

Supplementary Information for:

Synapsin Condensation Controls Synaptic Vesicle Sequestering and Dynamics

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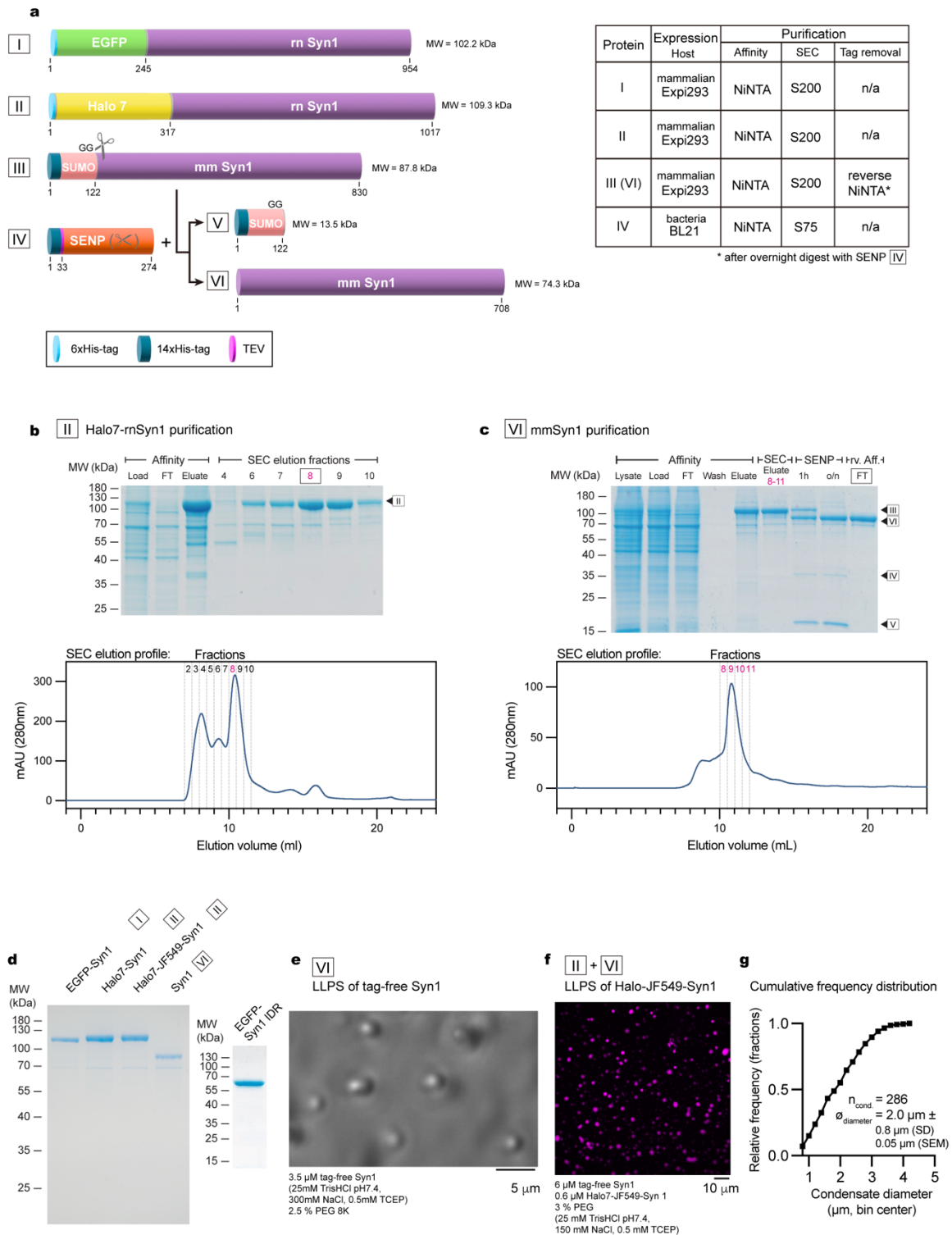
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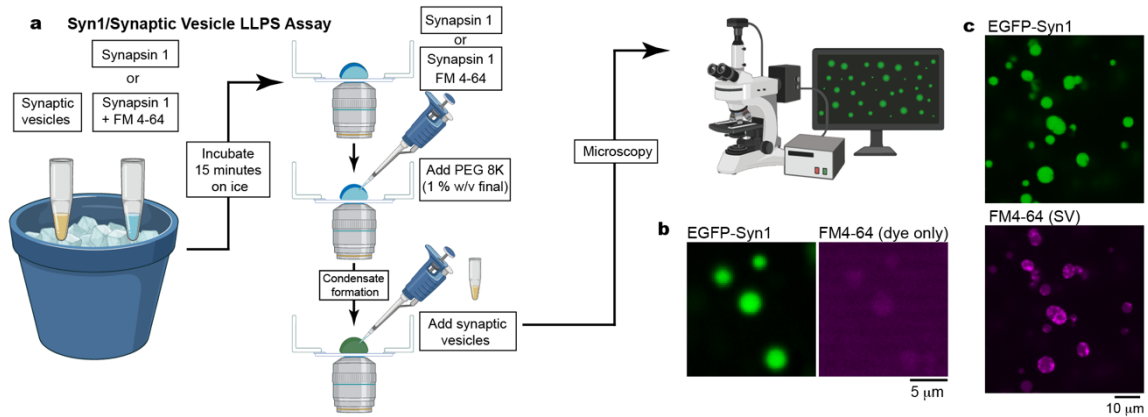
Supplementary Data contains:

- Supplementary Figures 1-14
- Supplementary Tables 1-2
- Supplementary Movies 1-3

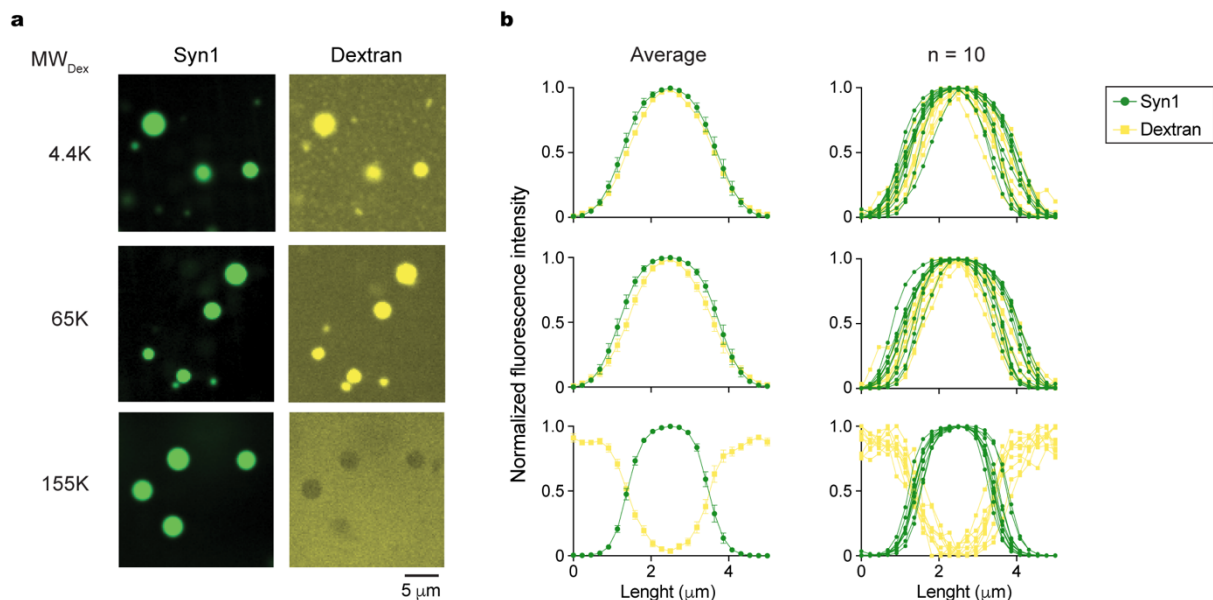


Supplementary Figure 1 | Recombinant proteins used for reconstitution of synapsin condensates. **a**, Left: Schemes of synapsin 1 proteins variants used for reconstitution. The cartoons are to scale. Right: Overview of the purification strategy for each construct. For details, see Material and Methods. **b**, SDS-PAGE gel for recombinant purified Halo7-synapsin 1. **c**, SDS-PAGE gel showing the purification of tag-free synapsin 1. **d**, SDS-PAGE

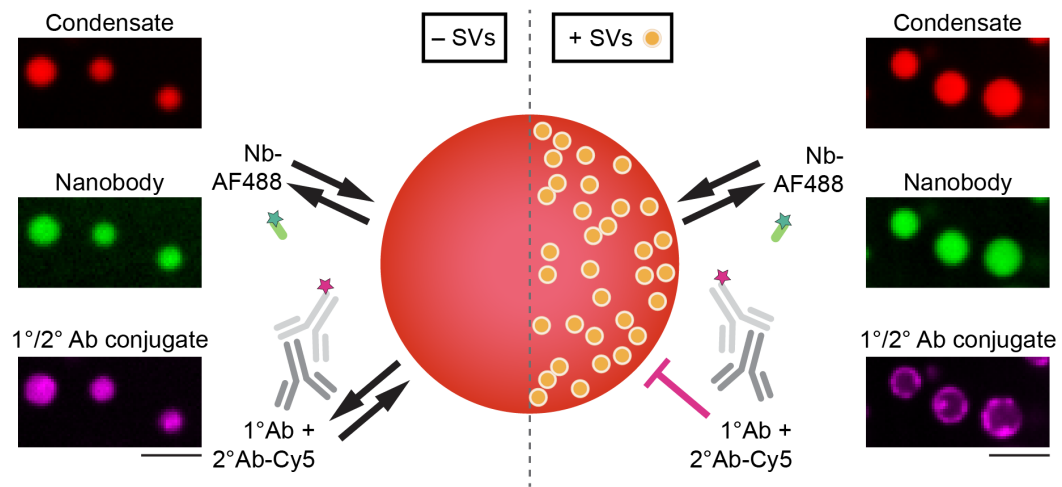
gel with all recombinant proteins used in this study (I: EGFP-synapsin 1; II: Halo7-synapsin 1; II*: Halo7*JF549-synapsin 1; VI: tag-free mmSynapsin 1). Right: EGFP-synapsin 1 IDR (amino acids: 416-705) **e**, Tag-free synapsin 1 forms condensates as visualized by phase contrast microscopy. **f**, JF549 labeled Halo7-synapsin 1 forms condensates. **g**, Cumulative size distribution of synapsin 1 condensates (mean 2 μm , standard deviation (SD) 0.8 μm , standard error of the mean (SEM) 0.05 μm).



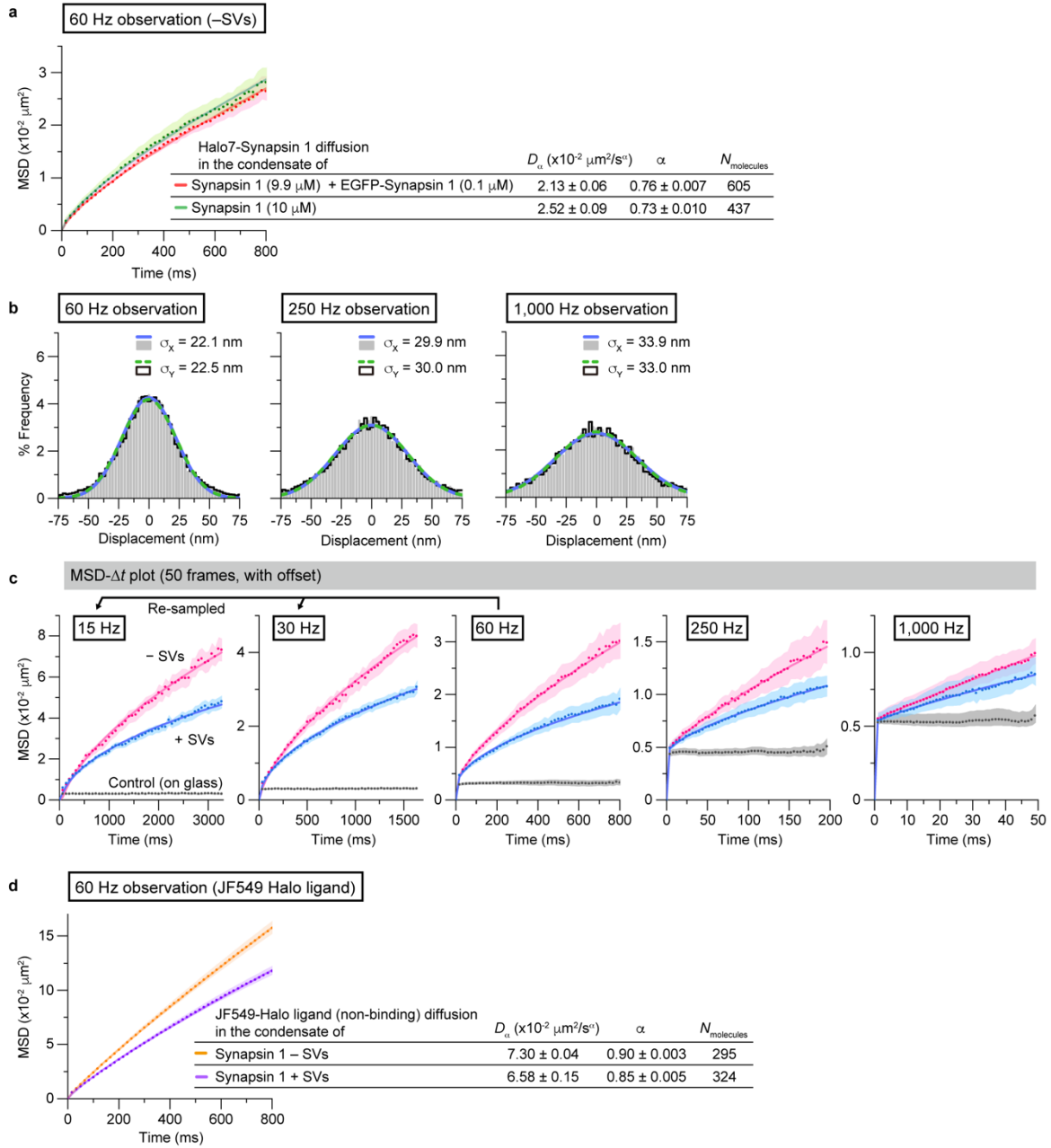
Supplementary Figure 2 | Reconstitution assay for visualizing SV/synapsin condensates. **a**, Scheme of the experimental pipeline. **b**, Exemplary fluorescence images of synapsin condensates with FM4-64 dye without SVs. **c**, Exemplary fluorescence images of synapsin 1/SV condensates.



Supplementary Figure 3 | Synapsin condensates contain a meshwork that acts as a molecular sieve. **a**, Confocal images of EGFP-synapsin 1 condensates upon incubation with fluorescently-labeled dextran of 4.4 kDa (top), 65.85 kDa (middle), and 155 kDa (bottom) panels. Scale bar, 5 μm . **b**, Line profiles indicating either enrichment (for 4.4 kDa and 65.85 kDa) or exclusion (for 155 kDa) of fluorescently-labeled dextran. Left: an average line profile $\pm$ SEM; right: exemplary line profiles. For each condition ten condensates of similar size from three independent reconstitutions were analyzed.

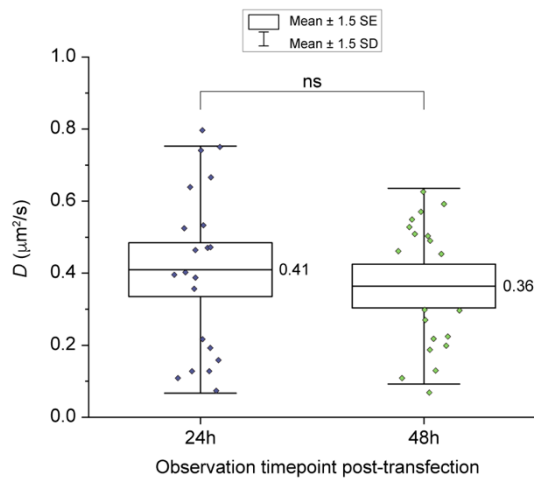


Supplementary Figure 4 | Differential partition of molecules into synapsin-drive condensates. Confocal images of Halo7*JF549-synapsin 1 condensates in the absence (left) or presence of synaptic vesicles (right). Condensates were co-incubated with a mixture of nanobody (Alexa Fluor 488-labeled nanobody against mouse IgG) and a primary/secondary antibody conjugate (primary: rabbit anti-LAMP1; secondary: Cy5-labeled goat-anti-rabbit). For each condition three independent reconstitutions were analyzed. Scale bars, 5 μm .

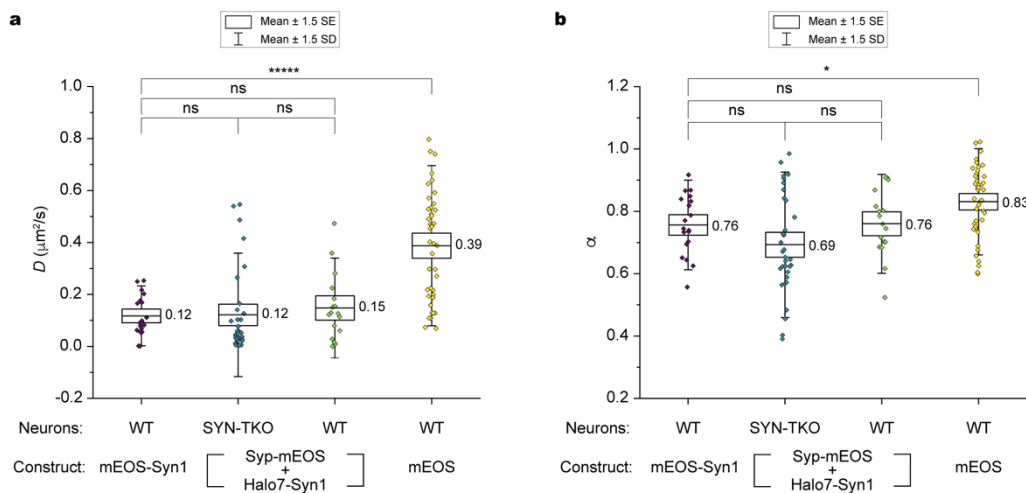


Supplementary Figure 5 | Control data for SMT employed for evaluating synapsin 1 diffusion in the SV/synapsin condensates. **a**, Halo7-synapsin 1 diffusion is not influenced by the presence of EGFP-synapsin 1 in the condensate (mean $\pm$ SEM). See Methods for details. **b**, Single-molecule localization precisions of JF549-labeled Halo7-synapsin 1 at three recording frame rates, determined for the molecules attached to the poly-L-lysine-coated glass-bottom dish. The standard deviations of the best-fit Gaussian functions provide the localization precisions. The number of observed spots were 23,800, 19,850, and 11,950 for frame rates of 60, 250, and 1,000 Hz, respectively. **c**, The MSD- Δt plots for Halo7-synapsin 1 located in the inner volume of the condensate (mean $\pm$ SEM). In these plots, the offset values are not subtracted data. The plots after subtraction are shown in *Fig. 2e*. **d**, The MSD- Δt plots for free JF549-Halo ligand molecules (not bound to Halo7 protein) located in the inner volume of the condensate (mean $\pm$ SEM). The diffusion is faster with less anomalies, compared with that of

the synapsin 1-bound dyes, but the effect of the SV inclusion in the synapsin 1 condensate is clearly visible; a decrease of the diffusion coefficient and an increase of the diffusion anomaly.

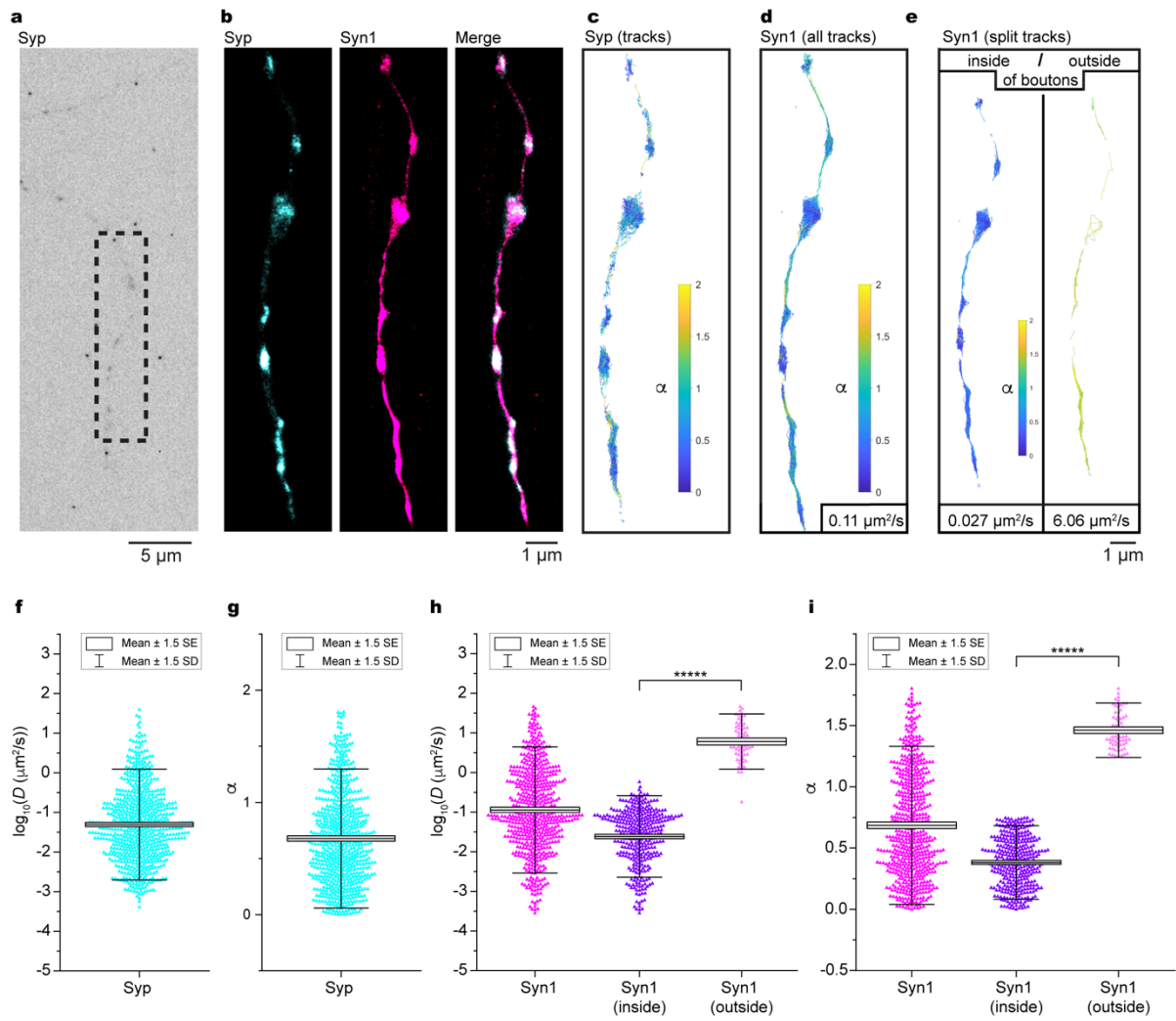


Supplementary Figure 6 | Confinement of expressed proteins in synaptic boutons is independent of the duration of expression. Mouse hippocampal neurons (14 days in culture) were transfected with mEOS3.2. Single-molecule tracking of mEOS3.2 was performed (100 Hz for 50 s) at either 24 h or 48 h post-transfection. Boxplot (mean $\pm$ 1.5 x SD (whiskers) and 1.5 x SE (box)) showing the geometric mean diffusion coefficients per tracking experiment (24 h, $n = 21$; 48 h, $n = 20$) of three independent neuronal preparations. Significance was tested using Mann-Whitney test ($p = 0.71$); ns = not significant.

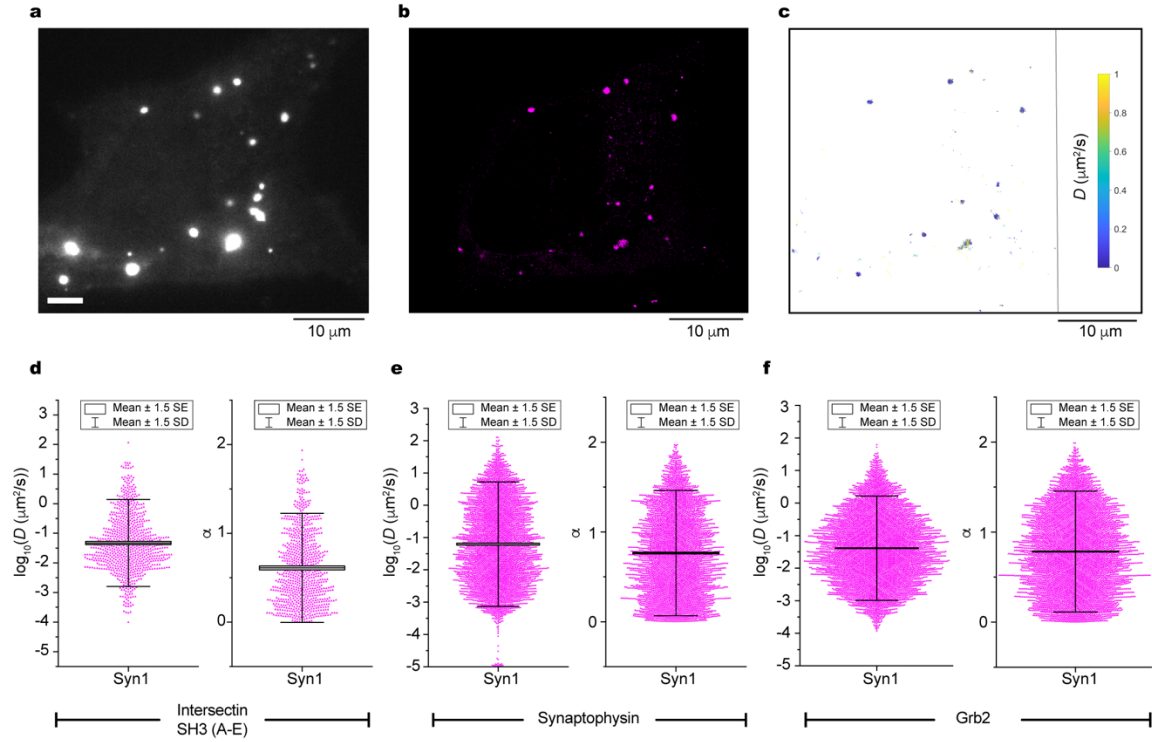


Supplementary Figure 7 | Synapsin confinement and diffusion rates are independent of synaptophysin/SVs. Single-molecule tracking experiments of synapsin 1 performed at 100 Hz for 50 s. (a) Boxplot (mean $\pm$ 1.5 x SD (whiskers) and 1.5 x SE (box)) showing the geometric mean diffusion coefficients per tracking experiment (WT mEos-Syn1, $n = 19$; SYN-TKO Syp-mEos and Halo7-Syn1, $n = 33$; WT Syp-mEos and Halo7-Syn1, $n = 17$; WT mEos, $n = 41$). Significance was tested using Mann-Whitney test (WT mEos-Syn1 vs WT mEos,

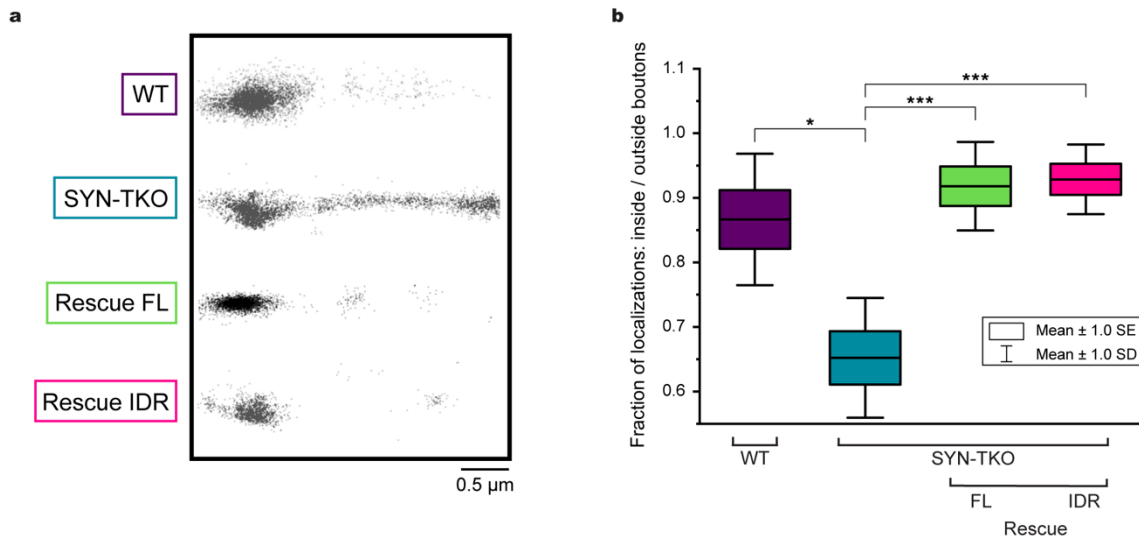
$p = 2.0 \times 10^{-6}$; WT mEos-Syn1 vs WT Syp-mEos Halo7-Syn1, $p = 0.59$; WT mEos-Syn1 vs SYN-TKO Syp-mEos and Halo7-Syn1, $p = 0.12$; SYN-TKO Syp-mEos and Halo7-Syn1 vs WT Syp-mEos and Halo7-Syn, $p = 0.18$; asterisks indicate significance, ns = not significant. **(b)** Boxplot (mean $\pm$ 1.5 x SD (whiskers) and 1.5 x SE (box)) showing the mean coefficients of confinement (α) per tracking experiment (WT mEos-Syn1, $n = 19$; SYN-TKO Syp-mEos and Halo7-Syn1, $n = 34$; WT Syp-mEos and Halo7-Syn1, $n = 17$; WT mEos, $n = 42$). Significance was tested using Mann-Whitney test (WT mEos-Syn1 vs WT mEos, $p = 0.011$; WT mEos-Syn1 vs WT Syp-mEos Halo7-Syn1, $p = 0.95$; WT mEos-Syn1 vs SYN-TKO Syp-mEos Halo7-Syn1; $p = 0.083$; WT Syp-mEos and Halo7-Syn1 vs SYN-TKO Syp-mEos and Halo7-Syn1; $p = 0.084$); asterisks indicate significance, ns = not significant. Tracking data was acquired from at least three independent neuronal preparations. Mouse hippocampal neurons (14 days in culture) from wild-type (WT) or synapsin triple knockout (SynTKO) animals were transfected with mEOS3.2-synapsin 1. Rescue experiments were done by co-transfecting SynTKO neurons with synaptophysin-mEOS3.2 and Halo7-synapsin 1. Overexpression experiments were performed by co-transfecting wild-type neurons with Synaptophysin-mEOS3.2 and Halo7-synapsin 1. Additionally, neurons were transfected with soluble mEOS3.2 to assess the diffusion rate and confinement of a free diffusing protein in the axon. Of note, Halo7-synapsin 1 and mEOS3.2-synapsin 1 are significantly slower and more confined than soluble mEOS3.2 while not being affected by synaptophysin-mEOS3.2 expression. Thus, a molecular crowding alone is insufficient to account for the diffusion coefficients and coefficient of confinement of synapsin in synaptic boutons.



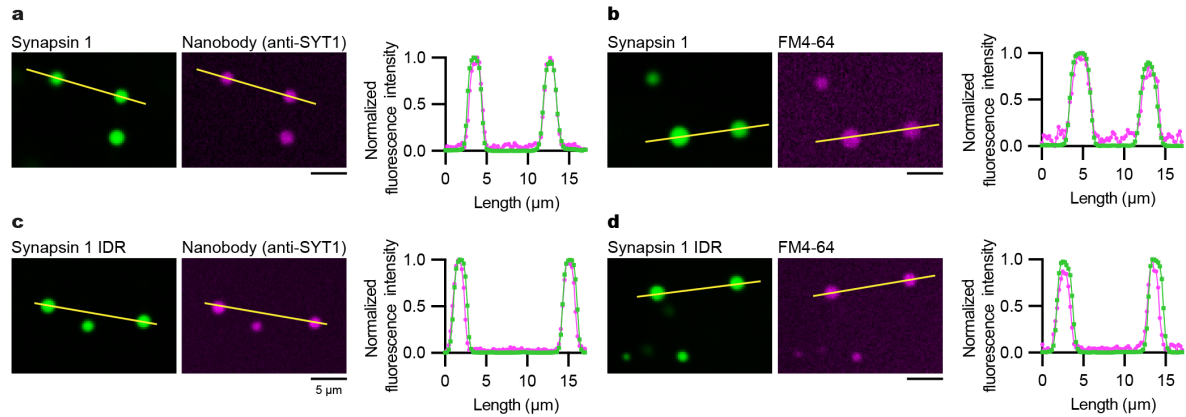
Supplementary Figure 8 | Synapsin 1 and synaptophysin are confined at synaptic boutons while maintaining their high motility. **a**, Widefield image of a neuron expressing synaptophysin-mEOS3.2. Mouse hippocampal neurons (14 days in culture) were transfected with Synaptophysin-mEOS3.2 (Syp) and Halo7-synapsin 1 (Syn1). **b**, Single-molecule localization reconstructions of proteins localized within dashed box in (a). Dual-color single-molecule tracking of Syp or Syn1 coupled to JF635 were performed at 100 Hz for 50 s. **c**, Map of all tracks ($n = 690$) of Syp within dashed box in (a) color-coded for coefficient of confinement (α). **d**, Map of Syn1 within dashed box in (a) color-coded for coefficient of confinement (α) ($n = 596$). **e**, Tracks of Syn1 filtered according to α (inside boutons (left, $n = 355$): $0.75 > \alpha > 0$ or outside boutons (right, $n = 68$): $2 > \alpha > 1.25$). Values indicate the geometric mean diffusion coefficient for each panel. Boxplot (mean $\pm 1.5 \times$ SD (whiskers) and $1.5 \times$ SE (box)) showing diffusion coefficients (**f**) and α (**g**) for all tracks of Syp shown in (c). Boxplot (mean $\pm 1.5 \times$ SD (whiskers) and $1.5 \times$ SE (box)) showing diffusion coefficients (**h**) and α (**i**) for all tracks of Syn1 shown in (d,e). Significance was tested using Mann-Whitney-Test; asterisks indicate significance; ns = not significant. Note that Syn1 is significantly slowed down (**h**, $p = 7.1 \times 10^{-39}$) and confined (**i**, $p = 5.0 \times 10^{-39}$) in synaptic boutons.



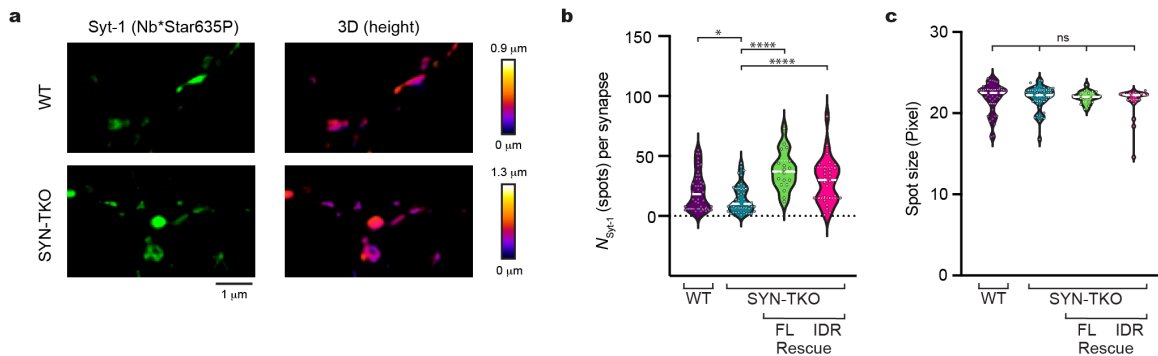
Supplementary Figure 9 | Synapsin 1-containing condensates reconstituted in non-neuronal cells recapitulate the motility pattern measured in synaptic boutons. CV-1 cells were transfected with EGFP-SH3(A-E) concatemer of Intersectin and mEOS3.2-synapsin 1 (Syn1). **a**, Widefield fluorescence image of synapsin 1-containing condensates in the cytosol. Single-molecule tracking measurements of Syn1 were performed at 100 Hz for 50 s. **b**, Reconstruction of proteins localized for cell in (a). **c**, Map of all tracks (n=768) for cell in (a) color-coded for diffusion coefficient. **d**, Boxplots (mean $\pm$ 1.5 x SD (whiskers) and 1.5 x SE (box)) showing diffusion coefficients (left) and the coefficient of confinement (α) (right) for all tracks of Syn1 shown in (c). **e**, the as in **d** but for the cells co-expressing Syn1 and untagged synaptophysin, **f**, the same as in **d** but for the cells co-expressing Syn1 and Grb2.



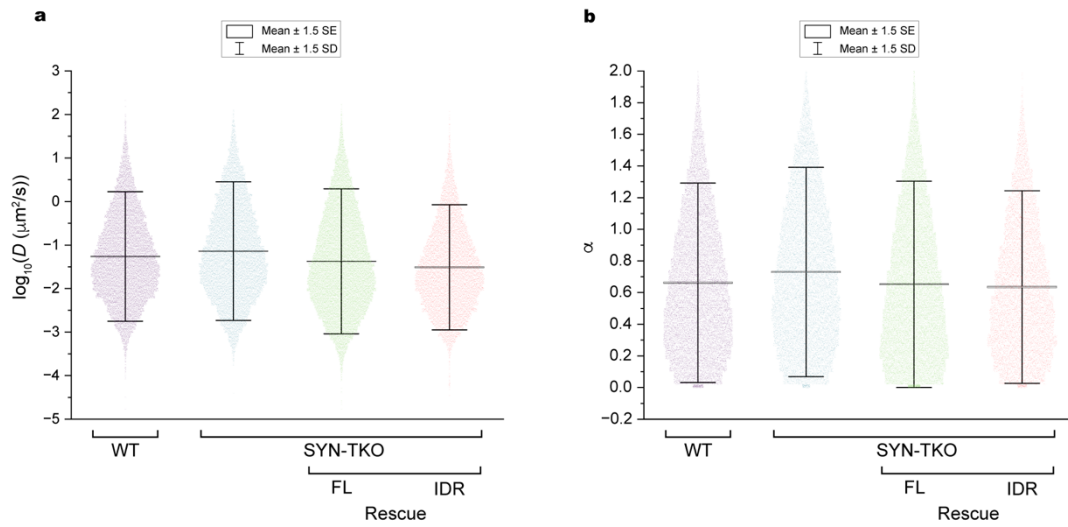
Supplementary Figure 10 | Quantification of the synaptic vesicle accumulation inside boutons. **a**, Representative single-molecule localization reconstructions of mouse hippocampal neurons (14 days *in vitro*) expressing synaptophysin-mEos3.2 (5000 frames, 10 ms exposure time) in neurons with different synapsin expressions: wild-type (WT), synapsin triple knock out neurons (SYN-TKO), SYN-TKO neurons rescued by expressing mCherry-synapsin 1 full-length (Rescue FL) or mCherry-synapsin 1 intrinsically disordered region amino acids 416-705 (Rescue IDR). Scale bar, 0.5 μm . **b**, Ratios of total number of single-molecule localizations of synaptophysin-mEos3.2 inside and up to 2 μm outside of boutons shown as a boxplot (mean $\pm$ 1.0 $\times$ SD (whiskers) and 1.0 $\times$ SE (box)) under the same synapsin expression patterns described in **a**. Significance was tested using unpaired *t*-test with equal variance assumed (WT vs SYN-TKO, $p = 8.3 \times 10^{-3}$; SYN-TKO vs Rescue-FL, $p = 8.8 \times 10^{-4}$; SYN-TKO vs Rescue-IDR; $p = 4.3 \times 10^{-4}$); $n = 5$ independent neuronal preparation for each expression pattern; asterisks indicate significance; ns = not significant.



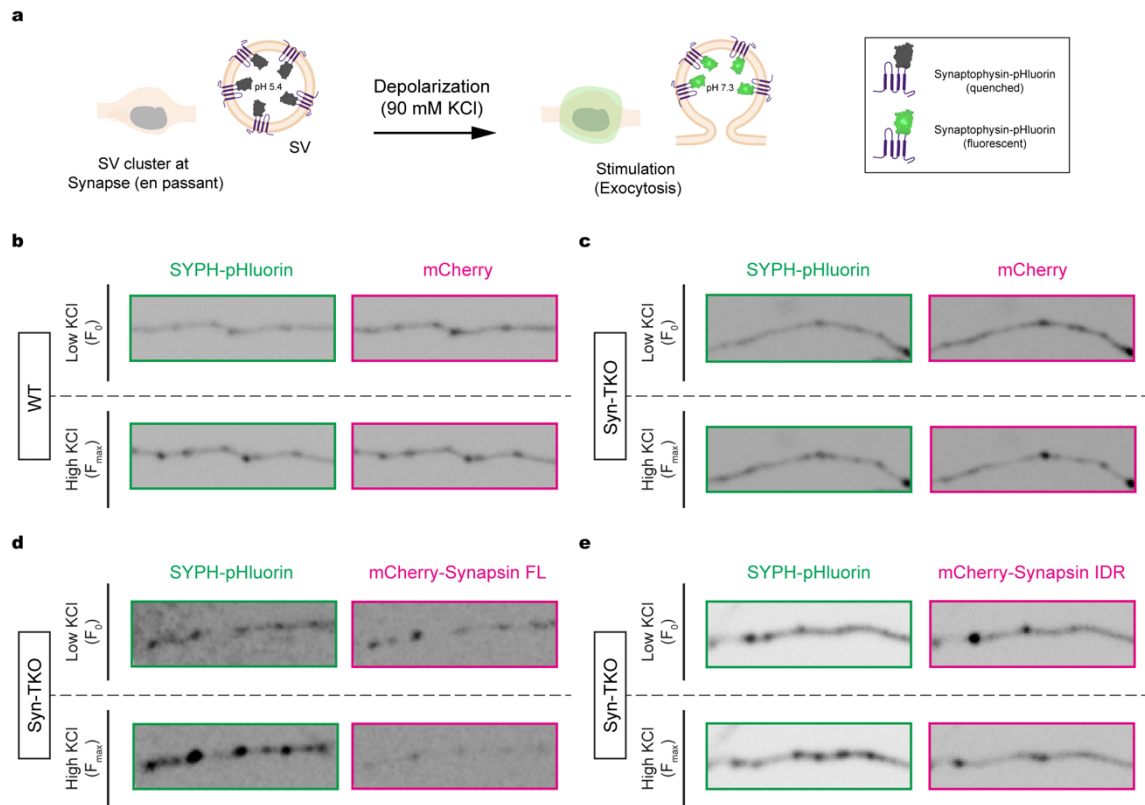
Supplementary Figure 11 | The full-length and the intrinsically disordered region of synapsin 1 sequester synaptic vesicles. **a**, Representative confocal images of the recombinant full-length synapsin 1 (10 μM) coincubated with synaptic vesicles (SVs, 3 nM) visualized using fluorescently-labeled Abberior STAR 635P nanobody against synaptotagmin 1 (anti-SYT1). Left: Images, right: line profiles. **b**, The same as in **a** but SVs were visualized with lipophilic dye FM4-64. **c**, Representative confocal images of the recombinant intrinsically disordered region (IDR, amino acids 416-705) of synapsin 1 (10 μM) coincubated with synaptic vesicles (SVs, 3 nM) visualized using fluorescently-labeled Abberior STAR 635P nanobody against synaptotagmin 1 (anti-SYT1). Left: Images, right: line profiles. **d**, The same as in **c** but SVs visualized with lipophilic dye FM4-64. Scale bars, 5 μm. All reconstitutions have been independently performed at least three times.



Supplementary Figure 12 | Quantification of the synaptic vesicle accumulation using 3D STED microscopy. **a**, Representative STED images of primary mouse hippocampal neurons stained with Aberrior STAR 635P-labeled nanobodies (Nb*Star635P) against synaptotagmin-1 (Syt-1), a *bona fide* synaptic vesicle protein. **b**, Quantification of number of Syt-1 positive spots in boutons of wild-type (WT; N = 41) neurons or synapsin triple knock-out (SYN-TKO; N = 48) neurons transfected with mCherry-synapsin 1 full-length (Rescue FL; N = 19) or mCherry-synapsin 1 intrinsically disordered region (IDR, amino acids 416-705; N = 28). **c**, Spot size distribution in all conditions analyzed in **b** indicating the consistent labeling pattern of Syt-1 nanobody. Significance was tested using unpaired, two-tailed *t*-test. Asterisks, significance; *, *p* < 0.05; ****, *p* < 0.0001; ns, not significant.



Supplementary Figure 13 | Analysis of all tracks confirms that synapsin determines the confinement and diffusion rates of synaptic vesicles (SVs). Single-molecule tracking experiments of synaptophysin-mEOS3.2 (Syp) performed at 100 Hz for 50 s. Mouse hippocampal neurons (14 days in culture) from wild-type or synapsin triple knockout (SYN-TKO) animals were transfected with Syp. Rescue experiments were done by co-transfecting SYN-TKO neurons with Syp and either full-length synapsin 1 (Rescue FL) or the intrinsically disordered region of synapsin 1 (Rescue IDR). Boxplots (mean $\pm$ 1.5 x SD (whiskers) and 1.5 x SE (box)) showing the diffusion coefficients (a) and the coefficient of confinement (α) (b) of all tracks (N = 77,675) of at least four independent neuronal preparations per condition.



Supplementary Figure 14 | Functional assay for measuring SV release. **a**, Scheme of the assay. SV release measured as an increase in fluorescence of pH-sensitive EGFP variant tagged to the luminal region of synaptophysin (SYPH-pHluorin) upon chemical stimulation with KCl (90 mM). Representative images of wild-type neurons co-transfected with only a soluble red fluorophore (**b**) or SYN-TKO neurons co-transfected with a soluble red fluorophore (**c**), synapsin FL (**d**) or the synapsin IDR (**e**).

Supplementary Table 1. DNA sequences of (a) His14-SUMO_Eu1-mmSynapsin1a and (b) SYPH-pHluorin expression cassettes

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b) ...cmv- <u>SYPH(M1-T190)</u> -p <u>Hluorin-SYPH(C191-A240)</u> -Linker- <i>SV40</i> ...	
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Supplementary Table 2. Primer sequences for plasmid construction

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#DML0438+0439 (<i>NheI</i> , <i>AgeI</i>)	FW: CTAGC GCCACCATGCATCACCATCACCATCACA RV: CCGGT GTGATGGTGGTGGTGGTGGTGGC G