RIM1 and RIM2 redundantly determine Ca\textsuperscript{2+} channel density and readily-releasable pool size at a large hindbrain synapse

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Abstract

The localization and density of voltage-gated Ca\(^{2+}\) channels at active zones is essential for the amount and kinetics of transmitter release at synapses. RIM proteins are scaffolding proteins at the active zone which bind to several other presynaptic proteins, including voltage-gated Ca\(^{2+}\) channel \(\alpha\) subunits. The long isoforms of RIM proteins, which contain N-terminal Rab3 and Munc13 interacting domains, as well as a central PDZ domain and two C-terminal C2 domains, are encoded by two genes, \(\text{Rim1}\) and \(\text{Rim2}\). Here, we used the ideal accessibility of the large calyx of Held synapse for direct presynaptic electrophysiology, to investigate whether the two \(\text{Rim}\) genes have redundant, or separate functions in determining the presynaptic Ca\(^{2+}\) channel density, and the size of a readily-releasable vesicle pool (RRP). Quantitative PCR showed that cochlear nucleus neurons, which include calyx of Held generating neurons, express both RIM1 and RIM2. Conditional genetic inactivation of RIM2 at the calyx of Held led to a subtle reduction in presynaptic Ca\(^{2+}\) current density, whereas inactivation of RIM1 was ineffective. The release efficiency of brief presynaptic Ca\(^{2+}\) "tail" currents, and the RRP were unaffected in conditional single RIM1 and RIM2 knock-out (KO) mice, whereas both parameters were strongly reduced in RIM1/2 double KO mice. Thus, despite a somewhat more decisive role for RIM2 in determining presynaptic Ca\(^{2+}\) channel density, RIM1 and RIM2 can overall replace each other's presynaptic functions at a large relay synapse in the hindbrain, the calyx of Held.
Introduction

Neurotransmitter release takes place at active zones, which contain docked vesicles with their molecular fusion machinery, voltage-gated Ca\(^{2+}\) channels, and presynaptic scaffolding proteins (Schoch and Gundelfinger 2006). The immediate vicinity of readily-releasable vesicles to Ca\(^{2+}\) channels on the range of a few tens of nanometers is essential for fast transmitter release, because it allows short Ca\(^{2+}\) diffusion times (Neher 1998). However, the molecular mechanisms which determine the enrichment of Ca\(^{2+}\) channels at active zones, and the exact Ca\(^{2+}\) channel-vesicle co-localization, are only beginning to be understood (Südhof 2012). RIM proteins are presynaptic scaffolding proteins specifically localized to the active zone, and found to bind several presynaptic proteins, like Munc13-1, Rab3a and voltage-gated Ca\(^{2+}\) channels (Betz et al. 2001; Kaeser et al. 2011; Schoch et al. 2002; Wang et al. 1997). RIM proteins are encoded by four genes (Rims1-4); the Rim1 and 2 genes give rise to five RIM isoforms, called RIM1\(\alpha\), RIM1\(\beta\), and RIM2\(\alpha\), RIM2\(\beta\) and RIM2\(\gamma\) (see Fig. 1A, B; Kaeser et al. 2008b; Wang and Südhof 2003). Studies using RIM1\(\alpha\) constitutive knock-out (KO) mice and RIM mutants in *C. elegans* found roles for RIM1 in transmitter release, presumably via determining RRP size (Calakos et al. 2004; Koushika et al. 2001; Schoch et al. 2002). Constitutive genetic inactivation of both RIM1\(\alpha\) and RIM2\(\alpha\) in mice leads to postnatal lethality not visible in the individual KOs, indicating redundant functions of the two long isoforms for mouse survival (Schoch et al. 2006). This redundancy has initially hindered a more detailed understanding of the presynaptic functions of RIM proteins.

More recently, floxed alleles of both the Rim1 and Rim2 genes (also called Rims1 and Rims2 genes) have been produced to allow conditional removal of both genes (Kaeser et al. 2011; Kaeser et al. 2008b). Experiments with conditional RIM1/2 double KO synapses of cultured neurons have shown that the central PDZ domain in RIM1/2 confers a high extracellular Ca\(^{2+}\) sensitivity on release, and mediates a high action-potential driven presynaptic Ca\(^{2+}\) influx;
furthermore, the PDZ domain was shown to bind to P/Q- and N-type Ca\(^{2+}\) channel \(\alpha\)-subunits (Kaeser et al. 2011). Conditional inactivation of \textit{Rim1} and \textit{Rim2} at the calyx of Held, a synapse accessible to direct presynaptic recordings, has shown that RIM proteins determine a high Ca\(^{2+}\) current density in the nerve terminal (Han et al. 2011). Together, these data showed that the central PDZ domain and a more C-terminal proline-rich motif (PxxP; Fig. 1) of the long RIM1 and RIM2 isoforms are critical for anchoring and enriching Ca\(^{2+}\) channels at the active zone. In addition, the RIM1/2 double KO studies revealed a role for RIM proteins in vesicle docking (Han et al. 2011; Kaeser et al. 2011). The reduced RRP size at the calyx of Held might be largely caused by the docking deficit (Han et al. 2011).

The contribution of the individual \textit{Rim1} and \textit{Rim2} gene products to presynaptic Ca\(^{2+}\) channel density and RRP size has not been addressed in direct presynaptic recordings. A redundancy of RIM1 and RIM2 function has been found recently with more indirect methods at inhibitory synapses of cultured hippocampal synapses (Kaeser et al. 2012). Another recent study in mouse cerebellar slices found that genetic inactivation of RIM1\(\alpha\) causes a reduced release probability and decreased axonal Ca\(^{2+}\) influx, and suggested that RIM1\(\alpha\) has a predominant role in setting presynaptic Ca\(^{2+}\) channel density at many synapses (Kintscher et al. 2013). Using the ideal presynaptic accessibility of the calyx of Held synapse, we investigated the effect of conditional inactivation of the single \textit{Rim1} or \textit{Rim2} gene for presynaptic Ca\(^{2+}\) current density and RRP size. Our results suggest redundant roles for RIM1 and RIM2 isoforms in determining both the presynaptic Ca\(^{2+}\) channel density, as well as enabling a large RRP.
Materials and Methods

Conditional knock-out of RIM1, RIM2 and RIM1/2 in the calyx of Held

We used the \textit{Krox20::Cre} mouse line, which drives expression of Cre-recombinase in neurons descending from rhombomeres 3 and 5 (Han et al. 2011; Maricich et al. 2009; Renier et al. 2010; Voiculescu et al. 2000), to recombine the floxed RIM1 allele and the floxed RIM2 allele, either separately or in combination. For this purpose, we crossed the \textit{Krox20^{+/+}\text{-Cre}} mouse line with \textit{RIM1^{lox/lox}} mice (Kaeser et al. 2008b); or with \textit{RIM2^{lox/lox}} mice (Kaeser et al. 2011); or with both \textit{RIM1^{lox/lox}} and \textit{RIM2^{lox/lox}} mouse lines, respectively. Because of germline recombination in the Krox20\text{-Cre} mouse line, we obtained \textit{RIM1^{lox/Δ}, Krox20^{+/+}\text{-Cre}} mice (called RIM1 cKO for conditional KO); \textit{RIM2^{lox/Δ}, Krox20^{+/+}\text{-Cre}} mice (called RIM2 cKO); or else \textit{RIM1^{lox/Δ}, RIM2^{lox/Δ}, Krox20^{+/+\text{-Cre}}} mice (called RIM1/2 double cKO for conditional double KO); the "Δ" symbols indicate removal of the allele in the germline. The Cre negative littermates were used for control experiments; the detailed breeding schemes were described before (Han et al. 2011). Since the data from all three control groups did not significantly differ, we pooled the control data and refer to it as "control mice".

Slice preparation and Electrophysiology

Postnatal days 9 - 11 (P9 - P11) mice were decapitated and transverse brainstem slices of 200 µm thickness were prepared with a LEICA VT1000S slicer. Slices were kept at 37 °C in a bicarbonate-buffered extracellular solution containing (in mM): 125 NaCl, 25 NaHCO₃, 1.25 KCl, 2 CaCl₂, 1 MgCl₂, 25 glucose, 3 myo-inositol, 2 Na-pyruvate, and 0.4 ascorbic acid, pH 7.2 when bubbled with 95% O₂/5% CO₂. During recordings, slices were perfused with the same solution at room temperature (21–23 °C). For paired recordings (Figs 2, 3), tetraethylammonium chloride (TEA, 10 mM), tetrodotoxin (TTX, 1 µM), D-2-amino-5-phosphonopentanoic acid (D-AP5, 50 µM), γ-D-glutamylglycine (γ-DGG, 2 mM) and cyclothiazide (CTZ, 100 µM) were added to the extracellular solution. For fiber stimulation experiments (Fig. 4), excitatory
postsynaptic currents (EPSCs) were evoked by stimulating presynaptic axons with a custom-made bipolar stimulation electrode placed medially to the MNTB, in the presence of D-AP5 (50 μM), bicuculline (10 μM) and strychnine (2 μM) in the extracellular solution. In these experiments, we reversibly applied 500 μM γ-DGG as a rapid-off antagonist at AMPA receptors, to probe whether the cleft glutamate concentration was different between control synapses, and RIM1/2 double cKO synapses (Clements et al., 1992; Wadiche and Jahr 2001). The choice of the relatively low concentration of γ-DGG was dictated by practical considerations. Since EPSCs are small in RIM1/2 double cKO mice (~ 1 nA), we aimed for a γ-DGG concentration that would block EPSCs down to ~ one thirds of their initial amplitude.

Calyceal terminals and MNTB principle cells were visually identified with an upright microscope (Zeiss Axioskop 2 FS, Germany) equipped with a 60× objective (Olympus LUMPlanFI; NA, 0.9) and gradient contrast infrared visualization (Luigs and Neumann, Germany). Pre- and/or postsynaptic whole-cell recordings at the calyx of Held synapse were made with an EPC10/2 double patch-clamp amplifier (HEKA, Germany). The intracellular (pipette) solutions contained (in mM): 140 Cs-gluconate, 20 TEA, 10 Hepes, 5 Na2-phosphocreatine, 4 MgATP, 0.3 Na2GTP, pH 7.2, to which either 0.1 mM or 5 mM EGTA was added for pre- or postsynaptic recordings, respectively. Pre- and postsynaptic series resistances (Rs) were ~ 10 - 25 MΩ, or 3 - 10 MΩ respectively, and compensated during the experiment such that the remaining Rs did not exceed 10 and 3 MΩ in pre- and postsynaptic recordings, respectively. Postsynaptic currents were corrected offline for the remaining Rs error (Meyer et al. 2001). For the measurement of release evoked by presynaptic Ca2+ "tail" currents (Fig. 3), 0.3 ms ramps from -70 to +60 mV, followed by a variable duration at +60 mV and by a 0.3 ms ramp back to -70mV, were applied. Presynaptic Ca2+ currents were corrected for leak and capacitative currents with a P/5 protocol. Presynaptic membrane capacitance (Cm) was estimated by cancelling the first exponential decay component of the capacitative transients in response to 10
mV hyperpolarizing voltage-clamp steps. This procedure estimates the resting $C_m$ value of a first membrane compartment represented by the giant terminal and proximal axon, whereas more distal axonal compartments will not influence this estimate (Wölfel and Schneggenburger 2003). Ca$^{2+}$ currents amplitudes measured at 0 mV were divided by the $C_m$ estimate to calculate Ca$^{2+}$ current density (Fig. 2B1 - B3).

Reverse transcription and quantitative PCR

The expression level of different RIM isoforms was determined by quantitative PCR with isoform-specific probes. For this, acute brainstem slices were made as described above and cochlear nucleus tissue including the ventral cochlear nucleus (VCN) and the dorsal cochlear nucleus (DCN) was dissected with fine forceps in half frozen RNase-free phosphate-buffered solution. The tissue from each mouse was pooled and RNA was extracted with an RNeasy® Micro Kit (Qiagen, Switzerland). In total, $n = 3$ C57Bl6 mice were used at P14. A recent gene expression study in the auditory brainstem suggests that the expression of RIM1 and RIM2 should not significantly change between P9 - P11 (the age range used for the recordings; see above), and P14 (Körber et al., 2014). The samples from each mouse were processed separately; each sample was processed in triplicate for qPCR. 100 ng RNA from each animal was used to make complementary DNA (cDNA), using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Switzerland). The reverse transcription (RT) reaction was incubated at 25 °C for 10 min, at 37 °C for 120 min, and at 85 °C for 5 s. Real-time PCR was performed separately for each gene in triplicate with TaqMan® Universal PCR Master Mix (Applied Biosystems, Switzerland). RT products and PCR reagents were distributed to a 384-well-plate by a Hamilton liquid handling system (Hamilton, Switzerland). Quantitative real-time PCR was done by a 7900HT Fast Real-Time PCR System (Applied Biosystems, Switzerland). The cycle threshold (CT) was defined at a level of 0.2 fluorescence unit.
As mRNA probes, we used the Taqman assays (Applied Biosystems; Rotkreutz, Switzerland) targeting exon-exon boundaries containing the isoform-specific first exons (see Fig.1 A and B, blue brackets; Kaeser et al. 2008b; Wang and Südhof 2003). In addition, the total amount of RIM1 or RIM2 transcripts was determined by using assays targeting exon-exon boundaries that are shared by all isoform (see Fig.1 A and B, red brackets). The following assays are available in Applied Biosystem: β-actin (Rn00667869_m1), RIM1α (Mm00694774_m1), RIM2α (Mm00453603_m1), RIM2total (Mm00453627_m1). The assays for RIM1total, RIM2β, and RIM 2γ were custom-designed. We also attempted to detect RIM1β mRNA (Kaeser et al. 2008b) with a custom-designed probe which, however, failed to detect any signal (not shown). The efficiency of each qPCR reaction was validated using a dilution series of brainstem cDNA, and the mRNA concentrations relative to β-actin were corrected for qPCR efficiency of each probe. All assays showed similar amplification efficiencies (0.88 - 0.93).

Data analysis and statistics

All data are reported as mean ± S.D. Statistical significance was calculated with student's non-paired two-tailed t-test with unequal variance if not noted otherwise. For the data in Fig. 2, a one-way ANOVA was first applied, which showed that the population means were significantly different for all analyzed parameters (Fig. 2B1-B4; Fig. 2C1 - C5; Fig. 2D1), except for the number of slowly released vesicles (Fig. 2D2; p = 0.23). Then, a student's t-test with Bonferroni correction for multiple comparisons, was used for a pairwise comparison between the different mutant genotypes (RIM1 cKO, RIM2 cKO, or RIM1/2 double cKO), versus control mice. In all cases, statistical significance was accepted at the p < 0.05 level. The star symbols in the Figures indicate the statistical significance of pairwise comparisons, with p > 0.05 (not significant) indicated by no symbol; and levels of significance of p < 0.05, p < 0.01, and p < 0.001 indicated by one, two and three star symbols, respectively.
Results

RIM1 and RIM2 are expressed in the cochlear nucleus

We first analyzed the expression level of the major isoforms of Rim1- and Rim2 genes in the cochlear nucleus, by using qPCR approaches and tissue samples from P14 mice (Fig. 1). The ventral cochlear nucleus (VCN) contains bushy cells which account for a sizeable fraction of the large neuron types; a subpopulation of the bushy cells, most likely corresponding to globular bushy cells, make calyces of Held (Cant and Benson 2003; Körber et al. 2014; Saul et al. 2008; Xiao et al. 2010). All RIM probes showed cycle threshold (CT) values 6 - 10 cycles higher than the abundantly expressed β-actin (Fig. 1C). The RIM2_total probe, designed to detect the RIM2 mRNA common for all isoforms (Fig. 1B, red bracket; Materials and Methods), and the probe for RIM2γ gave the lowest CT values (Fig. 1C). After normalization for qPCR efficiency and relative to the expression of β-actin, these two probes and the RIM1_total probe gave the strongest signal, with estimated mRNA content of ~ 1.5% relative to β-actin (Fig. 1D).

The probes for RIM1α, RIM2α and RIM2β gave weaker, but well-detectable signals (Fig. 1C, D). Taken together, qPCR indicates that both Rim1 and Rim2 genes are expressed in the cochlear nucleus, with a strong signal for the short RIM2γ isoform. In addition, the two long isoforms RIM1α and RIM2α are also clearly expressed in VCN neurons (Fig. 1D).

RIM1 and RIM2 redundantly regulate presynaptic Ca²⁺ channel density and RRP

To study the roles of the individual Rim1 and Rim2 genes in determining presynaptic Ca²⁺ channel density, and RRP size at the calyx of Held synapse, we produced conditional single RIM1 or RIM2 KO mice (called RIM1 cKO and RIM2 cKO), and conditional RIM1/2 double KO mice (called RIM1/2 double cKO; see Materials and Methods). We made paired pre- and postsynaptic recordings at the calyx of Held synapse and applied long presynaptic
depolarizations (50 ms to 0 mV), to study both presynaptic Ca\textsuperscript{2+} currents, and transmitter release evoked by such pool-depleting stimuli (Fig. 2A). Ca\textsuperscript{2+} currents at 0 mV, close to the peak of current - voltage relationships, had large amplitudes in control mice (1.19 ± 0.32 nA; Fig. 2B1; black data points). In calyces of Held from RIM1 cKO mice, Ca\textsuperscript{2+} currents had a tendency to be larger than in control mice, whereas in RIM2 cKO mice Ca\textsuperscript{2+} currents were somewhat smaller, but neither of these two datasets was significantly different from the control group (p > 0.05; Fig. 2B1). To obtain Ca\textsuperscript{2+} current densities, we normalized the absolute Ca\textsuperscript{2+} current amplitudes by the measured presynaptic membrane capacitance (C_m), which is a proxy of membrane area (Fig. 2B2; see Material and Methods). This revealed an unchanged Ca\textsuperscript{2+} current density in RIM1 cKO mice, whereas RIM2 cKO mice had smaller Ca\textsuperscript{2+} current densities as compared to control mice (p < 0.05). In RIM1/2 double cKO terminals, there was a more marked, ~40% reduction of presynaptic Ca\textsuperscript{2+} current density (Fig. 2B3; p < 0.01), in good agreement with our previous observations (Han et al. 2011). Taken together, conditional inactivation of RIM1 alone did not affect presynaptic Ca\textsuperscript{2+} channel density, and conditional deletion of RIM2 led to a partial deficiency in presynaptic Ca\textsuperscript{2+} channel density. Thus, the Rim1 and Rim2 genes have redundant functions in determining the Ca\textsuperscript{2+} channel density at active zones of calyx of Held synapses, with a somewhat larger role for Rim2 (see Discussion).

We next analyzed the amount and kinetics of transmitter release in response to pool-depleting long depolarizations (Fig. 2A, lower panels). EPSCs were measured in the presence of CTZ (100 µM) and γDGG (2 mM) to suppress the desensitization and saturation of postsynaptic AMPA-receptors (Neher and Sakaba, 2001). Presynaptic depolarizations caused large EPSCs in RIM1 - and RIM2 cKO mice, indistinguishable from EPSCs measured in control mice (Fig. 2A, middle panels; Fig. 2C1). Similarly, the peak release rates, and the number of vesicles released in the fast component were unchanged (Fig. 2C2, C3). In RIM1/2 double cKO mice, on the other hand, EPSCs in response to pool-depleting stimuli were strongly reduced in amplitude (Fig. 2C1). We
showed previously that the miniature EPSC amplitudes were unchanged in RIM1/2 double cKO mice (Han et al. 2011); therefore, the reduced EPSC amplitudes reflect less transmitter release. Correspondingly, the peak release rates and the fast component of the RRP were smaller than in control mice (Fig. 2A; Fig. 2C2, C3). In addition, the EPSC rise times and the fast release time constants, which both reflect the immediate speed of transmitter release, were slowed in RIM1/2 double cKO mice (Fig. 2A; Fig. 2C4, C5). The amount of vesicles released in the slow component was unchanged in single RIM cKO (Fig. 4D2), but the slow release time constant was slowed in RIM1/2 double cKO mice (Fig. 2D1; p > 0.01). Thus, removal of RIM1 or RIM2 alone did not significantly change RRP size, nor the release kinetics of RRP vesicles.

RIM1 and RIM2 redundantly determine Ca2+ channel-vesicle coupling

We showed above that Rim1 and Rim2 genes have a redundant role in determining RRP size and vesicle release kinetics (Fig. 2). It is possible, however, that removal of single Rim1/2 genes affect the spatial coupling between readily-releasable vesicles and Ca2+ channels in more subtle ways. To test this possibility, we investigated Ca2+ current - release coupling in RIM1/2 single cKO mice, as well as in RIM1/2 double cKO and control mice. We used brief ramp-like depolarizations with different durations at + 60 mV (Fig. 3A, top; Materials and Methods), thereby varying the number of Ca2+ channels that were open during the repolarization phase (Ca2+ "tail" current protocol; Borst and Sakmann 1999; Fedchyshyn and Wang 2005). In recordings of RIM1- and RIM2- single cKO mice, such pulses evoked well-measurable Ca2+ tail currents and EPSCs, similar to control mice (Fig. 3A). In double-logarithmic plots of EPSC amplitude versus QCa, we found steep relations for both the RIM1- and the RIM2 cKO data sets, which largely overlayed with the data set obtained in control mice (Fig. 3B1, B2). For each data set, we analyzed the slope ("Ca2+ current cooperativity") by line fits in the double logarithmic space, and the QCa value for which an EPSC of 2 nA was observed. While the slope was 4.6 ± 0.6 for the control data set (n = 11 paired recordings), it was slightly reduced in RIM1 and RIM2
cKO mice (to 3.9 ± 0.7 and 4.3 ± 0.4 respectively; n = 5 and 4, respectively), but these changes were not statistically significant (Fig. 3C; p > 0.05). Similarly, the Q_{Ca} value for an EPSC of 2 nA was slightly increased in RIM1 cKO mice, but this comparison did not reach statistical significance (Fig. 3D; p > 0.05). Therefore, based on Ca^{2+} tail current experiments in paired recordings, we conclude that removal of RIM1- or RIM2 alone does not significantly change the coupling efficiency between Ca^{2+} channels and readily-releasable vesicles at the calyx synapse.

For the RIM1/2 double cKO synapses, Ca^{2+} tail currents were reduced, and EPSC amplitudes for maximal tail currents were always below 0.5 nA (Fig. 3A; note different scale bars for control and RIM1/2 double cKO mice). The smaller Ca^{2+} tail currents are expected because of the smaller Ca^{2+} current amplitudes in calyx of Held terminals in RIM1/2 double cKO mice (Fig. 2). The small amplitudes of EPSCs did not allow us to measure reliable slope values between EPSC amplitudes and Q_{Ca} (Fig. 3B1, red data points). The low efficiency of AP-like depolarizations in inducing phasic release in RIM1/2 double cKO synapses is consistent with a slowed intrinsic release speed, and an additional coupling deficit in RIM1/2 double cKO mice (Han et al. 2011).

A small decrease in the cleft glutamate concentration in RIM1/2 double cKO synapses

In the absence of both RIM1 and RIM2, calyx synapses have a reduced pool size as tested by prolonged presynaptic depolarizations (Fig. 2), and the number of docked vesicles at the active zone was reduced, whereas active zone size was unchanged (Han et al. 2011). Thus, RIM1 and RIM2 proteins ensure a high density of docked vesicles at each active zone, and in turn the probability of release at a given active zone should be higher in wild-type mice than in RIM1/2 double cKO synapses. If a significant amount of multivesicular release happens at single active zones in control mice, then the decreased release probability in RIM1/2 double cKO mice might decrease the amount of multivesicular release, and therefore, the cleft glutamate concentration.

To test this prediction, we measured the blocking efficiency of a low-affinity competitive
antagonist at AMPA-receptors, γDGG, in RIM1/2 double cKO and control synapses. The amount of block by such rapid-off antagonists depends on the effective glutamate concentration in the synaptic cleft (Clements et al. 1992; Wadiche and Jahr 2001).

Fiber-stimulation evoked EPSCs recorded at calyx of Held synapses of control mice were large (7.0 ± 4.8 nA; n = 8 recordings), and 500 µM γDGG blocked the EPSCs reversibly to 42 ± 4 % of their amplitude under control conditions (Fig. 4A, C, D). In RIM1/2 double cKO mice, EPSCs had amplitudes of only 1.3 ± 1.3 nA, much smaller than in control mice (p < 0.05). The rapid-off antagonist γDGG reversibly blocked EPSCs in RIM1/2 double cKO mice to 35 ± 8 % of their control amplitude (Fig. 4B, C, D; n = 9); this block was slightly, but significantly stronger than in control mice (Fig. 4D; p < 0.05). This indicates that the amount of postsynaptic receptor saturation is lower in RIM1/2 double cKO mice as compared to control, suggesting that less multivesicular release occurs in RIM1/2 double cKO synapses than in wild-type mice. The relatively small difference of the blocking efficiency of γDGG might indicate that in wild-type calyx synapses, the probability of multivesicular release is not high under our recording conditions. Indeed, a recent study showed that the amount of multivesicular release depends on the release probability as set by the extracellular Ca²⁺ concentration, and that at 2 mM Ca²⁺, the condition used here, multivesicular release starts to appear (Budisantoso et al. 2013).

Discussion

Using conditional genetic elimination of the single Rim1 or Rim2 gene, or of the combination of both genes, we find that these genes have largely redundant presynaptic functions at the calyx of Held synapse. The mRNA transcripts of various RIM1 and RIM2 isoforms were expressed in the cochlear nucleus of young hearing mice (Fig. 1). Genetic inactivation of the individual Rim1 or Rim2 gene did not lead to noticeable changes in the readily-releasable pool of vesicles as studied by prolonged presynaptic depolarizations (Fig. 2), nor in the efficiency of brief presynaptic
depolarizations to induce release (Fig. 3). Similarly, the presynaptic Ca\(^{2+}\) current density was largely unchanged in the single KOs, although RIM2 inactivation reduced the Ca\(^{2+}\) channel density to some degree, maybe indicating a more prominent role for RIM2 in anchoring Ca\(^{2+}\) channels to the active zone. Overall, we conclude that at a large excitatory hindbrain synapse, the calyx of Held, RIM1 and RIM2 have redundant functions in determining the readily-releasable vesicle pool, and in anchoring presynaptic Ca\(^{2+}\) channels to the active zone.

Role of RIM proteins for anchoring Ca\(^{2+}\) channels at the active zone

Experiments in cultured hippocampal neurons and at the calyx of Held, a synapse uniquely accessible to direct presynaptic recordings, have shown that RIM proteins determine a high density of presynaptic voltage-gated Ca\(^{2+}\) channels (Han et al. 2011; Kaeser et al. 2011). These observations, together with biochemical experiments have shown that RIM proteins anchor Ca\(^{2+}\) channels at the active zone, probably via a direct interaction of the central PDZ domain with voltage-gated Ca\(^{2+}\) channel \(\alpha\) subunits (Kaeser et al. 2011). At the calyx synapse the overall Ca\(^{2+}\) current density was significantly reduced in RIM1/2 double cKO calyces (~ 40%), but the voltage-dependence of Ca\(^{2+}\) current activation was unchanged, and a large part of the Ca\(^{2+}\) currents in RIM1/2 double cKO mice were mediated by \(\omega\)-agatoxin sensitive P/Q-type channels, similarly as in wild-type mice (Han et al. 2011). Together with the previous results, this suggests that RIM proteins contribute to anchoring Ca\(^{2+}\) channels at the presynaptic active zone. Earlier studies have shown an interaction between the C2B domain of RIM1\(\alpha\) with the accessory Ca\(^{2+}\) channel \(\beta4\) subunit, and this interaction was also found for the C2B domains of the short RIM2, RIM3, and RIM4 \(\gamma\) isoforms. This interaction was found to modulate the inactivation of Ca\(^{2+}\) currents in heterologous expression systems (Kiyonaka et al. 2007; Uriu et al. 2010). However, this function of RIM proteins is independent from the Ca\(^{2+}\) channel anchoring function of the long RIM1- and RIM2 isoforms, and cannot account for the presynaptic deficits observed in RIM1/2 KO synapses (Kaeser et al. 2012).
When we inactivated either the *Rim1* or the *Rim2* gene alone, we found an unchanged presynaptic Ca\(^{2+}\) current density in the RIM1 cKO mice, and a somewhat smaller presynaptic Ca\(^{2+}\) current in RIM2 cKO mice (Fig. 2). Thus, it seems that at the calyx of Held, the RIM2 protein is more relevant than RIM1 for anchoring Ca\(^{2+}\) channels at the active zone. However, the reduced Ca\(^{2+}\) channel density in RIM2 cKO mice did not go along with significant changes of maximal EPSC amplitudes, pool size, or the release efficiency of Ca\(^{2+}\) tail currents (Figs 2, 3). Whole-cell recordings of a nerve terminal cannot distinguish between Ca\(^{2+}\) currents arising from the active zone, or from extrasynaptic membrane areas. Therefore, if genetic deletion of RIM2 had resulted in a stronger reduction of extrasynaptic Ca\(^{2+}\) channels as compared to synaptic ones, then such a reduction would be functionally less relevant. Overall, therefore, the present work suggests that the *Rim1*- and *Rim2* genes can replace each other in anchoring Ca\(^{2+}\) channels at the presynaptic active zone of the calyx synapse.

These findings are different from the situation at cerebellar parallel fiber synapses where constitutive inactivation of RIM1\(\alpha\) alone led to a reduced release probability and reduced presynaptic bouton Ca\(^{2+}\) influx (Kintscher et al. 2013). A somewhat predominant role for the RIM2 isoforms in determining the Ca\(^{2+}\) channel density at the calyx synapse might also explain the slightly larger Ca\(^{2+}\) currents observed in the RIM1 cKO mice, if it is assumed that early tissue-specific inactivation of RIM1 would lead to some compensatory upregulation of RIM2. In wild-type mice, RIM2 mRNA transcripts were well-detectable in the cochlear nucleus of P14 mice (Fig. 1). The Ca\(^{2+}\) channel anchoring function of RIM1 and RIM2 is mediated by the PxxP motif and PDZ domain (Kaeser et al. 2011)(Fig. 1). Thus, at the calyx of Held synapse, all isoforms which contain at least the PDZ domain and the PxxP motif (*Rim1\(\alpha\), *Rim1\(\beta\); and *RIM2\(\alpha\) and *RIM2\(\beta\); see Fig. 1) could function redundantly in anchoring Ca\(^{2+}\) channels at the presynaptic active zone.
Redundancy of RIM1 and RIM2 function depends on synapse identity

We found that the RRP, studied by prolonged presynaptic depolarizations, was not significantly affected in the single RIM1 or RIM2 cKO mice, whereas the RIM1/2 double cKO synapses showed a significantly reduced RRP and slowed release kinetics (Fig. 2; Han et al. 2011). Removal of all RIM1/2 isoforms at the calyx of Held also led to a strong reduction of the docked vesicle number which paralleled the reduced pool size (Han et al. 2011). Therefore, a large component of the reduction in RRP likely reflects a reduced number of docked vesicles in the double cKO mice. Here, we studied the RRP functionally, and we therefore cannot distinguish between possible docking - and priming roles of RIM proteins. Our data show that in RIM1 and RIM2 single cKO synapses, the RRP was not affected, suggesting that neither docking, nor priming are strongly affected by inactivating the individual Rim1/2 genes. Previous work showed that the priming function of RIM1 depends on the interaction of the N-terminal Zn-finger domain with Munc13-1 (Betz et al. 2001; Deng et al. 2011; Dulubova et al. 2005); the latter domain is present in RIM1α, RIM1β, and RIM2α (Fig. 1). This suggests that in the conditional RIM1 KO mice in which expression of both the RIM1α and RIM1β isoforms is abrogated (Kaeser et al. 2008b), RIM2α can replace the docking/priming function of the RIM1 isoforms.

Our results, together with recent reports, suggest that the redundancy of RIM isoforms depends on synapse identity. Whereas at the calyx synapse (this study) and in cultured hippocampal synapses (Kaeser et al. 2012) a predominant role of the RIM1 isoforms in anchoring Ca\(^{2+}\) channels at active zones was not apparent, a reduction of axonal Ca\(^{2+}\) transients were found in cerebellar synapses of conventional RIM1α KO mice (Kintscher et al. 2013). In agreement with the absence of a dominant role for RIM1α at the calyx, we found earlier that constitutive RIM1α KO mice do not have an obvious release phenotype in fiber stimulation experiments at the calyx.
of Held (F. Felmy and R.S., *unpublished observations*). This again suggests at most a redundant role of RIM1α with either RIM1β, or the RIM2 isoforms at the calyx of Held.

Taken together, experiments with single Rim 1/2 gene inactivation at the large calyx of Held synapse show largely overlapping roles of the two major Rim genes in vesicle priming/docking. The presynaptic Ca\(^{2+}\) channel anchoring function also depended on both genes, with a slight predominance of the Rim2 gene. In comparison with recent work (Kaeser et al. 2012; Kintscher et al. 2013), it is seen that the redundancy of Rim gene function likely depends on the expression regulation of RIM proteins in individual neuron - and synapse types. It is possible that the fast releasing calyx synapse makes use of most of the RIM1 and RIM2 isoforms to maximize fast and fail-safe synaptic transmission (Taschenberger et al. 2002). On the other hand, at some synapses with presynaptic forms of long-term plasticity, fewer RIM isoforms might be present, and maybe predominantly those which are permissive for long-term regulation of release (Castillo et al. 2002; Kaeser et al. 2008a). Future studies could disentangle the role of individual RIM protein isoforms in long-term regulation of transmitter release, and the mechanisms which control the degree of co-expression of distinct RIM isoforms in specific neurons and at specific synaptic connections.
Figure legends

Figure 1. Expression of RIM1 / RIM2 isoforms in the cochlear nucleus as detected by qPCR analysis.

A. Scheme of the mouse Rim1 gene with numbered exons (upper panel), and protein domains of the resulting RIM1α and β isoforms (lower panel). The isoform-specific 5’ exons are shown in blue and the first common exon shared by all isoforms is shown in red. The exon-exon boundaries targeted by isoform-specific and generic RIM1 PCR probes were marked by blue and red brackets respectively. Zn, Zinc-finger domain; PxxP, proline-rich region.

B. Schemes of mouse Rim2 gene and protein domain structure of RIM2α/β/γ.

C. Example of CT values analyzed in triplicate from the cochlear nuclei of a C57/Bl6 mouse at P14.

D. Quantification of the relative expression level, normalized to the β-actin values, and averaged for n = 3 mice at P14.

Figure 2. Presynaptic Ca²⁺ currents and readily-releasable pool are not strongly affected in RIM1- and RIM2 single KO mice.

A. Transmitter release in response to prolonged presynaptic depolarizations (0 mV for 50 ms) in representative RIM1 single cKO (blue), RIM2 single cKO (green), RIM1/2 double cKO (red) and control synapses (black). Presynaptic Ca²⁺ currents, EPSCs, release rates, and traces of cumulative release are shown from top to bottom panels. The time constant of the fast release component, as analyzed by double-exponential fits to the cumulative release traces, is indicated (τf). cDKO, conditional double KO.

B. Quantification of presynaptic Ca²⁺ current properties. Peak Ca²⁺ current amplitude at 0 mV (B1), presynaptic membrane capacitance Cm (B2), Ca²⁺ current density (B3) and Ca²⁺ current activation time constant (τIca; B4) are shown. In this panel and in C and D, the numbers of paired recordings for each observation are indicated below each bar.
C. Quantification of EPSC - and release parameters. The average values and individual data points for maximal EPSC amplitude \((C1)\), peak release rate \((C2)\), number of vesicles released in the fast component \((A_{fast}; C3)\), 20-80% rise time of EPSCs \((C4)\), and time constant of the fast release component \((\tau_f; C5)\) are shown. Note that none of the EPSC - and release parameters were significantly different in RIM1- or RIM2 single KO mice with respect to control mice, whereas all parameters were significantly different between RIM1/2 double cKO and control mice.

D. Average values and individual data points for the slow release time constant \((D1)\) and the number of slowly released vesicles \((D2)\).

**Figure 3.** \(\text{Ca}^{2+}\) channel - release coupling as measured by tail current protocols is unaffected in single RIM KOs but strongly impaired in the RIM1/2 double cKO mice.

A. Presynaptic voltage-clamp protocol (upper panel), \(\text{Ca}^{2+}\) tail currents (middle panel; time scale of the lower panel applies), and the corresponding EPSCs (lower panel) upon presynaptic depolarizations to +60 mV of varying durations. A representative paired recording is shown for each genotype. The *insets* show the peak of the \(\text{Ca}^{2+}\) currents on expanded scales.

B. Double-logarithmic plots of the EPSC amplitudes as a function of the presynaptic \(\text{Ca}^{2+}\) charge \((Q_{\text{Ca}})\) for RIM1 cKO (B1, blue), RIM2 cKO (B2, green) and control (black) group. A line fit to the logarithmized data set is superimposed for each data set from a given cell. For clarity, the results from only \(n = 3\) representative paired recordings out of larger data sets (see C) are shown for each group.

C. The slope values, or \(\text{Ca}^{2+}\) current cooperativity, obtained from line fits to log - log plots of EPSC amplitude versus \(Q_{\text{Ca}}\). Note the large values of 4 to 5 for the single RIM1- and RIM2 cKO synapses, and for the control data set. The number of paired recordings was: RIM1 cKO mice, \(n = 5\); RIM2 cKO mice, \(n = 4\), and control mice, \(n = 11\).
D. The presynaptic Ca\(^{2+}\) charge, \(Q_{\text{Ca}}\), needed to evoke an EPSC of 2 nA, as a measure of the absolute efficiency of small Ca\(^{2+}\) currents in evoking release.

**Figure 4.** The fast AMPA-receptor antagonist \(\gamma\)-DGG reveals a slightly lower cleft glutamate concentration in RIM1/2 double cKO mice.

A. Fiber stimulation - evoked EPSCs were suppressed by 500 µM \(\gamma\)-DGG in a representative control calyx. *Left*, individual (gray) and average (black) EPSC traces before ("baseline") and after applying \(\gamma\)-DGG. *Right*, time course of reversible block of EPSCs by \(\gamma\)-DGG. The black horizontal bar indicates the time of \(\gamma\)-DGG application. Individual EPSC amplitudes and the average value for baseline, steady - state block and wash-out are shown as gray circles and black lines, respectively.

B. As A, but for a RIM1/2 double cKO mouse. Note the smaller baseline EPSC amplitude, and the stronger steady- state block of EPSCs by \(\gamma\)-DGG as compared to the control cell in A.

C. Quantification of EPSC amplitudes under baseline conditions (*left* bars) and during steady - state block by \(\gamma\)-DGG (*right* bars) in control mice (*left* panel), and in RIM1/2 double cKO mice (*right* panel).

D. Relative \(\gamma\)-DGG block, normalized to the baseline EPSC amplitude measured in each cell, in control mice (*left* bar) and in RIM1/2 double cKO mice (*right* bar; \(p < 0.05\)).

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Author contributions:

Y.H., R.S., P.K. and T.S. conceived the study; P.K. created reagents; Y.H. and N.B. performed experiments and analyzed data; Y.H., P.K. and R.S. wrote the manuscript.
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