

**Expression and function of Rab3 interacting molecules  
and clarin-1 in inner hair cells**

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# Declaration

I hereby declare that I wrote this thesis for the degree of Doctor rerum naturalium  
'Expression and function of Rab3 interacting molecules and clarin-1 in inner hair cells'  
independently and with no other sources and aids than quoted.

Göttingen, 2013

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# Introduction

## **1. Introduction**

### **1.1 Hearing and the auditory system**

Hearing is one of the five senses and is essential for communications with others. It enables humans to transfer information and exchange ideas and thoughts. In other vertebrates, sound localization is also indispensable for survival and reproduction.

Sound is an oscillation of pressure waves propagated via a gas, liquid or solid medium. Its frequency and intensity are fundamental features for analyzing voices and for comprehending speech. Animals equipped with a pair of hearing organs utilize two additional features of sound, i.e. interaural time difference and interaural level difference, for localizing a sound source and analysis of an acoustic scene. Hearing is enabled by the auditory system, which includes the outer, middle, and inner ear, the auditory nerve as well as the central auditory pathway up to the auditory cortex.

#### **1.1.1 The mammalian ear**

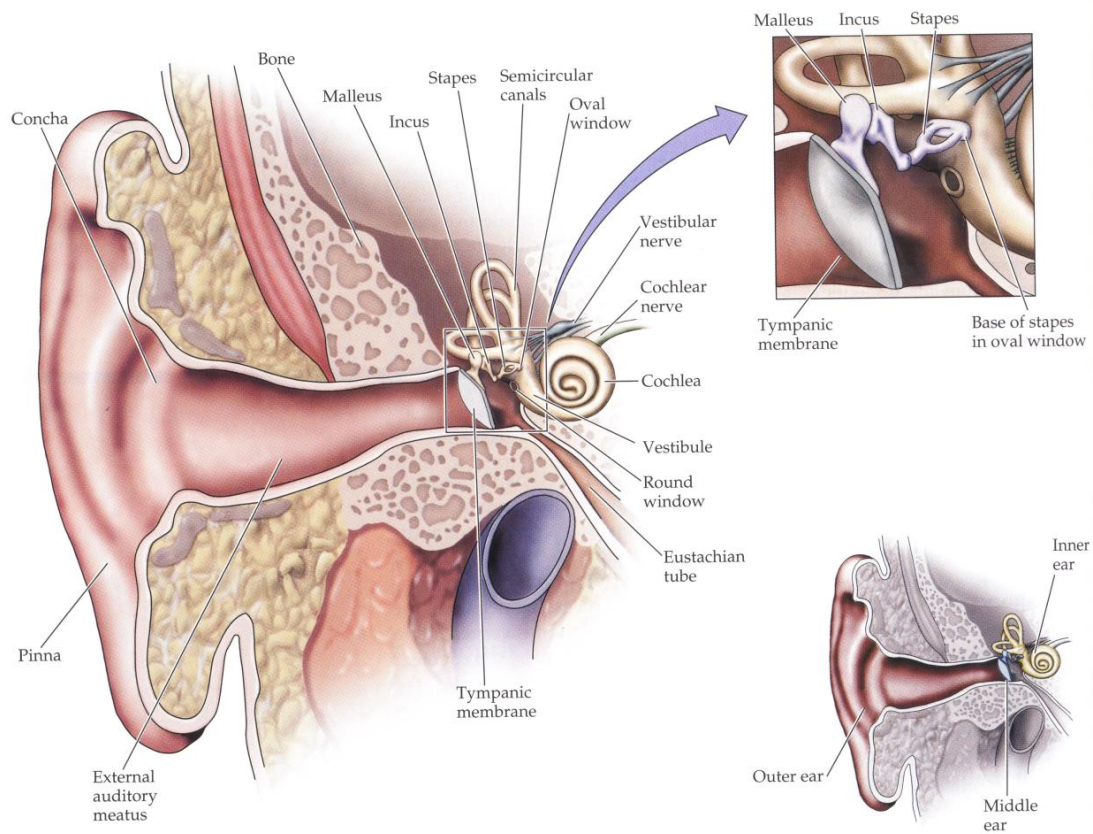
The external ear, which is composed of the auricle and external auditory meatus, gathers acoustic stimuli and focuses them on the tympanic membrane. In humans, the external ear has a resonant frequency of around 3 kHz (Shaw, 1974). The external auditory meatus ends at the tympanic membrane, which compartmentalizes the outer and middle ear (see Figure 1 for a schematic representation).

The middle ear is an air-filled cavity, containing three tiny ossicles called malleus, incus, and stapes. These ossicles transmit the sound vibration from the comparatively large, low impedance tympanic membrane to the much smaller, high impedance oval window. This converts the low pressure, large displacement of the airborne acoustic stimuli into a high pressure, small displacement of the fluid in the cochlea. Thus, the middle ear plays an important role known as impedance matching (Wever and Lawrence, 1954).

The inner ear is a complex structure, which consists of two parts: the organ of hearing, the cochlea, and the vestibular apparatus. The vestibular apparatus further consists of the saccule and the utricle, which detect linear acceleration and head position,

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as well as of the semicircular canals, which detect angular acceleration (I hereafter mention nothing about the vestibular apparatus in this thesis).



**Figure 1 The human ear**

The cross section of the human ear exposes the outer, middle, and inner ear as well as the eighth cranial nerve comprising the cochlear nerve and the vestibular nerve (left). After the sound waves vibrate the tympanic membrane, this energy is transferred via the three ear ossicles (malleus, incus, and stapes) to the oval window of the cochlea (upper right).

Adopted from Purves et al., 2004.

### 1.1.2 The cochlea

The cochlea derives its name from the *Cochlea*, the Latin word for snail, because of its unique coiled structure. The number of turns varies among species: in human it is 2.5, in mouse 1.75, and in guinea pig 4. The size of the human cochlea is 10 mm in width and

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5 mm from the base to the apex, and the coiled basilar membrane is approximately 35 mm in length, when uncoiled.

Inside the bony wall of cochlea, there are three partitioned compartments: scala vestibuli, scala media, and scala tympani. The scala vestibuli stands superior to the scala media with Reissner's membrane dividing them and the scala tympani stands inferior to the scala media with the basilar membrane dividing them. The scala vestibuli and the scala tympani communicate with each other via an opening known as helicotrema located at the very top of the cochlea apex, contain a common fluid called perilymph, which is similar to the extracellular fluid in composition, and are equipotential to the extracellular fluid elsewhere in the body. The scala media forms the middle compartment of the cochlea. However, it does not communicate with the other two compartments and contains a special fluid called endolymph, which is similar to the intracellular fluid with a high concentration of  $K^+$  (157 mM, Wangemann and Schacht, 1996). This is produced by active transport of  $K^+$  through the stria vascularis, generating a high positive potential (+ 80 mV, von Békésy, 1952) called endocochlear potential in the endolymph, which serves as a strong driving force for  $K^+$  movement across the stereocilia on the hair cells (Hibino et al., 2010).

When the vibration of the stapes is transmitted to the scala vestibuli via the membranous opening called oval window, the fluid in the cochlea is displaced towards the other side, which contains another membranous opening, the round window, and is thus transmitted onto the scala tympani. This flow causes vertical movements of the basilar membrane, creating a travelling wave of the basilar membrane towards the cochlear apex. Here, the basilar membrane is tuned in its properties; it is thick, narrow and stiff in the base, but gets thinner, broader and more elastic towards the apex. Due to this gradation of the properties, high frequency sound leads the maximal basilar membrane vibration in the base and low frequency sound leads the maximal vibration in the apex. This micromechanical process is the first stage for the sound analysis in the auditory system, and even without active cellular contribution results in a rough mapping of sound onto tonotopic locations of the basilar membrane (for review, see Purves et al., 2004).



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### 1.1.3 The organ of Corti

The organ of Corti is a highly specialized structure situated on the basilar membrane with the function to convert acoustic signals into electrical signals. It contains a single row of inner hair cells (IHCs), three rows of outer hair cells (OHCs) and several kinds of supporting cells such as Deiter's cells, Hensen's cells, phalangeal cells, and pillar cells (Figure 1).

The OHCs have a cylindrical shape and play a role in amplification of sound energy via an active process (for review, see Hudspeth et al., 1997; Dallos and Fakler, 2002). On the apical surface, they have V- or W-formed hair bundles (stereocilia) attaching to the tectrial membrane. Vertical movement of the basilar membrane turns into deflection of stereocilia, causing opening/closing of mechanotransduction (MET) channels to depolarize/hyperpolarize the OHCs via cation influx (mainly  $K^+$ ). This change in membrane potential drives an electromotility in the OHCs: depolarization causes cell contraction, in contrast, hyperpolarization causes cell elongation via a conformational change in the motor protein prestin (Zheng et al., 2000). The electromotility in the OHCs, indeed, underlies the cochlear amplification (Liberman et al., 2002).

Supporting cells play roles in homeostasis and mechanical support of the organ of Corti. In addition to these functions, recent studies revealed that they have a potential to trans-differentiate into new hair cells under specific conditions (White et al., 2006).

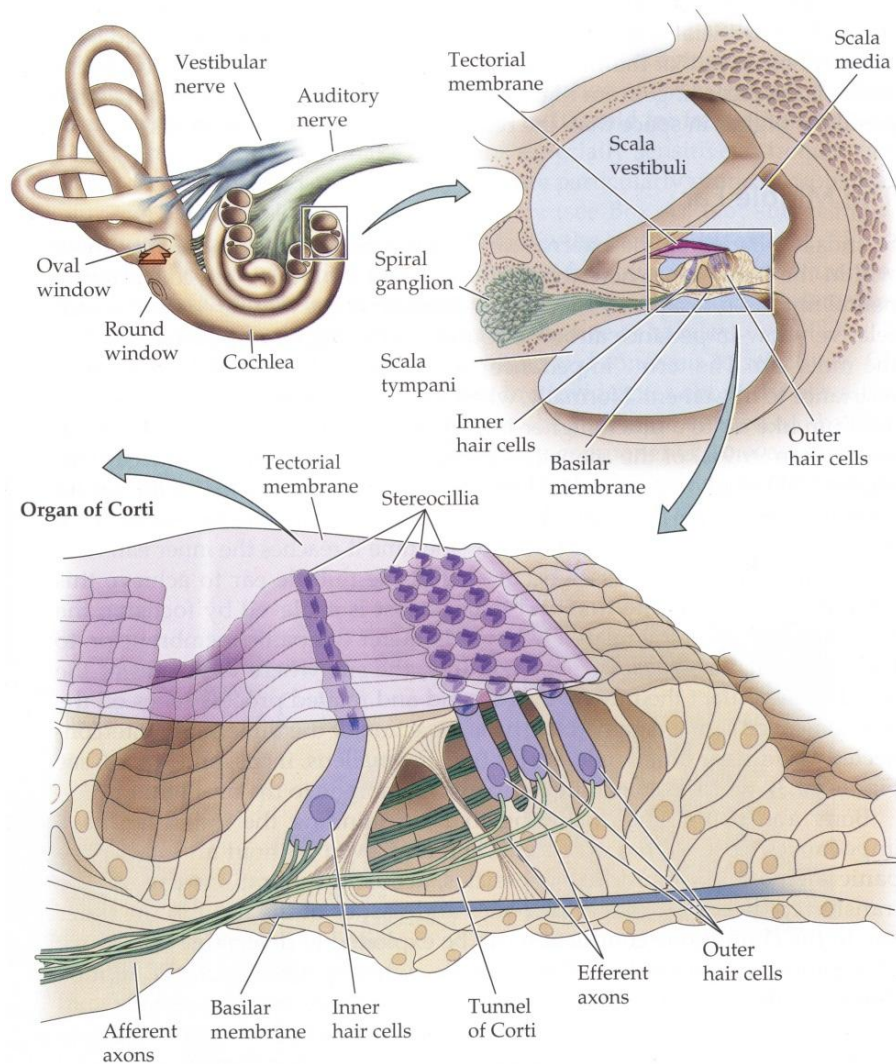
The organ of Corti receives afferent innervation by type I and type II spiral ganglion neurons (SGNs), which form auditory nerve fibers. In mature mice each IHC contacts with 5- 20 afferent dendrites of type I SGNs, a single type I SGN seems to receive input from a single IHC through a single active zone of the ribbon-type afferent synapse (e.g. Meyer et al., 2009). *L*-glutamate, a neurotransmitter at this synapse, is released from IHCs through the process of synaptic vesicle exocytosis and activates AMPA-type glutamate receptors on the type I SGNs to generate action potentials. This signal is relayed through the auditory pathway up to the auditory cortex. Type II SGNs receive inputs from 15- 20 OHCs (Spendlin et al., 1972), which also release *L*-glutamate (Weisz et al., 2010).

The organ of Corti also receives efferent innervation from the olivocochlear system.

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The olivocochlear system has two pathways: the medial olivocochlear (MOC) pathway arising from the contralateral ventral nucleus of the trapezoid body and the lateral olivocochlear (LOC) pathway arising from the ipsilateral lateral superior olive (Guinan et al., 1984). The projection pattern of the MOC fibers and the LOC fibers varies with development. At the early stage of development, the MOC fibers project to the IHCs alone, while the projection of LOC fibers is unknown. At the intermediate stage, the MOC fibers project to both IHCs and OHCs, while the LOC fibers project to IHC region alone. Finally at the late stage of development, the MOC fibers project to OHCs, while the LOC fibers project to the afferent dendrites of IHCs (for review, see Simmons, 2002). The olivocochlear system employs predominantly acetylcholine (ACh) as its neurotransmitter to hyperpolarize the targeting cells via activation of  $\alpha 9$  (Elgoyhen et al., 1994; Vetter et al., 1999) and  $\alpha 10$  (Vetter et al., 2007) nicotinic ACh receptors concomitant with subsequent activation of small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels (Yugas and Fuchs, 1999; Oliver et al., 2000). However, the LOC system additionally seems to employ dopamine as its neurotransmitter to activate dopamine receptors on the afferent dendrites of type I SGNs (Eybalin et al., 1993; Darrow et al., 2006). Efferents are believed to modulate the spontaneous activity of IHCs before the hearing onset (Glowatzki and Fuchs, 2000) and type I SGN activity after the hearing onset (Ruel et al., 2001) as well as the motility of OHCs for negative control of the cochlear amplifier via a motor protein prestin (Zheng et al., 2000).

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**Figure 2 The cochlea and the organ of Corti**

The cross section of the membranous labyrinth (top left) exposes three partitions of cochlea: scala vestibuli, scala media, and scala tympani (top right). The organ of Corti, a specialized structure for auditory transduction, is located between the tectorial membrane and the basilar membrane (bottom). A single row of inner hair cells, three rows of outer hair cells, and surrounding supporting cells are seen.

Adopted from Purves et al., 2004.

### 1.1.4 The inner hair cell (IHC)

The IHC is the genuine sensory cell of the organ of Corti, transducing auditory stimuli into a neural signal at its synapses with SGNs. It has a pear-like shape and 2-3 rows of

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stereocilia on the apical surface (hair bundle). The stereocilia amount to 20-50 in each IHC, depending on species and location (for review, see Raphael and Altschuler, 2003), and contain actin-filament cores (Sobin and Flock, 1983). The top of each stereocilium is connected to the lateral surface of its neighboring hair bundle through a tip-link filament, which is primarily composed of Cadherin 23 (Siemens et al., 2004; Sollner et al., 2004) and Protocadherin 15 (for review, see Müller, 2008). Since the tip links and mechanoelectrical transduction (MET) channels are directly connected to each other, deflections of the hair bundle leading to changes in tension of the tip link filaments directly gate the MET channels. A displacement towards the kinocilium (or longest stereocilia) increases tension of tip links, opens MET channels, and allows cation influx (mainly  $K^+$ ) to cause a depolarization in the IHC, which is termed a receptor potential. On the contrary, a deflection towards the opposite side (shortest stereocilia) decreases tension of tip links, closes MET channels, and shuts off cation influx to set the membrane potential to more negative values (for review, see Hudspeth, 1997). This receptor potential triggers the opening of voltage-gated  $Ca^{2+}$  channels at the ribbon-type synapses, further leading to exocytosis of synaptic vesicles at the active zones.

In addition to the MET channels, the IHCs express voltage-gated  $Ca^{2+}$  channels and  $K^+$  channels (for review, see Kros, 1996).

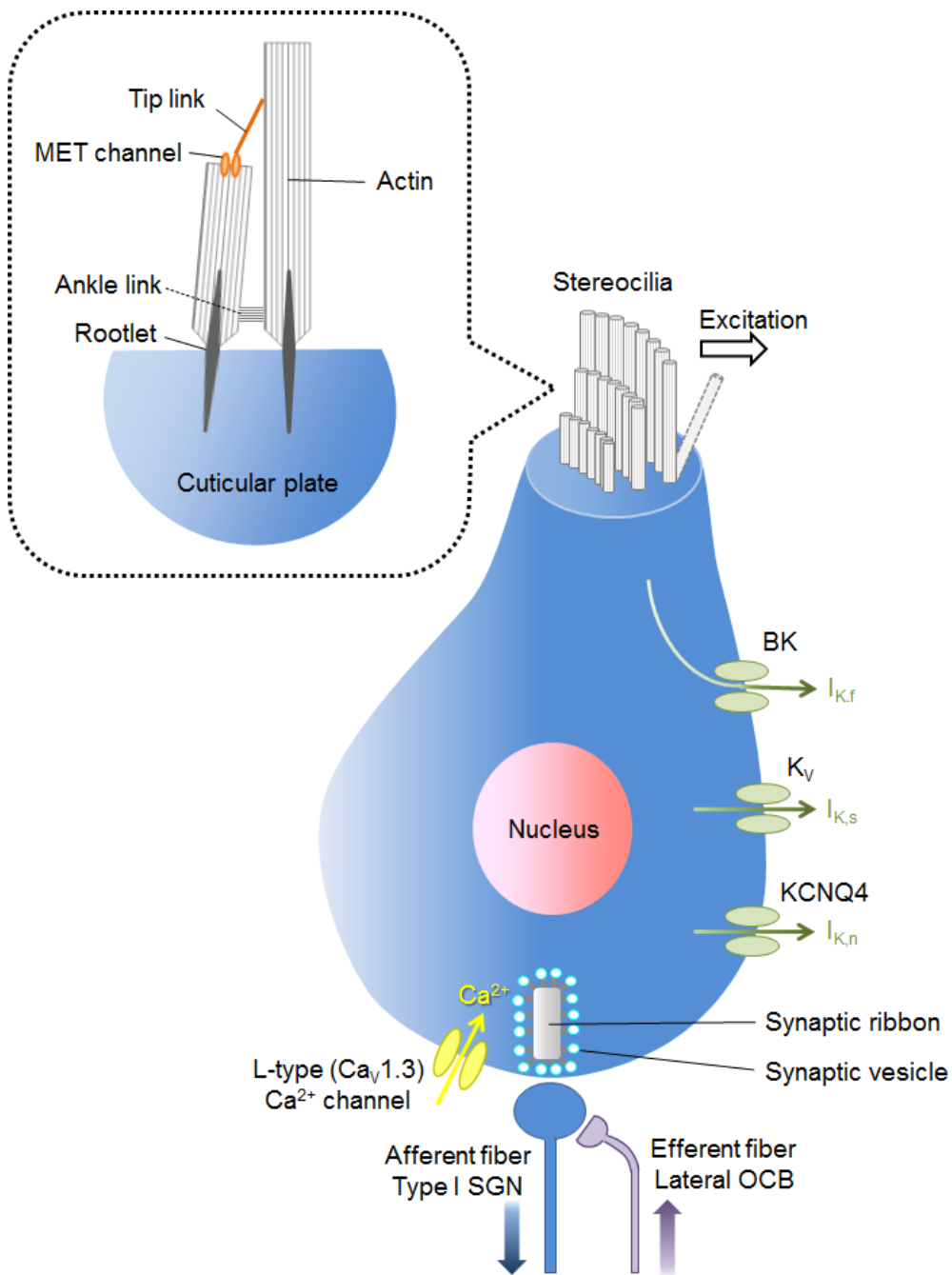
While presynaptic  $Ca^{2+}$  currents at the conventional CNS synapses are carried by P/Q-, N-, and R-type  $Ca^{2+}$  channels, that at the IHC afferent synapse is carried by L-type  $Ca^{2+}$  channels (predominantly  $Ca_v1.3$  ( $\alpha1D$ )  $Ca^{2+}$  channels, Platzner et al., 2000; Brandt et al., 2003). The L-type  $Ca^{2+}$  channels have an advantageous feature: they remain active upon prolonged depolarization by their relatively little inactivation (Moser and Beutner, 2000; Ricci and Schnee, 2003; Yang et al., 2006; Cui et al., 2007; Grant et al., 2008). The L-type  $Ca^{2+}$  channels are clustered at the release sites of the IHC ribbon synapse (Roberts et al., 1990; Zenisek et al., 2003; Brandt et al., 2005), allowing nanodomain control of synaptic vesicle exocytosis (Brandt et al., 2005). Interestingly, the number of L-type  $Ca^{2+}$  channels varies among ribbon synapses within a single IHC, which could contribute to the heterogeneous response properties of the auditory nerve fibers to the sound. The  $Ca^{2+}$  current density undergoes a developmental

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change; it reaches the maximum level at P6 and decreases afterwards (Beutner and Moser, 2001). Just recently, two families were identified that have sinoatrial node dysfunction and deafness (termed SANDD syndrome) due to a mutation in CACNA1D encoding  $Ca_v1.3$  ( $\alpha1D$ )  $Ca^{2+}$  channels (Baig et al., 2011).

Presynaptic  $K^+$  currents are carried by large-conductance  $Ca^{2+}$ -activated potassium (BK) channels,  $K_v$ -type voltage-gated potassium channels, voltage-gated potassium channel of the KQT-like subfamily, member 4 (KCNQ4), and small-conductance  $Ca^{2+}$ -activated potassium (SK) channels. The BK current, which is also known as  $I_{K,f}$ , due to a fast activation, shapes the sound-evoked receptor potential in the mammalian IHCs (Oliver et al., 2006) and contributes to frequency-tuning in the non-mammalian vertebrates IHCs (Ramanathan et al., 1999). Interestingly, it is not until the onset of hearing that they begin to be expressed (Kros et al., 1998). The  $K_v$ -type potassium current, which is known as  $I_{K,s}$  due to a slower activation, mediates a delayed rectifier conductance (Kros et al., 1998; Marcotti et al., 2003). The KCNQ4 ( $K_v7.4$ ) potassium channel, which is affected in autosomal dominant hereditary deafness type 2 (DFNA2), carries a low-voltage activated, slow delayed rectifier  $K^+$  conductance,  $I_{K,n}$ , and contributes to the resting membrane potential (Marcotti et al., 2003; Oliver et al., 2003; Kharkovets et al., 2006). SK channels are expressed on IHCs transiently until the onset of hearing (Katz et al., 2004; Marcotti et al., 2004b), and are essential for cholinergic function of efferent inhibition (Fuchs and Murrow, 1992; Kong et al., 2008).

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**Figure 3 The inner hair cell with its ion channels as well as afferent and efferent innervating fibers**

The IHC expresses the mechano-electrical transduction (MET) channel (depicted in orange), three classes of  $K^+$  channels (BK,  $K_v$ , and KCNQ4, green), L-type ( $Ca_v1.3$ )  $Ca^{2+}$  channels (yellow). Note that the IHC is innervated by afferent fibers of type I spiral ganglion neurons (blue), which further innervated by lateral olivocochlear efferents (purple). Modified from Bulankina and Moser, 2012.

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### **1.1.5 Molecular anatomy and physiology of the inner hair cell ribbon synapse**

The most characteristic property of the IHC afferent synapse is the synaptic ribbon, a submicron-sized electron-dense structure where 125 (in electron-microscopic study) or 200 (in 4pi study) synaptic vesicles are tethered (for review, see Nouvian et al., 2006). The synaptic ribbon is found among the retinal photoreceptors and bipolar cells, auditory and vestibular hair cells, and pinealocytes (for review, see Lenzi and von Gersdorff, 2001; Fuchs et al., 2003; Lagnado et al., 2003; Sterling and Matthews, 2005; Matthews and Fuchs, 2010). It is also found in the hair cells and electroreceptors of the lateral line in fish and amphibians (Katz et al., 1993). Thus, the synaptic ribbon seems to be present wherever the system requires exocytosis evoked by graded depolarization to cover a wide dynamic range of stimuli and where a high, sustained rate of release is needed.

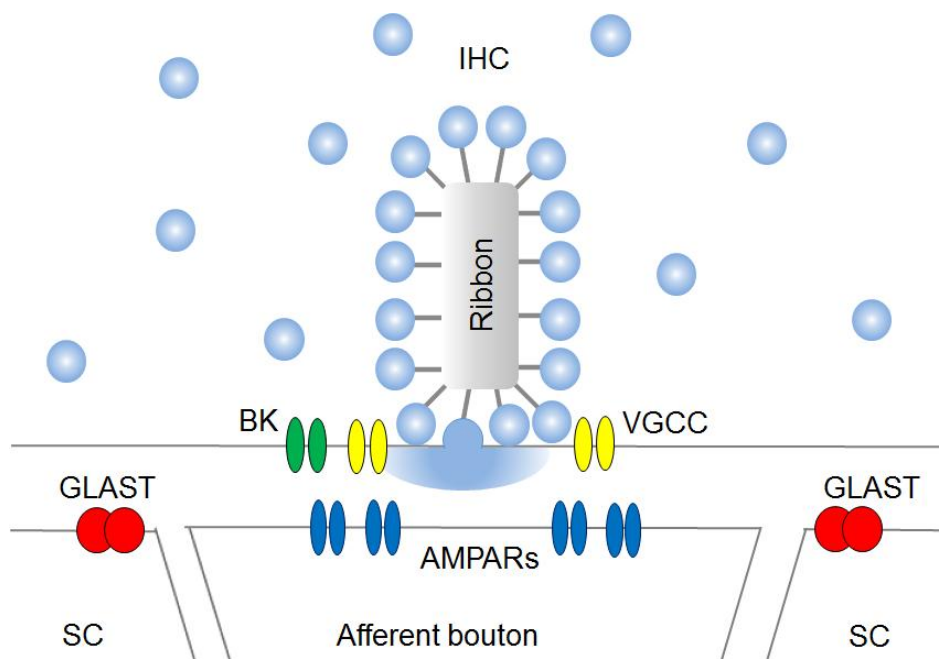
The synaptic ribbons in the mammalian cochlea are relatively small (less than 200 nm in width), Moser et al., 2006). In the mammalian cochlea, more than 100 synaptic vesicles are tethered to the ribbon (Nouvian et al., 2006) and several tens of L-type  $\text{Ca}^{2+}$  channels are present at these active zones (Brandt et al., 2005; Meyer et al., 2009; Zampini et al., 2010), where synaptic transmission occurs. The number of docked synaptic vesicles is estimated to be approximately 10-20 (Pangršič et al., 2010) and this seems enough to support a rapid burst of release at the onset of stimulus.

The molecular components of the synaptic ribbon have recently begun to be deciphered. The protein RIBEYE is a major constituent of the ribbon and has two domains. Its B domain is almost identical to a ubiquitous transcription factor, C-terminal binding protein 2 (CtBP2) and bears enzymatic activity (Schwarz et al., 2011), while its A domain mediates homophilic interactions (Magupali et al., 2008). In addition, scaffolding proteins such as bassoon and piccolo are present in synaptic ribbons. Recent studies have shown that bassoon anchors the ribbons to the active zones (Dick et al., 2001, 2003; Khimich et al., 2005; Frank et al., 2010). Moreover, Rab3 interacting molecules (RIMs, Wang et al., 1997) are present at the active zones of retinal photoreceptors (tom Dieck 2005) and immature IHCs (Gebhart et al., 2010),

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while it was stated to be absent from the active zones of mature IHCs (Gebhart et al., 2010).

Although the precise functions of the synaptic ribbon remain to be addressed, there have been a few hypotheses for them. One is that the ribbon functions as a ‘conveyor belt’ to move the vesicles in the upper rows down to the active zone for continuous vesicle supply (Bunt, 1971). Another is that the ribbon functions to enable a large RRP of synaptic vesicles at the active zone (Khimich et al., 2005; Moser et al., 2006b).



**Figure 4 The inner hair cell ribbon synapse**

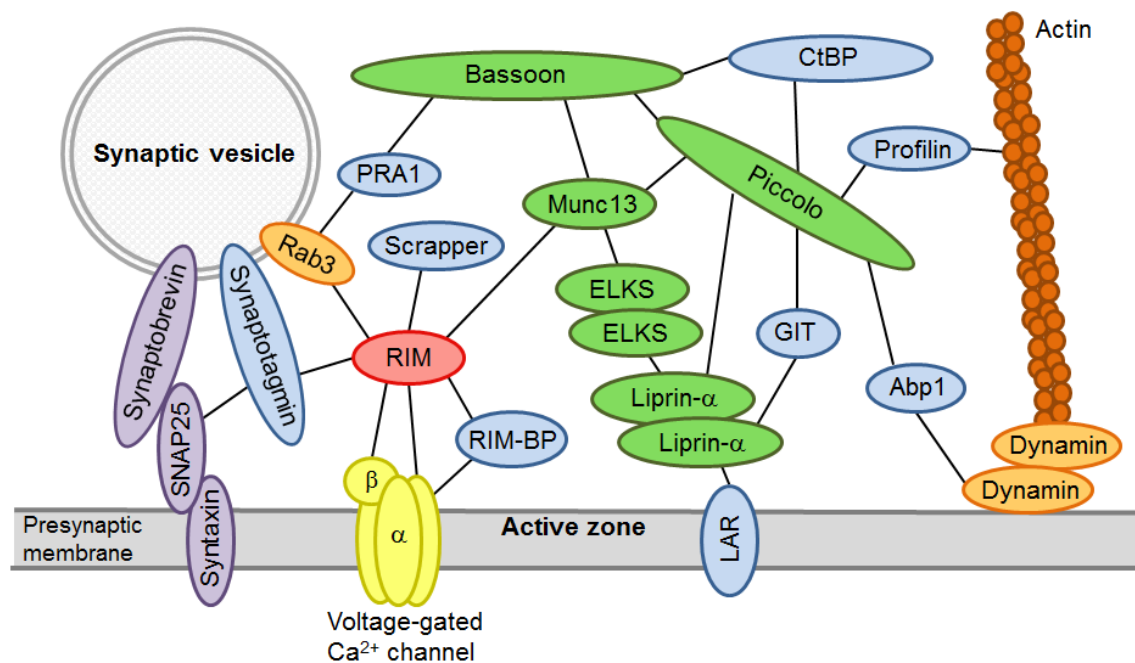
The synaptic ribbon, an electron-dense structure located beneath the presynaptic plasma membrane, tethers a number of synaptic vesicles (pale blue). L-type ( $\text{Ca}_v1.3$ )  $\text{Ca}^{2+}$  channels (VGCCs, yellow) and large-BK-type  $\text{K}^+$  channels (green) are clustered near the synaptic ribbons. AMPA-type glutamate receptors (blue) are expressed on the surface of postsynaptic afferent boutons for binding to neurotransmitter glutamate, while glutamate transporters (GLAST, red) are expressed on the surface of supporting cells (SCs) for clearance of glutamate in the synaptic cleft. Modified from Fuchs et al., 2003.



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### 1.2 Expression and function of RIMs at presynaptic active zones of the IHCs

The active zone is a specialized structure in the presynaptic plasma membrane where synaptic vesicles undergo docking, priming, and fusion in order to release their neurotransmitters onto the postsynaptic neurons. A variety of proteins are involved in this process (for review, see Südhof and Rizo, 2011; Schoch and Gundelfinger, 2006). Among these are the Rab3-interacting molecules (RIMs), which are major active zone proteins and are ubiquitously expressed at central synapses.

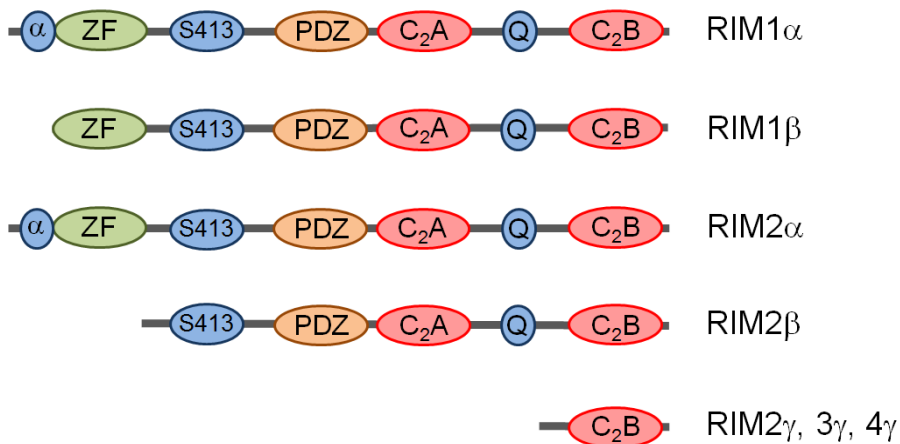


**Figure 5 The active zone proteins**

Modified from Mittelstadt et al., 2010

The RIMs are composed of the multi-domain  $\alpha$ -RIMs and  $\beta$ -RIMs (RIM1 $\alpha$ , RIM1 $\beta$ , RIM2 $\alpha$  and RIM2 $\beta$ ) as well as the shorter  $\gamma$ -RIMs (RIM2 $\gamma$ , RIM3 $\gamma$  and RIM4 $\gamma$ ).  $\alpha$ -RIMs contain a full set of domains: a helix  $\alpha$ 1 and a zinc finger domain in the N-terminus, a PDZ-domain in the center, two C<sub>2</sub>-domains (C<sub>2</sub>A and C<sub>2</sub>B) and a proline-rich sequence in the C-terminus (Wang et al., 2000). RIM1 $\beta$  is similar to RIM1 $\alpha$  except that RIM1 $\beta$  lacks the helix  $\alpha$ 1, while RIM2 $\beta$  is similar to RIM2 $\alpha$  except that RIM2 $\beta$  lacks the helix  $\alpha$ 1 and the zinc finger domain.  $\gamma$ -RIMs only contain an isoform-specific N-terminus and the C<sub>2</sub>B domain (Wang et al., 1997).

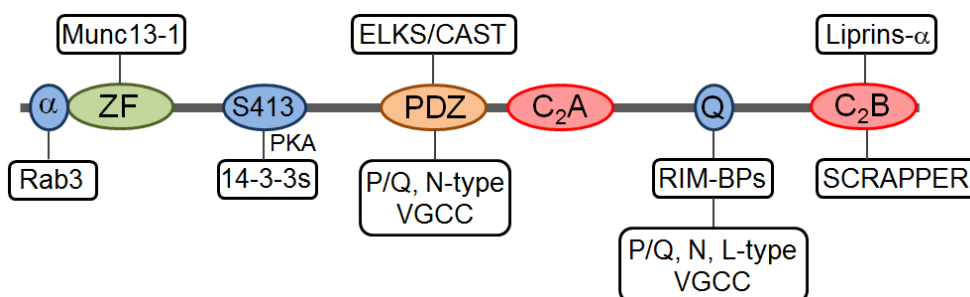
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**Figure 6 Domain components of RIM isoforms**

(a) helix  $\alpha$ 1; (ZF) zinc finger domain; (S413) serine 413, phosphorylation site for PKA; (PDZ) PSD95, Dlg, and ZO-1/2-like domain; (C<sub>2</sub>) PKC conserved region 2; (Q) proline-rich sequence. Modified from Mittelstadt et al., 2010.

Remarkably RIMs interact with remaining active zone proteins such as Munc13, Bassoon, Piccolo, ELKS and Liprin- $\alpha$  (for review, see Mittelstaedt et al., 2010). Moreover, PDZ domains of RIMs directly bind to the  $\alpha$ -subunits of P/Q- and N-type Ca<sup>2+</sup> channels (Kaeser et al., 2010). In addition, RIMs are linked to P/Q-, N-, and L-type Ca<sup>2+</sup> channels via an interaction with RIM-binding proteins (RIM-BPs) through their proline-rich domains (Hibino et al., 2002).



**Figure 7 RIMs interact with multiple other active zone proteins in central synapses**

(a) helix  $\alpha$ 1; (ZF) zinc finger domain; (S413) serine 413, phosphorylation site for PKA; (PDZ) PSD95, Dlg, and ZO-1/2-like domain; (C<sub>2</sub>) PKC conserved region 2; (Q) proline-rich sequence. Modified from Mittelstadt et al., 2010.

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The RIMs play essential roles in neurotransmitter release and synaptic plasticity. Several recent studies have shown that RIM1 and 2 regulate the docking/priming step of the release process and tether P/Q- and N-type  $\text{Ca}^{2+}$  channels to the active zones in hippocampal cultured neurons (Kaeser et al., 2010) as well as at the calyx of Held synapse (Han et al., 2011). RIM1 $\alpha$  alone not only controls release probability but also changes both short- and long-term synaptic plasticity in the hippocampus (Schoch et al., 2002; Castillo et al., 2002, Calakos et al., 2004). However, deletion of RIM2 $\alpha$  failed to show any synaptic phenotypes in the hippocampus. Knockout of both RIM1 $\alpha$  and RIM2 $\alpha$  causes perinatal lethality without changing synaptic structure and formation, although deletion of either RIM1 $\alpha$  or RIM2 $\alpha$  does not cause this perinatal lethality (Schoch et al., 2006).

Compared to CNS synapses, the expression and function of RIMs in the sensory epithelium have not been characterized. Expression of RIMs was detected in the mouse retina, although their function was not elucidated (tom Dieck et al., 2005; Deguchi-Tawarada et al., 2006). Notably the distribution profiles of the RIM proteins are different between the wild-type retina and the ribbon-deficient Bassoon mutant retina: both RIM1 and RIM2 colocalize with RIBEYE at the wild-type photoreceptor terminal, while only RIM1, not RIM2, colocalizes with RIBEYE at the mutant photoreceptor, suggesting that RIM1 localizes to the synaptic ribbon, but RIM2 is present at the plasma membrane of the active zone (tom Dieck et al., 2005). A recent study indicated that RIM2 $\alpha$ , but not other isoforms of RIMs, is expressed in IHCs, however, only before hearing onset (Gebhart et al., 2010). In order to provide more information on the role of RIMs in hearing, I probed the role of RIMs at the first auditory synapse employing RIM knockout animals.

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### 1.3 Clarin-1 and Usher syndrome type IIIA

#### 1.3.1 Usher syndrome and Usher proteins

Usher syndrome (USH) is an autosomal recessive disorder, which was named after the English medical doctor Charles Usher (Usher, 1914), although it had been first reported by the German medical doctor Albrecht von Graefe as retinitis pigmentosa in combination with deafness (von Graefe, 1858). Among the around 40 kinds of hereditary disorders which present deafness and blindness, USH is the most frequent with approximately 50 % of all cases (Saihan et al., 2009). USH is clinically classified into three types according to the extent and the onset of deafness as well as the presence of vestibular dysfunction (Smith et al., 1994). To date, at least 10 genetic loci have been mapped for USH and in most cases, the responsible genes have been identified (Weil et al., 1995; Verpy et al., 2000; Bitner-Glindzicz et al., 2000; Wayne et al., 1996; Bork et al., 2001; Bolz et al., 2001; Chaib et al., 1997; Ahmed et al., 2001, 2009; Alagramam et al., 2001; Mustapha et al., 2002; Weil et al., 2003; Kimberling et al., 1990; Eudy et al., 1998; Pieke-Dahl et al., 2000; Weston et al., 2004; Ebermann et al., 2007, 2010; Sankita et al., 1995).

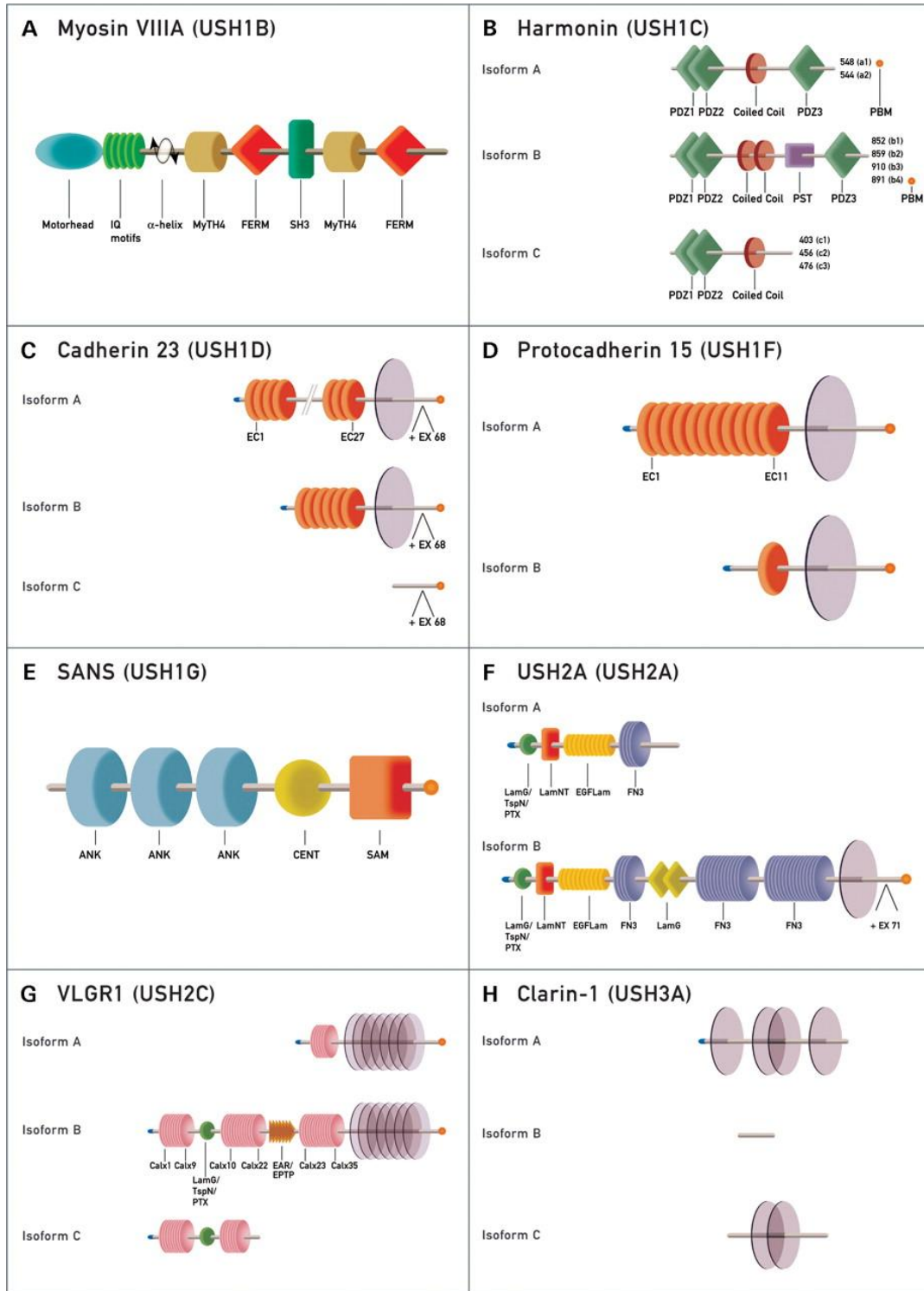
USH type I patients are born profoundly deaf and have severe vestibular dysfunction. They present with progressive vision impairment afterwards by the age of ten. To date, seven genetic loci (USH1B-H) have been mapped for this subtype, and five causative genes for USH type I have been identified, namely myosin VIIa (*MYO7A*, USH1B), harmonin (*USH1C*, USH1C), cadherin 23 (*CDH23*, USH1D), protocadherin 15 (*PCDH15*, USH1F), and sans (*SANS*, USH1G).

USH type II patients are born moderately to severely deaf without vestibular dysfunction. Vision problems in USH type II progress more slowly compared to USH type I. The causative genes for USH type II are usherin (*USH2A*, USH2A), VLGR1 (*VLGR1*, USH2C) and whirlin (*WHRN*, USH2D).

USH type III patients are not born deaf, but may develop progressive hearing loss afterwards. The severity of auditory, vestibular or vision symptoms varies from person to person. The only identified causative gene for USH type III is clarin-1 (*CLRN1*), whose genetic locus is 3q21-q25 (Sankila et al., 1995). In USH type III patients, at least

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10 mutations in the CLRN1 gene have been detected (Joensuu et al., 2001; Adato et al., 2002; Fields et al., 2002; Ness et al., 2003; Aller et al., 2004; Sadeghi et al., 2005; Ebermann et al., 2007; Herrera et al., 2008).



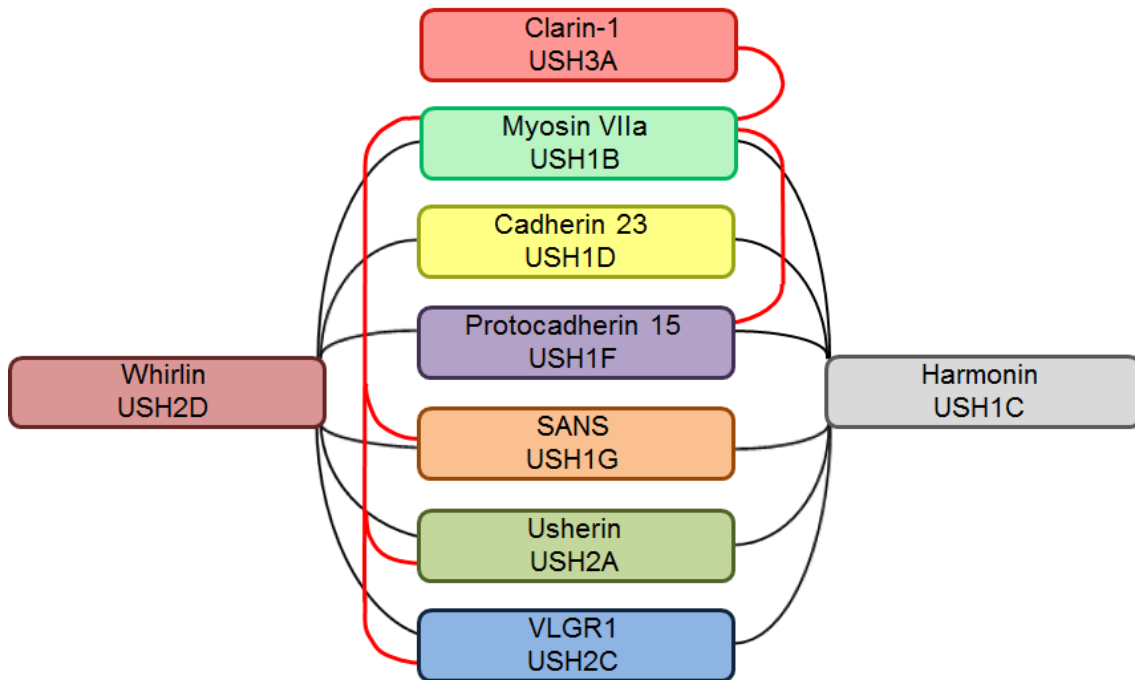
## Introduction

### Figure 8 Usher proteins and their different isoforms

(A) The USH1B protein, myosin VIIa, is comprised of a motor head domain, five calmodulin-binding IQ motifs, two FERM domains, two MyTH4 domains and a Src homology 3 (SH3) domain. (B) The USH1C protein, harmonin, is comprised of at least two PDZ (PSD95, discs large, ZO-1) domains (PDZ1 and 2) and a coiled-coil domain. The class A isoform contains an additional PDZ domain (PDZ3). The class B isoform also contains this PDZ3 domain, a second coiled-coil domain and a proline, serine, threonine-rich region (PST). Isoforms A1 and B4 contain a C-terminal class I PDZ binding motif (PBM). (C) The USH1D protein, cadherin 23, has three different isoforms. The isoform A is comprised of 27  $\text{Ca}^{2+}$ -binding extracellular cadherin domains (EC1-27), a transmembrane domain (grey disks) and a short intracellular domain with a C-terminal class I PBM. The isoform B is similar to the isoform A, but only contains the last six EC domains. The isoform C is comprised of the intracellular domain and C-terminal PBM. (D) The USH1F protein, protocadherin 15, is comprised of either eleven (isoform A) or one (isoform B) EC domain, a transmembrane domain and a C-terminal class I PBM. (E) The USH1G protein, SANS, is comprised of three ankyrin domains (ANK), a central region (CENT), a sterile alpha motif (SAM) and a C-terminal class I PBM. (F) Isoform A of the Usher 2A protein (USH2A) is comprised of an N-terminal thrombospondin/pentaxin/laminin G-like domain, a laminin N-terminal (LamNT) domain, ten laminin-type EGF-like (EGF Lam) and four fibronectin type III (FN3) domains. In addition to this region, Isoform B contains two laminin G (LamG), 28 FN3, a transmembrane domain and an intracellular domain with a C-terminal class I PBM. (G) Isoform B of the USH2C protein, the very large G-coupled protein receptor VLGR1, contains a thrombospondin/pentaxin/laminin G-like domain, 35  $\text{Ca}^{2+}$ -binding calcium exchanger  $\beta$  (Calx) domains, seven EAR/EPTP repeats, a seven-transmembrane region and an intracellular domain containing a C-terminal class I PBM. (H) Clarin-1, the USH3A protein, contains four (isoform A), none (isoform B), or one transmembrane (isoform C) domain. Adopted from Kremer et al., 2006.

## Introduction

Usher proteins undergo a wide range of mutual interactions (Figure 9). Among them, the USH1C protein harmonin plays a central role, interacting with most of the remaining USH proteins. Moreover, a recent study reported that harmonin reduces synaptic  $\text{Ca}^{2+}$  influx through  $\text{Ca}_v1.3$ -type  $\text{Ca}^{2+}$  channels in IHCs (Gregory et al., 2011).



**Figure 9 The Usher protein network**

Currently known protein–protein interactions between different Usher proteins. Note that clarin-1 interacts with Myosin VIIa (Adato et al., 1999). Modified from Brown et al., 2008.

### 1.3.2 Expression and function of clarin-1 at the IHC ribbon synapse

Clarin-1, a member of four- transmembrane superfamily also containing tetraspanins and claudins, had been proposed to regulate hair cell synaptic transmission as well as the organization of actin filaments, on the basis of its expression at the synaptic and stereociliar sites (Adato et al., 2002; Tian et al., 2009; Geng et al., 2009; Zallocchi et al., 2009 and 2012). A preceding study found that the *CLRN1* knockout mouse, an animal model for USH type IIIA, develops profound hearing loss as well as vestibular dysfunction, and that the hair bundles are disrupted in *CLRN1*<sup>-/-</sup> mice (Geng et al., 2009).

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Although the hair bundle disruption seemed to cause the mechano-electrical transduction deficit and mostly underlies the elevated ABR thresholds, a further involvement of synaptic deficits at the IHC afferent synapse was suggested on the basis of the observed delay in the peak latencies of ABRs in *CLRNI*<sup>-/-</sup> mice (Geng et al., 2009). To test this hypothesis, I probed the presynaptic functions of clarin-1 knockout mice.



# Materials and Methods

## 2. Material and Methods

### 2.1 Animals

The generation of knockout mice was previously described for *RIM2 $\alpha$* <sup>-/-</sup> (Schoch et al., 2006) and for *Clrn1*<sup>-/-</sup> (Geng et al., 2009), respectively. As controls, age-matched wild-type littermates were used. All experiments were complied with national animal care guidelines and approved by the University of Göttingen Board for Animal Welfare and the Animal Welfare Office of the State of Lower Saxony.

### 2.2 Single-cell nested RT-PCR

Wild-type C57BL6 mice at the age of postnatal days (P) 14 through 16 were used in this study. IHCs from the apical coils of freshly dissected organs of Corti were harvested after cleaning off supporting cells at a high bath perfusion rate (3 ml/min). Each individual IHC was aspirated and the pipette content was transferred into first strand cDNA synthesis mix containing after the dilution: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 100 units of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 40 units RNaseOUT Ribonuclease inhibitor (Invitrogen). Reverse transcription was performed with oligo(dT)primers according to the manufacturer's instructions. Aspirated bath solution was used as a negative control. Each cDNA mix was used as a template for two subsequent PCR reactions with nested primers specific for RIM1 $\alpha$ - $\beta$ , RIM2 $\alpha$ - $\gamma$ , RIM3 $\gamma$ , RIM4 $\gamma$ , and otoferlin or RIBEYE (specific) cDNA. Primer sequences are listed in Table 1 and 2.

## Materials and Methods

	fwd/rev	1st	nested
RIM1	forward	GAGGAACGAACGAGACAGATGAAA	GTCCGCCAAGTCATCAGATAGTGA
	reverse	TTTTTAACTTCTTGTGGCCGGACT	TTCTGCTTCTTCGAGACACAATGG
RIM2	forward	GCCTCTCAACTCAGCCAAAC	GATGGCAGCATGAACAGCTA
	reverse	CAGAGACGATTGGGAAGCTC	TAGGGAGGAAGGAGGGAAGA
RIM3	forward	TGGGAGCACCAACAGTAACA	AAGCCAGTTCAGTGACTTTCTGGA
	reverse	CATGTTTTCTTGGCCACCTT	GTTCTCCAGCAGGTAAGCCTTGAT
RIM4	forward	AACTGCCAGCTGCCTATATCAAG	TGTCTGCATTGCCAAGAAGAAAAC
	reverse	CGTAGTTTCCCCACACGATTACCT	GGGACTCTCAGGAAACAGAAGCAC

**Table 1. Primers for RT-PCR/nested RT-PCR**

	fwd/rev	1st	nested
RIM1 $\alpha$	forward	CTCCCCCTATGCAAGAACTG	ACCGAGGAGGAGAGGAACAT
	reverse	GACCTTGATCGCTCTTGGAG	TTGTTCGATCGCAGAGACAC
RIM1 $\beta$	forward	CAGAAGCTGTCCCATTTTCC	CCTTCTGGAGCTTTCTGAGC
	reverse	GACCTTGATCGCTCTTGGAG	TTGTTCGATCGCAGAGACAC
RIM2 $\alpha$	forward	AGCAAGAGCAGAAGGGTGAT	CTGCAGCAACCTGATCAAAA
	reverse	TCCACATCTTCATCATCCACA	ATTGAGGCTCACGCTGAGAT
RIM2 $\beta$	forward	GCCAGGTCTGCAATTCTGTT	CGCTGAACAATGCAAGAAAA
	reverse	TCCACATCTTCATCATCCACA	ATTGAGGCTCACGCTGAGAT
RIM2 $\gamma$	forward	TCCATGCAGCGCTCTCAG	CAGCCTCTCTGCCTCTTTTG
	reverse	TAGCTGTTTCATGCTGCCATC/ TCCAGGAAATCACTGAACTGG	CCTCCTCCTTCTCCTTCATCT

**Table 2. Subtype-specific primers for RT-PCR/nested RT-PCR**

### 2.3 Immunohistochemistry

The freshly dissected apical cochlear turns from P9-17 wild-type C57Bl6 mice were fixed with 100 % methanol for 20 min at  $-20^{\circ}\text{C}$ . Thereafter, the tissue was washed three

## Materials and Methods

times for 10 min in PBS and blocked for 1 h in goat serum dilution buffer (GSDB) (16% normal goat serum, 450 mM NaCl, 0.3% Triton X-100, and 20 mM phosphate buffer, pH 7.4) in a wet chamber at room temperature. Primary antibodies were dissolved in GSDB and applied overnight at +4°C in a wet chamber. After washing three times for 10 min (wash buffer: 450 mM NaCl, 20 mM phosphate buffer, and 0.3% Triton X-100), the tissue was incubated with secondary antibodies in GSDB in a wet light-protected chamber for 1 h at room temperature. Then the preparations were washed three times for 10 min in wash buffer and one time for 10 min in 5 mM phosphate buffer, placed onto glass microscope slides with a drop of fluorescence mounting medium (Dako, Glostrup, Denmark), and covered with thin glass coverslips. The following antibodies were used: mouse IgG1 anti-CtBP2 (also recognizing the ribbon protein RIBEYE; 1:150; BD Biosciences), polyclonal rabbit antibody 1 against RIM 2, PDZ domain (1:200; Synaptic System, Göttingen, Germany), RIM3 $\gamma$  (1:200, kindly provided by Prof. Dr. Susanne Schoch at University of Bonn) as well as secondary AlexaFluor488- and AlexaFluor568-labeled antibodies (1:200; Invitrogen). Confocal images were acquired using a laser scanning confocal microscope (TCS SP5, Leica, Wetzlar, Germany) with 488 nm (argon) and 561 nm (helium–neon) lasers for excitation and a 63 $\times$  oil-immersion objective (1.4 numerical aperture; Leica). Whole-mount preparations of the organ of Corti provided the possibility to analyze several IHCs in a row (Khimich et al., 2005). Images were processed using NIH ImageJ software and assembled for display in Adobe Illustrator software (Adobe Systems).

### 2.4 Patch-clamp recording

For analyzing the IHC presynaptic function, I performed perforated patch-clamp recordings in apical coils of freshly dissected organs of Corti (Moser and Beutner, 2000) from *RIM2 $\alpha$ <sup>-/-</sup>* mice or *Clrn1<sup>-/-</sup>* mice and their wild-type littermates (P13-19 for RIM2 $\alpha$ -related animals, P14-20 for Clrn-1-related animals). The pipette solution contained (in mM): 130 Cs-gluconate, 10 TEA-Cl, 10 4-AP (4-aminopyridine; Merck KGaA, Darmstadt, Germany), 1 MgCl<sub>2</sub>, 10 HEPES (pH adjusted with HCl to 7.17, osmolarity ~ 290 mOsm/kg) and 300  $\mu$ g/ml amphotericin B (Calbiochem, La Jolla, CA)

## Materials and Methods

dissolved in dimethyl sulfoxide (Invitrogen). The extracellular solution contained (in mM): 104 NaCl, 35 TEA-Cl, 2.8 KCl, 10 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 1 Cs gluconate, 5 4-AP, 11.1 D-glucose (pH adjusted with NaOH to 7.2, osmolarity ~ 300 mOsm).

Pipettes were prepared by a puller (P-97, Sutter Instruments Company, Novato, CA) with a resistance of 3-5 MΩ coated with Sylgard (Dow Corning, Midland, MI). An EPC-10 amplifier controlled by Pulse software (HEKA Elektronik, Lambrecht, Germany) was used for recordings from IHCs visualized by BX-50WI (Olympus, Tokyo, Japan) with a 40X magnification by an objective lens (Olympus).

All voltages were corrected for liquid-junction potentials. Currents were sampled at 20 kHz and low-pass filtered at 2 kHz. Cells that displayed a leak current exceeding –30 pA were discarded from analysis. Ca<sup>2+</sup> currents were further isolated using a P/n protocol. Series resistance (R<sub>S</sub>) was below 30 MΩ. Patch-clamp data were analyzed with Igor software (Wavemetrics, Portland, OR).

### **2.5 Auditory brainstem responses (ABRs) and distortion-product otoacoustic emission (DPOAE)**

*RIM2α*<sup>-/-</sup> and *RIM2α*<sup>+/+</sup> mice (5-weeks-old to 8-weeks-old) were anesthetized by intraperitoneal injection of a combination of ketamine (100–125 μg/g) and xylazine (2.5–5 μg/g). The heart rate of anesthetized animals was constantly monitored by electrocardiogram (ECG). The body temperature was maintained constant at 37 °C using a rectal temperature-controlled heat blanket (Hugo Sachs Elektronik–Harvard Apparatus GmbH, Hugstetten, Germany). For stimulus generation, presentation, and data acquisition, we used the TDT II or III Systems (Tucker Davis Technologies, Alachua, FL) run by BioSig32 software (TDT) or MATLAB (Mathworks, Natick, MA) routines. Sound pressure levels are provided in decibels sound pressure level (SPL) rms (tonal stimuli) or decibels SPL peak equivalent (clicks) and were calibrated using a ¼ inch Brüel & Kjaer microphone (D 4039; Brüel & Kjaer, Nærum, Denmark). Stimuli were presented ipsilaterally in the free field using a JBL 2402 speaker (JBL, Northridge, CA).

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For recording ABRs, the difference potential between vertex and mastoid subdermal needles was amplified (50,000 times), filtered (low pass, 4 kHz; high pass, 100 Hz) and sampled at a rate of 50 kHz for 20 ms,  $2 \times 2000$  times to obtain two mean ABRs for each sound intensity. Hearing threshold was determined with 10 dB precision as the lowest stimulus intensity that evoked a reproducible response waveform in both traces by visual inspection.

For recording DPOAEs, a 24-bit sound card and the ED1/EC1 speaker system (Tucker David Technologies) were used to generate two primary tones  $f_1$  and  $f_2$  ( $f_2/f_1$  ratio: 1.2). Primary tones were coupled into the ear canal by a custom-made probe containing an MKE 2 microphone (Sennheiser, Barleben bei Magdeburg, Germany) and adjusted to an intensity of 60 dB sound pressure level at the position of the ear drum as mimicked in a mouse ear coupler. The microphone signal was amplified (DMP3; M-audio, Hallbergmoos, Germany) and analyzed by fast Fourier transformation.

### 2.6 Statistics

Means are presented with their standard errors and were statistically compared using Student's unpaired, two-tailed  $t$  test, unless otherwise noted.

## Results

### 3. Results

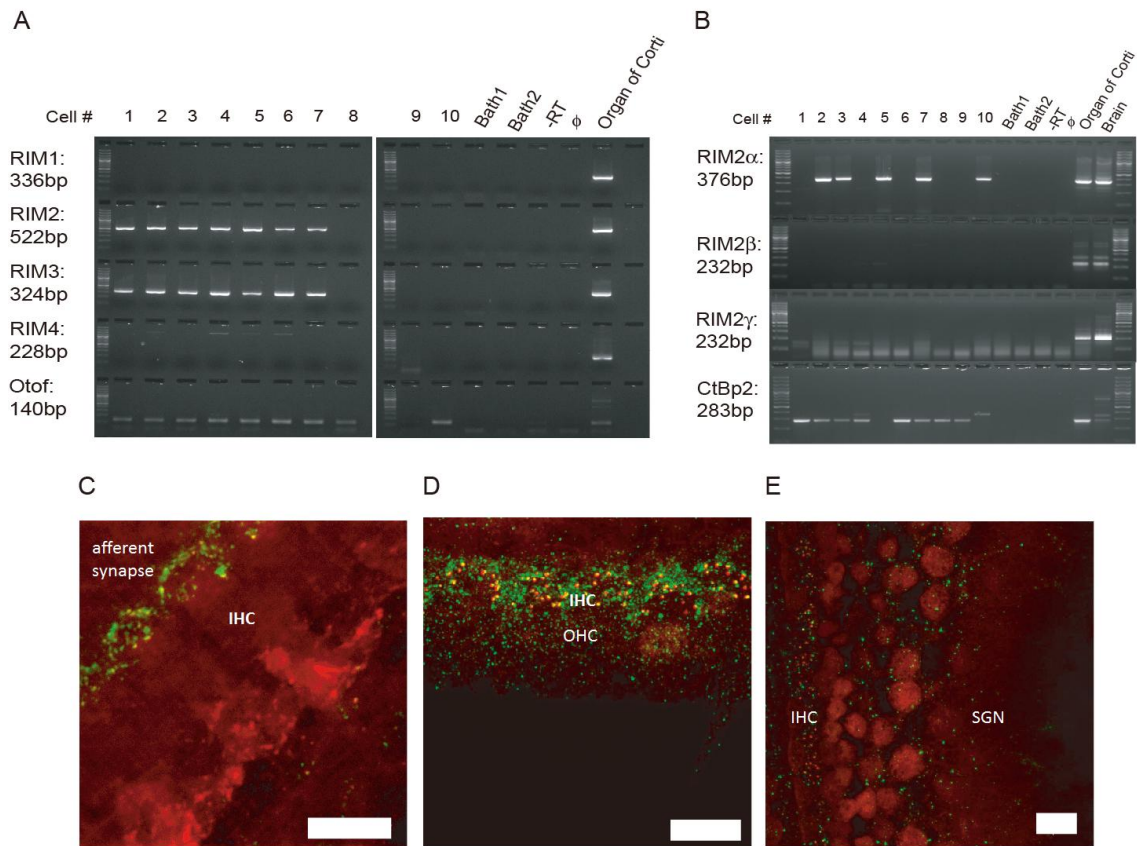
#### 3.1 Probing expression and function of RIM proteins in the IHCs

##### 3.1.1 Mature IHCs express RIM2 $\alpha$ and RIM3 $\gamma$ at their active zones after the onset of hearing

To detect Rab3-interacting molecules (RIMs) mRNAs in IHCs of hearing mice, I performed nested single cell RT-PCR in collaboration with Dr. Friederike Predöhl. Among the 10 IHCs examined, all cells expressed otoferlin (positive control), 7 cells expressed RIM2 and RIM3 mRNAs, but none contained RIM1 or RIM4. In contrast, nested RT-PCR analysis of the whole organ of Corti showed expression of all isoforms of RIMs (Figure 10A). To further clarify the isoform-specific expression of RIM2, we analyzed another 10 IHCs. Among them, 9 cells expressed CtBP2 (positive control), 5 cells expressed RIM2 $\alpha$  mRNAs, but none contained RIM2 $\beta$  or RIM2 $\gamma$  (Figure 10B). Taken together, these results suggest that the IHCs in hearing mice express the isoforms RIM2 $\alpha$  and RIM3 $\gamma$ .

To substantiate this finding, I performed immunohistochemical analysis of the expression of RIM2 $\alpha$  and RIM3 $\gamma$  on whole mounts of the organ of Corti from hearing mice using anti-RIM2 and anti-RIM3 $\gamma$  antibodies together with antibody directed against the nuclear protein CtBP2, which also detects RIBEYE, a major structural component of the synaptic ribbon (Schmitz et al., 2000, Khimich et al., 2005). As shown in Figure 10C-E, RIM2 and RIM3 $\gamma$  immunofluorescence was observed within and around the base of the IHCs. Specifically, we found RIM2 and RIM3 $\gamma$  immunofluorescence at CtBP2 labeled ribbon-type active zones, indicating that IHCs of hearing mice express RIM2 $\alpha$  and RIM3 $\gamma$  isoforms at their active zones. Additional RIM2 immunofluorescence outside the IHCs most likely represented RIM expression in efferent nerve terminals, which display small conventional active zones, as well as unspecific labeling.

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**Figure 10 Expression of RIM isoforms at the IHC ribbon synapse in hearing mice**

Expressions of RIM isoforms were detected by single cell nested RT-PCR (A and B) and immunohistochemistry (C, D, and E). (A) Expression of RIM isoforms in the C57BL/6 mouse after the hearing onset by single cell nested RT-PCR. Expected sizes of the PCR products are 336 bp for RIM1, 522 bp for RIM2, 324 bp for RIM3, 228 bp for RIM4, and 140 bp for Otoferlin, respectively. Otoferlin was used as a positive control. (B) Expressions of the RIM2 isoforms in IHCs of C57BL/6 mice. Only RIM2 $\alpha$  was detected in IHCs of C57BL/6 mice by single cell nested RT-PCR. The expected size of the PCR products are 376 bp for RIM2 $\alpha$ , 232 bp for RIM2 $\beta$ , 232 bp for RIM2 $\gamma$ , and 283 bp for CtBP2, respectively. PCR results were confirmed by sequencing. (C) Projection of confocal sections of C57BL/6 organ of Corti at P8. IHCs were immuno-labeled for ribbons (anti-CtBP2, red) and RIM2 (green). (D) Projection of confocal sections of C57BL/6 organ of Corti at P14. IHCs were immuno-labeled for ribbons (anti-CtBP2, red) and RIM2 (green). (E) Projection of confocal sections of

## Results

C57BL/6 organ of Corti at P14, C57BL/6. IHCs were immuno-labeled for ribbons (anti-CtBP2, red) and RIM3 $\gamma$  (green). Scale bar in C-E: 10  $\mu$ m.



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### 3.1.2 Disruption of RIM2 $\alpha$ reduces presynaptic Ca<sup>2+</sup> currents and exocytic membrane capacitance change

To probe the function of RIM2 $\alpha$  in the IHCs, I next performed perforated patch-clamp recordings on IHCs in RIM2 $\alpha$  knockout (*RIM2 $\alpha$ <sup>-/-</sup>*) mice and wild-type littermates (*RIM2 $\alpha$ <sup>+/+</sup>*). The mean peak amplitude of the Ca<sup>2+</sup> current was significantly smaller in *RIM2 $\alpha$ <sup>-/-</sup>* IHCs ( $-105.7 \pm 14.8$  pA,  $n = 5$ ) than in *RIM2 $\alpha$ <sup>+/+</sup>* IHCs ( $-157.2 \pm 15.3$  pA,  $n = 5$ ,  $p < 0.05$ , Figure 11A&B). This can result either from a reduced number of Ca<sup>2+</sup> channels or a reduced opening probability of Ca<sup>2+</sup> channels.

The exocytic membrane capacitance change ( $\Delta C_m$ ) evoked by 100 ms-long depolarizations, which is thought to reflect the number of synaptic vesicles fused during synchronous and sustained exocytosis, was also smaller in *RIM2 $\alpha$ <sup>-/-</sup>* IHCs (Figure 11C, D,  $42.8 \pm 14.3$  fF,  $n = 5$ ) than that in *RIM2 $\alpha$ <sup>+/+</sup>* IHCs ( $96.3 \pm 17.6$  fF,  $n = 5$ ,  $p < 0.05$ ).

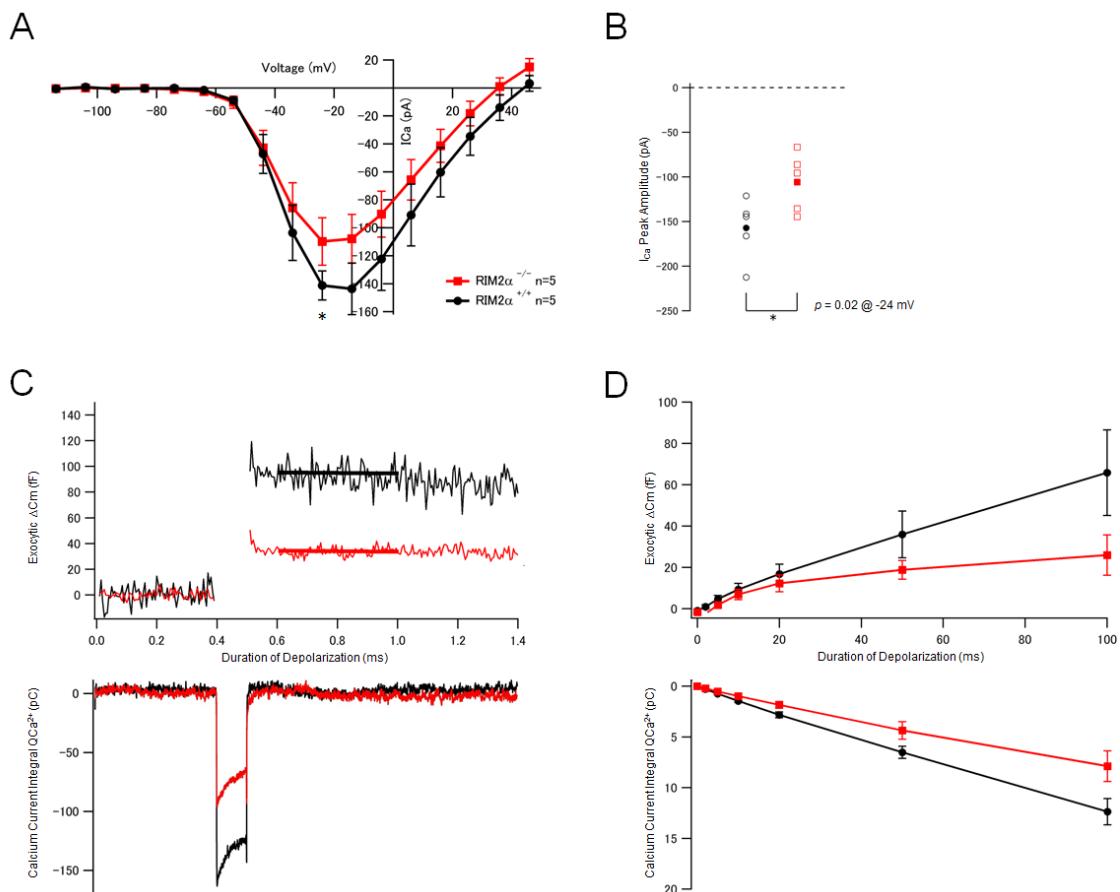


Figure 11 Patch-clamp analysis of presynaptic function of RIM2 $\alpha$ -deficient IHCs

## Results

*In vitro* perforated patch-clamp recording in *RIM2* $\alpha^{+/+}$  (black) and *RIM2* $\alpha^{-/-}$  (red) IHCs. All recording were done with P14-17 mice in the extracellular presence of 2 mM  $\text{Ca}^{2+}$ .

(A) Current-voltage relationship of *RIM2* $\alpha^{+/+}$  and *RIM2* $\alpha^{-/-}$  obtained from the initial 2-5 ms during 10 ms depolarizations.

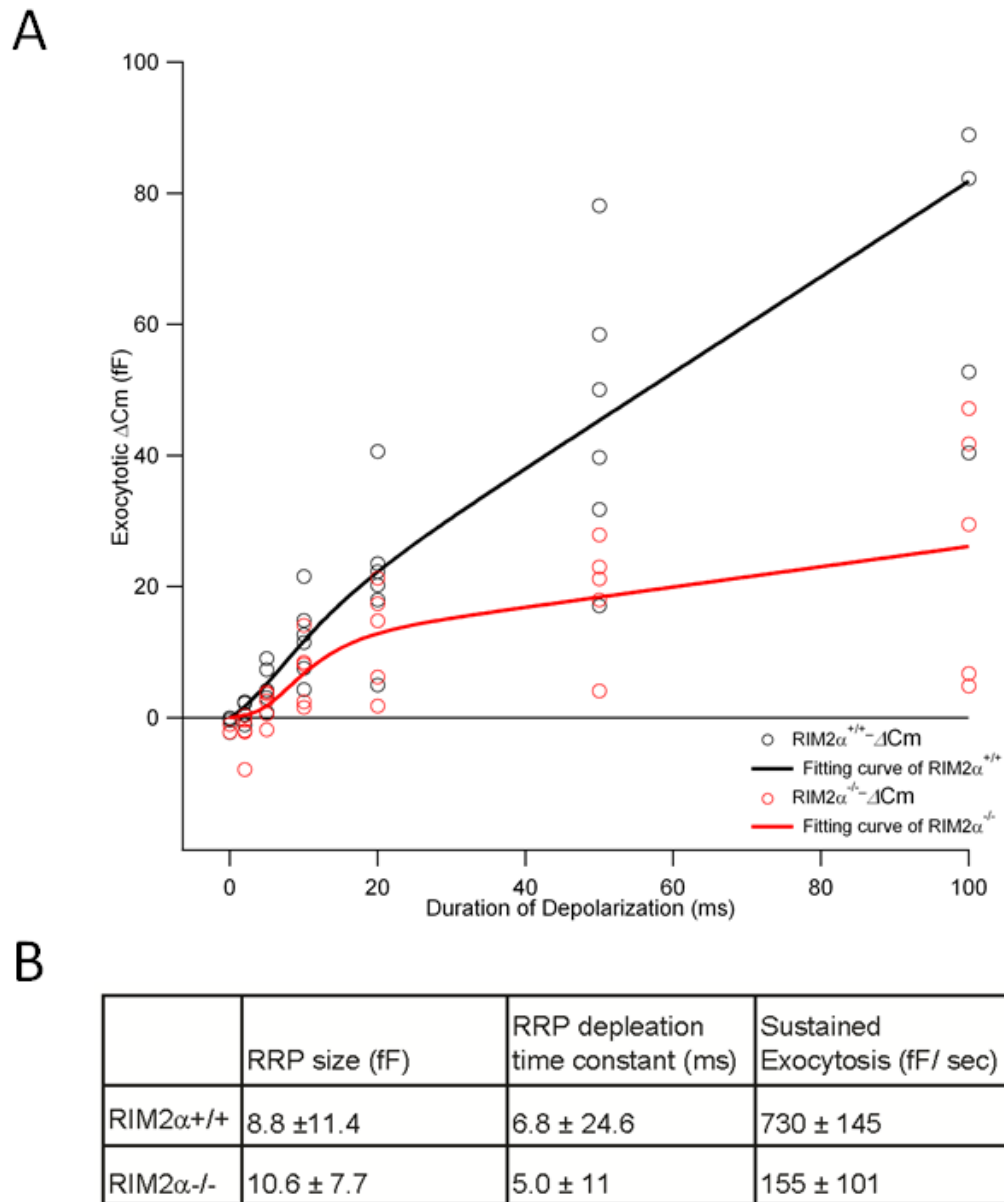
(B) The peak amplitude of presynaptic  $\text{Ca}^{2+}$  currents ( $I_{\text{Ca}}$ ) elicited by depolarization to -24 mV for *RIM2* $\alpha^{+/+}$  and *RIM2* $\alpha^{-/-}$ . Individual data points (open circles) and their averages (filled circles) are shown. Mean  $I_{\text{Ca}}$  peak amplitude was  $-157.17 \pm 15.26$  pA in *RIM2* $\alpha^{+/+}$  ( $n = 5$ ),  $-105.66 \pm 14.84$  pA in *RIM2* $\alpha^{-/-}$  ( $n = 5$ ).  $*p = 0.02$ .

(C) Sample traces of  $I_{\text{Ca}}$  and exocytic capacitance jump ( $\Delta C_m$ ) at 100 ms depolarization in *RIM2* $\alpha^{+/+}$  and *RIM2* $\alpha^{-/-}$ , lines indicate the average  $C_m$  before and after the depolarization

(D) Summary of integral of  $\text{Ca}^{2+}$  current ( $Q_{\text{Ca}}$ ) and  $\Delta C_m$  for depolarizing pulses of various durations. In *RIM2* $\alpha^{-/-}$ , both  $Q_{\text{Ca}}$  and  $\Delta C_m$  were significantly reduced compared to *RIM2* $\alpha^{+/+}$ . ( $p < 0.05$ ).

I analyzed the responses to depolarizations of different durations (Figure 11D) to further investigate the defect of exocytosis and fitted the cumulative exocytosis-time function by the sum of an exponential (raised to a power to accommodate the supralinear rise) and a linear term (Figure 12A). The size of the RRP was approximated as the amplitude of the initial exponential term (fast component), while sustained exocytosis was quantified as the slow linear term (slow component). The RRP size and kinetics of RRP fusion were not significantly different between IHCs of both genotypes (Figure 12B), arguing against a substantial role of *RIM2* $\alpha$  for establishing/stabilizing vesicular release sites and their close spatial coupling to  $\text{Ca}^{2+}$  channels at IHC active zones. However, the sustained exocytosis rate was reduced from  $730 \pm 145$  fF/sec for *RIM2* $\alpha^{+/+}$  IHCs to  $155 \pm 101$  fF/sec for *RIM2* $\alpha^{-/-}$  IHCs ( $p < 0.05$ ). This result indicated that *RIM2* $\alpha$  is essential for synaptic vesicle replenishment at the IHC ribbon synapse.

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**Figure 12 Estimation of vesicle pool size and dynamics**

(A) Fitting of  $\Delta C_m$  (t) relationship using the following function: for estimation of RRP size, RRP depletion time constant and sustained exocytosis rate. Individual data points represent averages for each cell.

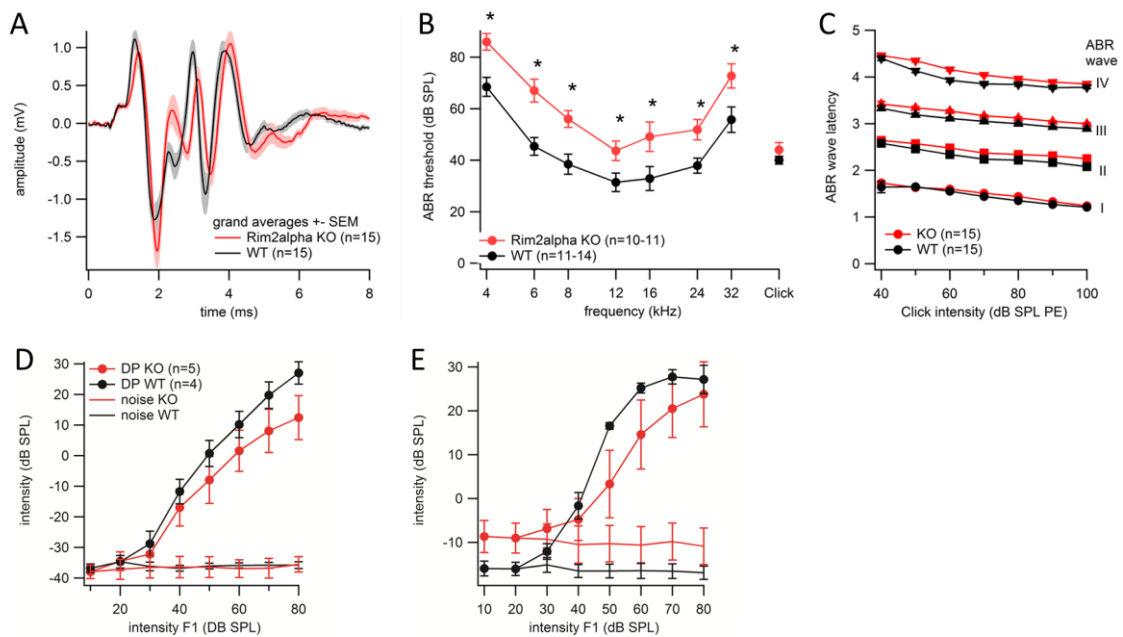
(B) The table for values obtained from fitting of  $\Delta C_m$  (t). Means  $\pm$  SEMs are shown.

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### 3.1.3 Auditory systems consequences of the disruption of RIM2 $\alpha$

Finally, we tested the hearing capability of *RIM2 $\alpha$ <sup>-/-</sup>* mice by measuring auditory brainstem responses (ABRs). Mice were anesthetized intraperitoneally with either ketamine plus xylazine or urethane plus xylazine, and the data was pooled because no significant difference in ABR thresholds was detected between ketamine/xylazine anesthetized group and urethane/xylazine anesthetized group. In ABR the first wave (wave I) is a representation of the compound action potential in the spiral ganglion neurons. Then the wave II is primarily generated by glubular cells in the cochlear nucleus (CN). The wave III is partly generated by spherical cells in the CN and partly by their targeting cells. The waves IV and V are generated by MSO principal neurons. (Melcher JR and Kiang NY. 1996c). Despite of unaltered amplitude of waves that were elicited by suprathreshold stimuli (Figure 13A), *RIM2 $\alpha$ <sup>-/-</sup>* mice showed higher thresholds than *RIM2 $\alpha$ <sup>+/+</sup>* mice at all frequencies examined (Figure 13B) as well as prolonged latencies to peaks of individual waves except for wave I (Figure 13C). DPOAE, which the active amplification by OHCs yields, were normally detected in *RIM2 $\alpha$ <sup>-/-</sup>* IHCs (Figure 13D). These results suggest that RIM2 $\alpha$  is necessary for normal hearing, but it is currently unclear whether or how much it can be attributed to the presynaptic deficit observed in IHCs *in vitro*, since neither amplitude nor latency of wave I was significantly altered in *RIM2 $\alpha$ <sup>-/-</sup>* mice. Altered synaptic transmission in the auditory brainstem might predominantly underlie the threshold shift observed in *RIM2 $\alpha$ <sup>-/-</sup>* mice.

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**Figure 13 The auditory brainstem response and the distortion product of otoacoustic emission from  $RIM2\alpha$  knockout mice**

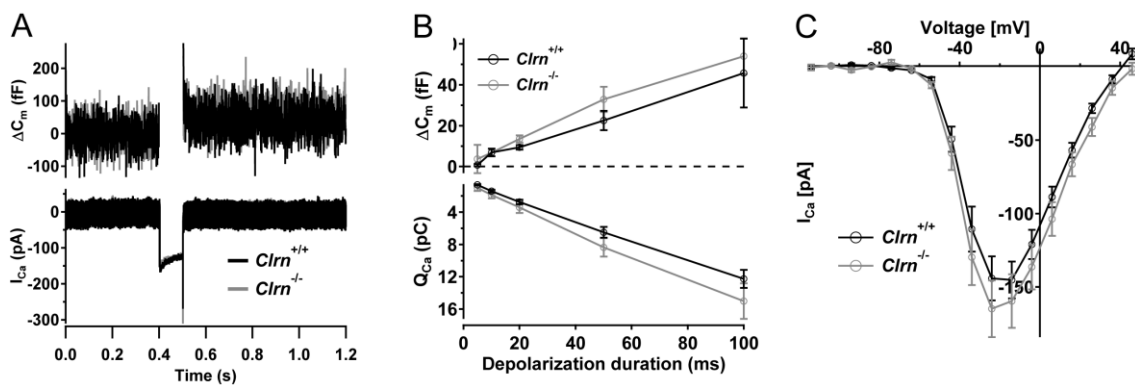
- (A) Auditory brainstem response (ABR) evoked by 80 dB clicks in  $RIM2\alpha^{+/+}$  (black) and  $RIM2\alpha^{-/-}$  (red) mice.
- (B) ABR audiograms obtained by tone burst or click stimulation in  $RIM2\alpha^{+/+}$  and  $RIM2\alpha^{-/-}$  mice. Asterisks (\*) show significant difference ( $p < 0.05$ ).
- (C) Latency to peak of the each wave at various sound pressure levels.  $RIM2\alpha^{-/-}$  mice showed significantly longer latencies to wave peak in all waves except for wave I.
- (D, E) Input-output relationship of DPOAE at 12 kHz (D) and 16 kHz (E) tone bursts derived from  $RIM2\alpha^{+/+}$  and  $RIM2\alpha^{-/-}$  mice. No significant difference.

### 3.2 Probing presynaptic function of clarin-1 at the IHC ribbon synapse

The elevated ABR thresholds in clarin-1 knockout ( $Clrn1^{-/-}$ ) mice shown by a previous study (Geng et al., 2009) and reports on a presumptive IHC synaptic phenotype in  $Clrn1^{-/-}$  mice (Bitner-Glindzicz et al., 2000; Zallochi et al., 2009) motivated our investigation of their presynaptic function in IHCs. Our collaborators in parallel studied

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mechanoelectrical transduction at hair bundles and assessed the number of ribbon synapses (Geng et al., 2012). I explored the physiological impact of the null mutation in clarin-1 on exocytic membrane capacitance changes ( $\Delta C_m$ ) in response to  $Ca^{2+}$  currents ( $I_{Ca}$ ) evoked by step depolarizations by perforated-patch recordings from IHCs in hearing mice (P14-20). I found that neither the integral of  $Ca_v1.3$ -mediated  $Ca^{2+}$  currents upon depolarization nor corresponding  $\Delta C_m$  was different between  $Clrn1^{-/-}$  and  $Clrn1^{+/+}$  IHCs (Figure 14C). In summary,  $Clrn1^{-/-}$  IHCs showed normal  $I_{Ca}$  and  $\Delta C_m$  in response to hair cell depolarizations. These results argue against an essential function of clarin-1 at the IHC ribbon synapse.



**Figure 14 Patch-clamp analysis of presynaptic function of clarin-1 at the IHC ribbon synapse**

(A) Representative exocytic membrane capacitance changes ( $\Delta C_m$ , top) and  $Ca^{2+}$  currents ( $I_{Ca}$ , bottom) in response to 50 ms depolarization from a resting potential of -84 mV to -14 mV in  $Clrn1^{-/-}$  (grey) and  $Clrn1^{+/+}$  (black) IHCs.

(B) Grand average  $\Delta C_m$  and  $Q_{Ca}$  for step depolarizations to 14 mV of variable durations. All responses are given as grand averages (calculated from the means of the individual cells)  $\pm$  SEM.

(C) Current-voltage relationship for  $Ca_v1.3$   $Ba^{2+}$  currents in control (black,  $n=12$  IHCs) and mutant ( $n=10$  IHCs) IHCs. Currents were evoked by 10 ms step depolarizations from a resting potential of -84 mV to variable potentials and their peak current was plotted against test potential.

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#### 4.1 RIM2 $\alpha$ regulates L-type Ca<sup>2+</sup> current and Ca<sup>2+</sup>-triggered exocytosis at the IHC ribbon synapse

I demonstrated that the IHCs of hearing mice express RIM2 $\alpha$  and RIM3 $\gamma$ , but not other isoforms of RIMs, at their active zones and that RIM2 $\alpha$  plays a regulatory role in synaptic transmission at the IHC ribbon synapse. This study, for the first time, demonstrates functional roles of RIM2 $\alpha$ , after previous studies failed to assign any roles to it (Schoch et al., 2006). Strikingly, the IHC ribbon synapse and central synapses do not share common isoform(s) of  $\alpha$ -RIMs to control Ca<sup>2+</sup>-triggered release, presumably due to their different origins (sensory epithelium versus neuron). This is in line with other previous findings, which show that the IHC ribbon synapse differs from other synapses in some respects. In addition to using Cav1.3 L-type Ca<sup>2+</sup> channels for secretion control, the IHCs likely utilize Otoferlin instead of the conventional Ca<sup>2+</sup> sensor synaptotagmins to mediate Ca<sup>2+</sup>-regulated vesicle fusion (Roux et al., 2006) and vesicle replenishment (Pangršič et al., 2010). Moreover, the IHC ribbon synapse seems to operate without neuronal SNARE proteins (Nouvian et al., 2011). Thus, the IHC ribbon synapse operates in a specialized manner so that it can keep pace with submillisecond-order stimuli, in order to perform fast and faithful sound encoding (for review, see Pangršič et al., 2012).

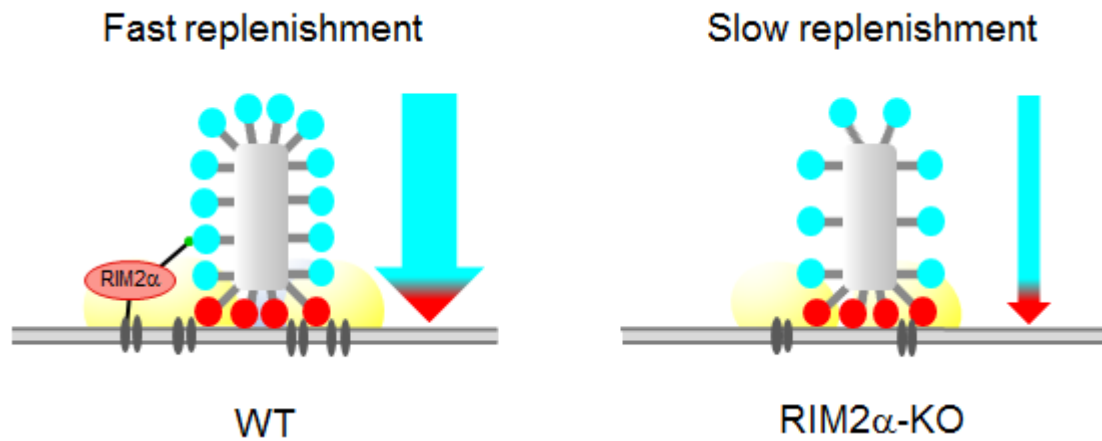
Previous work studied the functions of RIMs at conventional active zones using conventional or conditional knockout mouse lines. At the hippocampal CA3-CA1 synapse of RIM1 $\alpha$  knockout mice, Ca<sup>2+</sup>-triggered release was reduced (Calakos et al., 2004) and short-term synaptic plasticity was altered (Schoch et al., 2002). Deletion of RIM1 $\alpha$  reduced release probability in excitatory synapses in the amygdala (Fourcaudot et al., 2008) just like it did in the inhibitory synapses in the hippocampus (Kaeser et al., 2008a). In the absence of RIM1 $\alpha$ , LTP was abolished in the excitatory synapses in the hippocampus (Castillo et al., 2002; Huang et al., 2005; Pelkey and McBain, 2008) and the cerebellum (Castillo et al., 2002; Lonart et al., 2003; Simsek-Duran et al., 2004) as well as it was in the inhibitory synapses in the cerebellum (Lachamp et al., 2009). LTD was also abolished in inhibitory synapses in the hippocampus and the amygdala

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(Chevaleyre et al., 2007). Kaeser et al. (2008a) showed that double-knockout of RIM1 $\alpha$  and RIM1 $\beta$  reduced release probability in the CA3-CA1 synapse and further abolished LTP in the DG-CA3 synapse and LTD in the inhibitory interneuron synapses of the hippocampus. Moreover, double-knockout of RIM1 $\alpha$  and RIM2 $\alpha$  reduced the amplitude and increased failure rate of evoked release at the embryonic neuromuscular junction (Schoch et al., 2006). Furthermore, conditional double knockout of RIM1 $\alpha$  and RIM2 $\alpha$  disrupted synaptic transmission by decreasing Ca<sup>2+</sup> channel density and number of docked and readily releasable vesicles at the active zone of the calyx of Held synapse in the auditory brainstem (Han et al., 2011). As already stated, knockout of RIM2 $\alpha$  alone caused no synaptic deficits (Schoch et al., 2006).

In this study, however, I demonstrated that RIM2 $\alpha$ , as a key player, regulates the Ca<sub>v</sub>1.3-mediated Ca<sup>2+</sup> current and synaptic vesicle replenishment at the IHC ribbon synapse (Figure 15). I postulate that the reduced Ca<sub>v</sub>1.3 Ca<sup>2+</sup> current reflects a reduction in the number of Ca<sup>2+</sup> channels, which also impairs synaptic Ca<sup>2+</sup> influx. This is consistent with findings at the calyx of Held (Han et al., 2011) and excitatory synapses of hippocampal neurons in culture (Kaeser et al., 2011). While not yet tested by electron microscopy, I conclude from the unaltered RRP size that the number of membrane-proximal vesicles is not changed in the absence of RIM2 $\alpha$ . Thereby the IHC afferent synapse deviates from the calyx of Held synapse, which showed a reduced RRP in the absence of  $\alpha$ -RIMs (Han et al., 2011). Moreover, my finding of an impaired vesicle replenishment reports a yet unknown RIM function.





**Figure 15 Schematic representation of putative functions of RIM2 $\alpha$  at the IHC ribbon synapse**

Membrane-associated vesicles (red), ribbon tethered vesicles (blue) are shown. The width of arrows indicates the rate of synaptic vesicle replenishment. It is postulated that RIM2 $\alpha$ -knockout IHCs show a normal number of membrane-associated vesicles, but reduced density of Ca<sub>v</sub>1.3-type Ca<sup>2+</sup> channels (black) and slower replenishment of synaptic vesicles (arrows). Yellow clouds around the Ca<sup>2+</sup> channels shows Ca<sup>2+</sup> nanodomains for triggering synaptic vesicle fusion. RIM2 $\alpha$  might interact with Ca<sub>v</sub>1.3-type Ca<sup>2+</sup> channels as well as synaptic vesicles via Rab3 (green).

#### **4.2 Discrepancy between the *in vitro* finding of impaired vesicle replenishment with reduced Ca<sup>2+</sup> currents and the *in vivo* finding of relatively intact auditory brainstem responses**

Table 3 is a summary of the exocytic parameters obtained by presynaptic capacitance recordings from IHCs in RIM2 $\alpha$  knockout mice, Bassoon mutant mice with a deletion of exon 4/5 (*BSN* <sup>$\Delta$ Ex4/5</sup>, Khimich et al., 2005), and Otoferlin mutant mice with a single amino acid substitution of D1767G in the C<sub>2</sub>F domain (*Pachanga*, Pangršič et al., 2010).

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	RRP size (fF)	RRP depletion time constant (ms)	Sustained exocytosis (fF/sec)	Ref
<i>RIM2α</i> <sup>+/+</sup>	8.8	6.8	730	this study
<i>RIM2α</i> <sup>-/-</sup>	10.6	5.0	155	
<hr/>				
<i>BSN</i> <sup>wt</sup>	15-18	8-9	265	Khimich et al., 2005
<i>BSN</i> <sup>ΔEx4/5</sup>	5	11	?	
<hr/>				
<i>Otof</i> <sup>+/+</sup>	7.0	5.2	391	Pangršič et al., 2010
<i>Otof</i> <sup>Pga/Pga</sup>	9.4	3.9	98	

**Table 3 Quantification of exocytosis in *RIM2α*, Bassoon, and Otoferlin mutant IHCs**

The *RIM2α*<sup>-/-</sup> and *Otof*<sup>Pga/Pga</sup> IHCs share a common phenotype of slower sustained exocytosis with intact RRP size and RRP depletion rate. The slower sustained rate in both mutants is compatible with their roles in synaptic vesicle replenishment potentially related to priming or active zone clearance. The intact RRP size and RRP depletion time constant in the *RIM2α*<sup>-/-</sup> IHCs suggest that *RIM2α* is not essential for docking and fusion of synaptic vesicles at the active zones of IHC ribbon synapse. In contrast, the *BSN*<sup>ΔEx4/5</sup> IHCs have a unique phenotype of reduced synaptic complement of Ca<sup>2+</sup> channels, fewer docked vesicles resulting in a reduced RRP size and slower sustained exocytosis (Khimich et al., 2005; Frank et al., 2010). Finding impaired sustained exocytosis in *RIM2α* and otoferlin mutants is interesting also because those mice show very different hearing. While ABRs are relatively maintained in *RIM2α*<sup>-/-</sup>, the *Pachanga* mice essentially lack ABRs. The difference could be a quantitative one: the rate of sustained exocytosis of *RIM2α*<sup>-/-</sup> IHCs was almost twice as high as in the *Pachanga* IHCs. This might be caused by higher probability of spike generation in the type I spiral ganglion neurons at the *RIM2α*<sup>-/-</sup> IHC afferent synapse due to following mechanisms: down-regulated K<sup>+</sup> channels leads enhanced excitability of afferent

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dendrites, and/or impaired developmental pruning of synaptic contacts (for review, see Bulankina et al., 2012). Additional works on single auditory nerve fiber responses and synaptic ultrastructure in  $RIM2\alpha^{-/-}$  will be needed to further clarify the issue.

Finally, a recent study has shown that  $\gamma$ -RIMs are expressed in a variety of neurons in the cortex, cerebellum, olfactory bulb, and retina. Remarkably,  $RIM3\gamma$ , but not  $RIM4\gamma$ , exhibits a synaptic expression pattern. In contrast,  $RIM4\gamma$  is ubiquitously expressed along axons and dendrites. Despite the differential localization, either of them controls the neuronal branching. With regard to the synaptic function of  $\gamma$ -RIMs, their knock-down decreases miniature EPSC amplitude recorded from primary cortical neurons (Fuentes, 2010). Thus,  $\gamma$ -RIMS might play substantial roles at other synapses including the cochlear hair cell synapses. However, further insight into the function of  $\gamma$ -RIMs at the IHC ribbon synapse is out of scope of the current study.

### **4.3 Clarin-1 is dispensable for the ribbon synapse development and function**

In the present study, I provided evidence against a function of clarin-1 at the IHC ribbon synapse in hearing mice. Although previous studies predicted its involvement in hair cell synapse function (Adato et al., 2002, Geng et al., 2009, Zallocchi et al., 2009 and 2012), I found no evidence of synaptic dysfunction in the absence of clarin-1. The  $Clrn1^{-/-}$  mouse cochlea (Zallocchi et al., 2012) exhibited no morphological changes in the development of the IHC ribbon synapse (present study). Moreover,  $Clrn1^{-/-}$  IHCs did not show larger  $Ca^{2+}$  currents than wild-type controls, which was observed in athyroid IHCs at the same age (Sendin et al., 2007). These results further argue against a potential role of clarin-1 in the ribbon synapse development. Besides, a recent study ruled out an involvement of clarin-1 in photoreceptor function (Geller et al., 2009). Taken together, these sets of evidence suggest that clarin-1 is dispensable for ribbon synapse development and function.

In contrast, our collaborators have shown that clarin-1 is indispensable for development or maintenance of the hair bundles (Geng et al., 2012), as was indicated by preceding studies (Geng et al., 2009; Tian et al., 2009). The reduced amplitude of MET channel-mediated currents due to the disrupted hair bundles in  $Clrn1^{-/-}$  mice likely

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underlies elevated thresholds and delayed latencies of peaks in ABRs. Thus, hearing loss in the USH type III mouse model is caused by a hair bundle deficit.

The cochlear implant, the only treatment for USH at present, showed satisfying outcomes in Finnish USH3 patients (Pietola et al., 2012). This also supports the fact that USH3 patients have no major synaptic deficits in the auditory ascending pathways after the SGNs, which the electrodes directly stimulate. Restoration of hair bundle function by gene therapy in USH3 patients would promise faithful sound encoding through the intact IHC ribbon synapse.

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### Abbreviations

ABR	Auditory brainstem response
ACh	Acetylcholine
BK channel	Large conductance voltage and $\text{Ca}^{2+}$ activated $\text{K}^+$ channel
$C_m$	Membrane capacitance
$\Delta C_m$	Membrane capacitance increment
CN	Cochlear nucleus
CtBP	C-terminal binding protein
DPOAE	Distortion-product otoacoustic emission
EGTA	Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid
GluR	Glutamate receptor
GSDB	Goat serum diluted buffer
GTP	Guanosine 5'-triphosphate
HEPES-HBSS	HEPES-buffered Hanks' balanced salts solution
IBa	$\text{Ba}^{2+}$ current
ICa	$\text{Ca}^{2+}$ current
IV	Current-voltage relationship
IHC	Inner hair cell
LOC	Lateral olivocochlear
MET	Mechanoelectrical transduction
MOC	Medial olivocochlear
MSO	Medial superior olive
OHC	Outer hair cell
PBS	Phosphate-buffered saline
PSTH	Poststimulus time histogram
RIM	Rab3 interacting molecule
RRP	Readily releasable pool
RS	Series resistance
RT-PCR	Reverse transcription polymerase chain reaction

## Abbreviations

SGN	Spiral ganglion neuron
SK2	Small conductance Ca <sup>2+</sup> activated K <sup>+</sup> channel
SNARE	Soluble NSF attachment protein receptor
SOC	Superior olivary complex
SPL	Sound pressure level
TEA-Cl	Tetraethylammonium chloride
VGCC	Voltage-gated Ca <sup>2+</sup> channel
WT	Wild-type

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