-90%. Data sets were checked for normal distribution by D’Agostino & Pearson omnibus normality test and for equality of variances. For normally distributed data, unpaired two-tailed student’s t-test was applied, and Mann-Whitney U test was used for non-normally distributed data. The Pearson correlation coefficient was used to test for linear correlation.
Figure 4.19 – Detection of Ca$^{2+}$ hotspots in IHCs. After the middle plane is set based on the baseline fluorescence of the iGluSnFR-expressing boutons, 5 planes separated by 0.5 μm were recorded in alternating steps to avoid photobleaching. A mean ΔF image was generated by mean projection of the 5 planes. The mean ΔF was then Gaussian filtered, and maximum entropy thresholding was applied to create a mask. Watershed segmentation was used to separate individual Ca$^{2+}$ hotspots. The defined ROI masks were applied to every plane and the plane with a biggest ΔF for a given ROI was taken for further analysis.
IHCs make overall 5-20 synapses depending on the tonotopic position with SGNs, where single synapse drives fiber activity (Liberman, 1980; Merchan-Perez and Liberman, 1996; Meyer et al., 2009). IHC synapse, the first relay station of the auditory system, is able to encode sound with sub-millisecond precision and without any signs of fatigue (Matthews and Fuchs, 2010; Pangršič et al., 2012; Safieddine et al., 2012). Consequently, the IHC synapse has one of the highest rates of neurotransmitter release per active zone, which requires mechanisms of fast exocytosis, and tight coupling to subsequent endocytosis. Given the peculiar and largely unknown molecular composition of the IHC synapse, synaptic transmission in this synapse is not fully understood. Furthermore, for a given frequency, single-AZ driven SGNs can collectively cover the whole audible spectrum of sound pressures, ranging within six orders of magnitude. How individual AZs of an IHC, which is believed to be isopotential, can drive activity of SGNs with different properties is also largely unknown. In this thesis, I provided further insight on the endocytic machinery of the IHC synapse, and dissected the mechanisms contributing to encoding of sound pressures over a wide dynamic range.

In the first part, my collaborators and I worked on the role of AP180 at IHC synaptic transmission. We found the expression of AP180 at the basal synaptic pole of the IHC. The genetic disruption of AP180 caused elevated hearing thresholds, showing its relevance for the auditory system. We observed reduced rates of slow component of endocytosis and fewer clathrin-coated vesicles, suggesting a role of AP180 in clathrin-mediated endocytosis. Furthermore, upon high K\(^+\) stimulation, there was less budding of clathrin-coated structures from ELVs, suggesting involvement of AP180 in SV reformation from ELVs following bulk retrieval. In addition, Ap180 KO IHCs showed a reduced rate of SV replenishment. Upon high K\(^+\) stimulation, however, the membrane-proximal SVs were present, but mostly stuck at the multi-tethered and docked state. This suggests that the slowed replenishment of SVs was primarily due to impaired AZ clearance. In conclusion, we propose that AP180 promotes release site clearance and clathrin-dependent vesicle reformation.

In the second part, my collaborators and I explored whether the planar polarity mechanisms establishing the hair bundle orientation at the apical part of IHC could contribute to the baso-
lateral gradient of IHC AZ properties (Jean et al., 2019). Upon hair cell specific expression of PTXa which blocks Gαi signaling (Tarchini et al., 2016), the modiolar-pillar gradient of maximal Ca\(^{2+}\) influx was disrupted, while the gradient of voltage dependent activation of synaptic Ca\(^{2+}\) influx was maintained. This indicates that two distinct mechanisms determine the amplitude and voltage-dependent activation of the synaptic Ca\(^{2+}\) influx. Furthermore, this study shows the contribution of IHC intrinsic mechanisms on establishing the AZ heterogeneity.

In the third part, I studied the synaptic transfer function of IHCs by dual-imaging of synaptic Ca\(^{2+}\) influx and glutamate release. I have established the optical detection of glutamate release at individual IHC synapses using an intensity-based glutamate sensor, iGluSnFR (Marvin et al., 2013). In addition to the heterogeneity of voltage dependent-activation of synaptic Ca\(^{2+}\) influx and glutamate release, I found a heterogeneity of Ca\(^{2+}\) dependence of release at individual IHC synapses. I showed that different modes of Ca\(^{2+}\) domain control (Ca\(^{2+}\) nano- or microdomain-like) co-existed within a single IHC. We propose that co-existence of linear and nonlinear synapses helps IHC partition the wide dynamic range of sound encoding together with the heterogeneous voltage-dependence of Ca\(^{2+}\) channel activation.

In this general discussion, I will consider the aspects of exocytosis-endocytosis balance in IHCs. Furthermore, I will discuss the potential mechanisms contributing to the diverse SGN spiking patterns and, thereby, allowing sound encoding over a wide dynamic range. These mechanisms include presynaptic and postsynaptic heterogeneity, and efferent modulation. I examine the presynaptic heterogeneity at two different levels: presynaptic heterogeneity of Ca\(^{2+}\) signaling and Ca\(^{2+}\) dependence of release.

### 5.1 Exocytosis-endocytosis coupling in IHCs

The IHC synapse is able to release large amounts of neurotransmitter, with high temporal precision and without any obvious signs of fatigue (Matthews and Fuchs, 2010; Pangršič et al., 2012; Safieddine et al., 2012). Even during a sustained stimulation, each SGN can fire at hundreds of Hz, by the input from a single IHC AZ. Sustained exocytosis rates were up to 70 Hz per release site (~680 vesicles per second; (Pangrsič et al., 2010)) or higher at physiological temperatures (Strenzke et al., 2016). Maintaining such high rates of release requires a fine balance between exocytosis and endocytosis. How this is managed at this first synapse of the auditory system is not fully understood.

Interestingly, IHC synapse is equipped with peculiar set of proteins, which are not necessarily involved in the fusion machinery of the conventional excitatory synapses. IHCs express an unconventional vesicular glutamate transporter Vglut3 for the glutamate loading of SVs (Ruel et al., 2008; Seal et al., 2008). Moreover, they lack the major SV proteins, such as synaptophysin, complexin and synapsin (Safieddine and Wenthold, 1999; Strenzke et al., 2009). Surprisingly, exocytosis in mouse IHCs operate without the SNARE proteins, which mediate otherwise membrane fusion in conventional synapses (Nouvian et al., 2011). Mouse IHC exocytosis, probed by C\(_m\) measurements, was insensitive to genetic ablation (SNAP-25, synaptobrevin-1
5.1. Exocytosis-endocytosis coupling in IHCs

or 2 and 3) or neurotoxin-mediated cleavage of neuronal SNAREs. In addition, mRNA of the SNARE proteins was found in IHCs (Safieddine and Wenthold, 1999), but synapticly located protein was not detected by immunohistochemistry (Nouvian et al., 2011). Moreover, SV priming factors of the CAPS and Munc13 families are also missing from IHCs (Vogl et al., 2015). In IHC synapses, \( \text{Ca}^{2+} \) influx is mainly (\(~90\%) mediated via \( \text{Ca}_V 1.3 \) channels (Brandt et al., 2003; Dou et al., 2004; Platzer et al., 2000). Furthermore, IHC synapse does not express the classic neuronal \( \text{Ca}^{2+} \) sensors synaptotagmin 1 and 2, except for a transient period during development (Beurg et al., 2010; Reisinger et al., 2011). A multi-C\(_2\) domain protein, otoferlin, was suggested as the \( \text{Ca}^{2+} \) sensor for exocytosis in IHCs (for review; see Pangršič et al., 2012).

IHCs seem to operate with more conventional set of proteins for membrane retrieval (Jung et al., 2015a; Kroll et al., 2019; Neef et al., 2014) in comparison to the fusion machinery. Three modes of endocytosis were found in IHCs by \( C_m \) measurements: slow, fast and ultrafast. The slow endocytosis shows a linear \( C_m \) decline upon short depolarizations eliciting RRP; likely reflecting the clathrin-mediated endocytosis. Indeed, this slow decline was sensitive to manipulations of dynamin and clathrin inhibitors (Neef et al., 2014). Longer depolarizations elicited a fast and exponentially decaying \( C_m \) component with a time constant of \(~6\) s (Neef et al., 2014). This fast component was clathrin and dynamin independent and it is believed to reflect the bulk endocytosis (Neef et al., 2014). In addition, an ultrafast endocytosis with a time constant of \(~250\) ms was observed when global \( [\text{Ca}]^{2+} \) reached to 15 \( \mu \)M or more (Beutner et al., 2001; Neef et al., 2014).

The high rate of exocytosis is balanced by endocytosis, likely starting with a lateral clearance of the exocytotic proteins from the release sites of the AZ (for review; see (Neher and Sakaba, 2008)). The lateral clearance might involve interactions between the endocytic and exocytotic proteins. Indeed, an interaction between otoferlin and adaptor protein 2 (AP-2) was found (Duncker et al., 2013; Jung et al., 2015a). AP-2 is a heterometric adaptor protein that was shown to initiate the CME together with \( \text{PI}(4,5)P_2 \) in conventional synapses (for review; see (Traub and Bonifacino, 2013)). AP-2 links various cargo to the clathrin coat and other proteins. The \( AP-2\mu \) KO mice was deaf, and it was suggested that the binding of AP-2 to otoferlin promotes the replenishment of release sites, by speeding the AZ clearance of exocytosed material (Jung et al., 2015a). In addition, a role of AP-2 in SV reformation was found based on the EM analysis. Interestingly, however, the membrane retrieval was intact in \( AP-2\mu \) KO IHCs (Jung et al., 2015a). A recent work showed the involvement of endophilin-A in IHC endocytosis (Kroll et al., 2019). In conventional synapses, endophilin is a membrane-bending protein that is involved in CME, via recruiting dynamin (for review; see (Saheki and Camilli, 2012)). In IHCs, an interaction between endophilins and otoferlin was suggested, and disruption of endophilins caused a mild reduction of otoferlin levels (Kroll et al., 2019). Furthermore, CME was disrupted in the \( endophilin \) KO IHCs.

Next, my collaborators, and I tested whether AP180 is involved in IHC endocytosis by combining patch-clamp \( C_m \) measurements, high-pressure freezing and electron tomography, confocal microscopy and systems physiology. AP180 is an adaptor protein and was shown to
have synaptobrevin-2 as a cargo in conventional synapses (Koo et al., 2015). First, we found the presence of AP180 in the basal synaptic pole of the IHC. Unlike the decreased otoferlin levels observed in AP-2µ and endophilin KO IHCs, otoferlin levels were unchanged in AP180 KO IHCs. We observed decreased replenishment rate and slowed membrane retrieval. Upon high K⁺ stimulation, however, the membrane-proximal SVs seemed intact, suggesting the slowed SV replenishment is due to impaired AZ clearance rather than the SV resupply to the AZ. In conclusion, we suggest a role of AP180 in release site clearance, in addition to its role in clathrin recruitment in IHCs. Since the conventional cargo of AP180 is synaptobrevin-2 (Koo et al., 2015) and it is not expressed in IHCs, the interaction studies would be of interest to identify AP180’s cargo in IHCs, which is likely to be an exocytotic protein. Further work on this would not only shed light into the endocytic mechanisms, but also into the largely unknown fusion machinery of IHCs.

5.2 Potential mechanisms driving diverse SGN spiking behavior

5.2.1 Presynaptic heterogeneity

One of the candidate mechanisms of the diverse SGN spiking behavior is the heterogeneity of the presynaptic AZs in IHCs. A number of morphological and functional studies provided evidence for this candidate mechanism so far. A classical ultrastructural study in the cat cochlea on a small sample of back traced SGNs showed that the low SR-high threshold fibers contact synapses with bigger and/or multiple ribbons preferentially at the modiolar IHC side and high SR-low threshold fibers contact synapses with smaller ribbons preferentially at the pillar IHC side (Merchan-Perez and Liberman, 1996). Another follow-up study in cats used only the spatial information of the innervation side of the IHC and found that modiolar synapses exhibit more SVs between the ribbon and the membrane, and at the AZ (Kantardzhieva et al., 2013). Further evidence for the diversity of the presynaptic ribbon size comes from the studies in mice where CtBP2-binding peptide was used to fluorescently label the ribbon (Frank et al., 2009; Meyer et al., 2009; Ohn et al., 2016). Most interestingly, live confocal imaging of synaptic Ca²⁺ influx showed substantial heterogeneity of Ca²⁺ domains in their maximal amplitude and voltage dependence even within an IHC (Frank et al., 2009). A positive correlation between the ribbon size and synaptic Ca²⁺ influx was also found. Frank et al. (2009) further investigated the putative mechanisms contributing to the AZ heterogeneity and found little if any contribution of mitochondrial Ca²⁺ uptake, Ca²⁺-induced Ca²⁺ release, cytosolic buffering, and open probability of Ca²⁺ channels on the amplitude variability. In keeping with the morphological differences, the larger modiolar synapses showed larger maximal Ca²⁺ influx amplitude than the smaller pillar synapses (Meyer et al., 2009; Ohn et al., 2016). Ohn et al. (2016) took the study into another level by fast 3D live imaging of the most, if not all, AZs of IHCs as a function of position within an IHC. By applying voltage ramps, they probed the voltage dependence of synaptic Ca²⁺ influx. The pillar synapses showed activation at slightly more negative potentials (∼1.5 mV) than the modiolar synapses. This position-dependent difference in the voltage dependence of synaptic Ca²⁺ influx was hypothesized to contribute the differences in the SR
and sound threshold of SGNs. As the IHC is believed to be isopotential, it is not clear how the AZ heterogeneity is established within IHCs, and whether potential heterogeneity of other presynaptic mechanisms could contribute.

What factors could set the differential voltage sensitivity and abundance of Ca\(^{2+}\) channels of AZs within an IHC? The possibilities are the Ca\(^{2+}\) channel splice variants with different biophysical properties, preferential abundance of auxiliary subunits and diverse combinations of Ca\(^{2+}\) channel modulators (for review; see (Pangrsic et al., 2018)). The splice variants of the pore-forming \(\alpha 1\) subunit with short and long C-terminus are expressed by IHCs (Scharinger et al., 2015). In HEK293 cells, the short isoforms exhibited activation in more negative potentials, and higher open probability (Bock et al., 2011; Tan et al., 2011). In IHCs, however, when the long C-terminus was disrupted with a HA-tag, heterogeneity of the AZs were unchanged (Ohn et al., 2016). The observed higher maximal Ca\(^{2+}\) influx on cellular and synaptic level likely resulted from an increased open probability. The lack of an effect of the C-terminus manipulation on voltage dependence of Ca\(^{2+}\) influx might be due to the high regulation of the Ca\(^{2+}\) channels by CaBPs (Cui et al., 2007; Picher et al., 2017a; Schrauwen et al., 2012; Yang et al., 2006).

Several proteins have been tested for their putative contribution to position-dependent AZ Ca\(^{2+}\) influx properties. Gipc3, a PDZ domain containing scaffold protein, was probed as a regulator of Ca\(^{2+}\) channels (Ohn et al., 2016). The mutations in Gipc3 cause human deafness and progressive hearing loss and audiogenic seizures in mice (Charizopoulou et al., 2011; Rehman et al., 2011). Gipc3 mutant mice showed overall increased Ca\(^{2+}\) influx with a reversed pillar-modiolar gradient of maximal Ca\(^{2+}\) influx (Ohn et al., 2016). The pillar-modiolar gradient for the voltage dependence of Ca\(^{2+}\) channel activation was unaffected, while the activation of Ca\(^{2+}\) channels was shifted to more hyperpolarized potentials. Of the presynaptic hypothesis of neural diversity, the more negative activation of Ca\(^{2+}\) channels in Gipc3 mutant mice went along with an enhanced spontaneous SGN firing rate. Moreover, the Gipc3 mutant mice showed a narrower dynamic range, consistent with the previously described defects in the cochlear amplification. By the analogy to related protein Gipc1, which is involved in planar polarity mechanisms in IHCs, Gipc3 might contribute to the AZ in a spatially polarized way (Giese et al., 2012). Moreover, potential interaction of Gipc3 with the C-terminus of long Ca\(_{V} 1.3\) splice variants via its PDZ domain might contribute to setting the voltage-dependence of Ca\(^{2+}\) channel activation. Another PDZ domain containing protein, harmonin, was also tested as an interaction partner of Gipc3. The harmonin KO IHCs showed an overall hyperpolarized shift of the voltage dependence of Ca\(^{2+}\) channel activation while the pillar-modiolar gradient was preserved (Ohn et al., 2016). Next, my collaborators and I investigated whether the planar polarity mechanisms setting the hair bundle orientation might be involved in determining AZ properties in a position-dependent manner at the base of the IHC (Jean et al., 2019). We specifically tested the role of G\(\alpha\)i proteins and their binding partner LGN/Gpsm2, which were involved in cell-intrinsic planar polarity at the apex of the hair cells (Tarchini et al., 2013). We used a mouse line expressing PTXa in hair cells, which was shown to block G\(\alpha\)i signaling via ADP ribosylation (Tarchini et al., 2016). Upon the block of G\(\alpha\)i signaling, the pillar-modiolar
gradient of maximal Ca\(^{2+}\) influx was collapsed, while the gradient of voltage dependence was preserved (Jean et al., 2019). In line with Gipc3 phenotype, this result might indicate that different mechanisms regulate the spatial distribution of the number Ca\(^{2+}\) channels and their voltage dependence of activation per AZ. Furthermore, this study shows the role of IHC intrinsic mechanisms on establishing the spatial AZ heterogeneity.

In IHCs, deletion of RIBEYE caused a depolarized shift of Ca\(^{2+}\) influx activation and altered Ca\(^{2+}\) channel clustering compatible with broader presynaptic Ca\(^{2+}\) signals, while the maximal Ca\(^{2+}\) influx remained unchanged (Jean et al., 2018). Moreover, deletion of bassoon disrupted the Ca\(^{2+}\) channel organization (Frank et al., 2010). Several proteins such as bassoon, RIM2\(\alpha\), RIM2\(\beta\), RIM-BP2 were found to promote the Ca\(^{2+}\) channel number at IHC AZs (Jung et al., 2015a; Krinner et al., 2017). Furthermore, it was shown that RIM2\(\alpha\) and RIM3\(\gamma\) interacts directly with the C-terminus of the Ca\(^{2+}\) channel \(\alpha\) subunit and promotes its membrane expression (Picher et al., 2017b).

On a single channel level, the evidence from lower vertebrates supports similar biophysical properties of Ca\(^{2+}\) channels independent of the position within a cell (Rodriguez-Contreras et al., 2002; Zampini et al., 2006). The recordings from immature mouse IHCs and mature gerbil IHCs, however, show two gating modes of the Ca\(^{2+}\) channels: one with brief and infrequent openings, and the other with long-lasting clusters or bursts of long and brief openings (Zampini et al., 2010; Zampini et al., 2013). As the two gating modes did not seem to randomly distributed (Zampini et al., 2013), this hints a role of intracellular modulation. As suggested previously (Frank et al., 2009; Ohn et al., 2016), the AZ heterogeneity could stem from the differences in the gating kinetics of the Ca\(^{2+}\) channels; possibly modulated by the factors stated above. So far, the studies shed light into the factors regulating the number of Ca\(^{2+}\) channels per AZ or the overall voltage dependence of Ca\(^{2+}\) influx. The establishment of differential voltage sensitivity at AZs within IHCs, however, stays enigmatic.

To understand how the heterogeneous Ca\(^{2+}\) signaling translates into differences in release, the coupling of Ca\(^{2+}\) influx and exocytosis has to be understood. A tight functional coupling between Ca\(^{2+}\) channels and SVs has been suggested at IHC ribbon synapses (Brandt et al., 2005; Goutman and Glowatzki, 2007; Wong et al., 2014; Pangršić et al., 2015). Here, I employed dual-color imaging of synaptic Ca\(^{2+}\) influx and glutamate release to look for potential differences in Ca\(^{2+}\) channel-release coupling. I showed that there is a heterogeneity of Ca\(^{2+}\) dependence of release at individual IHC synapses. We propose this heterogeneity as a candidate mechanism contributing to diverse SGN spiking pattern.

In line with the previous finding of differential voltage sensitivity of synapses, I have found variability in the voltage of half-maximum of Ca\(^{2+}\) influx and also of glutamate release. A positive correlation between the \(V_{1/2}\) of synaptic calcium influx and glutamate release supports the translation of the heterogeneous Ca\(^{2+}\) signaling into release onto SGNs. Unexpectedly, I also found heterogeneity of apparent Ca\(^{2+}\) dependence of exocytosis at individual synapses. This introduces another level of regulation and could contribute to the several aspects of
5.2. Potential mechanisms driving diverse SGN spiking behavior

SGN behavior, such as SR, synchronous release and dynamic range of sound encoding. First, differential apparent Ca\(^{2+}\) dependence of release at IHC synapses could affect the SRs of SGNs. It is predicted that Ca\(^{2+}\) nanodomain control of release would increase the spontaneous release, as the stochastic opening of Ca\(^{2+}\) channels could trigger release in such a tight coupling scenario (for review; see (Moser et al., 2006; Eggermann et al., 2012)). Secondly, Ca\(^{2+}\) nanodomain control of release would increase the ratio of synchronous release over the asynchronous release. Given the observed differences in the ratio of synchronicity, the contribution of differential Ca\(^{2+}\) dependence of release can be argued. On the other hand, it is controversial whether they stem from the release of multiple vesicles or a single one (For MQR: see (Glowatzki and Fuchs, 2002); for UQR: see (Chapochnikov et al., 2014; Grabner and Moser, 2018; Huang and Moser, 2018)). Thirdly, Ca\(^{2+}\) nanodomain control of release could widen the dynamic range of coding via linear relation. Indeed, when relating the single synaptic Ca\(^{2+}\) influx to glutamate release, I found that synapses employing Ca\(^{2+}\) nanodomain-like control of release had significantly wider dynamic range compared to the synapses with looser Ca\(^{2+}\) influx-exocytosis coupling. Furthermore, this differential coupling provides the opportunity to further partition the wide dynamic range of sound encoding.

One interesting way to check whether differential coupling at AZs could contribute to SGN behavior would be via performing single-unit recordings in mutant mice that exhibits shift in the apparent Ca\(^{2+}\) dependence of release. One example comes from the postnatal development of the mice: the supralinear Ca\(^{2+}\) dependence of release turns into a near-linear one after the onset of hearing, and this time coincides with the appearance of high-SR SGN fibers (Wong et al., 2013; Wong et al., 2014).

What could be the possible factors establishing the differential coupling of Ca\(^{2+}\) channels and SVs? Putative factors establishing the modiolar-pillar gradient of synaptic Ca\(^{2+}\) influx could also affect the apparent Ca\(^{2+}\) dependence of release at individual AZs in a same or differential manner. These include preferential abundance of auxiliary subunits of Ca\(^{2+}\) channels and diverse combinations of Ca\(^{2+}\) channel modulators/interacting partners. Furthermore, the number of Ca\(^{2+}\) channels could also affect the coupling as it can change the topography of the AZ. In this study, however, no correlation was found between the amplitude of the synaptic Ca\(^{2+}\) influx and the Ca\(^{2+}\) dependence of release at a given AZ. RIM and RIM-BPs serve as an interesting candidate for possibly determining the distance between the Ca\(^{2+}\) channels and SVs. On the other hand, disruption of individual RIM or RIM-BP proteins did not change the coupling of Ca\(^{2+}\) influx to exocytosis on whole-cell level, when probed via \(C_m\) measurements (Krinner et al., 2017; Jung et al., 2015b).

5.2.2 Postsynaptic heterogeneity

The afferent fibers differ in their anatomy depending on their SR. In cats, high-SR fibers showed larger diameter preferentially innervating the pillar side of the IHCs, compared to the low-SR fibers innervating the modiolar side of the IHC (Liberman, 1982). It is not clear
whether larger diameter of the high-SR fibers can facilitate the action potential initiation and propagation. In cat and guinea pig, the afferent fibers contacting the modiolar side of the IHC have lower mitochondrial content than the ones contacting the pillar side (Francis et al., 2004). In mouse, even though there is a high variability in the SR, threshold and diameter, the SR distribution is not as clearly bimodal as in the other species (Taberner and Liberman, 2005). While the general relation between the SR and threshold sensitivity is preserved, they show rather a gradient. Furthermore, an opposing gradient of the AMPA receptor patches and ribbon size was proposed in mice, with smaller ribbons facing bigger AMPA receptor clusters (Liberman et al., 2011). This observation is, however, controversial since an opposite observation was observed in guinea pig (Zhang et al., 2018).

Two parallel studies identified three molecularly different subtypes of Type I SGNs using single-cell RNA sequencing (Shrestha et al., 2018; Sun et al., 2018). The subtypes differed in their cohorts of ion channels, receptors, synaptic proteins, and adhesion molecules. These differences suggested that the subtypes could differ in their glutamate responsiveness. Furthermore, by the fiber location, synapse location and morphology, the three identified subtypes of type I SGNs show some match with the anatomical features of the originally defined functional subtypes of high-, medium- and low-SR fibers (Shrestha et al., 2018; Sun et al., 2018; Taberner and Liberman, 2005). Interestingly, SGNs with distinct properties emerge during the first postnatal week in an activity-dependent manner: in the Vglut3 KO mice, where glutamate release from IHCs is abolished (Ruel et al., 2008; Seal et al., 2008), the SGNs subtypes failed to emerge (Shrestha et al., 2018; Sun et al., 2018). A third study also used single-cell RNA sequencing and identified similar subtypes (Petitpré et al., 2018). By patch-clamp recordings of the identified subtypes, fibers identified as the correlate of low-SR fibers showed both unitary- and multi-adapting responses, while the others solely showed unitary-adapting responses, showing differences in their intensity-coding behavior. Although the data obtained from the dissociated cultures of immature SGNs do not reflect the actual biophysical properties of SGNs, it shows the heterogeneity of their electrophysiological properties. Surprisingly, this study claimed that the neural diversity is already established at birth.

The recent updates on the molecular profiles of the type I SGN subtypes give further perspective on the heterogeneity of SGN spiking pattern. This shows the intrinsic properties of SGNs can as well alter the input and output properties, in addition to the heterogeneity of the presynapse and efferent modulation. The differences in their neuronal excitability can produce different outputs in response to same presynaptic input in terms of glutamate release. Moreover, the transcription factor Pou4f1, present distinctly in the subtype of SGNs innervating the modiolar side of the IHC, was shown to play an instructive role in presynaptic Ca$^{2+}$ signaling in IHCs (Sherrill et al., 2019). Furthermore, one of the most abundant markers of one SGN subtype, namely 1c, corresponding to low-SR fibers, is Lypd1 (Shrestha et al., 2018; Sun et al., 2018). This protein was shown to encode a transmembrane receptor involved in cholinergic signaling (Tekinay et al., 2009). This can create a unique response to acetylcholine released by the olivocochlear efferents (for detail; see next chapter).
5.2. Potential mechanisms driving diverse SGN spiking behavior

5.2.3 Efferent modulation

In the mature cochlea, medial olivocochlear (MOC) and lateral olivocochlear (LOC) efferent fibers innervate OHCs and type I SGNs. Before the onset of hearing, a LOC efferent innervation is found on IHCs transiently (Glowatzki and Fuchs, 2000). This inhibitory innervation is mediated by nicotinic cholinergic receptors, and Ca\(^{2+}\) influx through these receptors activates Ca\(^{2+}\)-dependent SK potassium channels (Glowatzki and Fuchs, 2000). This inhibitory synapse disappear at P12 at the onset of hearing, and mature IHCs contain few if any efferent contacts (Brandt et al., 2003; Katz et al., 2004; Liberman, 1990). After retracting from IHCs, LOC efferent fibers form axodendritic contacts to the afferent terminals (Pujol et al., 1998). In contrast to GABA and acetylcholine, which is present in the MOC system, dopamine is only present in the LOC system (for review; see (Eybalin, 1993)). Several studies show that LOC fibers modulate the activity of the afferent fibers. De-efferentation on the afferent fibers in cats preserved the rate-level functions and thresholds, with significant decrease in SR of SGNs (Liberman, 1990). A study applying acetylcholine and GABA in the vicinity of the IHC-afferent synapses reported increase and decrease in SR, respectively (Felix and Ehrenberger, 1992). Intracochlear application of dopamine and antagonists suggested a tonic inhibition of afferent fiber activity (Ruel et al., 2001). A later study used electrical stimulation of the LOC fibers at the floor of the 4th ventricle and showed enhancement or suppression of the afferent neural responses, possibly by comprising two functional subdivisions (Groff and Liberman, 2003). In addition, dopamine seems to colocalize with other neurotransmitters like acetylcholine and GABA (Safieddine and Eybalin, 1992; Safieddine et al., 1997). The colocalization of the putative LOC neurotransmitters creates an extra layer of complexity, which might explain the observed differences in the LOC modulation of afferent response.

Can the LOC modulation explain the position-dependent heterogeneity of SGN firing patterns? Some evidence show that the LOC modulation might at least partially contribute to the diversity. Interestingly, there is a spatial difference in the innervation density of the LOC fibers: they seem to primarily innervate the low-SR SGNs (Liberman, 1990). Furthermore, de-efferentation on the afferent fibers in mice disrupted the modiolar-pillar gradient of the ribbon size, showing the contribution of efferent innervation to diversity of type I SGNs (Yin et al., 2014). On the other hand, as de-efferentation on the afferent fibers in cats did not change the rate-level functions and the threshold of afferent fibers, it can be argued that efferent innervation is not the sole drive for the diverse SGN firing pattern. Further experiments is required to solve the complex LOC modulation of afferent response, and its contribution to the spatial heterogeneity of SGN firing properties.
A.1 List of Abbreviations

\( m \) \quad \text{Ca}^{2+} \) cooperativity
\( \Delta C_m \) \quad \text{Membrane capacitance changes}
\( \Delta F \) \quad \text{Fluorescence change}
\( [\text{Ca}^{2+}]_e \) \quad \text{Extracellular Ca}^{2+} \) concentration
\( \text{a.u.} \) \quad \text{Arbitrary unit}
\( \text{AAV} \) \quad \text{Adeno-associated virus}
\( \text{ABR} \) \quad \text{Auditory brainstem responses}
\( \text{AMPA} \) \quad \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
\( \text{AZ} \) \quad \text{Active zone}
\( \text{BAPTA} \) \quad 1,2-bis(2-aminophenoxy) ethane-N,N,N\text{',N\text{'-tetraacetic acid}
\( \text{Ca}_V \) \quad \text{Voltage-gated Ca}^{2+}
\( \text{CaBP} \) \quad \text{Calcium binding protein}
\( \text{CDI} \) \quad \text{Calcium-dependent inactivation}
\( \text{CME} \) \quad \text{Clathrin-mediated endocytosis}
\( \text{CtBP2} \) \quad \text{C-terminal binding protein 2}
\( \text{DPOAE} \) \quad \text{distortion product otoacoustic emission}
\( \text{EGTA} \) \quad \text{ethylene glycol-bis(2-aminoethylether)-N,N,N\text{',N\text{'-tetraacetic acid}
\( \text{ELV} \) \quad \text{Endosome-like vacoule}
\( \text{EPSC} \) \quad \text{Excitatory post-synaptic current}
\( \text{GFP} \) \quad \text{Green fluorescent protein}
\( \text{IHC} \) \quad \text{Inner hair cell}
### Appendix A. An appendix

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>MET</td>
<td>Mechanoelectrical transducer</td>
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<tr>
<td>OHC</td>
<td>Outer hair cell</td>
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<tr>
<td>$Q_{Ca}$</td>
<td>$\text{Ca}^{2+}$ charge</td>
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<tr>
<td>RIM</td>
<td>rab3-interacting molecule</td>
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<tr>
<td>RIM-BP</td>
<td>RIM binding protein</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>RRP</td>
<td>Readily releasable pool</td>
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<tr>
<td>SD</td>
<td>Standart deviation</td>
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<tr>
<td>SGN</td>
<td>Spiral ganglion neuron</td>
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<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptor</td>
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<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
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<tr>
<td>SPL</td>
<td>Sound pressure level</td>
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<tr>
<td>SR</td>
<td>Spontaneous rate</td>
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<tr>
<td>SV</td>
<td>Synaptic vesicle</td>
</tr>
<tr>
<td>TAMRA</td>
<td>carboxytetramethylrhodamine</td>
</tr>
<tr>
<td>$V_{1/2}$</td>
<td>Voltage of half-maximal activation</td>
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<tr>
<td>VDI</td>
<td>Voltage-dependent inactivation</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated $\text{Ca}^{2+}$ channel</td>
</tr>
<tr>
<td>Vglut</td>
<td>Vesicular glutamate transporter</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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