Rab Interacting Molecules 2 and 3 Directly Interact with the Pore-Forming CaV1.3 Ca\(^{2+}\) Channel Subunit and Promote Its Membrane Expression

Maria M. Picher\(^{1,2,3}\), Ana-Maria Oprisoreanu\(^4\), SangYong Jung\(^{1,2,5}\), Katrin Michel\(^4\), Susanne Schoch\(^4\)* and Tobias Moser\(^{1,2,3,6}\)*

\(^1\)Institute for Auditory Neuroscience and InnerEarLab, University Medical Center Göttingen, Göttingen, Germany, \(^2\)Synaptic Nanophysiology Group, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, \(^3\)Göttingen Graduate School for Neurosciences and Molecular Biosciences, University of Göttingen, Göttingen, Germany, \(^4\)Institute of Neuropathology and Department of Epileptology, University of Bonn, Bonn, Germany, \(^5\)Neuro Modulation and Neuro Circuity Group, Singapore Bioimaging Consortium (SBIC), Biomedical Sciences Institutes, Singapore, Singapore, \(^6\)Collaborative Research Center 889, University of Göttingen, Göttingen, Germany

Rab interacting molecules (RIMs) are multi-domain proteins that positively regulate the number of Ca\(^{2+}\) channels at the presynaptic active zone (AZ). Several molecular mechanisms have been demonstrated for RIM-binding to components of the presynaptic Ca\(^{2+}\) channel complex, the key signaling element at the AZ. Here, we report an interaction of the C\(_2\)B domain of RIM2\(\alpha\) and RIM3\(\gamma\) with the C-terminus of the pore-forming \(\alpha\)-subunit of CaV1.3 channels (CaV1.3\(\alpha1\)), which mediate stimulus-secretion coupling at the ribbon synapses of cochlear inner hair cells (IHCs). Co-expressing full-length RIM2\(\alpha\) with a Ca\(^{2+}\) channel complex closely resembling that of IHCs (CaV1.3\(\alpha1\)-CaV\(\beta2\a\)) in HEK293 cells doubled the Ca\(^{2+}\)-current and shifted the voltage-dependence of Ca\(^{2+}\) channel activation by approximately +3 mV. Co-expression of the short RIM isoform RIM3\(\gamma\) increased the CaV1.3\(\alpha1\)-CaV\(\beta2\a\)-mediated Ca\(^{2+}\)-influx in HEK293 cells, but disruption of RIM3\(\gamma\) in mice left Ca\(^{2+}\)-influx in IHCs and hearing intact. In conclusion, we propose that RIM2\(\alpha\) and RIM3\(\gamma\) directly interact with the C-terminus of the pore-forming subunit of CaV1.3 Ca\(^{2+}\) channels and positively regulate their plasma membrane expression in HEK293 cells.

Keywords: active zone, ribbon synapse, hair cell, channel clustering, exocytosis, hearing

INTRODUCTION

Ca\(^{2+}\)-influx through voltage-gated Ca\(^{2+}\) channels triggers the fusion of synaptic vesicles at the presynaptic active zone (AZ). The molecular mechanisms regulating the number and function of presynaptic Ca\(^{2+}\) channels are only partially understood but thought to involve presynaptic multidomain proteins such as Rab3 interacting molecule (RIM; Coppola et al., 2001; Kiyonaka et al., 2007; Han et al., 2011; Kaeser et al., 2011; Jung et al., 2015), RIM-binding protein (Liu et al., 2011; Acuna et al., 2015; Li and Kavalali, 2015; Müller et al., 2015) and Bassoon (Frank et al., 2010; Davydova et al., 2014). Four genes (RIMS1–4) encode the seven members of the RIM protein family.
(RIM1α, β; RIM2 α, β, γ; RIM3γ and RIM4γ), all exhibiting a C-terminal Ca2⁺B domain, while their complement of further domains differs. The long RIM isoforms (RIM1α, β; RIM2α, β) contain an additional Ca2⁺A domain, a PDZ domain, a zinc-finger domain and, for the α-isofoms, an N-terminal α-helix (Wang and Südhof, 2003). RIM1/2 interact with the pore-forming Ca2⁺-domain and, for the auxiliary β (CaVβ) subunit (Kiyonaka et al., 2007; Gebhart et al., 2010; Gandini et al., 2011) as well as to the “synaptic protein interaction” motif (synprint motif; cytoplasmic domain) indirectly linked to Ca2⁺-channels (Hibino et al., 2002; Kaeser et al., 2011). The extent of this regulation depended on the respective Ca2⁺-isoforms of CaV2.3 channels (Neef et al., 2009; Altier et al., 2011; Dolphin, 2012; Hoppa et al., 2012; Fell et al., 2016; Wang et al., 2016). In IHCs, for example, CaV2.2α is critical for establishing sufficient membrane expression of CaV1.3 (Neef et al., 2009) that mediates more than 90% of the IHC Ca2⁺-influx (Platzer et al., 2000; Brandt et al., 2003; Dou et al., 2004). However, despite the likely prevailing role of palmitoylated CaV2.2α in IHCs that occludes effects of RIM2 on CaV1.3 channels in heterologous expression systems (Gebhart et al., 2010), a dramatic loss of Ca2⁺-channels upon genetic disruption of RIM2 was observed in IHCs (Jung et al., 2015). Therefore, we reasoned that RIM2 might employ mechanisms beyond the CaVβ interaction to promote the large complement of synaptic Ca2⁺ channels in IHCs. Specifically, we were interested to explore whether RIM2 could directly interact with the CaV1.3α1 subunit. However, CaV1.3α1 neither contains the C-terminal PDZ-binding motif for the interaction with RIM1/2 PDZ-domains (Kaeser et al., 2011; DDWC (CaV2.1); DHWC (CaV2.2); DDKC (CaV2.3) vs. ITTL (CaV1.3), which binds other PDZ domain proteins of IHCs such as harmonin (Gregory et al., 2011)) nor a synprint motif, which was also found at IHC ribbon synapses (Jung et al., 2015) and the function of which at the presynaptic AZ has remained elusive. Here, we combined biochemical, physiological and morphological approaches to further investigate the interplay of RIMs and the CaV1.3 channel complex.

**MATERIALS AND METHODS**

**Animals**

Knock-out mice for RIM3γ were generated utilizing ES cells produced by the international Knockout Mouse Project (KOMP) consortium (Rims3tm1a(KOMP)Wtsi; ES cell line JMEA3.1; targeting project CDS34392). The line obtained after germ line transmission constitutes a “knock-out first” allele, in which insertion of a splice acceptor-lacZ gene trap cassette disrupts the endogenous RIM3γ transcript resulting in a constitutive knock-out (RIM3γ−/−). ES cells were injected into Balb/c mice. The resulting chimeric mice were monitored by coat color and genotyped by PCR. The following primers were used for the RIM3γ−/− line: RIM3γ 5’-GGACCCACACTGCAATGCTAA-3’ and 5’-CCCTTCAGCTTCTCTGTCCA-3’ product size 618 base pairs; RIM3γ+/+ 5’-GGACCCACACTGCAATGCTAA-3’ and 5’-ACCAGACTCCAAAGCCCTC-3’ product size 324 base pairs. All analyses were carried out with littersmates of heterozygous matings. In all animal experiments knock-out animals were compared to littermate controls, respectively. All experiments were performed in compliance with the national animal care guidelines and were approved by the board for animal welfare of the University Medical Center Göttingen, the University of Bonn and the animal welfare office of the state of Lower Saxony and North Rhine-Westphali.

**mRNA Isolation and cDNA Synthesis**

Total mRNA was obtained from microdissected mouse brain tissue using Dynabeads mRNA DIRECT Micro Kit according to the manufacturer’s (Life Technologies) instructions. cDNA was synthesized from purified mRNA by reverse transcription using the RevertAidH Minus Strand cDNA Synthesis Kit (Fermentas) and compromised oligo dT primers according to the manufacturer’s manual. cDNA samples were stored at −20°C. For quantitative real time PCR the Maxima Probe/Rox qPCR Master Mix (Thermo Fischer) together with Taqman gene expression assays (Applied Biosystem) was used according to the following protocol: experiments were performed in triplicates on an ABI Prism 9700HT system (PE Applied Biosystems, Foster City, CA, USA). Gene expression was analyzed as relative gene expression in comparison to the internal reference gene synaptophyisin. Therefore gene expression was calculated as 2−Δct (D cycle threshold value (ct) = ct of the analyzed gene − ct synaptophyisin).
Preparation of Protein Homogenates and Immunoblotting

Cell lysates from brain tissue were prepared from microdissected brain areas. Directly after preparation tissue samples were frozen in liquid nitrogen and either stored in −80°C or used directly. The frozen tissue samples were homogenized in 2 ml/mg tissue phosphate buffered saline pH 7.4 containing protease inhibitor cocktail (cOmplete, Roche) with the help of a tissue grinder. Cells in the homogenized tissue samples were lysed by adding 6× Laemmlı buffer (TRIS-hydrochlorid 378 mM, 30% glycerol, 12% SDS and 0, 06% Bromphenolblue, 10% β-mercaptoethanol) to the samples and a 1–5 min incubation. Proteins were denatured at 95°C for 5 min.

HEK293 cells were lysed in phosphate buffered saline pH 7.4 containing protease inhibitor cocktail (cOmplete, Roche) and 1% triton X-100. The lysis reaction was incubated 1 h at 4°C under rotation. After the lysis protein lysates were separated to the samples and a 1–5 min incubation. Proteins were denatured at 95°C for 5 min.

Protein homogenates were separated by SDS polyacrylamide gel electrophoresis (SDS PAGE) and blotted to nitrocellulose membrane overnight. Membranes were incubated 1.5 h in blocking solution of either 5% fish gelatin in PBS to avoid unspecific binding of antibodies and overnight at 4°C with a polyclonal antibody against RIM3y (1:100; cite Alvarez-Baron et al., 2013) and a monoclonal antibody against β-tubulin (1:10,000; BD Pharmigen). Antibody staining was visualized by incubation with IRDye anti rabbit 680 nm IgG and IRDye 800-anti mouse IgG (LI-COR) in a dilution of 1:20,000 for 1 h and an infrared scanning system (Odyssey, Licom). Quantification of western blots was carried out using the analyze gels plugin of the FIJI software.

Co-Immunoprecipitation

HEK293T cells were plated at a density of 1.5 × 10^5 cells/dish and co-transfected (Ca^{2+}-phosphate method) with the following plasmids: full-length untagged RIM2a and the HA-Cav.1.3 (aa 1509–2203), ZF-PDZ domain of RIM2a and HA-Cav.1.3 and C2A-C2B domain of RIM2a and HA-Cav.1.3. Forty-eight hours post-transfection cells were lysed for 1 h in ice cold lysis buffer (50 mM HEPES pH: 7.5, 150 mM NaCl, 1% Triton X-100) supplemented with proteinase inhibitors (Roche), followed by a short centrifugation step at 14,000 rpm/10 min/4°C. The clear supernatant was incubated for 2 h/4°C with HA-magnetic beads (Pierce) on a rotator. After the incubation time, beads were extensively washed with PBS-0.5% Triton X-100 buffer and boiled at 95°C/5 min in Laemmlı buffer supplemented with β-ME. Proteins were resolved in SDS-PAGE gel (8%), followed by the protein transfer to the nitrocellulose membrane (Millipore). The detection of the proteins was performed using primary antibodies anti-mouse HA (Covance; 1:1000), anti-rabbit RIM1/2 (1:1000; provided by Frank Schmitz), followed by secondary antibodies IRDye 1:10,000 (goat anti-mouse 800 and goat anti-rabbit 680). The detection was achieved with an infrared imaging system (Odyssey, Li-cor).

GST Pull-Down

The GST-fusion proteins (PDZ domain, C2A domain and C2B domain of RIM2α) were produced in *Escherichia coli* BL21-DE3 and purified using Glutathion-agarose beads (Sigma). The purification efficiency was assessed by Coomassie staining (Supplementary Figure S1). For the binding assay the HA-tagged C-terminal region of Cav.1.3α (aa 1509–2203) was overexpressed in HEK293T cells using either calcium-phosphate method or Lipofectamine2000 (Invitrogen). Forty-eight hours post transfection cells were lysed for 1 h in ice-cold lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% Triton X-100, Complete Protease Inhibitor Cocktail Tablets), centrifuged at 14,000 rpm/10 min/4°C and the resulting clear supernatant incubated for 2 h with GST and GST-fusion proteins. Beads were washed four times in PBS-0.5% Triton X-100 and proteins were eluted by boiling the beads in Laemmlı buffer. Proteins were analyzed by WB using the Odyssey infrared imaging system.

Patch-Clamp Recordings on Transiently Transfected HEK293/SK3-1 Cells

For electrophysiological recordings human embryonic kidney cells stably expressing the human small-conductance Ca^{2+}-activated K^{+} channel (HEK293/SK3-1) were transfected at 30% confluence using the transfection reagent ExGen500 (Bioimol) containing Cav.1.3A222Yα1 (Tan et al., 2011), β2a (GenBank accession number: NM053851), α281 (GenBank accession number: NM012919), RIM2α (GenBank accession number: NM_001256383) and RIM3γ (GenBank accession number: NM_182929.2) according to the manufactures protocol. Thirty-six to sixty hours after transfection I_{Ca}^{max} were acquired at room temperature using an external solution containing the following (in mM): 150 CholineCl, 1 MgCl2, 10 HEPES, 10 CaCl2, 100 nM Apamin; pH 7.4 (adjusted with methanesulfonic acid), 300–310 mosmol. The internal solution contained the following (in mM): 140 N-Methyl-D-glucamine, 5 EGTA, 10 NaCl, 1 MgCl2, 10 HEPES, 2 MgATP; pH 7.4 (adjusted with NaOH), 290 mosmol. I_{Ca}^{max} was recorded using an EPC 10 Amplifier controlled by "Patchmaster" software (HEKA), low-pass filtered at 5 kHz, sampled at 50 kHz with RSeries of ≤ 10 MΩ after 70% compensation. Conductance of Ca^{2+} channels was derived from the I–V curves G = I/(V - V_{rev}) (V_{rev} reversal potential of the Ca^{2+} current), was normalized to the maximal conductance (G_{max}) and fitted to the following equation: G/G_{max} = 1/(1 + exp(V_{rev} - V/k_{act})) to derive the potential of half maximal I_{Ca} activation (V_{50}) and the activation slope factor of the Boltzmann function (k_{act}).

Immunofluorescence on Transiently Transfected HEK293/hSK3-1 Cells

For immunostaining of RIMs and Ca^{2+} channels in co-transfected HEK293/hSK3-1 cells, cells were fixed for 2 min at −20°C with 99% methanol. Primary antibodies were rabbit anti-Cav.1.3 (1:50, Alomone Labs), goat anti-RIM2 (1:200, sc-16677, Santa Cruz Biotecnhology), mouse anti-RIM3 (1:100 (Alvarez-Baron et al., 2013)), which were detected by species-specific antibodies anti-mouse HA (Covance; 1:1000), anti-rabbit RIM1/2 (1:1000; provided by Frank Schmitz), followed by secondary antibodies IRDye 1:10,000 (goat anti-mouse 800 and goat anti-rabbit 680). The detection was achieved with an infrared imaging system (Odyssey, Li-cor).
Abberior STAR 580 and 635 for STED images (all secondary antibodies: 1:200). Specimens were imaged using a Abberior Instruments laser-scanning confocal/STED microscope with a 1.4 NA, 100× oil-immersion (STED) objective using excitation wavelengths of 561 and 640 nm. For STED microscopy a STED laser of 775 nm up to 1.2 W was used at a pulse rate of 40 MHz achieving a resolution of <30 nm. Every staining was repeated at least three times and representative images are shown.

**Patch-Clamp Recordings of IHCs**

These recordings were performed in the apical coil of the organ of Corti isolated from mice at the age of P15–P20 using the perforated-patch configuration at room temperature. The following solutions were used: extracellular solution (in mM): 135 NaCl, 2.8 KCl, 35 TEA-Cl, 1 CsCl, 1 MgCl₂, 2 CaCl₂, 10 NaOH-HEPES, 11.3 D-glucose at pH 7.3; intracellular solution (in mM): 135 Cs-gluconate, 10 TEA-Cl, 10 4-aminoypyridine, 1 MgCl₂, 10 CsOH-HEPES and 300 μg/ml amphotericin. Traces were low-pass filtered at 2.9 kHz recorded at a sampling rate of 50 kHz, underwent offline liquid junction potential correction and for being accepted required a R_series < 30 MΩ for analysis. Capacitance recordings were performed as previously published (Moser and Beutner, 2000).

**Auditory Brainstem Recordings**

For recordings of Auditory Brainstem Recordings (ABRs), mice were anesthetized with a combination of i.p.-administered ketamine (125 mg/kg) and xylazine (2.5 mg/kg). The core temperature was maintained constant at 37°C using a heat blanket (Hugo Sachs Elektronik–Harvard Apparatus). For stimulus generation, presentation and data acquisition, we used the TDT II System run by BioSig software (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. The difference potential between vertex and mastoid subdermal needles was amplified 50,000-fold, filtered (400–4000 Hz), and sampled at a rate of 50 kHz for 20 ms for a total of 1300 times to obtain two mean ABR traces 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 by two independent observers.

**Statistical Analysis**

Data are presented as mean ± SEM. For statistical comparisons Student’s t-test was used to compare normally distributed samples with indistinguishable variance or alternatively Wilcoxon rank-sum test was used as non-parametric test. For multiple comparisons of normally distributed data (assessed by Kolmogorov-Smirnov test) one-way ANOVA with post hoc Holm-Šídák were performed; p ≤ 0.05 was accepted as statistically significant and is indicated by * p < 0.01 by ** and p < 0.005 by ***.

**RESULTS**

**Biochemical Evidence for a Direct Interaction of RIM2α and RIM3γ with Cav1.3α**

We tested for a direct interaction of Cav1.3α and RIM2α by co-immunoprecipitation from transfected HEK293T cells and by GST-pull down assays (Figure 1, Supplementary Tables S1, S2). We found that full-length RIM2α was co-immunoprecipitated with an HA-tagged version of the C-terminus of Cav1.3α1 (Figure 1A). However, unlike for Cav1.2α1 and Cav1.2α2α1 (Kaeser et al., 2011), a construct containing the RIM2α-PDZ domain (here also including the ZF domain) did not bind the Cav1.3α-C-terminus (Figure 1C). Instead, the C-terminus of RIM2α, containing two C₂ domains, C₂A and C₂B, co-immunoprecipitated with the Cav1.3α-C-terminus (Figure 1C). In order to further narrow down the site of interaction of RIM2 we performed GST-pulldown assays. Only the GST-tagged RIM2α-C₂B domain but not the RIM2α-C₂A and—PDZ domains bound to the HA-tagged Cav1.3α1-C-terminus (Figure 1D). Similar findings were obtained for RIM3γ (Figure 1B) indicating that this interaction of the Cav1.3α1-C-terminus generalizes to C₂B domains of other RIMs.

**Co-Expression of RIM2α or RIM3γ Increases the Current Density Mediated by “IHC-Like” Cav1.3 Channel Complexes in HEK293/SK3-1 Cells**

In order to assess the functional relevance of the direct interaction of RIM isoforms with the Cav1.3α1 C-terminus, we studied the effect of full-length RIM2α or RIM3γ on voltage-gated Ca²⁺-currents mediated by “IHC-like” Cav1.3 channels (Cav1.3α1, Cav1.3β2a and Cav1.3α2β1) in HEK293 cells. We employed HEK293 cells that stably express the Ca²⁺-activated small-conductance K⁺ channel (SK3-1) as a negative feedback in an attempt to improve the viability of the cells by limiting toxic Ca²⁺-influx. We chose to work with the rat Cav1.3α clone used by Tan et al. (2011) (corrected for a C-terminal mutation) and the Cav1.3β2a in order to mimic the IHC Ca²⁺-channel complex (Platzer et al., 2000; Brandt et al., 2003; Neef et al., 2009) as closely as possible. Moreover, use of the Cav1.3β2a is expected to minimize possible effects of the RIM2α-Cav1β interaction on channel gating (Gebhart et al., 2010). Immunofluorescence analysis of Cav1.3 and RIM2 expression in HEK293/SK3-1 cells showed partial overlap of signals at or near the plasma membrane (Figure 2A), indicative of a co-localization of both proteins and compatible with their interaction.

For the electrophysiological analysis we only included recordings with current densities, the Ca²⁺-current normalized to the cell capacitance, exceeding 20 pA/pF in order to increase the signal-to-noise ratio. Under these conditions the current density was nearly doubled when co-expressing RIM2α (Figure 2B), suggesting a positive regulation of Cav1.3 channel plasma membrane expression. The voltage-dependence of
CaV1.3 channel activation was shifted toward more depolarized potentials by 3 mV (Figure 2C, Supplementary Table S2), while the inactivation of the Ca^{2+}-current was neither significantly changed for its early nor its later components (Figures 2D,E).

We then tested whether the RIM3γ that only contains the C_{2}B domain also promotes membrane expression of CaV1.3 channels. Immunofluorescence analysis of CaV1.3 and RIM3γ in HEK293/SK3-1 showed partial overlap of signals at or near the plasma membrane (Figure 3A), indicative of a co-localization of both proteins and compatible with their interaction. We found a mild but significant increase in maximum Ca^{2+}-current densities in HEK293/SK3-1 cells.
In previous work we showed that RIM2 and RIM3 directly interact with Ca$_{\alpha\beta\gamma}$2+ (Jung et al., 2015). In order to investigate the role of RIM3 in IHC synaptic transmission we generated and analyzed constitutive RIM3γ knock-out mice (RIM3γ$^{-/-}$). RIM3γ$^{-/-}$ mice were generated by targeting ES cells with a gene trap cassette, in which insertion of a splice acceptor-lacZ gene trap disrupts the endogenous RIM3γ gene (Figures 4A). In order to verify that the insertion of the splice acceptor-cassette indeed abolishes the expression of functional RIM3γ, we characterized transcripts and protein expression levels in brains of wild-type, heterozygous and homozygous RIM3γ$^{-/-}$ mice. The level of transcripts was assessed by quantitative real time RT-PCR of RIM3γ mRNA prepared from hippocampus (HC), cerebellum (CB) and cortex (CX). RIM3γ transcript levels were reduced to about 60% in heterozygous and almost completely abolished in homozygous RIM3γ$^{-/-}$ mice in all brain areas (Figure 4B). To analyze if these reduced transcript levels result in the ablation of the protein quantitative immunoblotting of homogenates from hippocampus, cerebellum and cortex were performed. Stainings of the immunoblot with a RIM3γ-specific antibody revealed, that the gene trap had successfully abolished RIM3γ protein expression (Figures 4C,D). Hearing was tested via auditory brainstem responses (ABR) by presenting acoustic stimuli and recording synchronized neuronal activity. Here, co-expressing RIM3γ (Figure 3B). The voltage-dependence of Ca$_{\alpha\beta\gamma}$1.3 channel activation and Ca$_{\alpha\beta\gamma}$2+ current inactivation remained unchanged (Figures 3C–E, Supplementary Table S2). In summary, both RIM2α and RIM3γ that are present at IHC AZs increase Ca$_{\alpha\beta\gamma}$2+ current densities in HEK293/SK3-1 cells expressing an IHC-like Ca$_{\alpha\beta\gamma}$2+ channel complex. Since we used the palmitoylated Ca$_{\alpha\beta\gamma}$2b subunit, for which previous work investigating the interaction of RIM and Ca$_{\alpha\beta\gamma}$β subunits found the least effect, we speculate that this increase reflects a positive regulation of membrane expression via the direct interaction between the C$_{\beta}$B domain of RIM2α and RIM3γ and the C-terminus of Ca$_{\alpha\beta\gamma}$1.3a1. As both RIM isoforms, RIM2α and RIM3γ, are present at IHC AZs, these interactions might be functionally relevant in IHCs.

### Does RIM3 have a Functional Role at IHC AZs?

In previous work we showed, that RIM2α, RIM2β and RIM3γ but not RIM1 are expressed in IHCs and localize at the ribbon synapse (Jung et al., 2015). In order to investigate the role of RIM3γ in IHC synaptic transmission we generated and analyzed...
trends towards reduced Ca$^{2+}$ patch configuration. However, we only found non-significant capacitance increments upon depolarizations in perforated-2015). Therefore, we recorded Ca$^{2+}$ as sustained exocytosis were reduced by 50% (Jung et al., RIM2 knock-out mice, while Ca$^{2+}$ sound encoding. 

Amplitude and latency of ABR wave I, which the individual ABR waves, indicated by roman letters, reflect the processing at various stages of the early auditory pathway (Figure 4E). Amplitude and latency of ABR wave I, which represents the synchronized firing activity of spiral ganglion neurons (compound action potential of the spiral ganglion), was unaffected by the disruption of RIM3γ. In addition, ABR thresholds were comparable between RIM3γ knock-out mice and age-matched controls (Figure 4F), suggesting a minor if any role of RIM3γ in sound encoding.

We note that a mild hearing impairment was found in RIM2 knock-out mice, while Ca$^{2+}$-current amplitudes as well as sustained exocytosis were reduced by 50% (Jung et al., 2015). Therefore, we recorded Ca$^{2+}$-currents and membrane capacitance increments upon depolarizations in perforated-patch configuration. However, we only found non-significant trends towards reduced Ca$^{2+}$-current amplitudes (Figure 4G) and a tendency towards enhanced Ca$^{2+}$-current inactivation (measured as ratio of the residual Ca$^{2+}$-current after 200 ms depolarizations and the initial current, i.e., peak-normalized I$_{200}$, Figure 4H) in RIM3γ-deficient IHCs. Moreover, we probed exocytic changes of membrane capacitance ($\Delta C_m$) in response to depolarizations of varying durations and found indistinguishable fast (< 20 ms depolarizations) and sustained exocytosis between genotypes (Figure 4I). In summary, while RIM3γ promotes Ca$_{v}$1.3 membrane expression in HEK293 cells and is expressed at IHC AZs, it seems largely dispensable for IHC presynaptic function.

DISCUSSION

RIM proteins are multifunctional proteins that positively regulate vesicle tethering and Ca$^{2+}$ channel clustering at AZs. Here, we studied whether RIM2α and RIM3γ, both expressed at IHC AZs, directly interact with the pore-forming Ca$_{v}$1.3α Ca$^{2+}$ channel subunit that mediates stimulus-secretion coupling at IHC synapses. Based on co-immunoprecipitation, GST-pull-down assays, fluorescence microscopy of protein co-localization at IHC AZs, the presence of RIM3γ directly bind to the C-terminus of the pore-forming Ca$_{v}$1.3α Ca$^{2+}$ channel subunit most likely via their C2B domain. Both, RIM2α and RIM3γ, enhance the Ca$_{v}$1.3 Ca$^{2+}$-current when co-expressed in HEK293/SK3-1 cells. While, RIM2α is required for establishing a large complement of Ca$_{v}$1.3 Ca$^{2+}$ channels at IHC AZs, the presence of RIM3γ seems to be dispensable for Ca$^{2+}$-influx and exocytosis in IHCs.
Interaction of RIMs and CaV1.3 Ca$^{2+}$ Channels in HEK293 cells

A RIM-mediated up-regulation of Ca$^{2+}$ channel density at AZ was reported in hair cells, hippocampal neurons and the calyx of Held and can be attributed to various modes of direct and indirect interaction between RIMs and Ca$^{2+}$ channels. To date, two direct interaction sites of RIMs and specific Ca$^{2+}$ channel isoforms were reported. RIMs were proposed to exhibit a PDZ-domain dependent interaction with the pore-forming CaV$\alpha_{1}$ subunit of CaV2.2 and CaV2.1 channels (CaV2.X, Kaeser et al., 2011) and bind to the synprint motif of CaV2.2 and CaV1.2 channels via the C-terminal C$_2$A and C$_2$B domains.
(Coppola et al., 2001). However, neither of the described mechanisms seems to apply to Cav1.3 channels (Coppola et al., 2001; Kaeser et al., 2011), the predominant Ca\(^{2+}\) channel isoforms at IHC ribbon synapses, that similar to the calyx of Held (Han et al., 2015) display a substantial Ca\(^{2+}\)-current reduction in the absence of RIM2 (Jung et al., 2015). For Cav1.3, as well as for Cav1.2, Cav2.1 and Cav2.2 channels, a C-terminal C2-domain dependent interaction of RIM with the auxiliary Ca\(\alpha\) subunit was shown to regulate the biophysical properties of Ca\(^{2+}\) channels in heterologous expression systems (Kiyonaka et al., 2007; Gebhart et al., 2010; Gandini et al., 2011). In addition, RIMs are indirectly linked to Ca\(^{2+}\) channels by RIM-binding proteins, which seem to be dispensable for the regulation of membrane expression of Ca\(^{2+}\) channels in central synapses (Hibino et al., 2002; Kaeser et al., 2011; Liu et al., 2011; Acuna et al., 2015). The findings of the present study support a direct interaction of the C\(\beta\) domain of RIM2\(\alpha\) and RIM3\(\gamma\) with the C-terminus of Cav1.3\(\alpha\)-subunit. In keeping with the notion of Kaeser et al. (2011), we did not observe binding of the RIM2 PDZ-domain to the ITTL-site of Cav1.3\(\alpha\), that also diverges from the consensus-motif for RIM1/2 PDZ-domains (Kaiser et al., 2011; DDWC (Cav2.1); DHWC (Cav2.2); DDKC (Cav2.3) vs. ITTL (Cav1.3)). This is interesting in the light of the established interaction of the Cav1.3\(\alpha\)-subunit with other PDZ-domain proteins (Cavin-Jageman et al., 2007; Gregory et al., 2011). The C\(\beta\) domain of all RIMs contains a short Lysine-rich amino acid sequence that is also found in Synaptotagmin 1 (Perin et al., 1999; Coppola et al., 2001; Wang and Südhof, 2003) and Munc13-1 (Calloway et al., 2015), which interacts with the synprint site of Cav1.2 channels. However, the synprint site characterized in Cav1.2 (Wiser et al., 1999), P/Q- (Catterall, 1999) and N-type (Sheng et al., 1997) Ca\(^{2+}\) channels seems to be lacking in Cav1.3\(\alpha\) (Coppola et al., 2001). Therefore, our results indicate the presence of a novel RIM binding motif in the C-terminus of Cav1.3\(\alpha\), which will have to be mapped in further studies.

Our analysis of biophysical Cav1.3\(\alpha\) properties in HEK293 cells took advantage of the constitutive presence of a negative feedback to Ca\(^{2+}\)-influx by the small conductance Ca\(^{2+}\)-activated K\(^+\) channel SK3-1, which increased the yield of Cav1.3\(\alpha\)-positive cells that were in good condition. This raised our confidence in interpreting an increase in current density as the enhanced membrane expression of Cav1.3\(\alpha\) when co-expressed with RIM2\(\alpha\) or RIM3\(\gamma\). We chose to compare current densities above a threshold of 20 pA/pF for signal to noise ratio and the previous study by Gandini et al. (2011) might result from deviating RIM proteins (RIM1 vs. RIM2\(\alpha\), RIM3\(\gamma\)) and Cav1.3\(\alpha\)-1 isoforms used for these experiments (corrected vs. uncorrected Cav1.3 rat clone) or the differing IV protocols applied (steady-state IV after 30 ms here vs. 2 s). Here, we favor the interpretation that the increased current density resulted from enhanced plasma membrane expression due to direct RIM-Ca\(\gamma\)B interaction with the C-terminus of Cav1.3\(\alpha\) but cannot rule out an additional effect of RIM via Ca\(\alpha\)B-dependent positive regulation of Cav1.3\(\alpha\) (Gebhart et al., 2010). While enhanced current density could in principle also reflect an increase in open probability by RIM-Cav1.3\(\alpha\) interaction, the depolarized shift of Cav1.3\(\alpha\) activation seems to argue against this. Instead, such a shift likely indicates a negative regulation of Cav1.3\(\alpha\) gating by RIM interaction potentially by impacting on the function of the Cav1.3\(\alpha\) C-terminus (Bock et al., 2011). In summary, experiments on heterologously co-expressed Cav1.3\(\alpha\) and RIMs indicate a functionally relevant interaction involving the C\(\beta\) domain of RIM and the C-terminus of Cav1.3\(\alpha\). Further studies will need to establish the precise molecular mechanism and affinity of this interaction.

### Role of RIMs in Promoting Synaptic Ca\(^{2+}\)-Influx in IHCs

The increased Cav1.3 Ca\(^{2+}\)-current density in HEK293/SK3-1 cells upon co-expression of RIM2\(\alpha\) and RIM3\(\gamma\) is consistent with the notion that RIMs are positive regulators of plasma membrane expression of Ca\(^{2+}\) channels as proposed based on genetic disruption of RIM function for several presynaptic terminals (Han et al., 2011, 2015; Kaeser et al., 2011; Kintscher et al., 2013; Jung et al., 2015). In IHCs, genetic deletion of all RIM2 isoforms caused a robust reduction of IHC Ca\(^{2+}\)-influx (by approximately 50%), while the selective disruption of RIM2\(\alpha\) diminished IHC Ca\(^{2+}\)-influx by only 17% (Jung et al., 2015). This indicated that RIM2\(\beta\) and/or RIM2\(\gamma\) promote the clustering of Ca\(^{2+}\) channels at IHC AZ in an additive manner with RIM2\(\alpha\) potentially facilitated by the formation of RIM dimers (Guan et al., 2007). Our present findings of an interaction of the RIM C\(\beta\) domain with the C-terminus of Cav1.3\(\alpha\) and a positive regulation of Cav1.3 Ca\(^{2+}\)-current density by RIM3\(\gamma\) in HEK293 cells suggests a putative presynaptic function, even though the protein is present pre- and post-synaptically (Liang et al., 2007; Alvarez-Baron et al., 2013). Indeed, we found expression of RIM3\(\gamma\) in IHCs at the mRNA and protein levels (Jung et al., 2015). However, genetic deletion of RIM3\(\gamma\) left IHC Ca\(^{2+}\)-influx and hearing unaffected. The lack of a significant sound coding phenotype in the RIM3\(\gamma\) knockout mice might be related to a predominant role of the long RIM2 isoforms that co-exist with RIM3\(\gamma\) at the IHC AZ. We speculate that their interaction with the various CAZ proteins poises them to critically determine the number of AZ tethered Ca\(^{2+}\) channels, while the short RIM3\(\gamma\) exerts more auxiliary function. A compensatory scenario was previously observed at the Calyx of Held synapse, where RIM1 and RIM2 possess the ability to largely replace each other (Han et al., 2015). Further studies investigating RIM2/RIM3 double-knock-out mice will be required to elucidate a potential contribution of RIM3\(\gamma\) in Cav1.3 clustering at IHC AZs.
AUTHOR CONTRIBUTIONS

MMP, SS and TM designed the study. MMP performed electrophysiological recordings, immunohistochemistry and STED microscopy of heterologously expressed Ca\(^{2+}\) channels and IHC electrophysiology. A-MO performed in vitro interaction studies and immunohistochemistry of HEK cells. SJ performed IHC electrophysiology. KM generated the RIM3 KO mice. MMP, A-MO, KM and SJ analyzed the data. MMP, A-MO, SJ, SS and TM prepared the manuscript.

ACKNOWLEDGMENTS

We thank N. Herrmann, S. Gerke and C. Senger-Freitag for expert technical assistance, G. Hoch for developing image analysis routines and Dr. J. Neef for help and discussion. We thank Dr. L. Pardo for providing HEK293/SK3-1 cells and Drs. T.W. Soong and Erwin van Wijk for providing Ca\(^{2+}\) expression plasmids. This work was supported by grants of the German Research Foundation: Priority Program 1608, MO889/3-1 to TM and Collaborative Research Centers 889 to TM (Project A2) and 1089 to SS (projects A01 and P02), as well as the German Federal Ministry of Education and Research (Independent groups in neurosciences, 01GQ0806 to SS).

REFERENCES

Acuna, C., Liu, X., Gonzalez, A., and Sudhof, T. C. (2015). RIM-BPs mediate tight coupling of action potentials to Ca\(^{2+}\)-triggered neurotransmitter release. Neuron 87, 1234–1247. doi: 10.1016/j.neuron.2015.08.027

Altit, C., Garcia-Caballero, A., Simms, B., You, H., Chen, L., Walcher, J., et al. (2011). The Ca\(^{2+}\)/\(\beta\) subunit prevents RFP2-mediated ubiquitination and proteasomal degradation of L-type channels. Nat. Neurosci. 14, 173–180. doi: 10.1038/nn.2712

Alvarez-Baron, E., Michel, K., Mittelstadt, T., Opitz, T., Schmitz, F., Beck, H., et al. (2013). RIM3\(\gamma\) and RIM4\(\gamma\) are key regulators of neuronal arborization. J. Neurosci. 33, 824–839. doi: 10.1523/JNEUROSCI.2229-12.2013

Bichet, D., Cornet, V., Gеб, S., Carlier, E., Volsen, S., Hoshi, T., et al. (2011). The I-II loop of the Ca\(^{2+}\) channel \(\alpha_1\) subunit contains an endoplasmic reticulum retention signal associated by the \(\beta\) subunit. Neuron 25, 177–190. doi: 10.1016/s0896-6273(01)00881-8

Bock, G., Gebhart, M., Scharinger, A., Jangsangthong, W., Busquet, P., Poggiani, C., et al. (2011). Functional properties of a newly identified C-terminal splice variant of Cav1.3 L-type Ca\(^{2+}\) channels. J. Biol. Chem. 286, 42736–42748. doi: 10.1074/jbc.M111.269951

Brandt, A., Strissnig, J., and Moser, T. (2003). Ca\(^{2+}\)1.3 channels are essential for development and presynaptic activity of cochlear inner hair cells. J. Neurosci. 23, 10832–10840.

Calin-Jageman, I., Yu, K., Hall, R. A., Mei, L., and Lee, A. (2007). Erbin enhances voltage-dependent facilitation of Cav1.3 Ca\(^{2+}\) channels through relief of an autoinhibitory domain in the Cav1.3 \(\beta_1\) subunit. J. Neurosci. 27, 1374–1385. doi: 10.1523/JNEUROSCI.5191-06.2007

Calloway, N., Gouzer, G., Xue, M., and Ryan, T. A. (2015). The active-zone protein Bassoon specifically controls presynaptic P/Q-type Ca\(^{2+}\) channels and vesicles to add release sites and promote refilling. Mol. Cell. Neurosci. 44, 246–259. doi: 10.1016/j.mcn.2013.03.011

Gregory, F. D., Bryan, K. E., Pangrsic, T., Calin-Jageman, I., Moser, T., and Lee, A. (2011). Harmonin inhibits presynaptic Cav1.3 Ca\(^{2+}\) channels in mouse inner hair cells and is essential for normal hearing. J. Neurosci. 31, 11024–11036. doi: 10.1523/JNEUROSCI.4648-14.2016

Han, Y., Baba, N., Kaeser, P., Sudhof, T. C., and Schneggenburger, R. (2015). RIM1 and RIM2 redundantly determine Ca\(^{2+}\) channel density and readily releasable pool size at a large hindbrain synapse. J. Neurophysiol. 113, 255–263. doi: 10.1152/jn.00488.2014

Huang, R., Dai, H., Tomchick, D. R., Dulubova, I., Machius, M., Sudhof, T. C., et al. (2007). Crystal structure of the RIM1\(\alpha\) C2B domain at 1.7 Å resolution. J. Biol. Chem. 282, 13577–13585. doi: 10.1074/jbc.M107.67757

Kim, S. H., Gwon, H. J., Rho, M. J., Ghosh, R., Khim, S. H., Jang, H. J., et al. (2017). Modulation of Ca\(^{2+}\)-triggered release by RIM3 and SNAP-25 in mouse inner hair cells and is essential for normal hearing. J. Neurosci. 27, 1374–1385. doi: 10.1523/JNEUROSCI.5191-06.2007

Kimmich, R., András, B., Simon, T., and Winkler, E. (2002). Functional coupling of Rab3-interacting molecule 1 (RIM1) and L-type Ca\(^{2+}\) channels in mouse insulin release. J. Biol. Chem. 286, 15757–15765. doi: 10.1074/jbc.M110.87757

Gandini, M. A., Sandoval, A., González-Ramírez, R., Mori, Y., de Waard, M., and misdemeanors, A. (2015). RIM2 and RIM3 Directly Interact with Ca\(^{2+}\) Channels and IHC Electrophysiology. A-MO performed expert technical assistance, G. Hoch for developing image analysis routines and Dr. J. Neef for help and discussion. We thank Dr. L. Pardo for providing HEK293/SK3-1 cells and Drs. T.W. Soong and Erwin van Wijk for providing Ca\(^{2+}\) expression plasmids. This work was supported by grants of the German Research Foundation: Priority Program 1608, MO889/3-1 to TM and Collaborative Research Centers 889 to TM (Project A2) and 1089 to SS (projects A01 and P02), as well as the German Federal Ministry of Education and Research (Independent groups in neurosciences, 01GQ0806 to SS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2017.00160/full#supplementary-material

FIGURE S1 | Exemplary SDS polyacrylamide gel electrophoresis (SDS-PAGES) after Coomassie staining. (A) Visualized GST-bound protein fraction obtained by GST-pulldown assays. Protein bands of the respective rab interacting molecules 2 (RIM2) domains are indicated by the blue arrow heads. Protein amount loaded is indicated below. (B) GST-bound protein fraction of RIM3\(\gamma\) visualized in exemplary SDS-PAGE after Coomassie staining. The RIM3\(\gamma\) specific protein band is indicated by the blue arrow.
Hoppa, M. B., Lana, B., Margas, W., Dolphin, A. C., and Ryan, T. A. (2012). α2δ expression sets presynaptic calcium channel abundance and release probability. *Nature* 486, 122–125. doi: 10.1038/nature11033

Jung, S., Oshima-Takago, T., Chakrabarti, R., Wong, A. B., Jing, Z., Yanmanbeva, G., et al. (2015). Rab3-interacting molecules 2a and 2b promote the abundance of voltage-gated Ca$_{V}$.1.3 Ca$^{2+}$ channels at hair cell active zones. *Proc. Natl. Acad. Sci. U S A* 112, E3141–E3149. doi: 10.1073/pnas.1417207112

Kaeser, P. S., Deng, L., Wang, Y., Dulabova, I., Liu, X., Rizo, J., et al. (2011). RIM proteins tether Ca$^{2+}$ channels to presynaptic active zones via a direct PDZ-domain interaction. *Cell* 144, 282–295. doi: 10.1016/j.cell.2010.12.029

Kintscher, M., Wozny, C., Johenning, F. W., Schmitz, D., and Breustedt, J. (2013). Role of RIM1α in short- and long-term synaptic plasticity at cerebellar parallel fibres. *Nat. Commun.* 4:2392. doi: 10.1038/ncomms3392

Kiyonaka, S., Wakamori, M., Miki, T., Utau, Y., Nonaka, M., Bito, H., et al. (2007). Kifs1 and kifs2 are two coiled-coil proteins involved in the development and function of the cochlear inner hair cell afferent synapse of the mouse. *Proc. Natl. Acad. Sci. U S A* 104, 10730–10740. doi: 10.1073/pnas.1001538107

Kiyonaka, S., Wakamori, M., Miki, T., Uriu, Y., Nonaka, M., Bito, H., et al. (2007). Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature* 345, 260–263. doi: 10.1038/345260a0

Kratsch, M., Engel, J., Schrott-Fischer, A., Stephan, K., Bova, S., Chen, H., et al. (2000). Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca$^{2+}$ channels. *Cell* 102, 89–97. doi: 10.1016/s0092-8674(00)0013-1

Sheng, Z.-H., Yokoyama, C. T., and Catterall, W. A. (1997). Interaction of the synprint site of N-type Ca$^{2+}$ channels with the C2B domain of synaptotagmin I. *Proc. Natl. Acad. Sci. U S A* 94, 5405–5410. doi: 10.1073/pnas.94.10.5405

Tan, B. Z., Jiang, F., Tan, M. Y., Yu, D., Huang, H., Shen, Y., et al. (2011). Functional characterization of alternative splicing in the C-terminus of L-type Cav1.3 channels. *J. Biol. Chem.* 286, 42725–42735. doi: 10.1074/jbc.M111.265207

Wang, T., Jones, R. T., Whippens, J. M., and Davis, G. W. (2016). α2δ-3 is required for rapid transsynaptic homeostatic signaling. *Cell Rep.* 16, 2875–2888. doi: 10.1016/j.celrep.2016.08.030

Wang, Y., and Südhofer, T. C. (2003). Genomic definition of RIM proteins: evolutionary amplification of a family of synaptic regulatory proteins. *Genomics* 81, 126–137. doi: 10.1016/s0888-7543(02)00024-1

Wiser, O., Trus, M., Hernández, A., Renström, E., Barg, S., Rorsman, P., et al. (1999). The voltage sensitive L-type Ca$^{2+}$ channel is functionally coupled to the exocytotic machinery. *Proc. Natl. Acad. Sci. U S A* 96, 248–253. doi: 10.1073/pnas.96.1.248

**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.