Supplementary Information

Actin is crucial for all kinetically distinguishable forms of endocytosis at synapses

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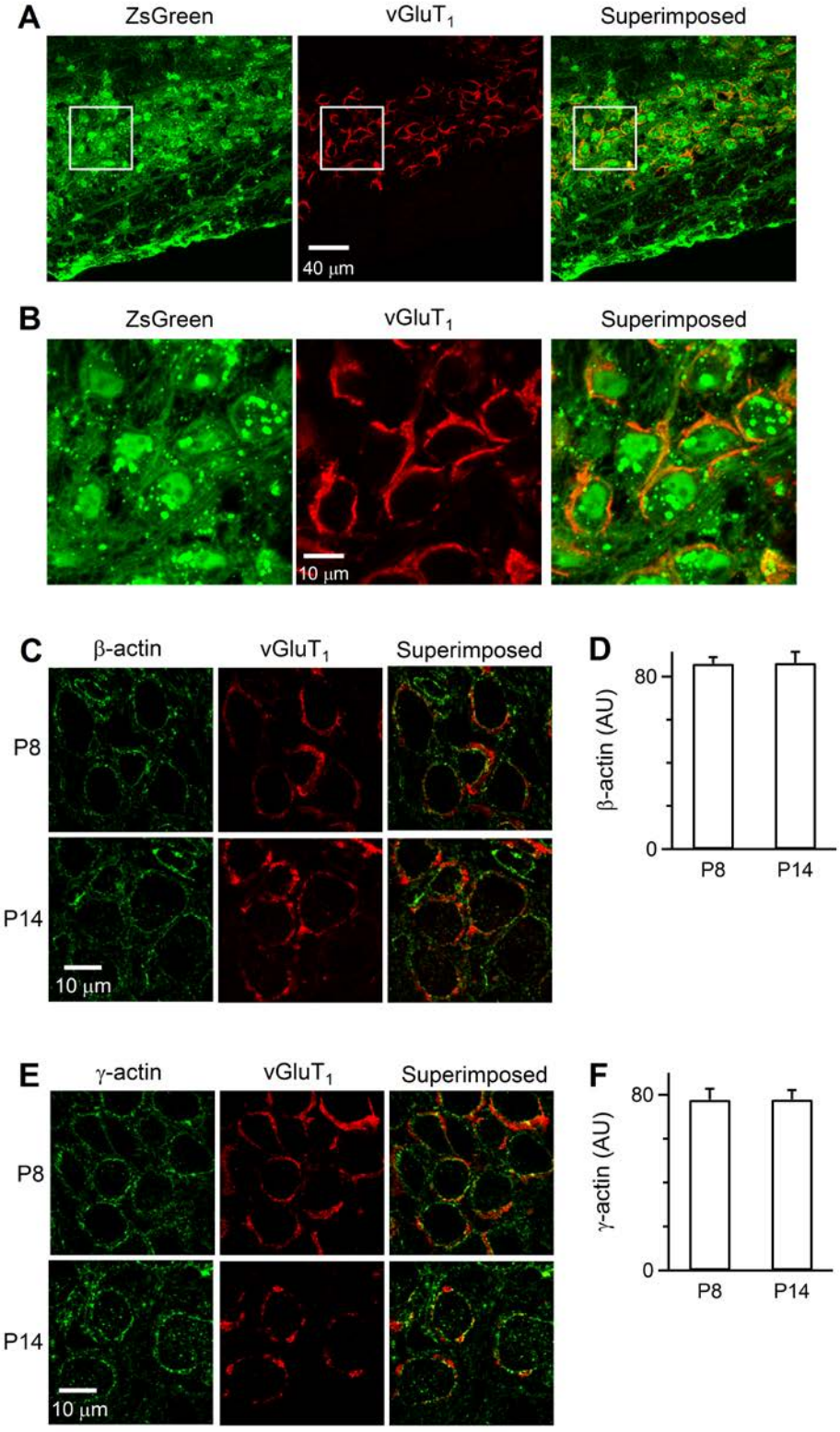
A
ZsGreen
vGluT1
Superimposed
40 μm
B
ZsGreen
vGluT1
Superimposed
10 μm
C
β-actin
vGluT1
Superimposed
P8
P14
10 μm
D
β-actin (AU)
80
0
P8
P14
E
γ-actin
vGluT1
Superimposed
P8
P14
10 μm
F
γ-actin (AU)
80
0
P8
P14

Figure S1, related to Figure 1. Krox20 Cre is present in mouse calyx-containing neurons and β-actin and γ-actin are present in immature and more mature mouse calyces

(A) Krox20 Cre expression, detected from antibody staining of ZsGreen (green, left), overlaps with $vGluT_1$ staining, which labels vesicles in the calyx (red, middle) in the medial nucleus of the trapezoid body in $Krox20^{Cre/+}$; $ZsGreen^{+/-}$ mouse (left and middle panel superimposed in the right).

(B) The white box region in panel A is enlarged.

(C) Antibody staining of β-actin (left) and $vGluT_1$ (middle) in P8 and P14 wild-type mouse. Left and middle panels are superimposed in the right panel.

(D) β-actin staining intensity (mean + s.e.m.; AU: arbitrary unit) in P8 (45 calyces, 3 mice) and P14 (38 calyces, 3 mice) wild-type mouse. No significant difference was detected.

(E-F) Similar to panel C-D, respectively, except that β-actin is replaced with γ-actin. P8, 40 calyces, 3 mice; P14, 37 calyces, 3 mice. No significant difference was detected in panel F.

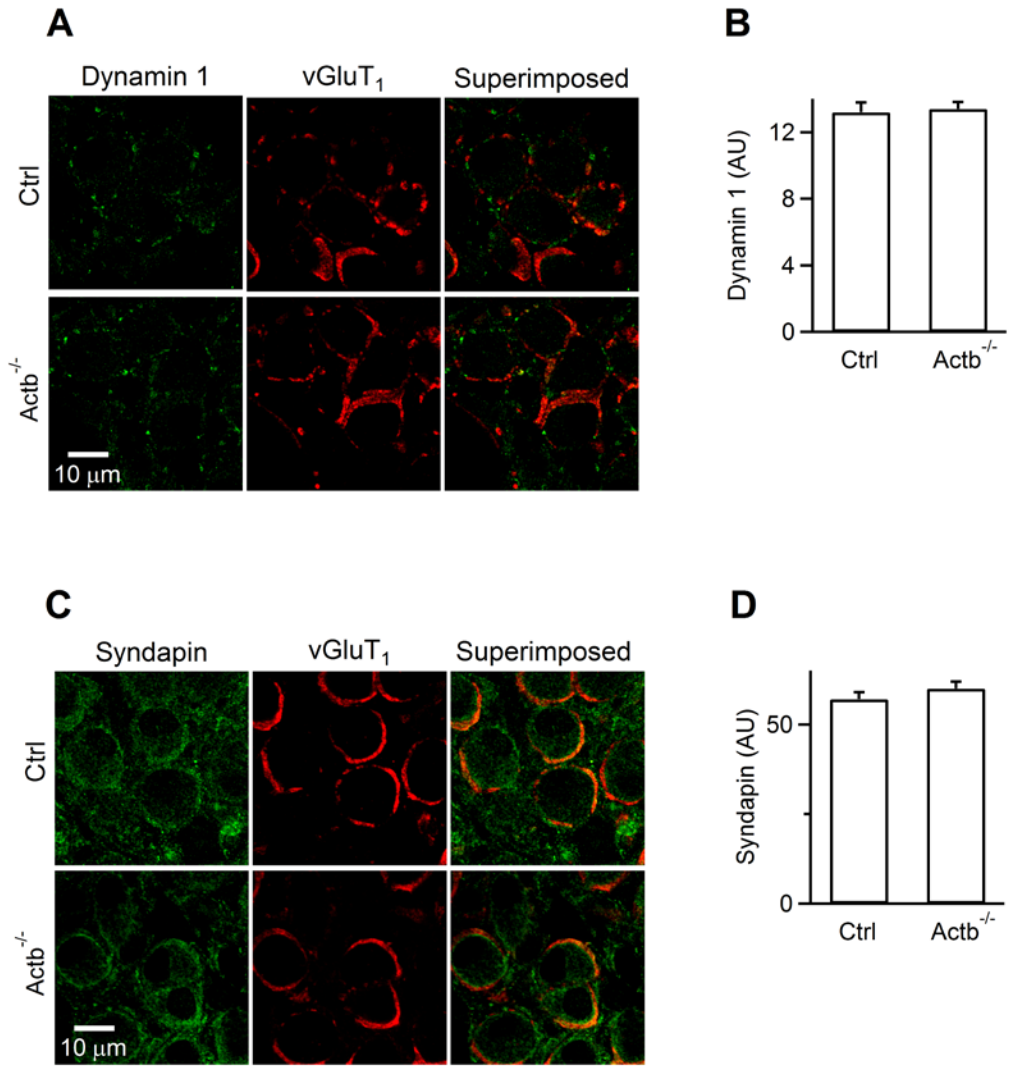

Figure S2, related to Figure 1. β-actin knockout does not affect dynamin and syndapin expression in calyces

(A) Antibody staining of dynamin 1 (upper) and $vGluT_1$ (middle) in Ctrl and $Actb^{-/-}$ calyces. Upper and middle images are superimposed in the bottom.

(B) Dynamin staining intensity (mean + s.e.m.) in Ctrl mice (74 calyces, 3 mice) and $Actb^{-/-}$ mice (66 calyces, 3 mice). No significant difference was detected.

(C-D) Similar to panels A-B, respectively, except that dynamin 1 is replaced with syndapin. Ctrl, 57 calyces, 3 mice; $Actb^{-/-}$, 60 calyces, 3 mice. No significant difference was detected.

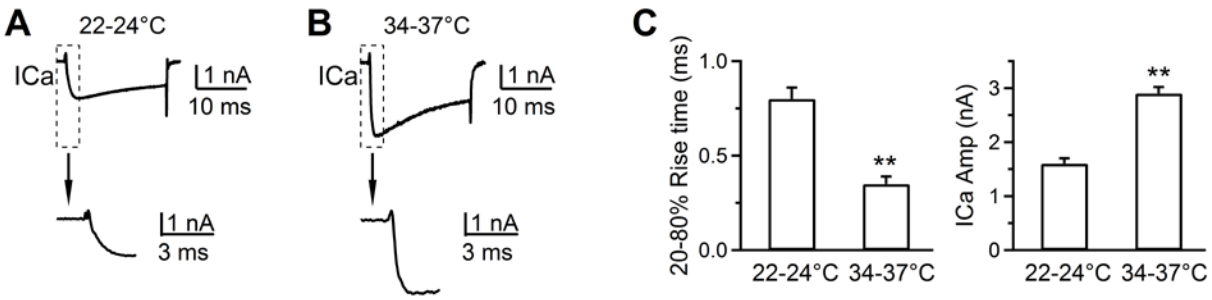

Figure S3, related to Figure 2. Increasing temperature increases the kinetics and the amplitude of calcium currents in wild-type mouse calyces

(A-B) Sampled ICa induced by $depol_{20ms}$ at 22-24°C (A) and 34-37°C (B) in wild-type mouse calyces. Traces within the box are enlarged (lower) to show the kinetics of ICa rise.

(C) The 20-80% rise time (left) and the amplitude (Amp, right) of ICa in response to $depol_{20ms}$ in wild-type mouse calyces at 22-24°C (n = 13 calyces) and 34-37°C (n = 8 calyces). **: $p < 0.01$, t test.

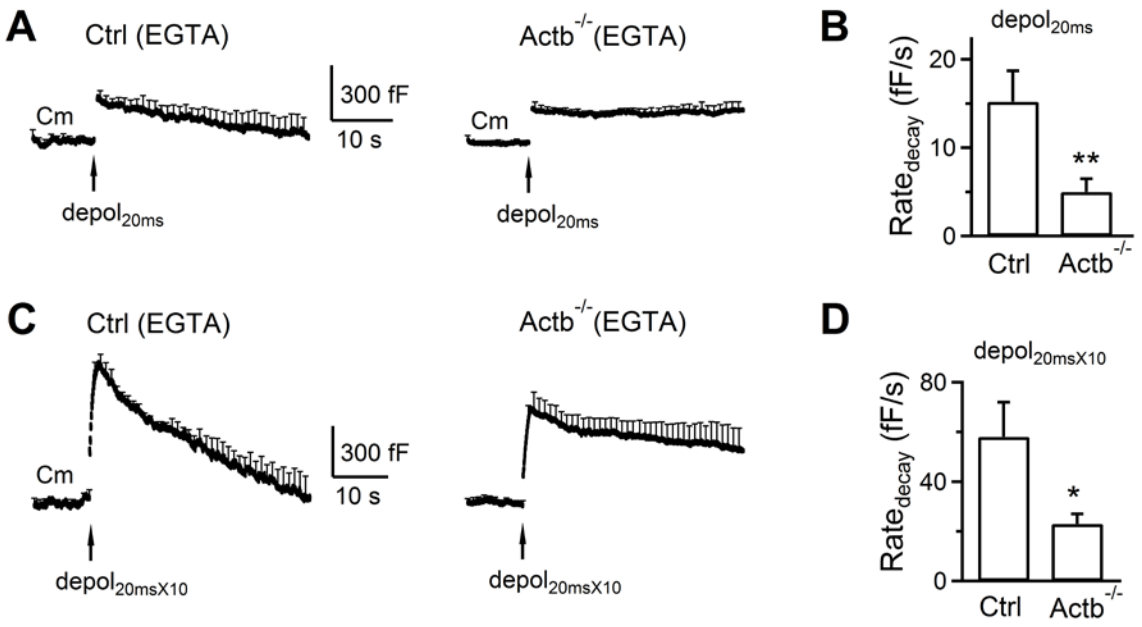

Figure S4, related to Figures 2 and 3. β-actin knockout inhibits slow and rapid endocytosis in calyces dialyzed with 1 mM EGTA

(A-B) The Cm trace (mean + s.e.m., A) and $Rate_{decay}$ (mean + s.e.m. B) induced by $depol_{20ms}$ (arrow) from Ctrl (7 calyces, 6 mice) and $Actb^{-/-}$ (8 calyces, 8 mice) calyces dialyzed with 1 mM EGTA *, $p<0.05$; **, $p<0.01$. s.e.m. is plotted every 1 s in panel A.

(C-D) Similar to A-B, respectively, except that $depol_{20ms}$ was replaced with $depol_{20msX10}$. Ctrl, n = 7 calyces, 6 mice; $Actb^{-/-}$, n = 8 calyces, 8 mice.

Conclusion: 1 mM EGTA blocks residual calcium induced by $depol_{20ms}$ or $depol_{20msX10}$, and thus asynchronous release caused by residual calcium. In the presence of 1 mM EGTA in the pipette solution, β-actin knockout still reduced $Rate_{decay}$, suggesting that the reduction is not due to changes in residual calcium that increase asynchronous release to counteract the ΔCm decay.

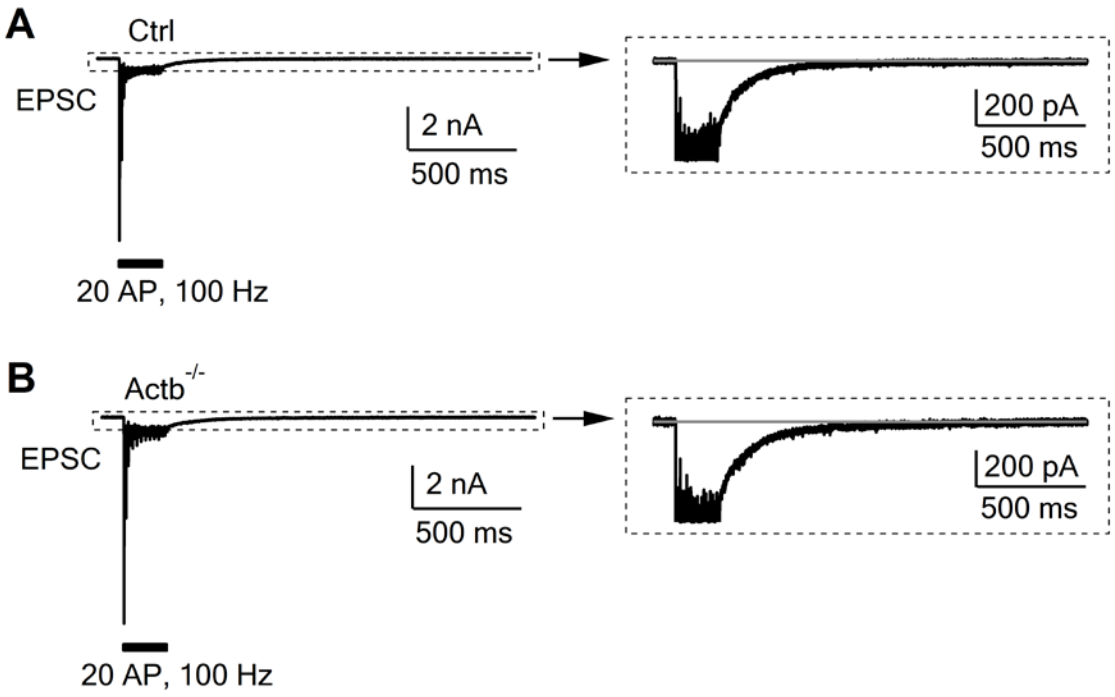

Figure S5, related to Figure 2. β-actin knockout does not prolong the EPSC decay in the calyx of Held synapse

(A-B) EPSCs induced by a presynaptic fiber stimulation train (20 AP at 100 Hz) in a Ctrl mouse (A) and a Actb$^{-/-}$ calyx (B). The box region is enlarged in the right to show that the EPSC completely returned to the baseline (gray line) within 1.5 s after stimulation in both A and B. The fiber stimulus at the midline of the trapezoid body, where presynaptic fibers connecting calyces pass through, induced an action potential at the calyx, which in turn induced an EPSC.

Conclusion: The EPSC decayed to baseline within 1.5 s, whereas the capacitance decay (Fig. 2D-F) did not return to baseline for 40 s after 20 AP at 100 Hz in Actb$^{-/-}$ calyces. Evidently, the prolonged capacitance decay is not due to substantial asynchronous release lasting for 40 s after stimulation.

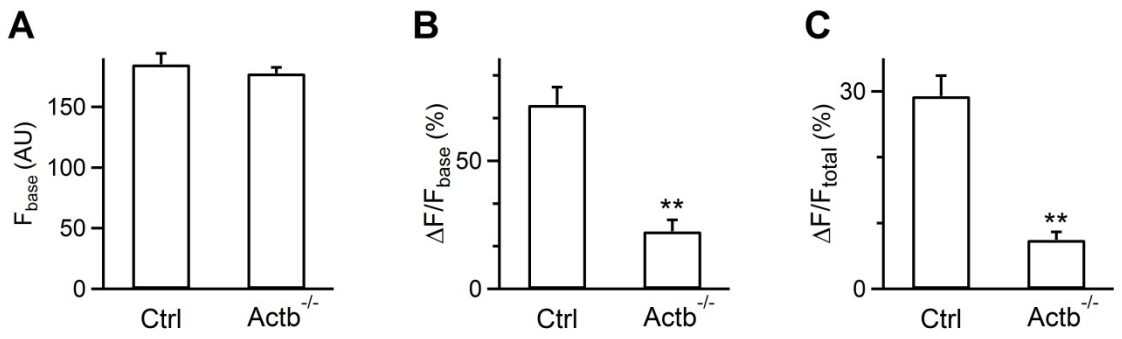

Figure S6, related to Figure 6. β-actin knockout reduces exocytosis induced by $Train_{10s}$ at hippocampal synapses

(A) Baseline F_{SypH} (F_{base}) in Ctrl boutons (n = 12 experiments) and in $Actb^{-/-}$ boutons (n = 8 experiments). No significant difference was detected.

(B) ΔF induced by $Train_{10s}$, normalized to F_{base} ($\Delta F/F_{base}$), in Ctrl boutons (n = 12 experiments) and in $Actb^{-/-}$ boutons (n = 8 experiments). **, $p < 0.01$ (t test).

(C) ΔF induced by $Train_{10s}$, normalized to the total F_{SypH} fluorescence (F_{total}) obtained in the presence of NH_4Cl that was applied at the end of the experiment ($\Delta F/F_{total}$), in Ctrl boutons (n = 5 experiments) and in $Actb^{-/-}$ boutons (n = 5 experiments). **, $p < 0.01$ (t test).

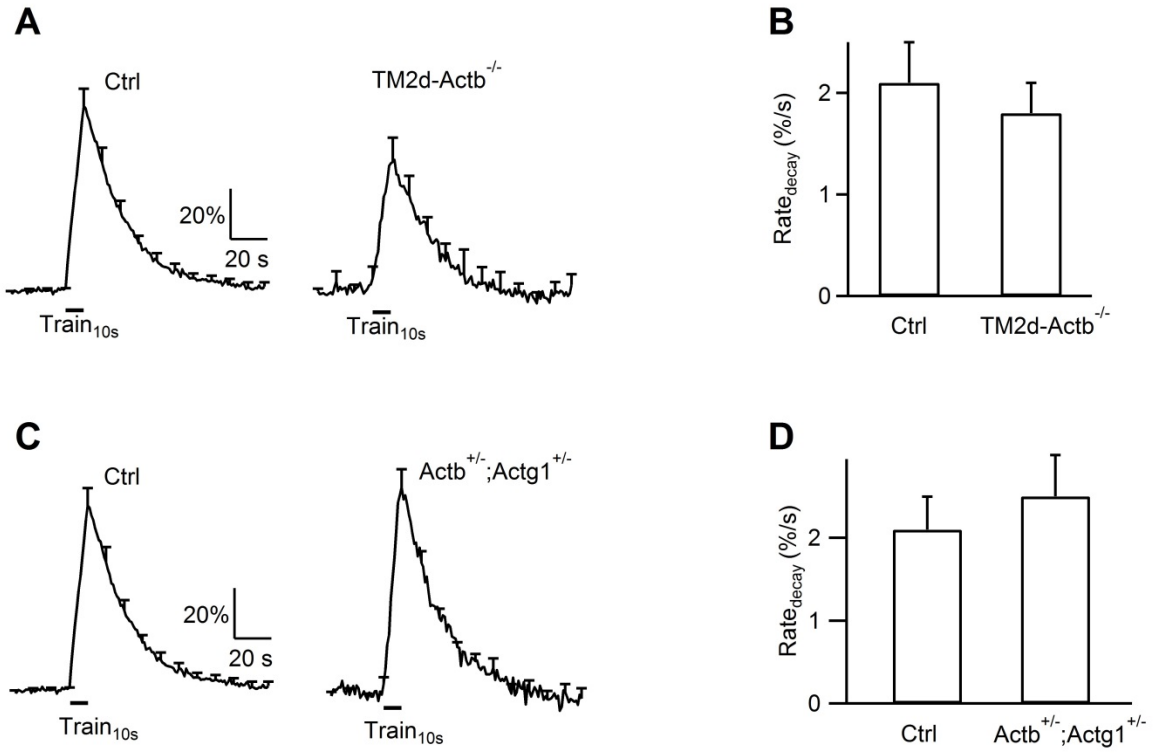

Figure S7, related to Figures 6 and 7. Endocytosis is not substantially inhibited by partial reduction of β-actin to ~55% of control or in heterozygotes of β- and γ-actin double knockout at hippocampal synapses

(A-B) F_{SypH} traces (mean + s.e.m., A) and $Rate_{decay}$ (mean + s.e.m., B) induced by $Train_{10s}$ in control (12 experiments) and at 2 days (6 experiments) after 4-OH-tamoxifen application to hippocampal neurons cultured from Cre-ER^{TM};Actb$^{LoxP/LoxP}$ mice (TM2d-Actb$^{-/-}$), which reduced β-actin to 55 ± 5% (n = 4) of control (see Fig. 6C).

Conclusion: since β-actin was reduced to 55 ± 5% (n = 4) of control at 2 day 4-OH-tamoxifen application (see Fig. 6C in the main text), we concluded that partial reduction of β-actin to ~55% of control does not inhibit endocytosis at hippocampal synapses.

(C-D) F_{SypH} traces (mean + s.e.m., C) and $Rate_{decay}$ (mean + s.e.m., D) induced by $Train_{10s}$ in Ctrl cultures (12 experiments) and in Actb$^{LoxP/+}$;Actg1$^{LoxP/+}$ hippocampal cultures transfected with a Cre plasmid to delete one allele of *Actb* and *Actg1* gene (Actb$^{+/-}$;Actg1$^{+/-}$ culture, 6 experiments).
Conclusion: deletion of one allele of *Actb* and *Actg1* gene is insufficient to cause inhibition of endocytosis.

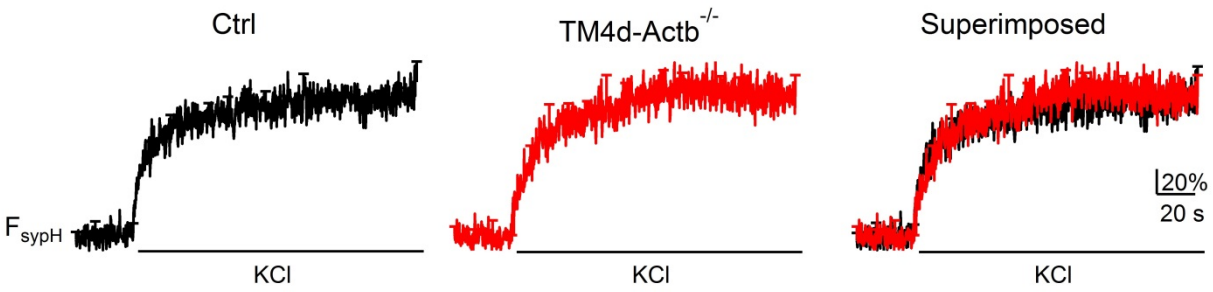

Figure S8, related to Figure 8. Exocytosis during 90 mM KCl application is not reduced by β-actin knockout at hippocampal synapses

F_{SypH} traces before and during application of 90 mM KCl in control hippocampal boutons (n = 4 experiments, left) and in Cre-ER^{TM};Actb$^{LoxP/LoxP}$ hippocampal boutons treated with 4-OH-tamoxifen (1 μM) for 4 days (n = 4 experiments, middle, TM4d-Actb$^{-/-}$). The bath solution contained bafilomycin (0.5 μM), which blocks vesicular re-acidification. Data are expressed as mean + s.e.m (s.e.m. plotted every 20 s). Traces in the left and the middle panel are superimposed in the right panel.