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SUPPLEMENTARY INFORMATION

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Synaptotagmin-1 drives synchronous Ca^{2+}-triggered fusion by C_2B-domain-mediated synaptic-vesicle-membrane attachment

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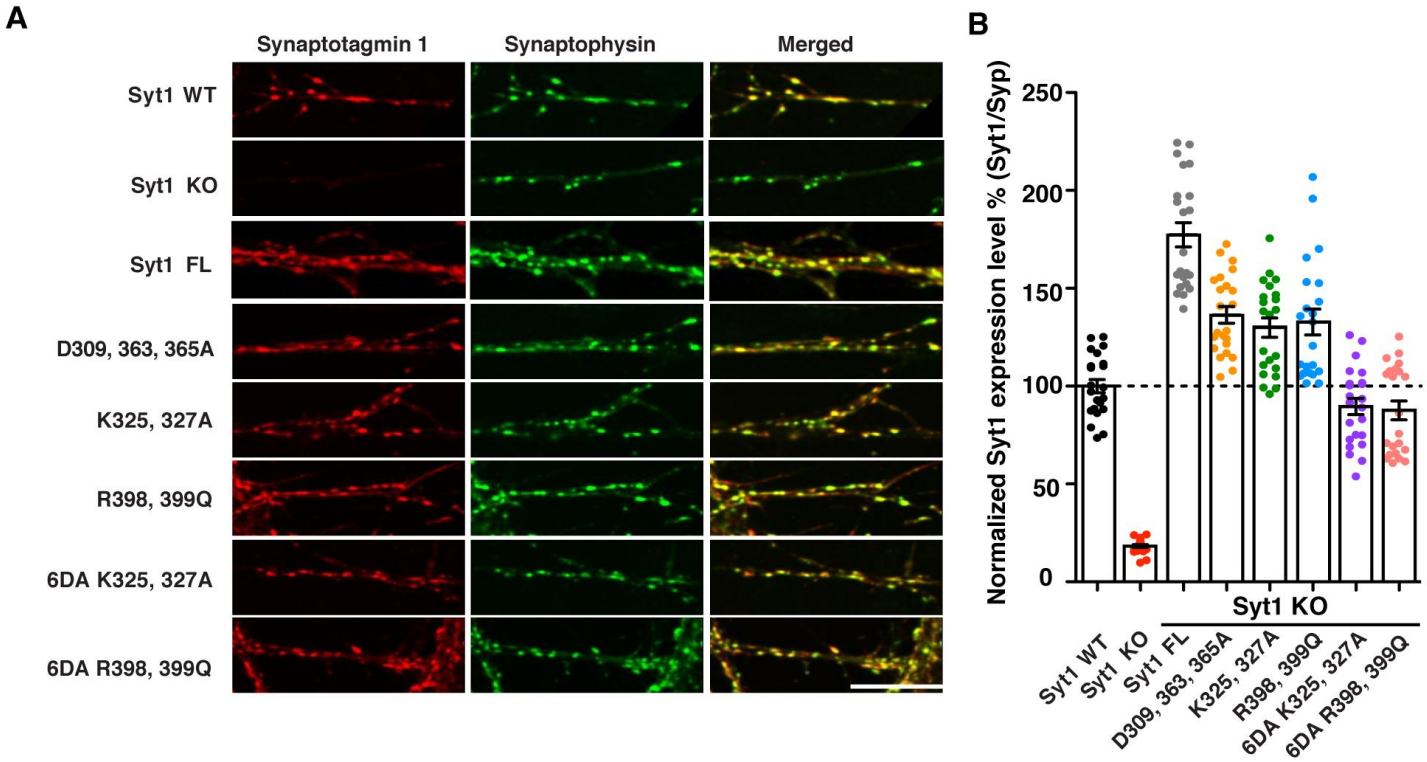
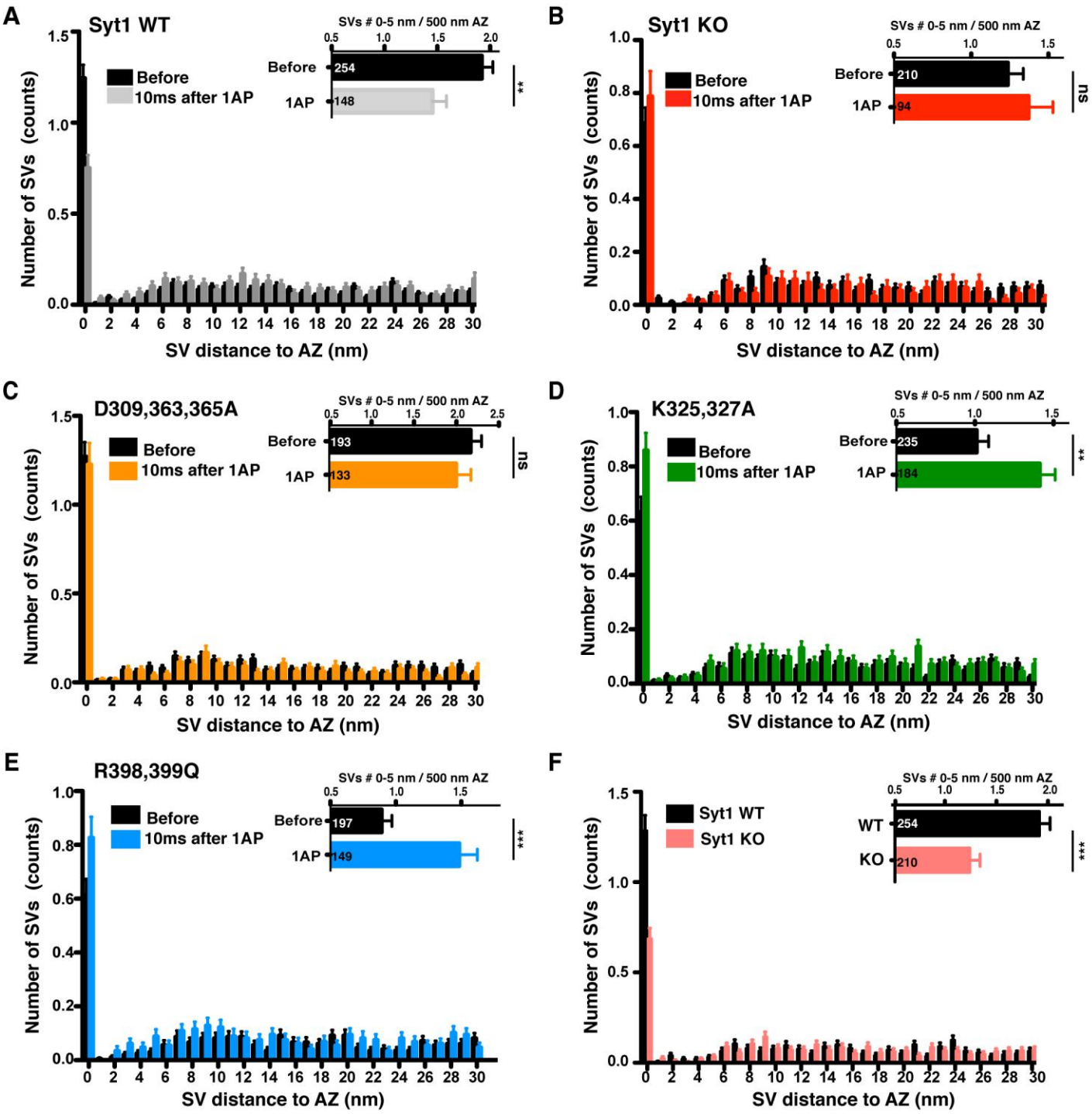


Supplementary Figure 1

Quantification of expression level of Syt1 mutants at the presynaptic terminals

(**A)** Representative images of Syt1 WT, KO and KO neurons rescued with wild-type Syt1 full length (FL) and mutant constructs immunostained for Syt1 (red) and a synaptic marker Synaptophysin (green). Scale bar: 20 μm. **(B)** Scatter plot shows averaged fluorescence intensity of Syt1 protein. Data were normalized to the intensity of endogenous Synaptophysin and to the Syt1 intensity in the Syt1 WT neurons (n = 22, 23, 22, 23, 22, 22, 23, 22; respectively). 6DA indicates mutations at both Ca^{2+} binding sites of the C_2A and C_2B domains. (C_2A: D178, 230, 232A; C_2B: D309, 363, 365A). Data were obtained from cultured neuron from 3 independent cultures and are shown as mean ± SEM.

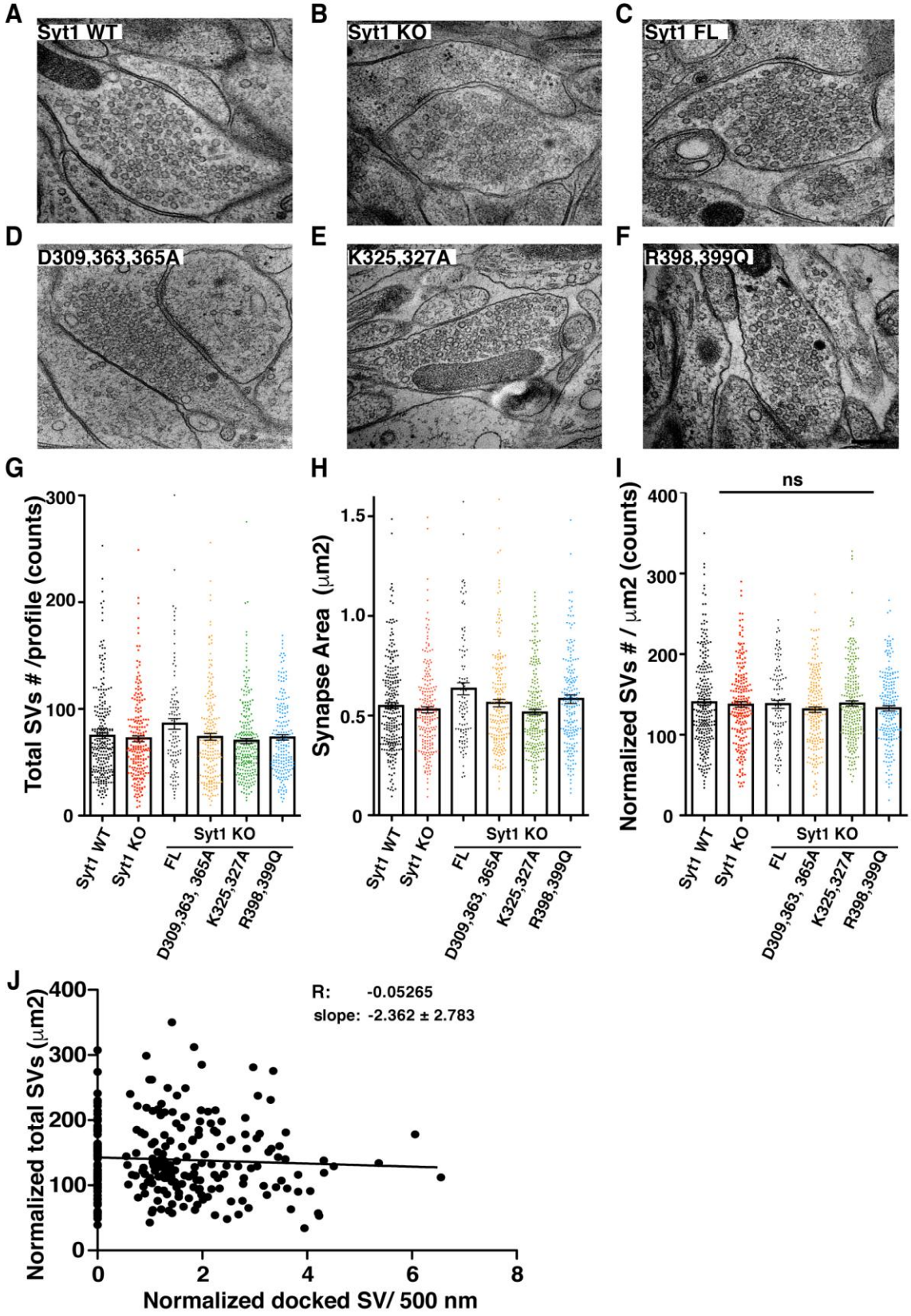


Supplementary Figure 2

Absolute distribution of synaptic vesicles within 30 nm of the AZ.

Averaged distribution of SVs before and 10 ms after AP stimulation in **(A)** Syt1 WT (**B**) Syt1 KO and Syt1 KO rescued with (**C**) D309, 363, 365A (**D**) K325, 327A or (**E**) R398, 399Q. SV numbers are displayed as a function of distance (bin size 1 nm). Bar graph on the right of each panel represent the average number of SVs within 0-5 nm to the AZ before and 10 ms after AP. (**F**) Averaged distribution of SVs in Syt1 WT (black) and KO (pink). Data were obtained from

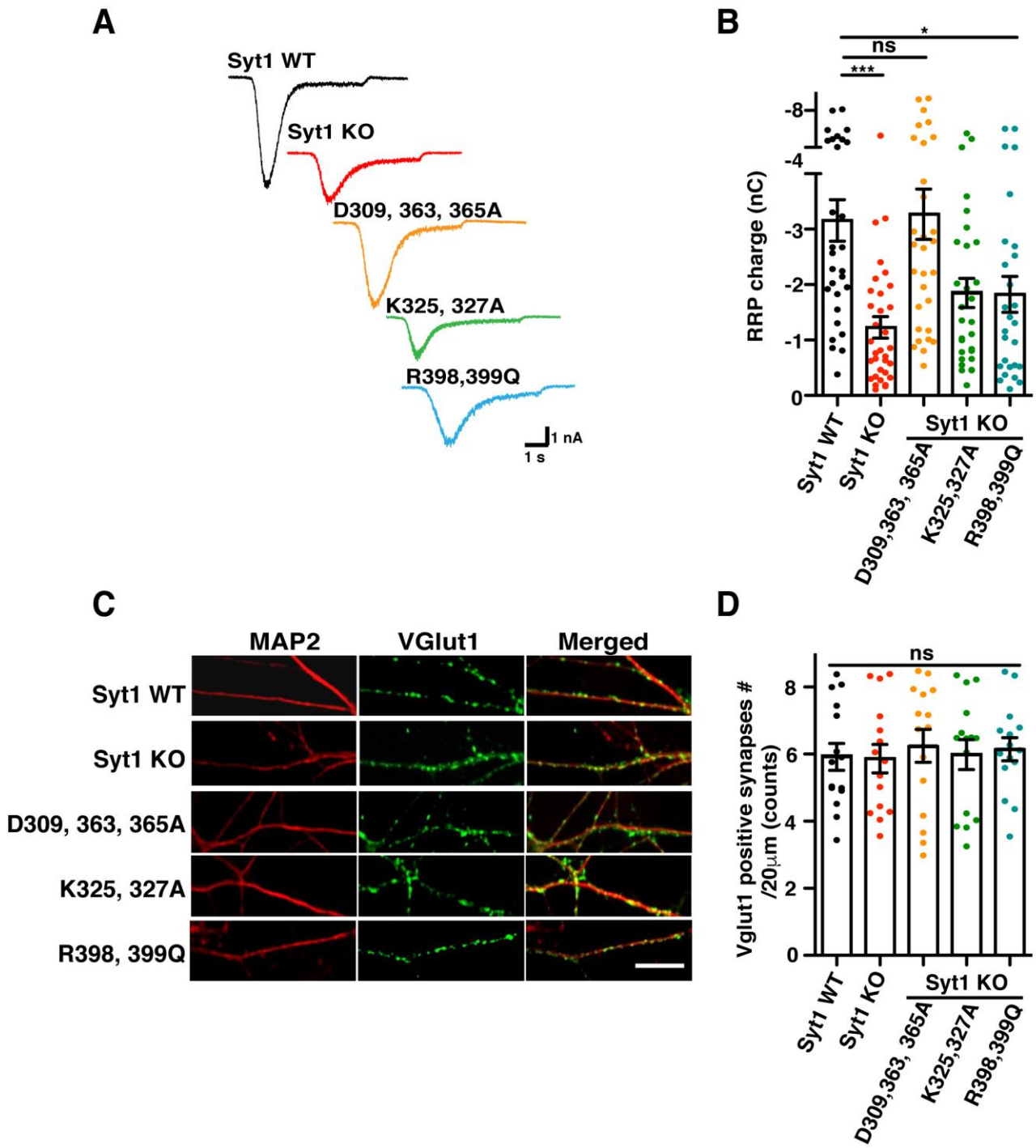
cultured neurons from at least 3 independent cultures and shown as mean ± SEM. Statistical significance was assessed by Mann-Whitney test. ***$p < 0.001$; **$p < 0.01$; ns: not significant. n represents the number of electron micrographs (synapses) analyzed.



Supplementary Figure 3

Quantification of total SV number and synapse area

(**A-F**) Representative electron micrographs of Syt1 WT, KO and KO rescued with Syt1 FL and mutant constructs. Scale bar: 200 nm. (**G**) Scatter plot shows averaged number of total SVs within synapse. (**H**) Averaged area of the synapses. (**I**) Normalized number of total SVs in synapse profile. Numbers were normalized to synapse area. (n = 254, 210, 102, 193, 235, 197; respectively) (**J**) Correlation plot of the number of docked vesicles (per 500 nm of AZ) versus total number of SVs (per μm^2 of synapse area) in Syt1 WT (n = 254). Linear regression fit shows poor correlation with indicated regression coefficient and slope. n represents the number of electron micrographs (synapses) analyzed. Data were obtained from cultured neurons from at least 3 independent cultures and are shown as mean ± SEM. Statistical significance was assessed by Kruskal-Wallis ANOVA test, followed by Dunn's multiple comparison test; ns: not significant. For detailed numbers and statistical analysis, see Supplementary Table 1B.

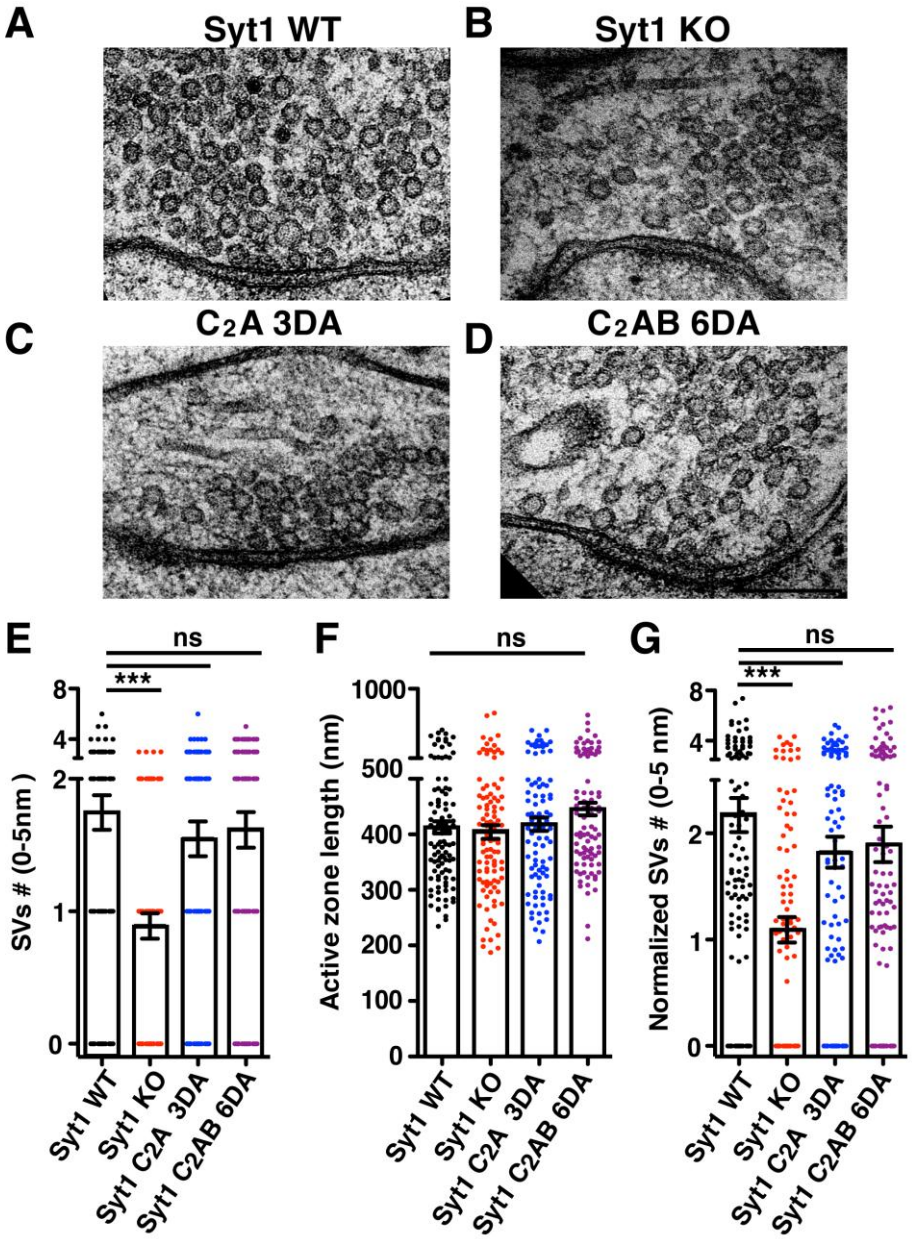


Supplementary Figure 4

Effects of Syt1 C_2B mutants on vesicle priming

(A) Representative traces of release induced by 5 seconds' hypotonic stimulation from mass-cultured Syt1WT, Syt1 KO, KO neurons rescued with mutant constructs. Responses from excitatory synapses were obtained by applying extracellular solution containing 500 mM sucrose with the presence of 0.1 μM TTX and 30μM bicuculline. **(B)** Averaged readily releasable pool (RRP) charge. (n=30,33,30,29,27;respectively). **(C)** Representative images of Syt1 WT, KO and KO neurons rescued with mutant constructs immunostained for MAP2 (red) and a synaptic marker for excitatory neuron Vglut1 (green). Scale bar: 20 μm. **(D)** Averaged synapse density. Synapses number was measured as the Vglut1 positive puncta. Numbers are normalized to dendritic lengths. (n=15,15,15,15,15;respectively). n

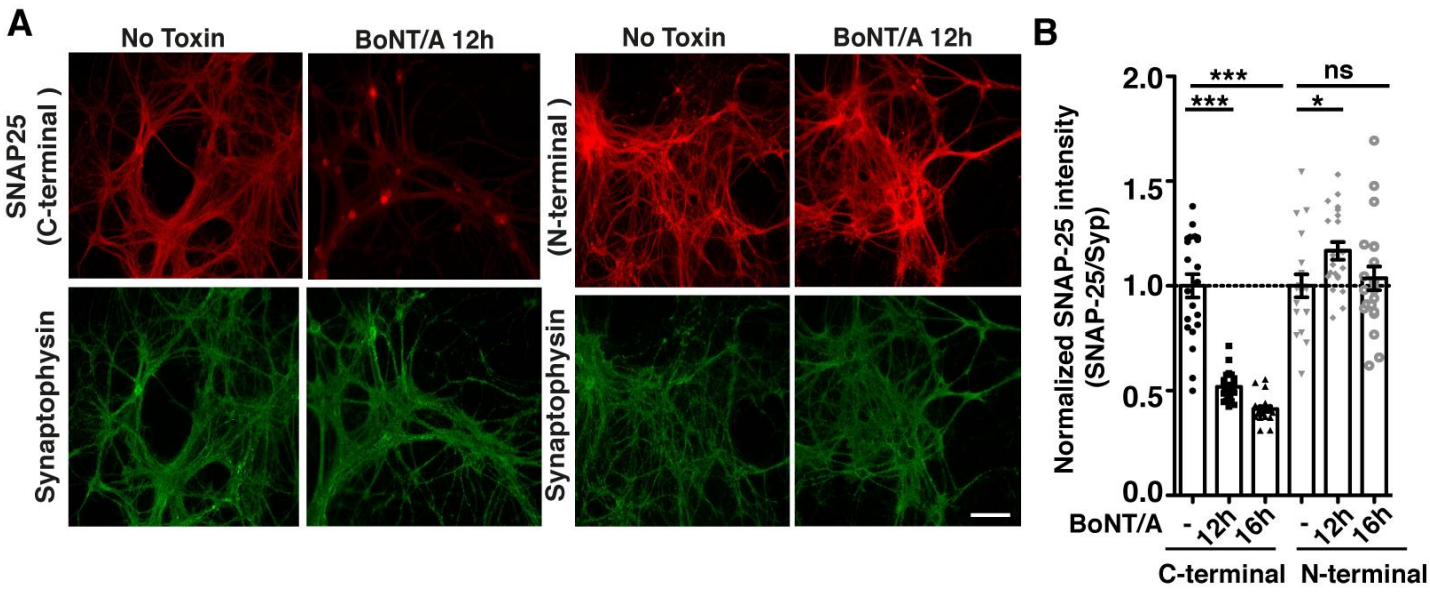
represents the number of cells analyzed or the number of images analyzed, as applies. Data were obtained from cultured neurons from 3 independent cultures and are shown as mean ± SEM. Statistical significance was assessed by Kruskal-Wallis ANOVA, followed by Dunn's multiple comparison test for (B) and one-way ANOVA with Dunnett's multiple comparisons test for (D). ***$p < 0.001$; *$p < 0.05$; ns: not significant.



Supplementary Figure 5

Mutations at Ca^{2+} binding site of C_2 domains do not impair the membrane attachment activity of Syt1 at rest

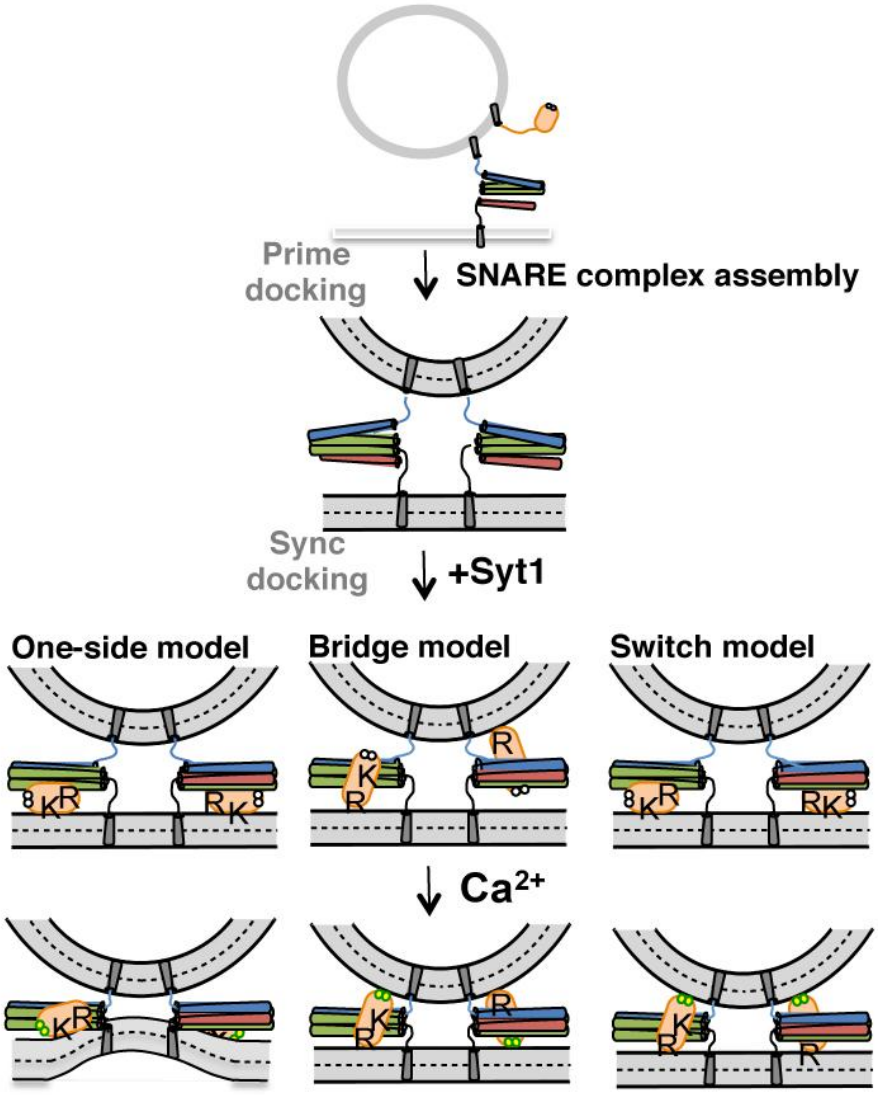
(A-D) Representative electron micrographs of synapses from Syt1 WT, KO and KO rescued with mutants of Ca^{2+}binding site at the C_2A domain (D178, 230, 232A; termed C_2A 3DA) or at both C_2A and C_2B domains (D178, 230, 232A; D309, 363, 365A; termed C_2AB 6DA mutant). Scale bar: 200 nm **(E)** Averaged vesicle number within 0-5 nm to the AZ (n=102,99,95,96; respectively).(**F**) Averaged AZ lengths. **(G)** Normalized vesicle number. Data were normalized to the AZ lengths. n represents the number of electron micrographs (synapses) analyzed. Data were obtained from cultured neuron from 2 independent cultures and shown as mean ± SEM. Statistical significance was assessed by Kruskal-Wallis ANOVA, followed by Dunn's multiple comparison test. ***p < 0.001; ns: not significant.



Supplementary Figure 6

N-terminal of SNAP-25 remains in the presynaptic terminal after BoNT/A cleavage

(A) Representative images of WT neurons before or 12h after BoNT/A treatment immunostained for N-or C-terminal specific antibodies of SNAP-25 (red) and a synaptic marker Synaptophysin (green). Scale bar: 20 μm. **(B)** Scatter plot shows averaged fluorescence intensity of SNAP-25 before and 12h or 16h after exposure to BoNT/A (n = 21, 21, 21, 19, 21, 21; respectively). Data are normalized to endogenous Synaptophysin expression and to the SNAP-25 intensity before toxin treatments. n represents the number of images analyzed. Data were obtained from cultured neurons from 2 independent cultures and are shown as mean ± SEM. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparison test. ***$p < 0.001$;*$p < 0.05$; ns: not significant.



Supplementary Figure 7

Scheme summarizes possible models for Syt1-mediated vesicle membrane attachment events

One-side model: Assumes that in the absence of Ca^{2+} the Syt1 C_2B domain is positioned parallel to the SNARE complex where the polybasic region (K) binds to the PIP_2 on the plasma membrane and the bottom residues (R) to the SNARE complex. Upon Ca^{2+} triggering, Ca^{2+}-bound top loops together with (K) and (R) bend the plasma membrane to reduce the repulsive force between membrane further trigger releases. **Bridge model:** Assumes that in the absence of Ca^{2+}, the bottom residues (R) binds to the plasma membrane, where the polybasic region (K) to the SNARE complex. This configuration restricts C_2B domain to turn into a perpendicular orientation to the plasma membrane, leading to an ideal position for the C_2B domain to bridge two membranes. **Switch model:** Assumes Syt1 C_2B domain can dynamically switch between two orientations. Upon Ca^{2+} stimulation, Syt1 rapidly switches its orientation and subsequently triggers release.