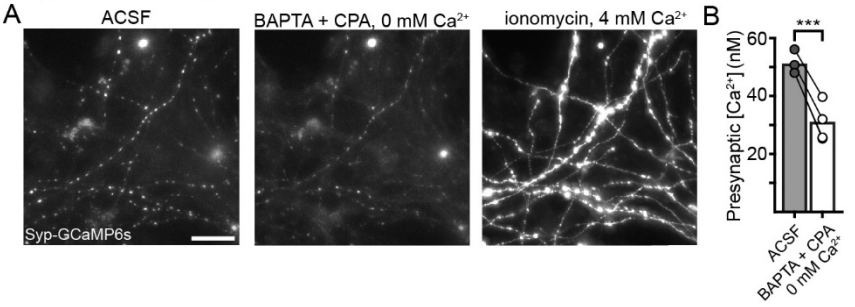
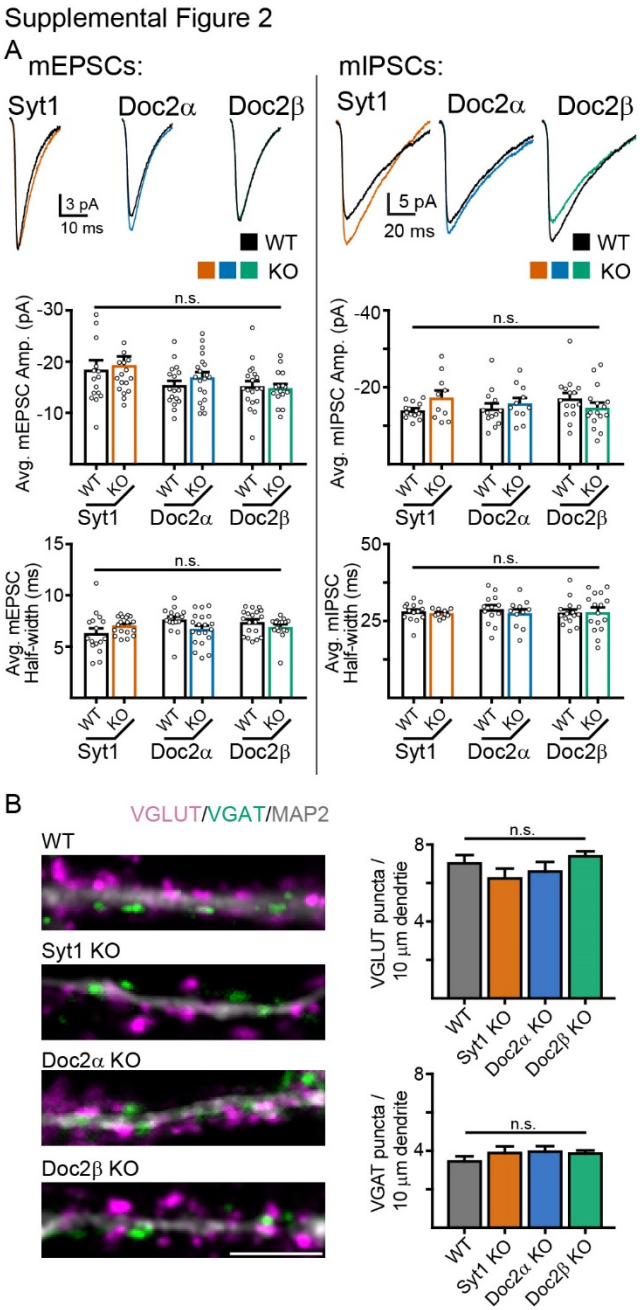
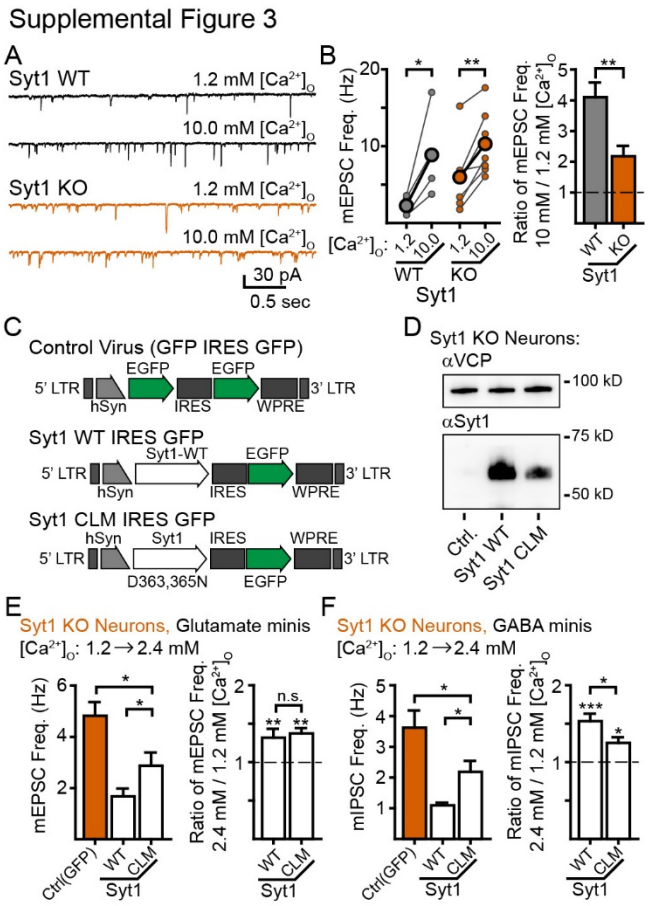
Supplemental Figure 1



Supplemental Figure 1. Presynaptic Ca^{2+} measurements. Related to Figure 1. **A.** Cultured neurons were transfected with synaptophysin-GCaMP6s (Syp-GCaMP6s) to optically measure basal free [Ca^{2+}] in the presynaptic compartment (please see Methods) at rest and after 20 min incubation in bath solution containing 0 mM Ca^{2+}, 30 µM BAPTA-AM, and 30 µM CPA. Scale bar, 20 µm. **B.** Quantification of presynaptic [Ca^{2+}] demonstrating that resting [Ca^{2+}] is reduced with the addition of BAPTA-AM and CPA in 0 mM Ca^{2+} (ACSF: 50.7 ± 1.9 nM; 0 mM Ca^{2+}/BAPTA/CPA: 30.7 ± 3.4 nM, n = 4, $p < 0.001$, paired t-test). *** indicates $p < 0.001$.

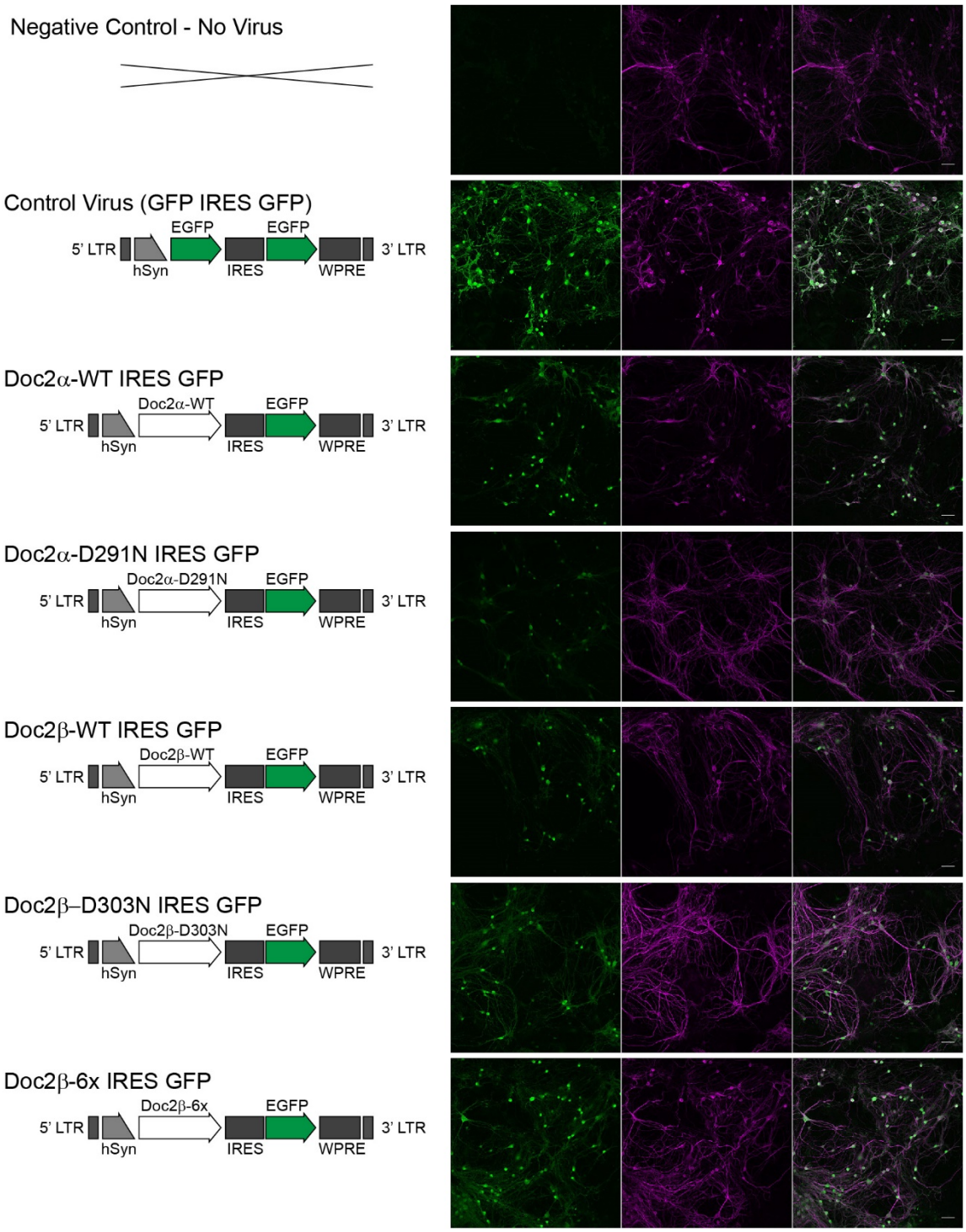


Supplemental Figure 2. Loss of each sensor did not affect the amplitude of minis, shape of minis, or the density of synapses. Related to Figure 1. **A.** Unitary mEPSCs (left) and mIPSCs (right) are unaltered by the loss of syt1 (orange), Doc2α (blue), or Doc2β (green). Representative average traces (top) of unitary mEPSCs and mIPSCs and their quantified average amplitudes (middle) and half-widths (bottom). Black traces and bars represent data from WT littermates and each dot (middle, bottom) represents the average value from an individual neuron. **B.** Representative ICC images (left) and quantification (right) demonstrating that the density of excitatory synapses (marked by VGLUT, magenta; ANOVA p = 0.55) and inhibitory synapses (marked by VGAT, green; ANOVA p = 0.28) per unit length of dendrite (marked by MAP2, white) were unchanged by loss of syt1, Doc2α, or Doc2β. 9 fields of view from 2 pups were quantified for each genotype. n.s. represents p > 0.05. Scale bar applies to all images and indicates 5 μm. Bar graphs represent mean ± SEM.



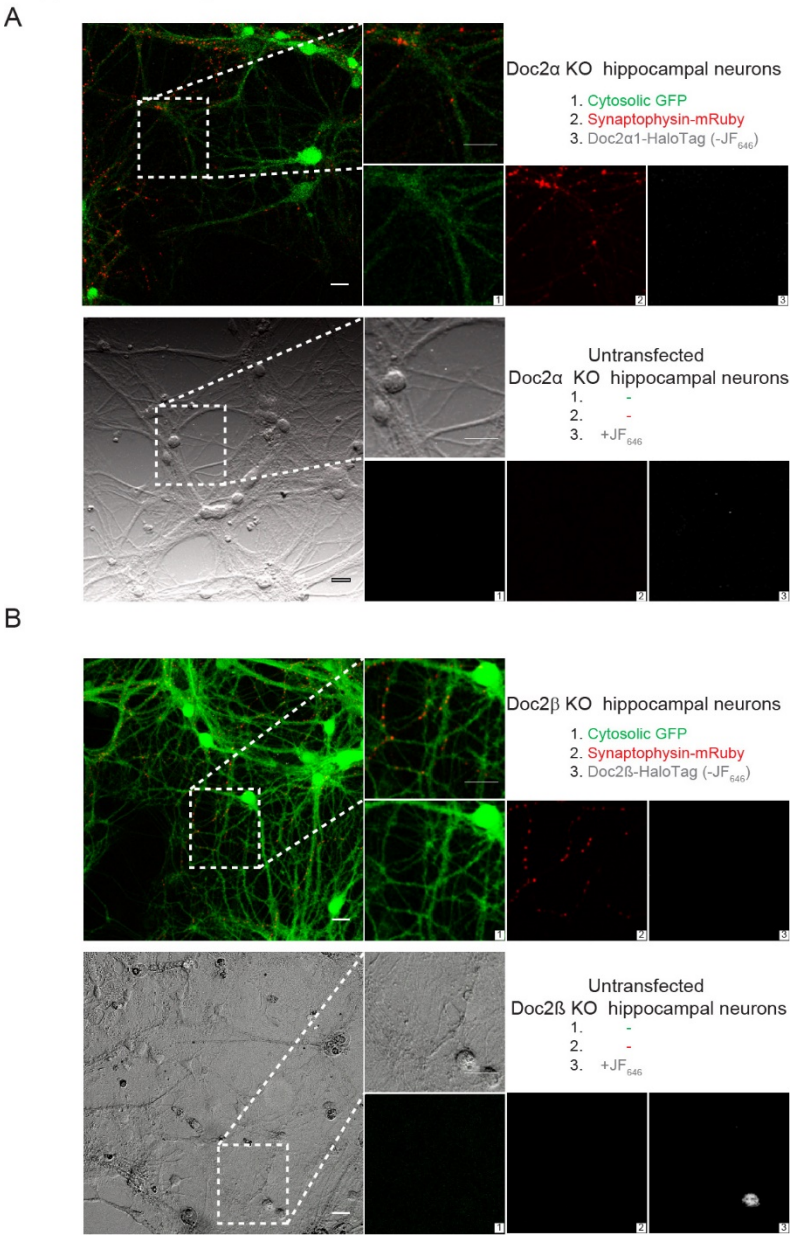
Supplemental Figure 3. At supra-physiological $[Ca^{2+}]_O$, syt1 can drive mEPSCs; syt1 must bind Ca^{2+} to drive mIPSCs. Related to Figure 2. **A.** Example traces and quantification (**B**) of the effect of increasing $[Ca^{2+}]_O$ from 1.2 mM to 10.0 mM on mEPSC frequency. **C.** Schematic of the lentiviral expression vector used to express GFP (control), wildtype syt1 (syt1 WT), or a calcium-ligand mutant form of syt1 (syt1 CLM, D363,365N). **D.** Western blot of syt1 KO neurons confirming the viral expression of syt1 WT and syt1 CLM. **E.** Left: quantification of mEPSC frequencies demonstrating that syt1 CLM partially clamped minis in KO neurons (1.2 mM $[Ca^{2+}]_O$; syt1 KO + Ctrl.: 4.8 ± 0.5 Hz, n = 10; syt1 KO + syt1 WT: 1.3 ± 0.3 Hz, n = 15; syt1 KO + syt1 CLM: 2.9 ± 0.5 Hz, n = 13; ANOVA $p < 0.001$; Ctrl. Vs. CLM: $p = 0.012$ and WT vs CLM: $p = 0.030$, Sidak's multiple comparisons test). Right: Ca^{2+}-induced increase in mEPSC frequency was similar for syt1 WT and syt1 CLM expressing neurons (WT: 31 ± 12% increase, n = 10, $p = 0.003$; CLM: 37 ± 7% increase, n = 9, $p = 0.005$; WT vs. CLM: $p = 0.71$). **F.** Left: quantification of mIPSC frequencies demonstrating the syt1 CLM, again, partially clamped minis in KO neurons (1.2 mM $[Ca^{2+}]_O$; syt1 KO + Ctrl.: 3.6 ± 0.6 Hz, n = 7; syt1 KO + syt1 WT: 1.1 ± 0.1 Hz, n = 11; syt1 KO + syt1 CLM: 2.1 ± 0.4 Hz, n = 11; ANOVA $p < 0.001$; Ctrl. Vs. CLM: $p = 0.017$ and WT vs CLM: $p = 0.043$, Sidak's multiple comparisons test). Right: Ca^{2+}-induced increase in mIPSC frequency was reduced in syt1 CLM expressing neurons as compared to syt1 WT expressing neurons (WT: 53 ± 10% increase, n = 11, $p < 0.001$; CLM: 25 ± 7% increase, n = 11, $p = 0.048$; WT vs. CLM: $p = 0.030$). * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$, n.s. signifies $p > 0.05$. Bar graphs represent mean ± SEM.

Supplemental Figure 4

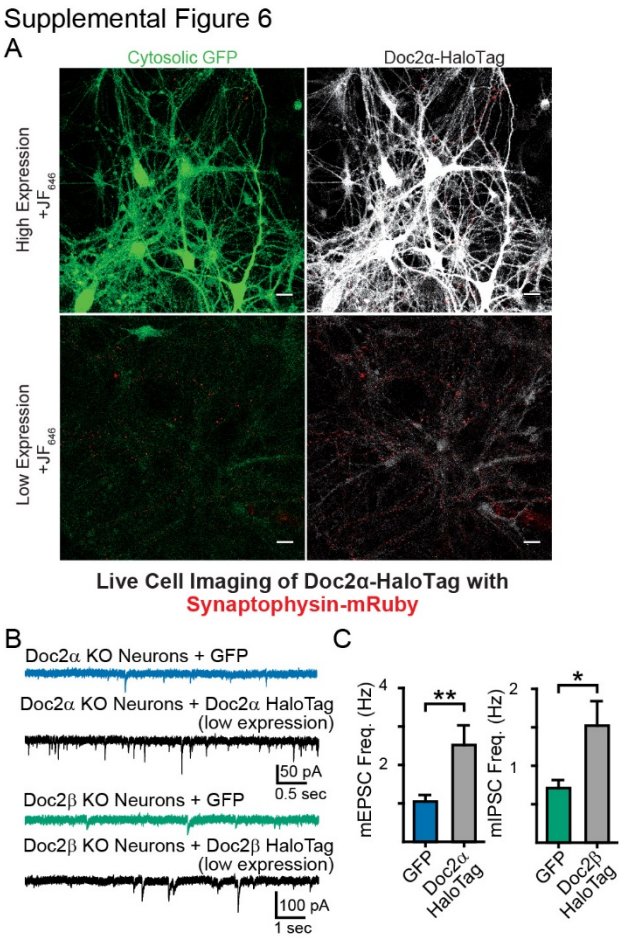


Supplemental Figure 4. Lentiviral expression of Doc2 constructs. Related to Figure 4 and 5. *Left*. Schematic of the lentiviral expression vector used to express these constructs. *Right*. Immunocytochemistry images staining for GFP (green) and MAP2 (purple). GFP was used as a positive marker for viral infection while MAP2 was used to identify neurons. In all cases, >95% of neuronal somas were positive for GFP expression. Scale bar represents 50 μm.

Supplemental Figure 5



Supplemental Figure 5. Imaging controls for the HaloTag-Doc2 constructs. Related to Figure 6. **A.** Controls for the live cell imaging experiments utilizing Doc2α and β (**B**). For each panel, the top images show infected neurons that have not been exposed to the JF646 ligand, demonstrating that bleed through, or otherwise non-specific fluorescence, was not detected in this far-red channel. The bottom panel shows uninfected neurons that were exposed to JF646, demonstrating that this ligand specifically labels the HaloTag. Scale bar represents 20 μm.



Supplemental Figure 6. Overexpression of cytosolic proteins occludes localization data. Related to Figure 6. **A.** Top panels depict GFP (green) and Doc2α-HaloTag-JF646 (white) in neurons that were infected by high titer viruses. The bottom panels depict the same viruses used at significantly lower titers to achieve lower protein expression. All images presented in this figure were acquired at the same settings and subjugated to the same adjustments for brightness and contrast. Scale bar represents 20 μm. **B.** Example traces and quantification (**C**) illustrating that both Doc2α-HaloTag (α KO + GFP: 1.1 ± 0.2 Hz, n = 17; α KO + Doc2α-HaloTag: 2.5 ± 0.5 Hz, n = 16; p = 0.014) and Doc2β-HaloTag (β KO + GFP: 0.7 ± 0.1 Hz, n = 11; β KO + Doc2β-HaloTag: 1.5 ± 0.3 Hz, n = 12; p = 0.031) rescued mini frequencies when expressed at the 'low expression' level. * indicates p <0.05 and ** indicates p < 0.01. Bar graphs represent mean ± SEM.