Figures and figure supplements

RIM-BP2 primes synaptic vesicles via recruitment of Munc13-1 at hippocampal mossy fiber synapses

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**Figure 1.** RIM-BP2 KO affects synaptic transmission specifically at MF synapses. (a) Immunostaining of RIM-PB2 in hippocampal brain slices (DG = dentate gyrus) and schematic illustration of recording configurations. (b) Input-output of synaptic transmission, plotted as PFV against fEPSP amplitude, of associative commissural (AC) and Schaffer collateral (SC) synapses showed no difference between RIM-BP2 WT and KO slices (AC: n(WT) =17 slices/6 animals; n(KO)=21 slices/6 animals) (SC: n(WT)=9 slices/3 animals; n(KO)=12 slices/3 animals). Sample traces show averages of 10 sweeps. Values represent mean ± SEM. (c) Input-output of synaptic transmission of MF synapses, plotted as PFV against fEPSP amplitude (MF: n(WT)=22 slices/7 animals; n(KO)=18 slices/7 animals). Sample traces show averages of 10 sweeps. (d) Frequency facilitation with 1 Hz stimulation of MF synapses (sweep 10–30). Sample traces show averages of five sweeps before (gray) and at the end of 1 Hz stimulation (black). For statistics please see Figure 1—source data 1.

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Figure 2. RIM-BP2 deletion does not alter the localization of Ca\textsubscript{v}2.1 clusters relative to the active zone protein RIM1 and the postsynaptic marker Homer1 at MF synapses. (a) Confocal (left) and gSTED (right) images of RIM-BP2, Munc13-1 and Bassoon (Bsn) at the active zone (AZ) of WT MF.
boutons (MFBs) in situ. Arrows indicate synapses in side view. (b) Example of k nearest neighbor distance analysis of protein clusters at MF synapses. Following image thresholding and Watershed segmentation, X and Y coordinates of each segmented cluster identified were retrieved and Euclidean distances of for example Munc13-1 clusters relative to a given RIM-BP2 cluster calculated with a custom-written MATLAB script. Several hundreds to thousands of clusters per image were analyzed and values averaged per animal (n = 6). (c) Upper, mean k nearest neighbor distances for Munc13-1 clusters relative to a given RIM-BP2 (left) or Bassoon (middle) cluster and for RIM-BP2 clusters relative to a given Bassoon cluster (right). Lower, mean k nearest neighbor distances for Munc13-1 clusters relative to Munc13-1 itself as center (left), for Bassoon clusters relative to itself (middle) and RIM-BP2 clusters relative to itself (right). Based on ultrastructural studies of MF AZ size, estimating an AZ diameter of 391 nm, at WT MFBs we detected at least three Munc13-1 clusters, two Bassoon clusters and four RIM-BP2 clusters within a single AZ, having a d_k < 391 nm. (d) gSTED images of CaV2.1, RIM1 and Homer1 clusters at MFBs of RIM-BP2 WT and KO brain slices. Arrows indicate synapses with two Cav2.1 clusters apposed to a single Homer1 cluster. (e) Average number of Cav2.1, RIM1 and Homer1 clusters found at MFBs and cluster ratio per each RIM-BP2 WT (n = 9) and KO (n = 9) mouse analyzed (f). No significant differences were observed between the two groups. (g) Example of k nearest neighbor distance analysis of protein clusters at MF synapses. Several hundreds to thousands of clusters per image were analyzed and values averaged per animal. (h) k nearest neighbor distances of the first and second closest RIM1 k neighbor (k = 1, k = 2) relative to a given Cav2.1 (first left), no significant differences were observed between RIM-BP2 WT and KO mice. No significant differences were observed also for the mean k nearest neighbor distance at which Cav2.1 clusters are located relative to a given Cav2.1 (second left). Based on ultrastructural studies of MF AZ size, estimating an AZ diameter of 391 nm, at WT MFBs we detected one RIM1 cluster and three Cav2.1 clusters per single active zone, having a d_k < 391 nm. No significant difference was observed for Cav2.1 and RIM1 localization in relation to Homer1 (third left and first right, respectively). Values represent mean ± SEM. For statistics please see Figure 2—source data 1.

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Figure 2—figure supplement 1. RIM-BP2 deletion does not alter P/Q type Ca\textsuperscript{2+}-channels localization at MF synapses. (a) in situ gSTED images of Cav2.1 and Homer1 at MF synapses in RIM-BP2 WT and KO mice. Dotted line represents an example of a synapse analyzed by line profile measurement. Line profile thickness was set to ~250 nm. (b) Intensity line profiles of the synapses displayed in (a). For display, intensity in each channel was normalized to its maximum after background subtraction. We analyzed 24–35 line profiles per mouse (n = 9 mice/genotype). (c) inter-Cav2.1 distances found averaged per mouse analyzed. (d) Frequency histogram for all inter-Cav2.1 distances found. Counts were normalized to obtain a probability density function with an integral equal 1. No significant difference between RIM-BP2 WT and KO mice was observed. Values represent mean ± SEM. For statistics please see Figure 2—figure supplement 1—source data 1.

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Figure 3. Loss of RIM-BP2 specifically reduces Munc13-1 levels at MF synapses but not at CA3-CA1 synapses. (a) Representative gSTED images of Ca_{2.1} and Munc13-1 clusters at MF boutons (MFB) identified by ZnT3 expression (confocal) in RIM-BP2 WT and KO brain sections. Arrows indicate Figure 3 continued on next page.
Munc13-1 clusters nearby Ca\textsubscript{v}2.1 clusters. (b) Example of \textit{k} nearest neighbor distance analysis of Munc13-1 clusters relative to a given Cav2.1 cluster at MF synapses. Following image thresholding and Watershed segmentation, X and Y coordinates of each segmented cluster identified were retrieved and Euclidean distances of for example Munc13-1 clusters relative to a given Cav2.1 cluster calculated with a custom-written MATLAB script (b, upper). Similarly, we retrieved the number of for example Munc13-1 clusters found at specific distance intervals (nm) from a given Cav2.1 cluster (b, lower). Several hundreds to thousands of clusters per image were analyzed and values averaged per animal. (c) Average number of Cav2.1 clusters within the ZnT3 + area found per each RIM-BP2 WT (n = 8) and KO (n = 6) animal analyzed. ZnT3 was used as marker to identify MF synapses. (d) Average number of Munc13-1 clusters within the ZnT3 + area found per each RIM-BP2 WT and KO animal analyzed. (e) Ratio of Munc13-1 clusters/Cav2.1 clusters in RIM-BP2 KO and WT mice. (f) The number of Munc13-1 clusters at determined distance intervals (nm) from a given Cav2.1 cluster decreased significantly at all distances analyzed in RIM-BP2 KO, while the distance of the first closest \textit{k} neighbor (\textit{k} = 1, g) up to the fourth (\textit{k} = 4) significantly increased. (h) Representative gSTED images of Cav2.1 and Munc13-1 clusters at CA3-CA1 synapses in RIM-BP2 WT and KO brain sections. Arrows indicate Munc13-1 clusters adjacent to Cav2.1 clusters. (i) Average number of Cav2.1 clusters and Munc13-1 clusters found within the field of view at CA3-CA1 synapses in RIM-BP2 KO (n = 6) and WT (n = 9) mice. (k) Ratio of Munc13-1 clusters/Cav2.1 clusters at CA3-CA1 synapses. (l) At CA3-CA1 synapses, loss of RIM-BP2 does not significantly alter either the number of Munc13-1 clusters at determined distance intervals (nm) from a given Cav2.1 cluster or the distance at which the first closest \textit{k} neighbor (\textit{k} = 1, m) is found. Values represent mean ± SEM. *\textit{p}<0.05, **\textit{p}<0.01. For statistics please see \textit{Figure 3—source data 1}. 

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**Figure 3—figure supplement 1.** Loss of RIM-BP2 leads to an increased distance between Munc13-1 and Cav2.1 clusters at MF synapses. (a) in situ gSTED images of Munc13-1 and Cav2.1 at MF synapses in RIM-BP2 WT and KO mice. Dotted line represents an example of a synapse analyzed by line profile measurement. Line profile thickness was set to ~250 nm. (b) Intensity line profiles of the synapses displayed in (a). For display, intensity in each channel was normalized to its maximum after background subtraction. We analyzed 30–48 line profiles per mouse (n (WT) = 8 mice and n (KO) = 6 mice). (c) Munc13-1-Cav2.1 distances averaged per mouse analyzed. A significant increase in the distance at which Munc3-1 clusters are found relative to Cav2.1 clusters was observed in RIM-BP2 KO as compared to WT mice. (d) Frequency histogram for all Munc13-1-Cav2.1 distances found. Counts were normalized to obtain a probability density function with an integral equal 1. Values represent mean ± SEM. *p<0.05. For statistics please see Figure 3—figure supplement 1—source data 1.

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Figure 3—figure supplement 2. Loss of RIM-BP2 leads to an increased inter-Munc13-1 clusters distance specifically at MF synapses. (a) in situ gSTED images of Munc13-1 and Homer1 at MF synapses in RIM-BP2 WT and KO mice. Dotted line represents an example of a synapse analyzed by line profile.
measurement. Line profile thickness was set to ~250 nm. (b) Intensity line profiles of the synapses displayed in (a). For display, intensity in each channel was normalized to its maximum after background subtraction. We analyzed 31–36 line profiles per mouse (n (WT) = 6 mice and n (KO) = 6 mice). (c) Inter-Munc13-1 distances averaged per mouse analyzed. A significant increase in the distance at which two Munc13-1 clusters are found relative to each other was observed in RIM-BP2 KO as compared to WT mice. (d) Frequency histogram for all inter-Munc13-1 distances found. Counts were normalized to obtain a probability density function with an integral equal 1. (e) In situ gSTED images of Munc13-1 and Homer1 at CA3-CA1 synapses in RIM-BP2 WT and KO mice. Dotted line represents an example of a synapse analyzed by line profile measurement. Line profile thickness was set to ~250 nm. (f) Intensity line profiles of the synapses displayed in (e). For display, intensity in each channel was normalized to its maximum after background subtraction. We analyzed 27–30 line profiles per mouse (n (WT) = 6 mice and n (KO) = 6 mice). (g) Inter-Munc13-1 distances averaged per mouse analyzed. At CA3-CA1 synapses, no significant difference was observed in the distance at which two Munc13-1 clusters are found relative to each other. (h) Frequency histogram for all inter-Munc13-1 distances found. Counts were normalized to obtain a probability density function with an integral equal 1. Values represent mean ± SEM. **p<0.01. For statistics please see Figure 3—figure supplement 2—source data 1.

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Figure 3—figure supplement 3. RIM-BP2 deletion results in significantly reduced Munc13-1 levels at MF synapses. (a) in situ confocal images of Munc13-1 immunoreactivity at MF synapses, in the CA3 stratum lucidum of RIM-BP2 WT (n = 8) and KO (n = 6) mice. (b) Munc13-1 intensity for MF synapses, normalized to RIM-BP2 WT mice. Loss of RIM-BP2 resulted in significantly lower Munc13-1 intensity in RIM-BP2 KO mice as compared to RIM-BP2 WT mice. (c) in situ confocal images of Munc13-1 immunoreactivity in the CA1 stratum radiatum (n(WT)=9; n(KO)=6). (d) Munc13-1 intensity for CA3-CA1 synapses, normalized to RIM-BP2 WT mice. At CA3-CA1 synapses, RIM-BP2 deletion did not significantly alter Munc13-1 intensity. Values represent mean ± SEM. *p<0.05. For statistics please see Figure 3—figure supplement 3—source data 1.

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Figure 3—figure supplement 4. Loss of RIM-BP2 does not alter Munc13-2 levels at both MF-CA3 and CA3-CA1 synapses. (a) Representative gSTED images of Cav2.1 and Munc13-2 clusters at MF boutons (MFB) identified by ZnT3 expression (confocal) in RIM-BP2 WT and KO brain sections. Arrows
indicate Munc13-2 clusters nearby Cav2.1 clusters. (b) Example of $k$ nearest neighbor distance analysis of Munc13-2 clusters relative to a given Cav2.1 cluster at MF synapses. Following image thresholding and Watershed segmentation, X and Y coordinates of each segmented cluster identified were retrieved and Euclidean distances of for example Munc13-2 clusters relative to a given Cav2.1 cluster calculated with a custom-written MATLAB script (b, upper). Similarly, we retrieved the number of for example Munc13-2 clusters found at specific distance intervals (nm) from a given Cav2.1 cluster (b, lower). Several hundreds to thousands of clusters per image were analyzed and values averaged per animal. (c) Average number of Cav2.1 clusters within the ZnT3 +area found per each RIM-BP2 WT (n = 6) and KO (n = 6) animal analyzed. ZnT3 was used as marker to identify MF synapses. (d) Average number of Munc13-2 clusters within the ZnT3 +area found per each RIM-BP2 WT and KO animal analyzed. (e) Ratio of Munc13-2- clusters/ Cav2.1 clusters in RIM-BP2 KO and WT mice. (f) Number of Munc13-2 clusters at determined distance intervals (nm) from a given Cav2.1 cluster and distance at which the closest $k$ neighbor ($k = 1,2,3,4$; g) is found. No significant difference was observed between RIM-BP2 KO and WT mice. (h) Representative gSTED images of Cav2.1 and Munc13-2 clusters at CA3-CA1 synapses in RIM-BP2 WT and KO brain sections. Arrows indicate Munc13-2 clusters nearby Cav2.1 clusters. (i) Number of Cav2.1 clusters and Munc13-2 clusters (j) found at CA3-CA1 synapses in RIM-BP2 KO (n = 6) and WT mice (n = 6). (k) Ratio of Munc13-2 clusters/Cav2.1 clusters at CA3-CA1 synapses. (l) At CA3-CA1 synapses, loss of RIM-BP2 does not significantly alter either the number of Munc13-2 clusters at determined distance intervals (nm) from a given Cav2.1 cluster or the distance at which the closest $k$ neighbor ($k = 1,2,3$) is found (m). Values represent mean ± SEM. For statistics please see Figure 3—figure supplement 4—source data 1.

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Figure 4. Loss of RIM-BP2 specifically affects vesicle docking at MF synapses. Representative EM images of MF synapses from acute hippocampal slices obtained from RIM-BP2 KO (black: three animals/each 2–3 slices/78 active zones) and WT (red: three animals/2–3 slices each/52 active zones) mice.

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(b) Representative EM images of CA1 synapses from acute hippocampal slices obtained from RIM-PB2 KO (black: three animals/each 2 slices/27 active zones) and WT (red: three animals/2 slices each/25 active zones) mice. (c) Frequency distribution and bar graph show no difference in the size in the post-synaptic density (PSD) in MF active zones from WT or RIM-BP2 KO slices. (d) Frequency distribution and bar graph show a reduction of docked vesicles per 100 nm of the active zone at RIM-BP2 KO MF synapse compared to WT MF synapses. (e) Frequency distribution and bar graph depict no difference in the number of docked large vesicles (LV) (vesicle diameter >70 nm) (f) Frequency distribution and bar graph of docked vesicles at CA3-CA1 synapses show no difference between WT and RIM-BP2 KO. Values represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. For statistics please see Figure 4—source data 1.

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**Figure 5.** RIM-BP2 KO affects synaptic transmission at granule autaptic neurons. (a) Sample traces of evoked EPSC amplitudes before (black) and after DCG IV application (gray) for RIM-BP2 WT and KO neurons. RIM-BP2 KO neurons were rescued by lentiviral transduction of RIM-BP2. (Number of experiments (cells/cultures); EPSC WT (70/4); KO (78/4); RIM-BP2 (24/3)) (b) Summary graphs of normalized EPSC amplitudes evoked by 2 ms depolarization (red arrow). (c) Sample traces and (d) summary graphs of normalized RRP responses elicited by a 5 s application of 500 mM sucrose. Summary graph of the P_{VR} calculated as the ratio of the EPSC charge and the RRP charge. (Sucrose WT (69/4); KO (59/4); RIM-BP2 (24/3)). (e) Sample traces of evoked EPSC amplitudes with an interstimulus interval of 25 ms. (f) Summary graph of paired-pulse ratio (PPR) of RIM-BP2 WT, KO and RIM-BP2 rescued autaptic granule neurons. (PPR WT (70/4); KO (74/4); RIM-BP2 (24/3)) (g) Summary graph of Paired-Pulse-Ratio (PPR) of granule cells with different inter-stimulus intervals of RIM-BP2 WT and KO granule autapses (PPR WT (28/2), PPR KO (29/2)). (h) Sample traces of miniature EPSCs (mEPSCs) and summary graph of mEPSC frequencies. (mEPSC WT (63/4); KO (43/4); RIM-BP2 (24/3)). Values represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. For statistics please see Figure 5—source data 1.

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Figure 5—figure supplement 1. Characterization of autaptic granule cells. (a) Representative EM image of a granule cell in culture. (b) Sample traces and summary graphs of evoked EPSCs of granule cells with an interstimulus interval of 25 ms before (black) and after DCG IV application (gray) of RIM-BP2 WT and KO neurons. Both WT and KO neurons respond to the DCG IV application. EPSC amplitudes were reduced in RIM-BP2 KO neurons. (c) Representative EM image of a small central synapse in culture. (d) Sample traces and summary graphs of evoked EPSCs of hippocampal autaptic neurons with an interstimulus interval of 25 ms from RIM-BP2 WT and KO neurons that did not respond to DCG IV application. Autaptic neurons that do not respond to DCG IV application showed no reduction in EPSC amplitude in RIM-BP2 KO autapses compared to WT.

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Figure 5—figure supplement 2. Loss of RIM-BP2 increases synaptic facilitation in autaptic granule neurons. Sample traces of miniature EPSCs (mEPSCs) and summary graph of mEPSC amplitudes. (mEPSC WT (48/4); KO (37/4); RIM-BP2 (24/3)) (b) Sample traces of normalized EPSC amplitudes to the first EPSC and (c) summary graph of EPSCs elicited by a 10 Hz stimulation train (WT (54/3); KO (44/3); RIM-BP2 (18/3). Values represent mean ± SEM. For statistics please see Figure 5—figure supplement 2—source data 1.
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Figure 6. Monomeric Munc13-1 rescues vesicle priming in RIM-BP2 KO granule autaptic neurons. (a) Sample traces of evoked EPSC amplitudes before (black) and after DCG IV application (gray) for RIM-BP2 WT and KO neurons and lentiviral-transduced RIM-BP2 KO rescues with either Munc13-1 WT (M13WT) or Munc13-1 K32E (M13K32E). (b) Summary graphs of normalized EPSC amplitudes evoked by 2 ms depolarization (red arrow) (EPSC WT (49/3); KO (47/3); M13WT (18/2); M13K32E (41/3)) and after DCGIV application. (EPSC-DCG WT (49/3); KO (44/3); M13WT (18/2); M13K32E (41/3)) (c) Sample traces and (d) summary graphs of normalized RRP responses elicited by a 5 s application of 500 mM sucrose (RRP WT (41/3); KO (36/3); M13WT (17/2); M13K32E (39/3)). Summary graph of the Pvr calculated as the ratio of the EPSC charge and the RRP charge (Pvr WT (41/3); KO (38/3); M13WT (16/2); M13K32E (37/3)). Values represent mean ± SEM. *p<0.05, **p<0.01. For statistics please see Figure 6—source data 1.

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