RIM-Binding Protein 2 positively regulates the abundance and release site coupling of presynaptic Ca²⁺ channels at a fast central synapse

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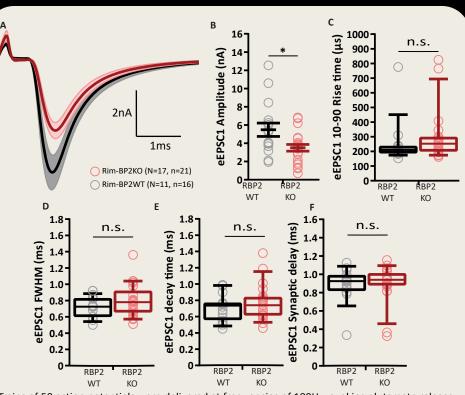
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1.Abstract

3. Unaltered CAZ molecular composition in Rim-BP2^{-/-}

RIM-Binding Protein 2 (RIM-BP2) is a multidomain protein of the presynaptic active zone (AZ). By binding to Rab-interacting protein (RIM), Bassoon and voltage-gated Ca²⁺ channels (CaV), it is considered a to be central organizer of the topography of CaV and release sites of synaptic vesicles (SVs). Here, we investigated the role of RIM-BP2 at the endbulb of Held synapse, a fast relay of the auditory pathway with high release probability. Disruption of RIM-BP2 reduced the amplitude of evoked excitatory ostsynaptic currents (EPSCs) and altered short-term plasticity due to reduced vesicular release probability. In addition, SV replenishment to the readily releasable SV pool was slowed. Augmenting Ca²⁺ influx by adding extracellular Ca²⁺ restored normal transmission. Presynaptic CaV channels were reduced and their topography altered. Moreover, there were fewer SVs in a distance of 2-20 nm to the AZ membrane. We conclude that RIM-BP2 positively regulates the clustering and SV coupling of CaV hannels at the endbulb of Held

2.Smaller eEPSCs in the Rim-BP2-/-



of the first eEPSC of a 100Hz train at Rim-BP2 deficient endbulbs of Held. (A) ed EPSCs eEPSCs in RBP2-WT (black) and RBP2-KO (wine-red) endbulb mplitude in the mutant. (**B**) reduced eEPSC amplitude in RBP2-KO ompared to RBP2-WT. Analysis of evoked glutamate release kinetics shows that: (\mathbf{C}) 10-90 rise ime, (**D**) full-width at half-maxima, (**E**) decay time and (**F**) synaptic delay are unaffected by t distributed data are presented as box and whisker plots (grand median of all BC means, lower/upper quartiles, 10-90th percentiles (C, D, E, F); n.s. p-value \geq 0.05, Mann-Whitney U-test). N: number of animals, n: number of BCs





Synaptology

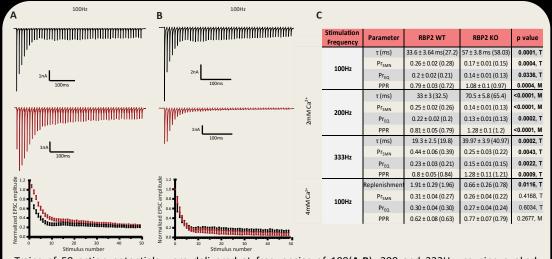
of Held. (A, C, E, G) Maximal z-projections of confocal image stacks of BCS in Rim-BP2WT and Rim-BP2KO shown on left and right respectively. 30µm coronal brainstem slices were immunolabeled for Rim-BP2 (A), Munc13-1 (C), RIM2 (E) and CAST (G) and co-stained for VGlut1 and Gephyrin, used as reference markers to indicate endbulbs and inhibitory synapses respectively. Slices were immunolabeled for Bassoon (I) and co-stained for Homer1 (excitatory synapses) and Gephyrin. (B, D, F, H, J) Quantification of fluorescence intensity of CAZ proteins at endbulbs and inhibitory synapses of BCs. A shows loss of Rim-BP2 immunoreactivity in the Rim-BP2KO prainstem sliced. The levels of Munc13-1 (D), RIM2 (F), CAST (H) and Bassoon (J) are unaltered at Rim-BP2KO endbulbs. Right insets of D, F, H, J show similar to WT number of CAZ protein spots localized to endbulb CAZs. Data information: N: number of animals, S: number of slices, n: number of BCs. All scale bars: 5μm.

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Rim-BP2WT(N=7, S=8, n=13) Rim-BP2KO(N=3, S=4, n=17) Rim-BP2WT(N=3, S=3, n=16) Coronal brainstem sections were obtained from RimBP2 WT and KO mice to assess the levels of Active zone proteins in AZs of endbulbs

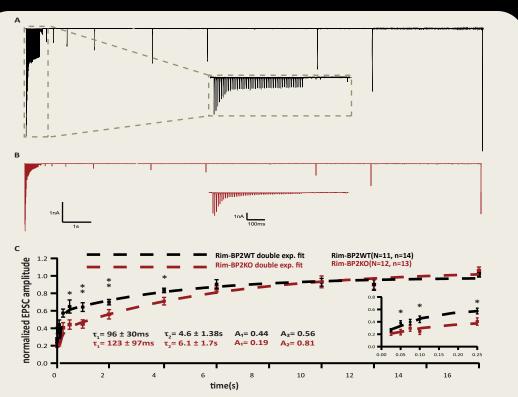
4. Reduced P_{_} rescuable at high Ca²⁺, where replenishment seems slower



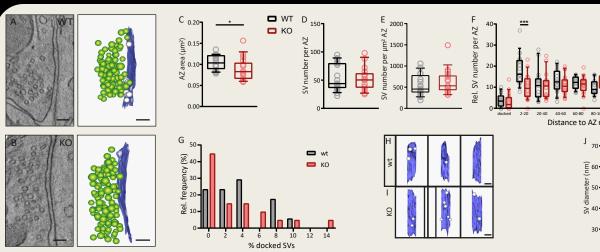


were delivered at frequencies of 100(A,B), 200 and 333Hz, causing evoked lutamate release. eEPSCs were recorded from Rim-BP2WT (top, black traces) and Rim-BP2KO (bottom, ine-red traces). Example traces are shown for extracellular Ca²⁺concentrations of 2mM (**A**) and 4mM (**B**). The ild type traces show the characteristic fast short-term depression of bushy cell EPSCs, which is altered in the nutant. The mutant BCs show a delayed short-term depression with the first EPSC amplitude not being the est in the train (higher PPR), hinting at a reduced release probability (**C**) The effect is abolished at 4mM Ca²⁺, hishment becomes prominent (C). instead a deficit in vesicle reple

5. Slowed recovery⁺ in the Rim-BP2^{-/-}

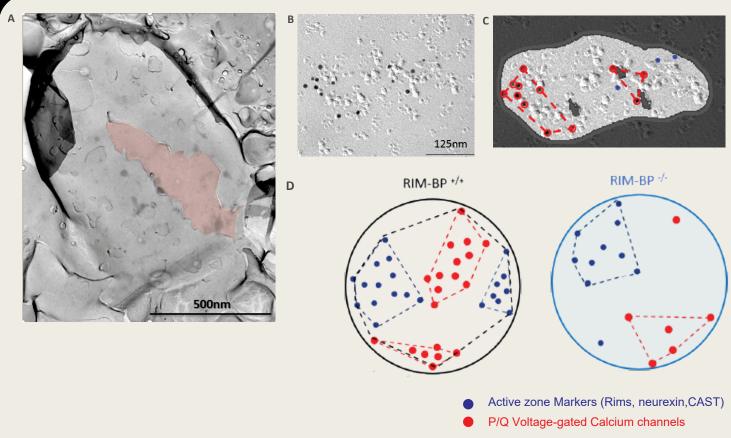


The deletion of Rim-BP2 leads to slower recovery from short-term depression at the endbulb of Held. Representative traces of RBP2-WT (A) and RBP2-KO (B), illustrate the recovery protocol. After a 100Hz conditioning train of 50stimuli, single test pulses were delivered at time intervals of (in ms) 25, 50, 75, 100, 250, 500 (further intervals in s), 1, 2, 4, 6, 10, 12 and 16. To assess recovery, the EPSC amplitude in response to the test pulse is normalised to the first EPSC amplitude of the conditioning train. Insets (A, B) show the time course of recovery during the first 5 test stimuli in sub-second detail. (C) Recovery is plotted as mean ± SEM EPSC amplitude in response to test pulses normalized to the first EPSC amplitude of the conditioning train. The double exponential fits are represented by the dashed lines for RBP2-WT (black) and RBP2-KO (wine-red). Inset shows the first five esponses in detail. Normality was tested with Jargue-Bera test. Info: N: number of animals, n: number of BCs



(A,B) Single virtual sections and corresponding models of representative tomograms of WT (A) and KO (B) active zones (AZs) showing the AZ membrane (blue), synapt vesicles (SVs) (green), and morphologically docked SVs (grey). Scale bars: 100 nm. (C) The AZ area is slightly smaller in the KO endbulb synapses. (D) Unaltered total number of synaptic vesicles (SVs) in mutant AZs. (E) Normalized to the AZ area, the SV number is unaltered in KO endbulbs. (F) Morphological docked SVs (0-2 nm) and SV numbers within 200 nm from the AZ membrane divided into 20 nm bins. *p-value < 0.05, ***p-value < 0.001*. Student's t test or Mann Whitney test. (G) Frequence distribution of the proportion of morphologically docked SVs (H,I) Topviews of representative tomogram models of WT (H) and RBP2-KO (I) AZs with docked SVs. Scale bars: 100 nm. (J) Mean SV diameter is unaltered in mutant synapses. Box and whisker plots present median, lower/upper quartiles, 10–90th percentiles. p-value > 0.05 dent's t test. Each data point represents the mean diameter of SVs of individual synapses.

7. The Rim-BP2 perturbation reduces the number of CaV particles per cluster in endbulb AZs



A: Scanning electron microscopy (SEM) overview of a separated enbulb to BC synaptic cleft. B: Snapshot of an active zone, defined by the intramembrane particles, analyzed for clustering (C) of VGCCs (10nm gold particles) and CAZ markers (5nm gold particles). The sketch in D showcases the two most significant differences in clustering between WT and RIM-BP2 KO endbulbs . Notably the nearest neighbour distance between AZ and VGCC clusters is significantly increased in the knock out. Even hough the cluster areas remain similar, the KO endbulbs contain significantly less VGCC particles per cluster, suggesting a role of RBP2 in their trafficking to the AZ.

