SUPPLEMENTARY INFORMATION

- 8 supp figs
- 1 supp table

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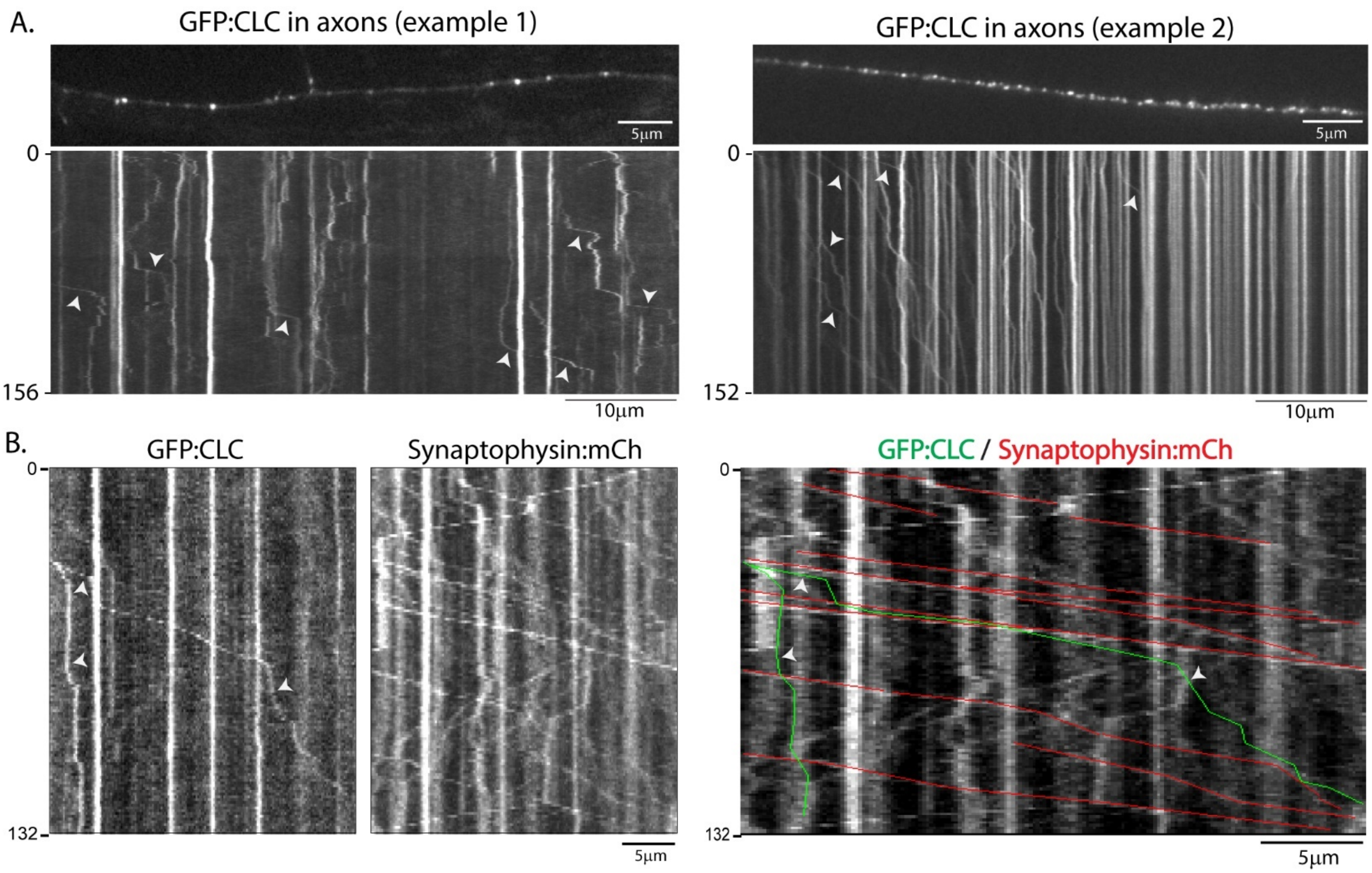
Figure S1, related to Figure 1



<u>**Figure S1:**</u> **Axonal clathrin dynamics.**

(A) Two examples of GFP:CLC dynamics in axons (image of axon on top, kymograph at bottom). Unlike dendrites, blinking (on/off) dynamics are rare in axons, and a fraction of particles move intermittently (arrowheads). Example 1 shows an axon with lower density of clathrin particles and example 2 shows an axon with higher particle density. Note that stationary and moving particles are seen in both cases.

(B) Kymographs from dual-imaging of GFP:CLC and synaptophysin:mCherry (left), with overlaid tracks (right). The movement of clathrin transport-packets was much less frequent than the fast transport of synaptophysin-vesicles. Though the extensive movement of synaptophysin made definitive conclusions difficult, we could see clear instances where clathrin-packets were transported independently of synaptophysin – see overlaid clathrin (green) and synaptophysin (red) tracks in kymograph below.

Time in seconds to the left of kymographs, and scale bar on lower right.

Figure S2, related to Figure 2

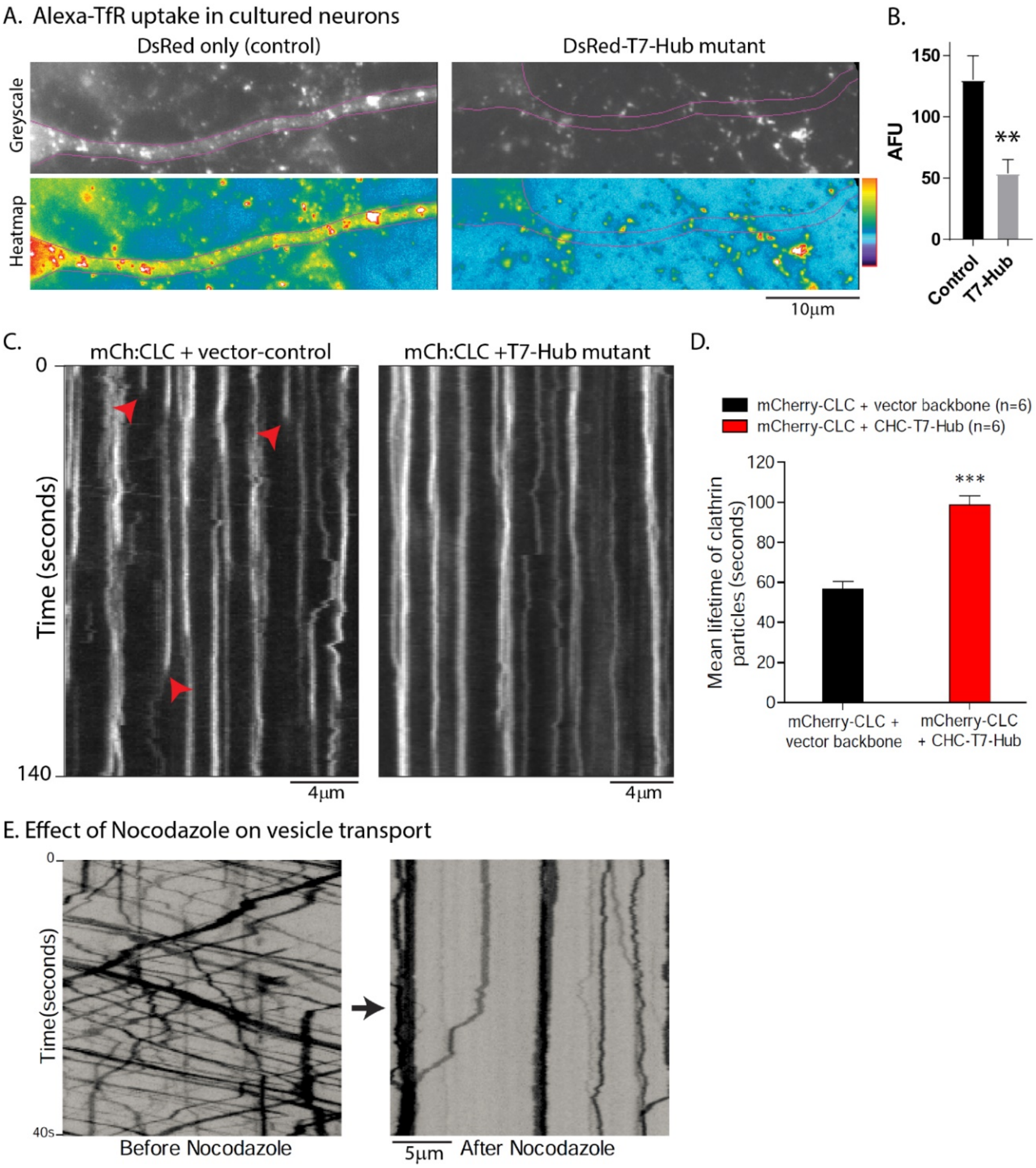


Figure S2: The clathrin T7-Hub mutant blocks receptor mediated endocytosis.

(A) Neurons were transfected with T7-Hub mutant (DsRed-tagged) or vector-control (DsRed-only), and Alexa-488-transferrin uptake into the transfected neurons was analyzed. As evident in the representative greyscale images (above) and pseudocolor heatmaps (below), the T7-Hub mutant attenuates transferrin-uptake. Data quantified in (B), 5-6 neurons per condition, from one culture; **$p<0.001$.

(C) Kymographs showing dynamics of mCh:CLC in dendrites co-transfected with the T7-Hub mutant (or vector control). Note that the blinking of clathrin fluorescence – due to receptor mediated endocytosis, some marked by arrowheads – is essentially abolished in the T7-Hub transfected neurons.

(D) Quantification of mCh:CLC lifetime in dendrites (100-150 particles from 6 dendrites were analyzed; ***$p<0.0001$).

(E) Kymographs showing movement of the pan-vesicle marker NPYss:mCherry (see Ganguly et al., 2015) in an axon before and after 10μg/ml Nocodazole for 30 min. Note complete stoppage of vesicle transport after adding the microtubule depolymerizing agent.

Time in seconds to the left of kymographs, and scale bar on lower right.

Figure S3, related to Figure 3

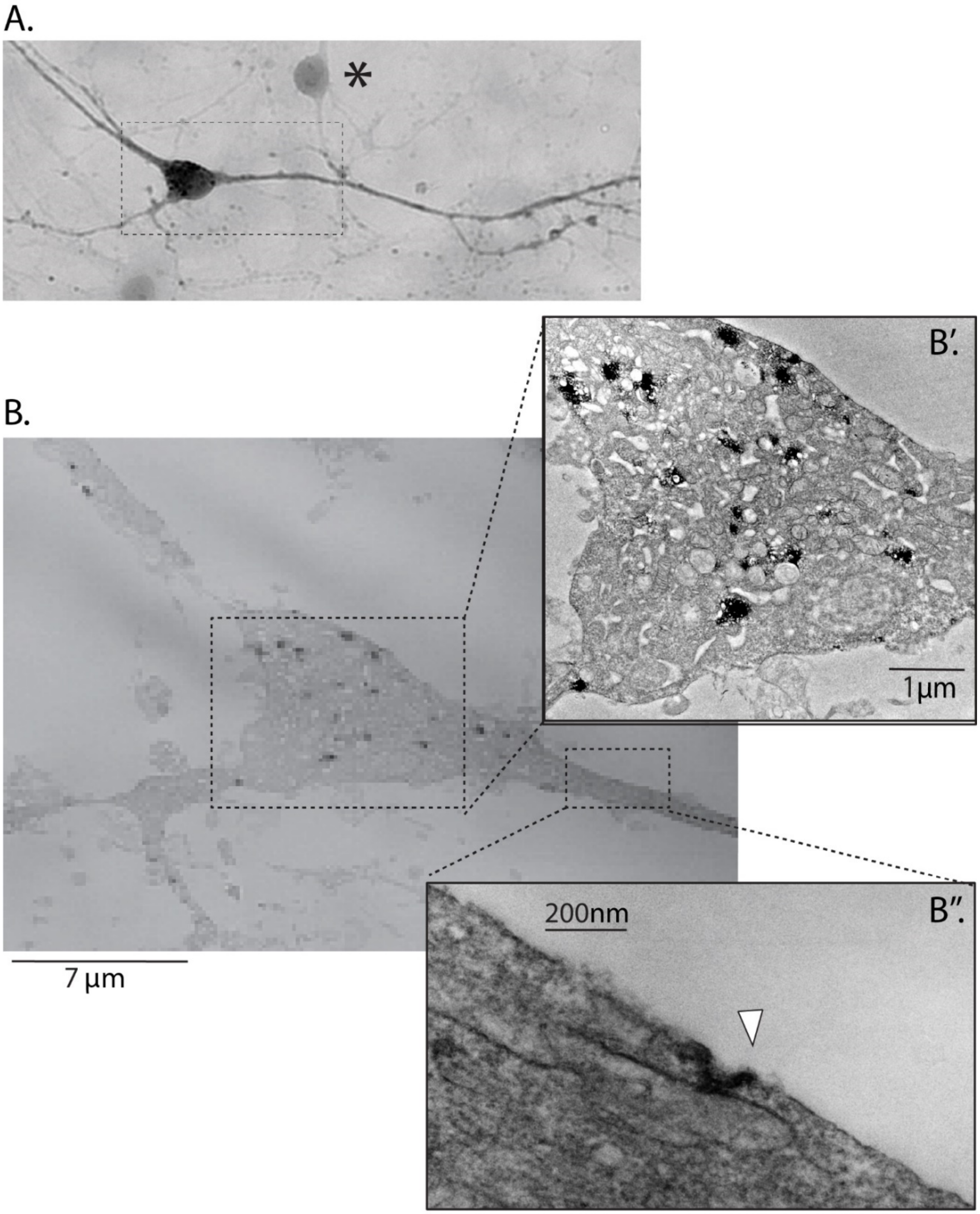


__Figure S3:__ **Apex-clathrin labeling and EM.**

(A) Neurons were transfected with Apex:GFP:CLC and processed for EM. Gridded coverslips were used to identify transfected neurons for EM processing, as described in methods. The DAB signal from an Apex:GFP:CLC transfected neuron is shown, along with an adjacent un-transfected neuron (marked with an asterisk).

(B) EM of rectangular region from (A), dark signals represent clathrin. Note that many clathrin structures are seen in the somatodendritic region (B'), some clearly representing coated clathrin pits (B'').

Figure S4, related to Figure 4

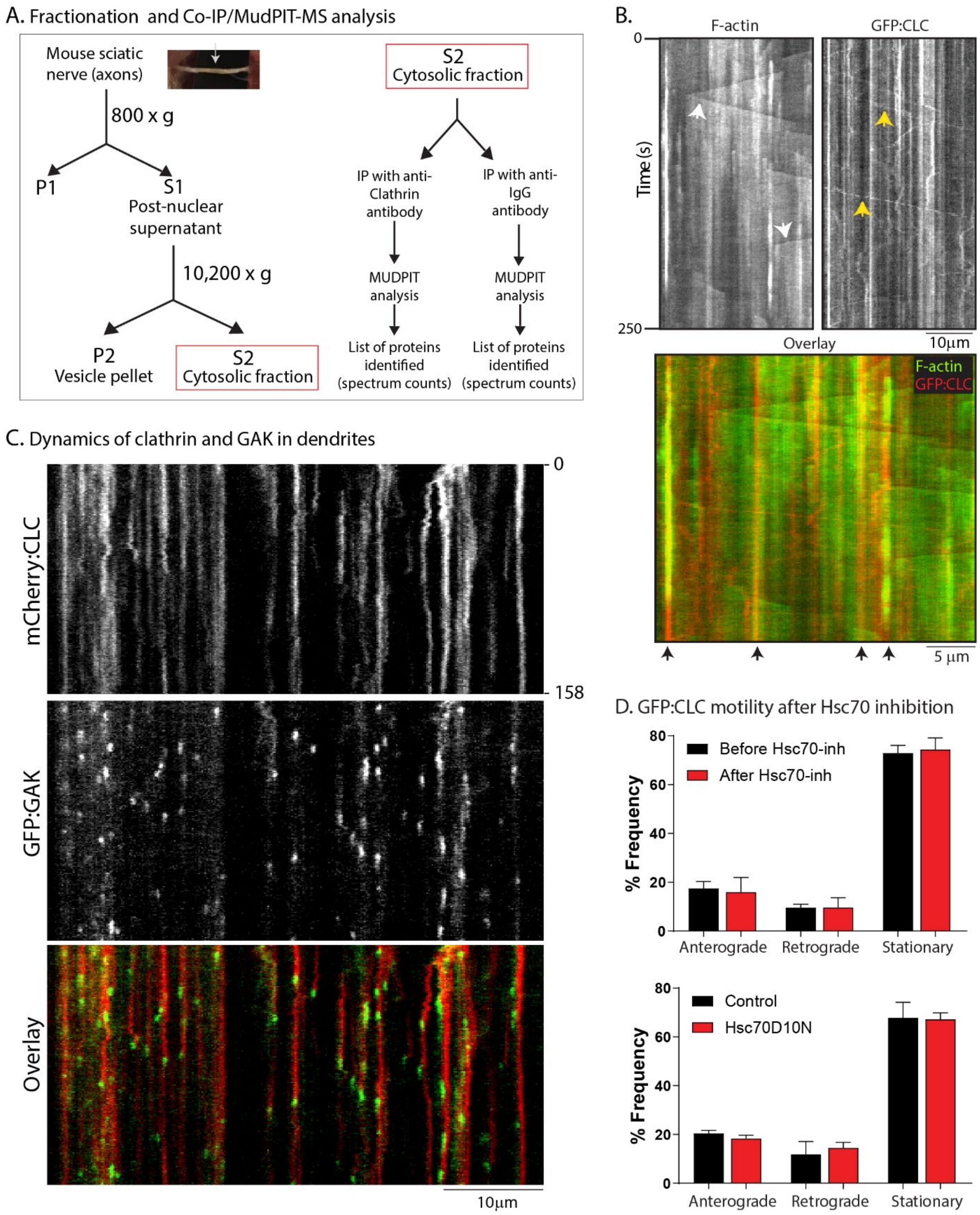


Figure S4: Composition of axonal clathrin packets.

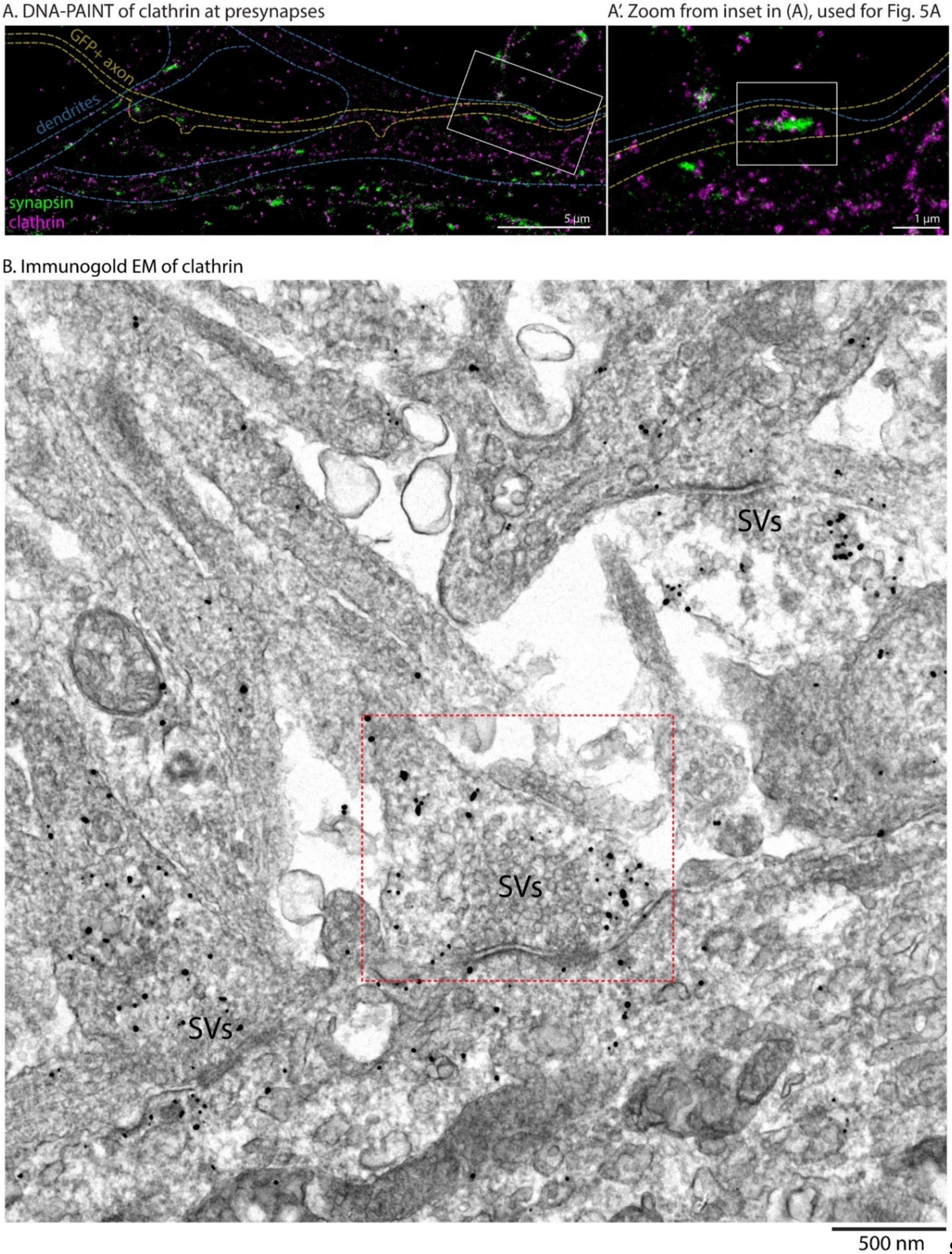
(A) Schematic for fractionation and Mudpit-MS. Clathrin was immunoprecipitated from cytosolic (S2) sciatic nerve fractions (dissected from mice, see inset picture and arrow), and associating proteins were identified by MS.

(B) Kymographs from two-color imaging of GFP:Utr-CH (labeling F-actin) and mCh:CLC – some 'actin trails' and clathrin transport-packets are marked by white and yellow arrowheads respectively. On the overlaid red/green kymographs on right, note that many stationary clathrin particles are colocalized with F-actin (black arrowheads, bottom).

(C) Simultaneous two-color imaging of mCherry:CLC and GFP:GAK in dendrites. Note that GAK particles only appear in kymographs when clathrin is disassembled, consistent with the role of GAK as a protein that dis-assembles clathrin cages. Also note that this behavior of GAK is very different from its dynamics in axons, where many GAK particles colocalize with clathrin and are intermittently transported (**Fig. 4D**).

(D) Quantification of GFP:CLC particle dynamics in axons before and after pharmacologic inhibition of Hsc70 with a small-molecule (VER155008); or genetic interference of Hsc70 function using a dominant-negative construct (Hsc70D10N). Note that motility of clathrin is unchanged after Hsc70 inhibition.

Figure S5, related to Figure 5



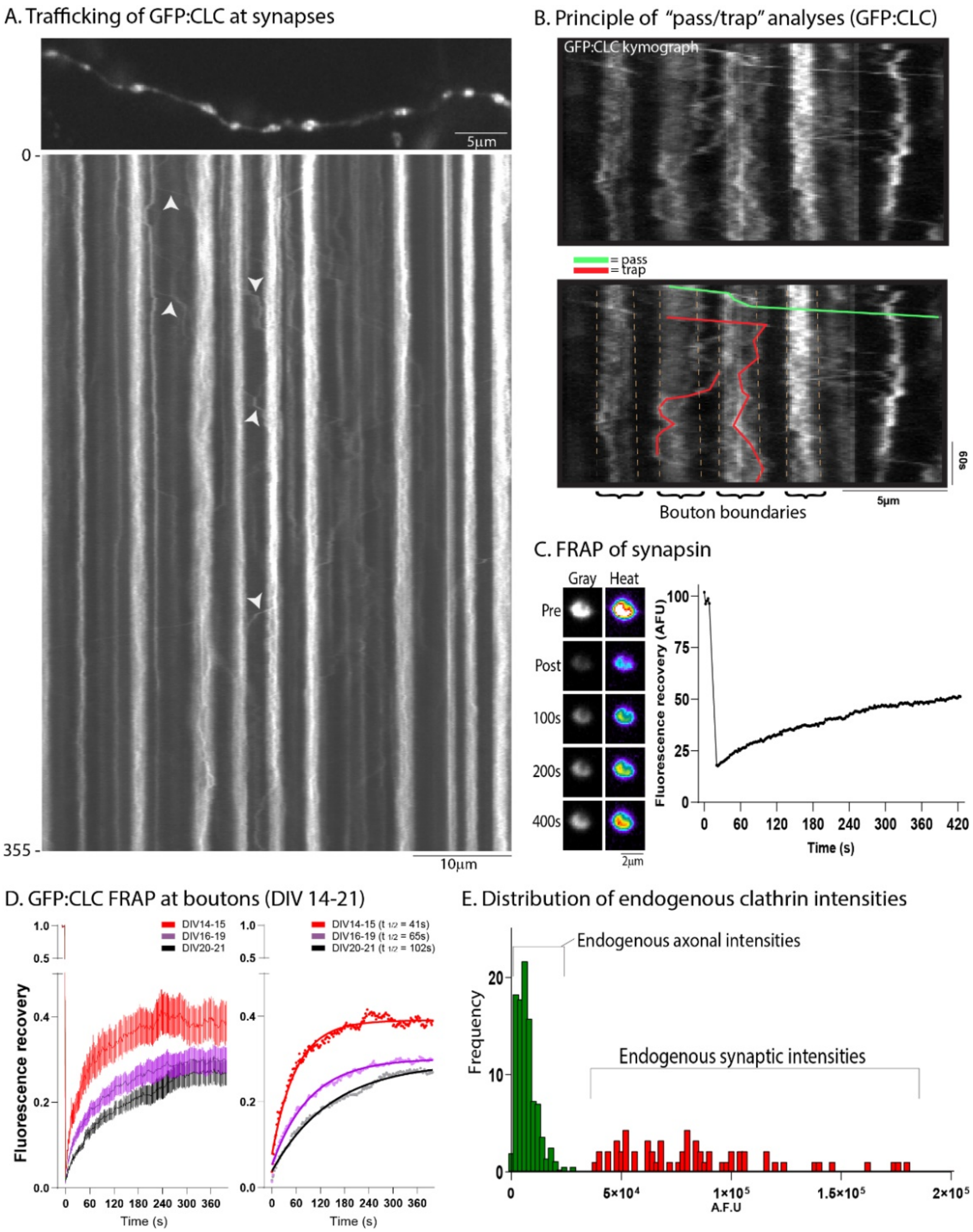
Supplementary

__Figure S5:__ **Supplements super-resolution and immuno-EM data shown in Fig. 5.**

(A) DNA-PAINT: Larger field of view showing axons and dendrites corresponding to the bouton shown in **Fig. 5A**. Blue dashed lines represent dendrites (identified by MAP2 staining), and dashed yellow lines represent an axon (identified by Actin:GFP transfection). A' shows the actual image shown in **Fig. 5A**.

(B) Immuno-EM of clathrin - Large field of view showing synapses and neurites around the synapse showed in **Fig. 5F1** (dashed red box marks ROI used in figure). Note that the immuno-gold particles do not bind to many other structures (tubulo-vesicular organelles, mitochondria, cytoskeletal structures, cytoplasmic matrix, etc.) and appears specific for clathrin.

Figure S6, related to Figure 6



<u>Figure S6:</u> Trafficking and synaptic targeting of clathrin particles.

(A) Image (top) and kymograph (bottom) of GFP:CLC trafficking in presynaptic boutons (varicosities) and flanking axons. Note that many clathrin particles are exchanged between boutons. Elapsed time on left, and scale-bar on lower right of kymograph.

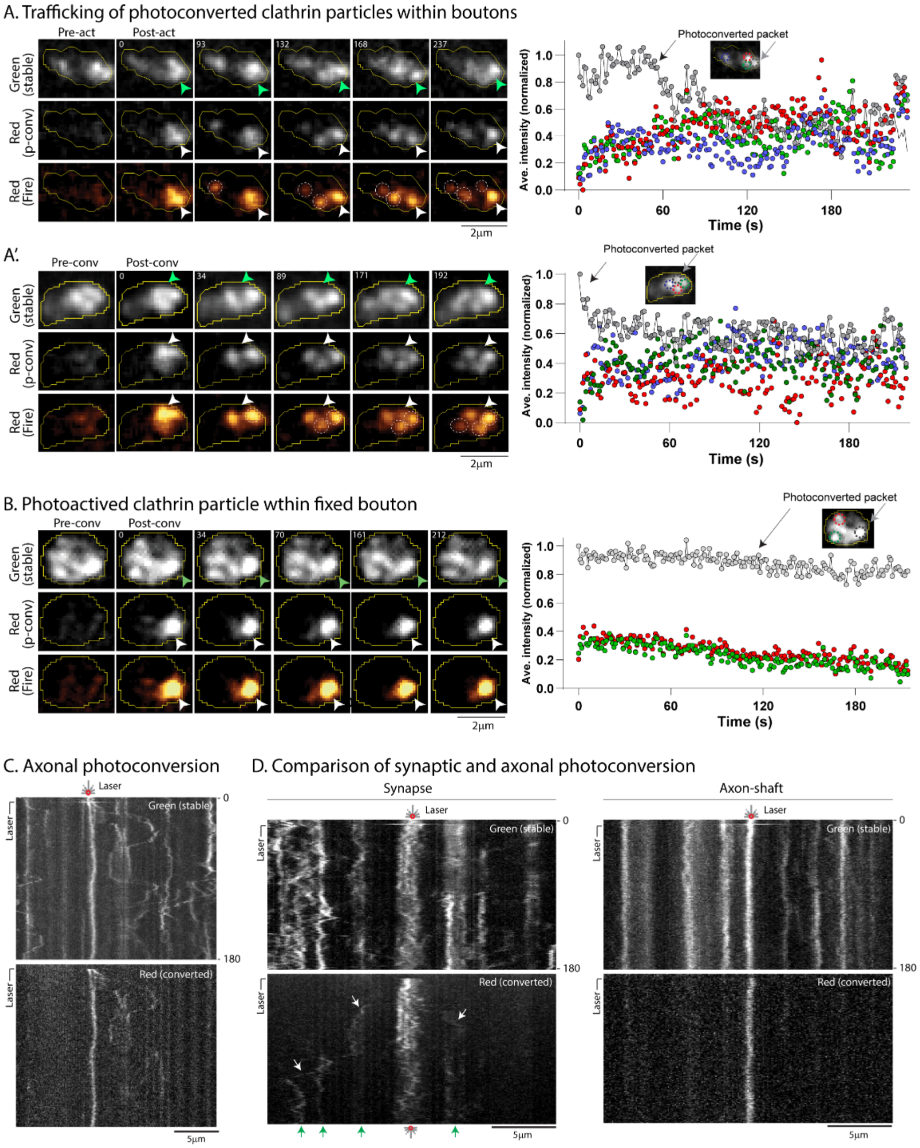
(B) Principle of "pass/trap analysis" to quantify moving clathrin particles trapped at boutons. Moving GFP:CLC (or Dendra2:CLC) particles that did not re-emerge from boutons after 60 seconds were considered trapped. Green and red lines show examples of passing and trapped GFP:CLC particles. Time/scalebar on lower right.

(C) Time-series grayscale and heatmap images of a bouton containing GFP:synapsin that was photobleached, with corresponding FRAP curve on right (time in seconds shown on left of images). Note the relatively smooth, gradual rise of GFP:synapsin fluorescence after photobleaching.

(D) FRAP-curves of GFP:CLC from boutons of neurons cultured for 14-21 days in vitro (DIV). Note that there is an earlier flattening of the FRAP-curves with increasing DIV – suggesting a larger immobile pool with age in cultures – likely reflecting synaptic maturity over time (data from 34 neurons, 5 separate cultures for DIV14-15; 51 neurons, 10 separate cultures for DIV16-19; and 44 neurons, 6 separate cultures for DIV20-21). All clathrin photobleaching and photoconversion experiments in main figures were done on DIV 13-14 neurons.

(E) Intensity distributions (histograms) of endogenous clathrin in axons and synapses, determined by immunostaining cultures with antibody to clathrin heavy chain (to mark synapses, see methods; 355 axonal particles and 92 synapses were analyzed from 2 separate cultures).

Figure S7, related to Figure 7



<u>Figure S7:</u> Photoconversion of single clathrin packets in synapses and axons.

(A, A') Photoconversion of single clathrin-packets (Dendra2:CLC) at presynaptic boutons – two more examples. **<u>Left</u>**: Dendra2:CLC was photoconverted in a single synaptic clathrin-packet (arrowhead, elapsed time in seconds on top left). Note that after photoconversion (middle and bottom panels), the photoconverted packet lost fluorescence, while there was an increase of fluorescence in adjacent packets, identified by stable green fluorescence (some marked by dashed circles in bottom panel). **<u>Right</u>**: Quantification of intensities in individual packets (normalized to highest post-conversion intensity). Colors in graph correspond to the colors of circles marking packets in inset-image. Note reciprocal fluctuations in photoconverted and non-photoconverted packets.

(B) Same experiment as above, but in fixed NEURONS. Note that while the Dendra2:CLC synaptic packet could be photoconverted, there was little to no loss of fluorescence from this packet, or gain of fluorescence in the other local clathrin packets.

(C) Kymographs from photoactivation of single Dendra2:CLC axonal packets. In this example, note that moving clathrin particles caused some disruption of the photoconverted clathrin, but there was no appreciable loss of clathrin molecules from the photoconverted axonal packet. Also note that the time-scale of dispersion in this case – seconds – is very different from the slower exchange of clathrin molecules at the synapse (over tens of seconds to minutes).

(D) A side by side comparison of synaptic and axonal Dendra2:CLC photoconversion. In the kymographs of photoconverted (red) fluorescence, note that in the case of synapses, single particles of clathrin emerged from the laser-illuminated bouton, and were trapped at adjacent boutons (white arrowheads on kymograph; green arrowheads at bottom mark the positions of non-activated boutons, as determined by the stable green fluorescence). On the other hand, no dispersion of fluorescence was seen when a single axonal clathrin-packet was photoconverted.

Key for kymographs: elapsed time in seconds on right and scale-bar on lower right (laser illumination also marked).

Figure S8, related to Figure 8

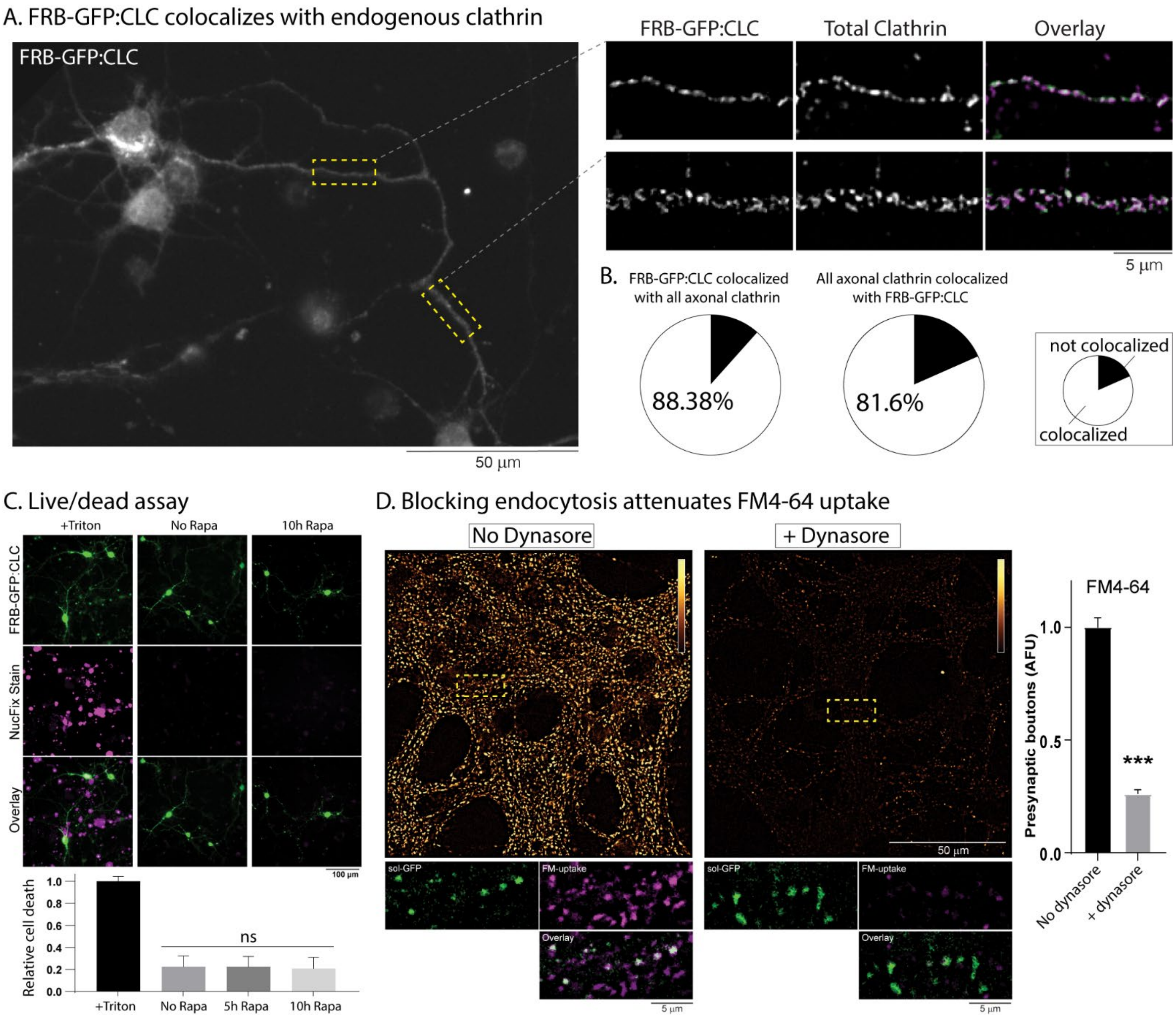


Figure S8: **Transfected FRB-GFP:CLC co-assembles with endogenous clathrin.**

(A) Neurons were transfected with FRB-GFP:CLC and immunostained with an antibody recognizing the clathrin heavy-chain (see 'methods"). In the insets magnified in A', note the extensive colocalization of transfected FRB-GFP:CLC with endogenous clathrin.

(B) Colocalization analysis indicates that the majority of transfected FRB-GFP:CLC (~ 80-90%) co-assembles with the endogenous clathrin in axons (~250 particles were analyzed from 10 neurons).

(C) Live/dead assay with NucFix stain (see "Methods") was validated in FRB-GFP:CLC transfected neurons by treating the cells with 0.01% Triton for one minute (left panel). Note that there was no staining above baseline after addition of Rapalog; quantified at bottom (12-16 fields of neurons were imaged for each condition from two separate cultures).

(D) Blocking endocytosis by Dynasore led to an attenuation of the FM4-64 dye uptake, as shown in the representative images on left (zoom from boxed regions shown below) and quantification of the data on right. For quantification, boutons were identified by varicosities in neurons transiently transfected with soluble GFP (~240-310 synapses were analyzed for each condition from 3 coverslips).

Table S1. Proteins associated with clathrin - MUDPIT data (related to Fig. 4)

Protein Families	UniProtKB ID (from MudPIT)	Gene IDs	Organism
ATP citrate synthase	Q3V117	Acly	Mus musculus (Mouse)
Actin	P68134, P60710, Q8BFZ3, P68033	Acta1, Actb, Actbl2, Actc1	Mus musculus (Mouse)
Argonaute	Q8CJG0	Ago2	Mus musculus (Mouse)
Ahnak2	E9PYB0	Ahnak2	Mus musculus (Mouse)
Adaptor Related Protein Complex	Q8CC13, Q8CBB7, Q3UHJ0, P17426, P17427, Q9DBG3, P84091, P62743, Q9Z1T1	Ap1b1, Ap1g1, Aak1, Ap2a1, Ap2a2, Ap2b1, Ap2m1, Ap2s1 , Ap3b1	Mus musculus (Mouse)
V-ATPase	Q9Z1G4	Atp6v0a1	Mus musculus (Mouse)
Bag3	Q9JLV1	Bag3	Mus musculus (Mouse)
Bcr	Q6PAJ1	Bcr	Mus musculus (Mouse)
Bmp	Q91Z96	Bmp2k	Mus musculus (Mouse)
Calcoco	Q8CGU1	Calcoco1	Mus musculus (Mouse)
Ccdc	D3YZP9	Ccdc6	Mus musculus (Mouse)
Clint1	Q5SUH7	Clint1	Mus musculus (Mouse)
Clathrin	Q6IRU5, Q68FD5	Cltb, Cltc	Mus musculus (Mouse)
Alpha-crystallin	P23927	Cryab	Mus musculus (Mouse)
Casein	P19228, Q02862	Csn1s1, Csn1s2a	Mus musculus (Mouse)
Dab2	P98078	Dab2	Mus musculus (Mouse)
Connecdenn	Q8K382	Dennd1a	Mus musculus (Mouse)
Dynamin	P39053, P39054	Dnm1, Dnm2	Mus musculus (Mouse)
Dynein	Q9JHU4	Dync1h1	Mus musculus (Mouse)
Edc	Q3UJB9	Edc4	Mus musculus (Mouse)
Eef	P58252	Eef2	Mus musculus (Mouse)
Epsin	Q80VP1, Q5NCM5	Epn1, Epn2	Mus musculus (Mouse)
Eps	P42567, Q60902	Eps15, Eps15l1	Mus musculus (Mouse)
Fatty acid binding protein	Q05816	Fabp5	Mus musculus (Mouse)
Fatty Acid Synthase	P19096	Fasn	Mus musculus (Mouse)
Fcho	Q3UQN2	Fcho2	Mus musculus (Mouse)
Fibrinogen A	E9PV24	Fga	Mus musculus (Mouse)
Filamin A	Q8BTM8	Flna	Mus musculus (Mouse)
Gak	Q99KY4	Gak	Mus musculus (Mouse)
Gulp1	Q8K2A1	Gulp1	Mus musculus (Mouse)
H1	P15864, P43274	H1-2, H1-4	Mus musculus (Mouse)
Hip1	Q8VD75, Q6ZQ77	Hip1, Hip1r	Mus musculus (Mouse)
Hnrnp	Q8BG05, Q8VDM6	Hnrnpa3, Hnrnpul1	Mus musculus (Mouse)
Heat shock proteins	Q80TZ3, Q71LX8, Q8K0U4, Q61696, P16627,P17156, Q3U2G2, P48722, P20029, Q504P4, Q61699	Dnajc6, Hsp90ab1, Hspa12a, Hspa1a, Hspa1l, Hspa2, Hspa4, Hspa4l, Hspa5, Hspa8, Hsph1	Mus musculus (Mouse)
Intersectin	E9Q0N0, B2RR82	Itsn1, Itsn2	Mus musculus (Mouse)
Kcp	Q3U492	Kcp	Mus musculus (Mouse)
Lactate Dehydrogenase	P16125	Ldhb	Mus musculus (Mouse)
Lrp1	Q91ZX7	Lrp1	Mus musculus (Mouse)
Maged1	Q9QYH6	Maged1	Mus musculus (Mouse)
Map7	A2AG50	Map7d2	Mus musculus (Mouse)
Myosin	Q3UH59, Q69ZX3, E9Q174	Myh10, Myh11, Myo6	Mus musculus (Mouse)
Numb	Q9QZS3, O08919	Numb, Numbl	Mus musculus (Mouse)
Osbp	D3YTT6	Osbpl3	Mus musculus (Mouse)
Pdcd	Q9WU78	Pdcd6ip	Mus musculus (Mouse)
Phgdh	Q61753	Phgdh	Mus musculus (Mouse)
Picalm	Q7M6Y3	Picalm	Mus musculus (Mouse)
Pik3	Q61194	Pik3c2a	Mus musculus (Mouse)
Prune	Q52KR3	Prune2	Mus musculus (Mouse)
Periaxin	O55103	Prx	Mus musculus (Mouse)
Reps	O54916, B9EI38	Reps1, Reps2	Mus musculus (Mouse)
Rundc3a	O08576	Rundc3a	Mus musculus (Mouse)
Sec Faily	Q9D1M0, E9QAT4, Q01405	Sec13, Sec16a, Sec23a	Mus musculus (Mouse)
Sh3d19	Q91X43	Sh3d19	Mus musculus (Mouse)
Slc12a4	Q9JIS8	Slc12a4	Mus musculus (Mouse)
Snap91	Q61548	Snap91	Mus musculus (Mouse)
Alpha-synuclein	O55042	Snca	Mus musculus (Mouse)
Sorting nexin	Q91VH2	Snx9	Mus musculus (Mouse)
Sos1	Q62245	Sos1	Mus musculus (Mouse)
Spectrin Beta	Q62261	Sptbn1	Mus musculus (Mouse)
Stambpl1	Q76N33	Stambpl1	Mus musculus (Mouse)
Ston2	E9PXP7	Ston2	Mus musculus (Mouse)
Synj1	D3Z656	Synj1	Mus musculus (Mouse)
Tfg	Q9Z1A1	Tfg	Mus musculus (Mouse)
Tnrc6b	Q8BKI2	Tnrc6b	Mus musculus (Mouse)
Tom1l2	Q5SRX1	Tom1l2	Mus musculus (Mouse)
Trim21	Q3U7K7	Trim21	Mus musculus (Mouse)
Tubulin	P68368, Q9CWF2, Q922F4	Tuba4a, Tubb2b, Tubb6	Mus musculus (Mouse)
Ubiquitin C	P0CG50	Ubc	Mus musculus (Mouse)
Utrophin	E9Q6R7	Utrn	Mus musculus (Mouse)
Vcp	Q01853	Vcp	Mus musculus (Mouse)

Ybx	P62960, Q9JKB3	Ybx1, Ybx3	Mus musculus (Mouse)
Ywhag	P61982	Ywhag	Mus musculus (Mouse)
FLJ45252 homolog	Q6PIU9	FLJ45252 homolog	Mus musculus (Mouse)