Uncoupling the roles of synaptotagmin I as a dual Ca^{2+} sensor during endo- and exocytosis of synaptic vesicles

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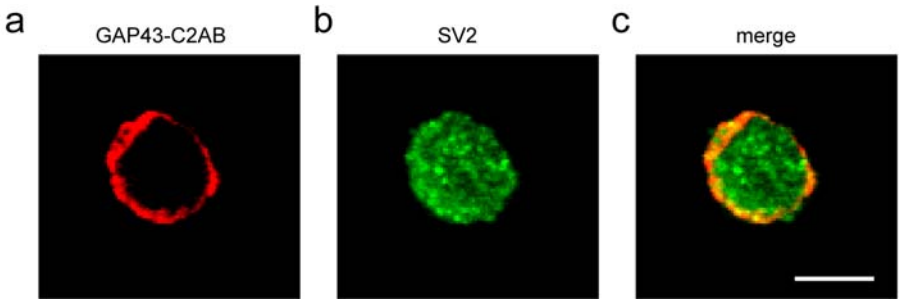
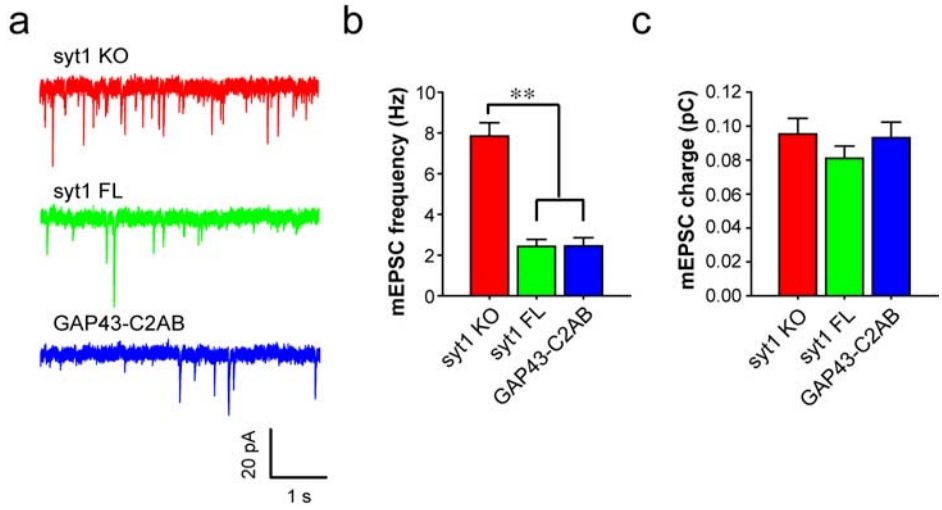
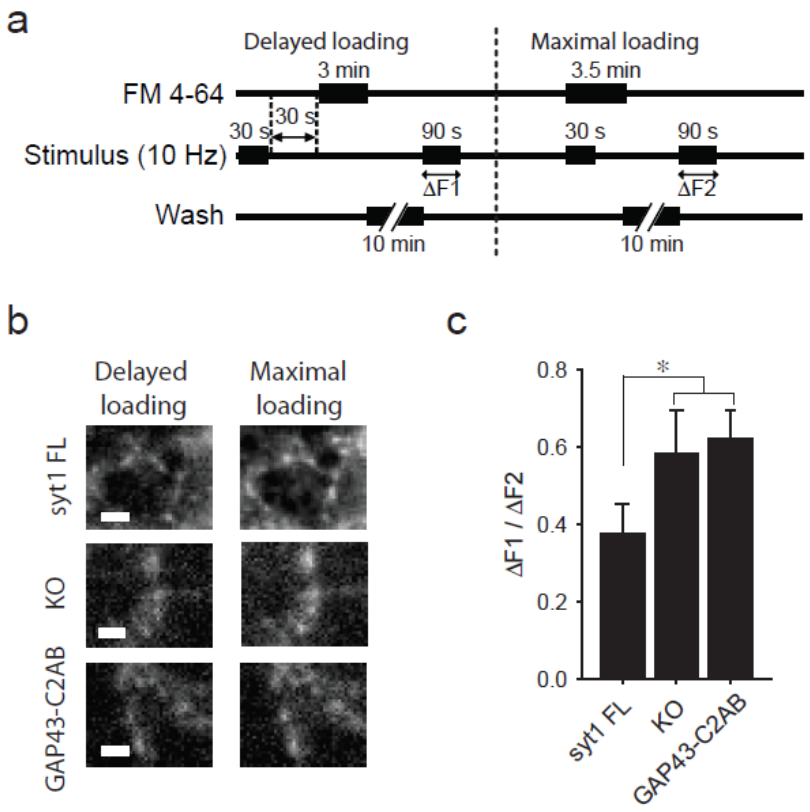
SUPPLEMENTARY FIGURES 1-9



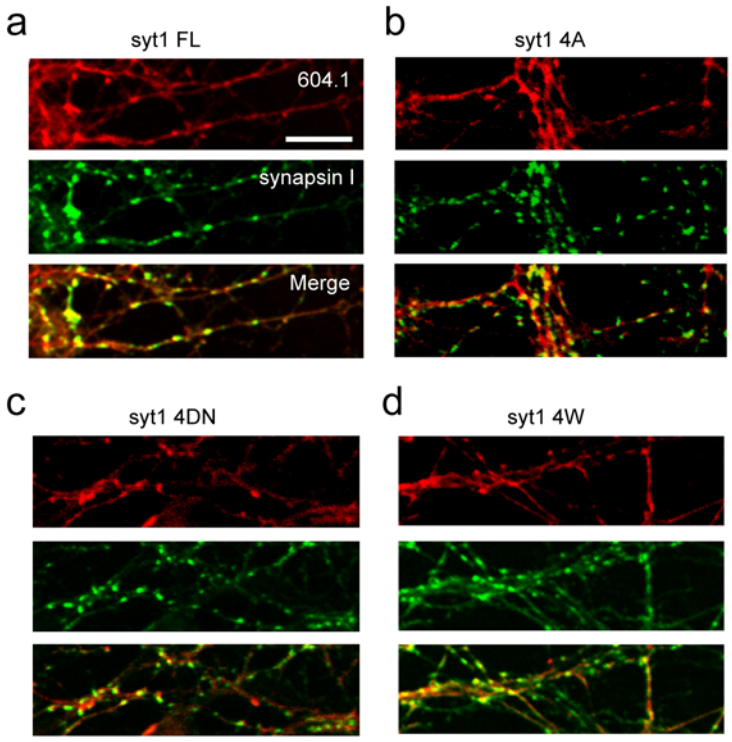
Supplementary Figure 1. GAP43-C2AB is localized to the plasma membrane of chromaffin cells. (a) Immunostaining with an antibody specific for the C2A domain of syt1 was used to visualize GAP43-C2AB. (B) Immunostaining with an anti-SV2A antibody was used to demarcate secretory vesicles. (C) Overlaid image; GAP43-C2AB exhibited a distinct ring-like localization pattern, consistent with targeting to the plasma membrane. Scale bar, 8 μm.



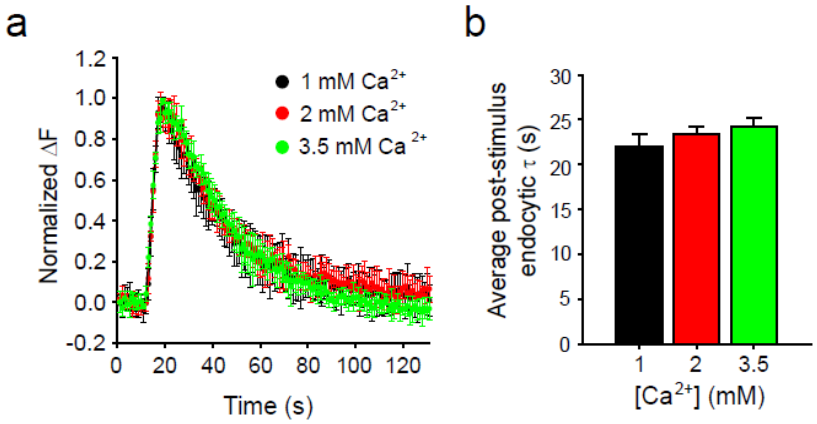
Supplementary Figure 2. GAP43-C2AB rescues the changes in mEPSC frequency that occur in syt1 KO neurons. (a) Representative traces of mEPSCs recorded from syt1 KO neurons and KO neurons with lentiviral expression of $syt1^{FL}$ or GAP43-C2AB. (b) Bar graph showing that neurons expressing $syt1^{FL}$ (n = 13) and GAP43-C2AB (n = 19) exhibited similar mEPSC frequencies, which were significantly lower than for syt1 KO neurons (n = 20). (c) Bar graph summarizing the charge transfer during mEPSCs. ** $p<0.001$. Error bars represent SEM.



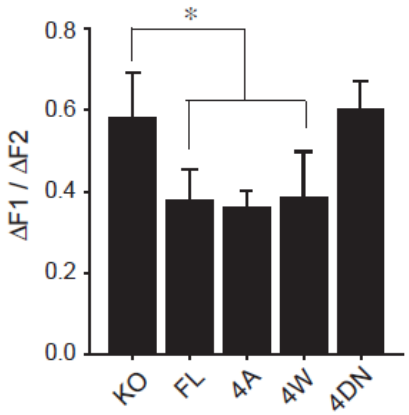
Supplementary Figure 3. GAP43-C2AB does not rescue defective SV endocytosis in syt1 KO hippocampal neurons. (a) Protocol for FM 4-64 pulse-chase experiments. Neurons were stimulated at 10 Hz for 30 s. After a short delay (30 s), neurons were exposed to FM 4-64 for 3 min and then washed in Ca^{2+} free solution for 10 min. A train of 900 pulses (10 Hz) was delivered to evoke unloading of FM 4-64. After a 10 min rest, neurons were stimulated at 10 Hz for 30 s in the presence of FM 4-64. This was followed by washing and unloading in the same manner as the first round. (b) Sample images showing boutons labeled with FM 4-64 during the delayed loading (left panels) and the maximally loaded boutons (right panels). Scale bar, 2 μm. (c) Average ΔF1/ΔF2 ratio of syt1 KO neurons (n = 35), and neurons expressing $syt1^{FL}$ (n = 40) or GAP43-C2AB (n = 30). GAP43-C2AB expressing neurons, similar to syt1 KO neurons, exhibited a larger extent of FM dye uptake during a delayed loading phase, demonstrating slower rates of endocytosis as compared to neurons expressing $syt1^{FL}$. 'n' is total number of boutons pooled from 3 coverslips. ∗ $p < 0.05$. Error bars represent SEM.



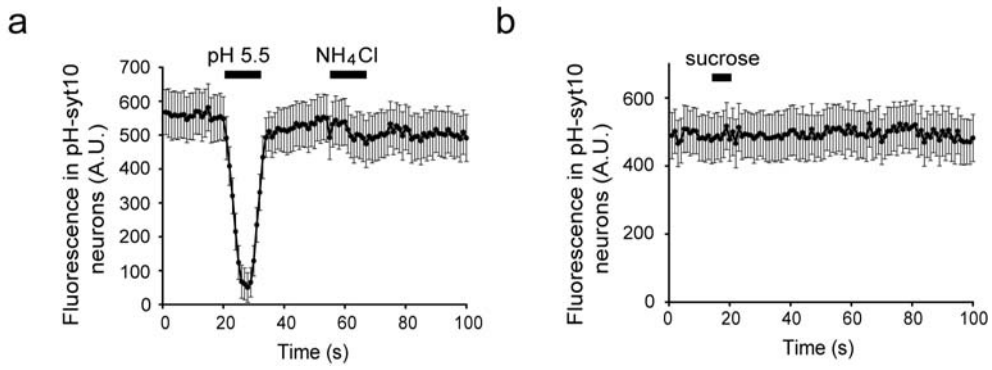
Supplementary Figure 4. Syt1^{4A}, syt1^{4W}, and syt1^{4DN}, are targeted to presynaptic boutons. (a-d) Typical immunostaining images of syt1 KO neurons expressing syt1^{FL} (a), syt1^{4A} (b), syt1^{4DN} (c), or syt1^{4W} (d), using an antibody specific for the luminal domain of syt 1 (604.1). Presynaptic boutons were labeled using an antibody against synapsin I. Scale bar, 5 μm.



Supplementary Figure 5. Varying the extracellular [Ca^{2+}] from 1-3.5 mM does not affect the kinetics of SV endocytosis. (a) Normalized fluorescence changes of sypHy in WT neurons in the presence of 1 mM (n = 11), 2 mM (n = 10), or 3.5 mM (n = 8) extracellular Ca^{2+}. 'n' is number of boutons from 1 coverslip. (b) Bar graph comparing the time constants for endocytosis at different extracellular [Ca^{2+}]. Error bars represent SD.

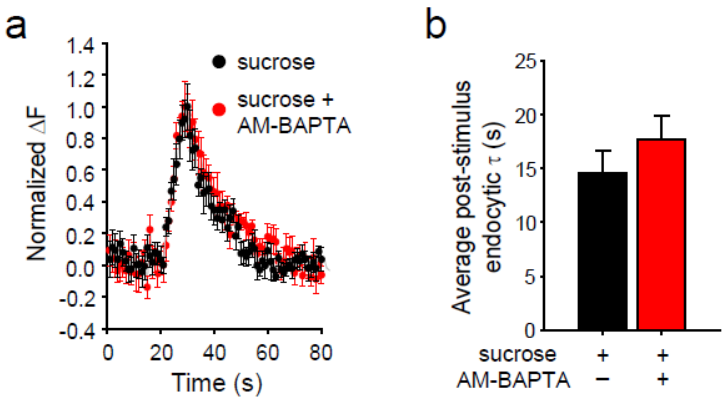


Supplementary Figure 6. FM imaging reveals that syt1 accelerates SV endocytosis via its Ca^{2+} binding activity. Average ΔF1/ΔF2 ratio (see Supplementary Fig. 3) of syt1 KO neurons (n = 35), KO neurons expressing $syt1^{FL}$ (n = 40), $syt1^{4A}$ (n = 34), $syt1^{4W}$ (n = 30), or $syt1^{4DN}$ (n = 30). $Syt1^{4A}$, $syt1^{4W}$ and $syt1^{FL}$ have the same degree of delayed dye loading estimated by ΔF1/ΔF2 ratio; in contrast, delayed loading was significantly enhanced in $syt1^{4DN}$ expressing neurons, thus confirming slower rate of endocytosis for this mutant. 'n' is total number of boutons pooled from 3 coverslips. The protocol for the FM 4-64 pulse-chase experiments to determine ΔF1/ΔF2, is described in Supplementary Fig. 3. * $p < 0.05$. Error bars represent SEM.

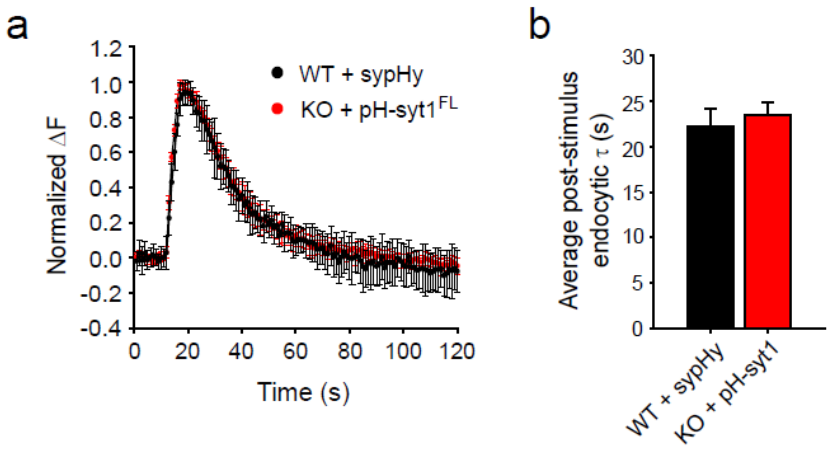


Supplementary Figure 7. Sucrose does not affect the fluorescence intensity of plasma membrane localized pH-syt10. To test whether the sucrose-induced changes in sypHy fluorescence intensity were secondary to mechanical effects, we investigated the influence of sucrose on a plasma membrane reporter, pHluorin tagged synaptotagmin X (pH-syt10). (a) We monitored the changes of pH-syt10 fluorescence intensity in response to application of low pH solution (pH 5.5) or NH_4Cl. If pH-syt10 was localized to the plasma membrane, low pH buffer would quench the fluorescence of pH-syt10; whereas in the case of SV localization, NH_4Cl application would neutralize the acidic luminal pH of SVs (or other acidic organelles) and subsequently dequench the fluorescence of pH-syt10. Strikingly, we found that the fluorescence intensity of pH-syt10 was markedly reduced by the low pH solution but was unaffected by NH_4Cl application, suggesting that pH-syt10 is localized to the plasma membrane in hippocampal neurons and not to SVs. (b) We measured fluorescence intensity of pH-syt10 in response to 0.5 M sucrose superfusion, and found that sucrose did not induce any obvious fluorescence change (n = 20 boutons, 1 coverslip). Hence, the sucrose-induced changes in sypHy fluorescence intensity reflect changes in SV retrieval but are not secondary to mechanical effects.

Note: the pH-syt10 construct is analogous to the well-characterized pH-syt1 construct [49] and will be described in detail elsewhere.



Supplementary Figure 8. Sucrose-induced endocytosis is Ca^{2+} independent. (a) Normalized fluorescence changes of sypHy in syt1 KO neurons in response to superfusion of 0.5 M sucrose (n = 12) in the presence of Ca^{2+}, or under Ca^{2+} free conditions (0 mM Ca^{2+} plus preincubation with 30 μM AM-BAPTA) (n = 8 boutons, 1 coverslip). (b) Bar graph comparing the endocytic time constants for vesicle retrieval, after hypertonic sucrose-induced exocytosis, under Ca^{2+} and Ca^{2+} free conditions. Error bars represent SD.



Supplementary Figure 9. PHluorin tagged syt1 and synaptophysin report identical rates of SV endocytosis when expressed in hippocampal neurons. (a) Normalized pHluorin responses showing that the kinetics of endocytosis were similar between syt1 KO neurons (n = 32) rescued by pH-syt1^{FL} and WT neurons expressing sypHy (n = 25). 'n' is total number of boutons pooled from 2 coverslips. (b) Bar graph showing similar endocytic time constants between WT neurons expressing sypHy and syt1 KO neurons expressing pH-syt1^{FL}. Error bars represent SEM.