Neuron, Volume *72*

Supplemental Information

Recruitment of Endophilin to Clathrin-Coated Pit Necks Is Required for Efficient Vesicle Uncoating after Fission

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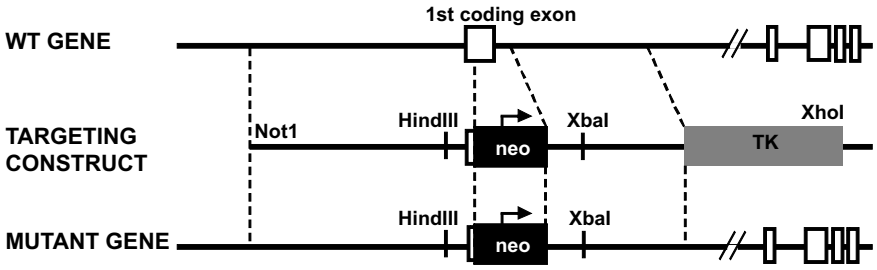
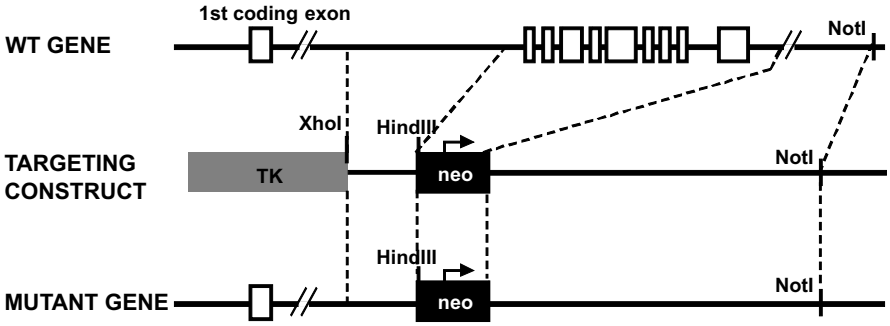
A

Endophilin 1 KO targeting strategy

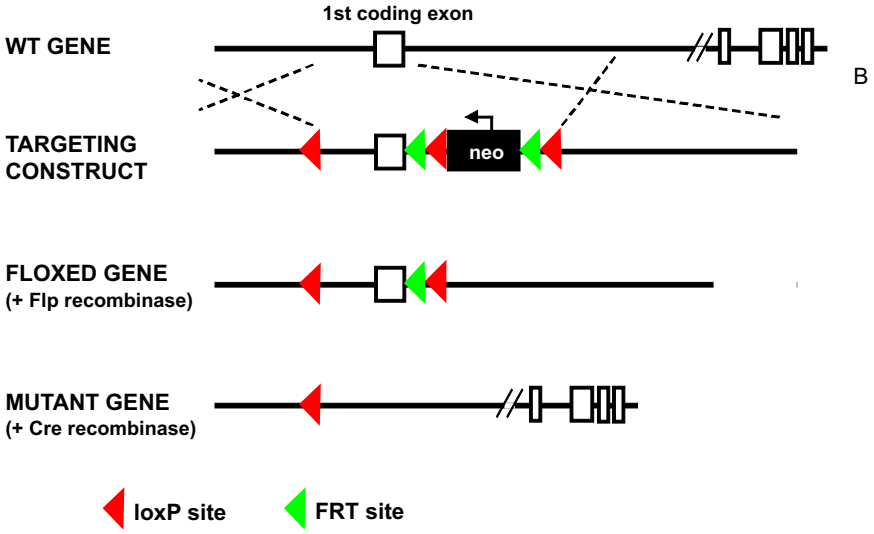


Endophilin 2 KO targeting strategy



1st coding exon encodes for 15 aa: MSVAGLKKQEYKASQ

Endophilin 3 conditional KO targeting strategy



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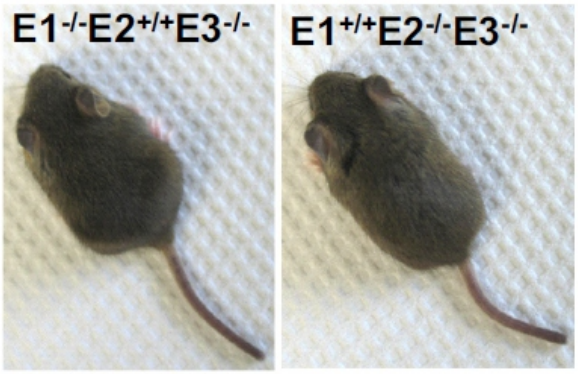

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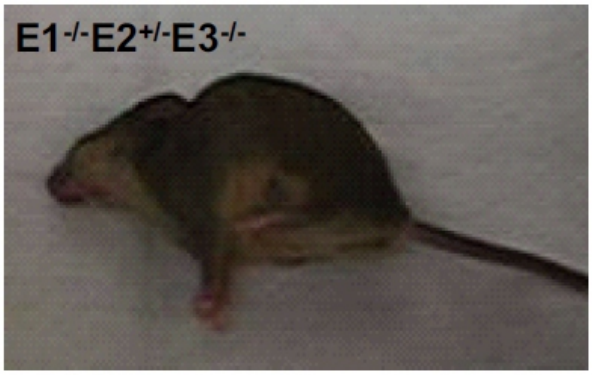

Figure S1. Endophilin 1, 2 and 3 single knock-out (KO) mice: gene deletion strategies.

(A) Schematic representation of endophilin 1 and 2 gene KO targeting strategies and of the endophilin 3 conditional KO gene targeting strategy. Mating of heterozygous mice produced pups with a typical Mendelian distribution for all three single KOs. Single KO mice did not have an obvious phenotype. (B) Two months old mice mice double KO for endophilin 1 and 2, or endophlin 1 and 3, respectively. These mice were phenotypically normal.

(C) Mice double KO mice for endophilin 1 and 3 and heterozygous for endophlin 2 (three months old) were viable, but showed spontaneous epileptic, sometimes lethal, seizures. A 3 month old mouse is shown during a seizure.

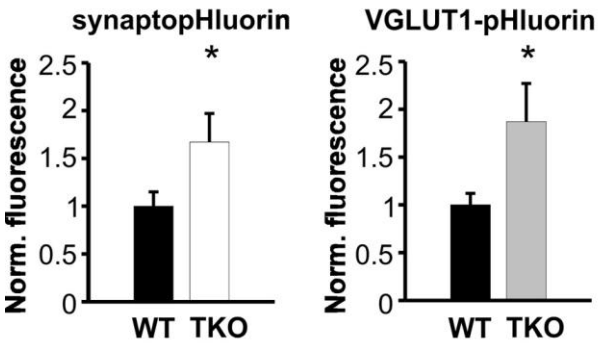

Figure S2. Increased accumulation of pHluorin-conjugated synaptic vesicle proteins in the plasma membrane of endophilin TKO neurons.

The fraction of synaptopHluorin and VGLUT1-pHluorin stranded at the plasma membrane was calculated by expressing the fluorescence of random neuronal processes in live cultures in Tyrode buffer as a fraction of the total fluorescence of the same processes after exposure to ammonium chloride (to collapse the low pH of the lumen of synaptic vesicles and of other endocytic compartments). Values were normalized to the surface fluorescence of WT cells. At least 25 images from 4 animals per condition were analyzed. *$p < 0.05$ compared to WT, error bars represent SEM.

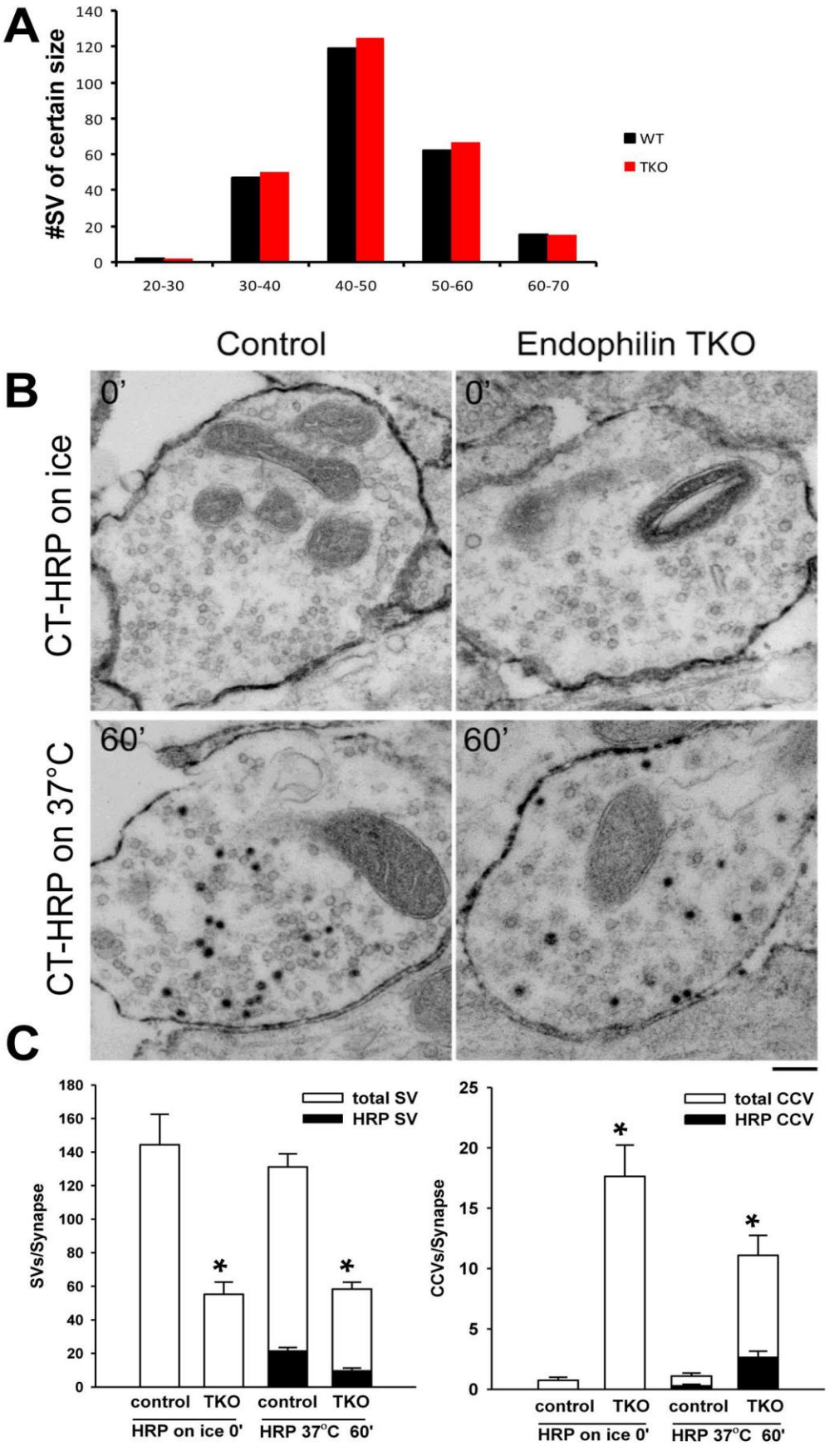
A
#SV of certain size
140
120
100
80
60
40
20
0
20-30
30-40
40-50
50-60
60-70
WT
TKO
Control
Endophilin TKO
B
CT-HRP on ice
CT-HRP on 37°C
0'
0'
60'
60'
C
SVs/Synapse
total SV
HRP SV
180
160
140
120
100
80
60
40
20
0
control
TKO
control
TKO
HRP on ice 0'
HRP 37°C 60'
CCVs/Synapse
total CCV
HRP CCV
25
20
15
10
5
0
control
TKO
control
TKO
HRP on ice 0'
HRP 37°C 60'

Figure S3. Ultrastructural analysis of endophilin TKO synapses.

(A) Size of synaptic vesicles at WT and endophilin TKO synapses in neuronal cultures (DIV 18-24). More than 250 vesicles from 2 independent experiments were analyzed for each condition.

(B) and (C) Neuronal cultures from WT and TKO mice were briefly incubated on ice with horseradish peroxidase-conjugated cholera-toxin (CT-HRP) and then either fixed or further incubated in Tyrode's buffer at 37°C for 1 hr before fixation. (B) Representative EM micrographs of the samples. Note that in the control sample the vesicles are SVs, while in the TKO sample they are primarily CCVs. (C) Quantification of the changes in total SV and CCV numbers, and of the HRP labeled and unlabeled fractions of the vesicles ($*p<0.001$, t-test). Error bars represent S.E.M.

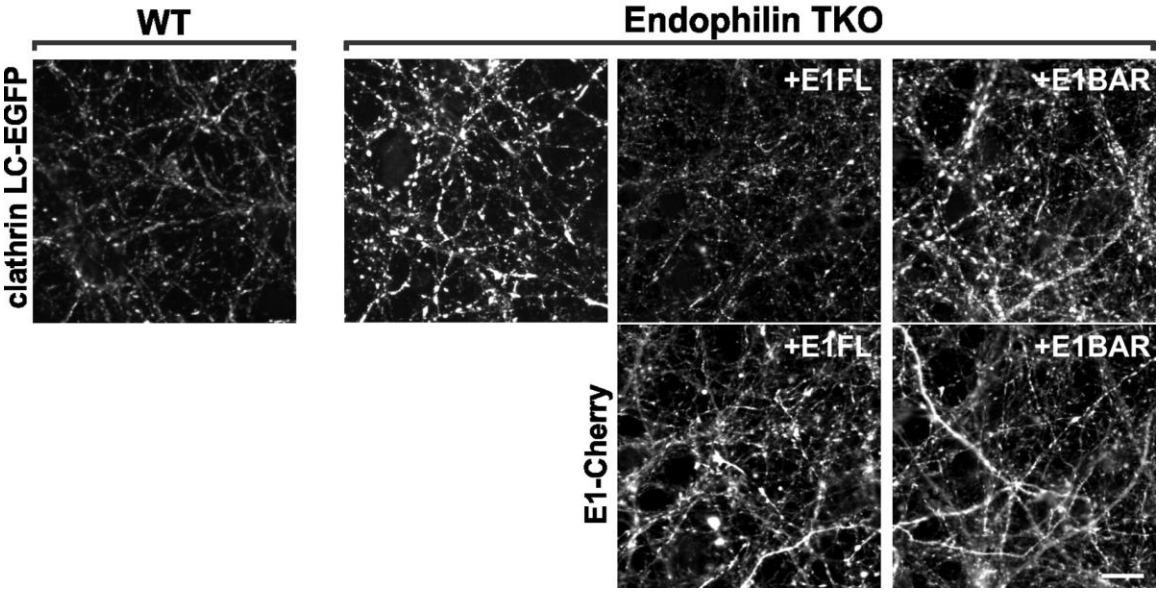

Figure S4. Expression of full-length endophilin 1, but not of its BAR domain, rescues the clustered localization of clathrin in endophilin TKO cultures. Fluorescence images of EGFP-clathrin LCa in cortical cultures (DIV 18-25) of WT mice, endophilin TKO mice, endophilin TKO mice expressing Cherry conjugated full length endophilin 1 (E1FL) and endophilin TKO mice expressing the Cherry conjugated endophilin 1 BAR construct (E1BAR). Note that the presence of large clusters of clathrin in endophilin TKO cultures could be rescued by expression of endophilin 1 full length, but not of its BAR domain. These types of images were used for the quantification of the clustered fluorescence in the four conditions as reported in Fig 6D. At least 10 images per condition from 6 independent experiments were analyzed.

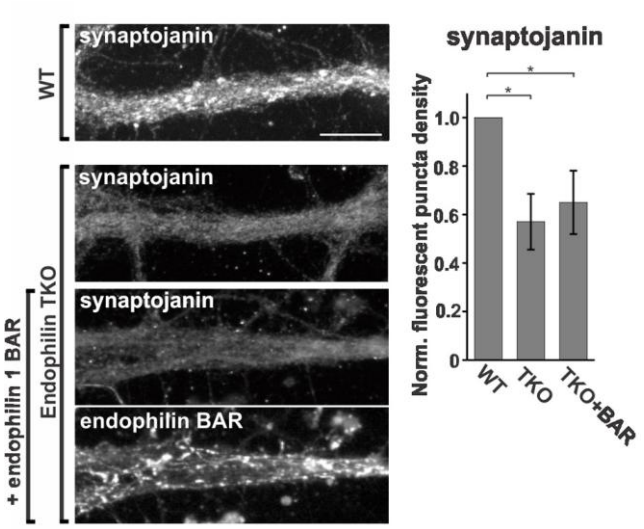

Figure S5. The altered localization of synaptojanin 1 in endophilin TKO neurons could not be rescued by overexpression of C-terminally Cherry-tagged endophilin BAR construct.

Representative images are shown at left. Quantification of the results (normalized to WT) are shown at right. At least 10 images per condition from 3 animals were analyzed, neuronal cultures were 17-22 days old. *$p < 0.05$, t-test, error bars represent S.E.M.

Table S1. LIST OF ANTIBODIES

endophilin 1	De Camilli Lab, Yale Univeristy, New Haven, CT
endophilin 2	De Camilli Lab, Yale Univeristy, New Haven, CT
endophilin 3	Santa Cruz, CA
pan-endophilin	De Camilli Lab, Yale Univeristy, New Haven, CT
actin	ICN Biomedicals, Aurora, OH
clathrin heavy chain (CHC)	Affinity Bioreagents, Rockford, IL
clathrin light chain (CLC)	Gift from R. Jahn, Max Planck Institute, Gottingen, Germany
α-adaptin	Affinity Bioreagents, Rockford, IL
AP-180	De Camilli Lab, Yale Univeristy, New Haven, CT
Dynamin I and pan	De Camilli Lab, Yale Univeristy, New Haven, CT
amphiphysin I	De Camilli Lab, Yale Univeristy, New Haven, CT
synapsin	De Camilli Lab, Yale Univeristy, New Haven, CT
synaptophysin	Chemicon, Temecula, CA
synaptotagmin 1	Gift from R. Jahn, Max Planck Institute, Gottingen, Germany
synaptobrevin 2	Synaptic Systems, Goettingen, Germany
Bassoon	Enzo Life Sciences, Plymouth Meeting, PA
rab3A	Gift from R. Jahn, Max Planck Institute, Gottingen, Germany
GAD65	De Camilli Lab, Yale Univeristy, New Haven, CT
syntaxin	De Camilli Lab, Yale Univeristy, New Haven, CT
synaptojanin	De Camilli Lab, Yale Univeristy, New Haven, CT
auxilin	De Camilli Lab, Yale Univeristy, New Haven, CT
Hsc70	Thermo Scientific, Rockford, IL
SNX9	De Camilli Lab, Yale Univeristy, New Haven, CT
pacsin	BD Biosciences - Transduction Lab, Franklin Lakes, NJ
vGLUT1	Synaptic Systems, Goettingen, Germany
vGLUT2	Chemicon, Temecula, CA
vGAT	Synaptic Systems, Goettingen, Germany
PSD-95	Millipore, Billerica, MA
γ-adaptin	BD Biosciences - Transduction Lab, Franklin Lakes, NJ

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Gene targeting strategies

The endophilin 1 (SH3GL2) gene was inactivated in 129Sv/J ES cells by deleting the 3' end of the first coding exon (see Fig S1A). The endophilin 2 (SH3GL1) gene inactivation was achieved by removing all coding exons following exon 1 (Fig S1A). ES cell procedures to generate chimeras were performed as described (Cremona et al., 1999). The endophilin 3 (SH3GL3) conditional KO targeting vector was made by inGenious Targeting Laboratory (Stony Brook, New York, USA) by flanking a 1.8kb region containing exon 1 with loxP sites and inserting an FRT site flanked neomycin cassette into intron 1 (Fig S1A). The targeting vector was electroporated into hybrid C57BL/6J-129S1/Sv mouse embryonic stem cells, which were then selected and screened by Southern blotting. Chimeric animals were mated to a FLPe recombinase deleter strain to remove the neomycin selection cassette (Rodriguez et al., 2000). The absence of the neomycin selection cassette in the FLPe-positive off-spring was confirmed by PCR. Conditional mutants were then crossed with a β-actin-Cre mouse (FVB/N-Tg(ACTB-cre)2Mrt/J; (Lewandoski and Martin, 1997) to disrupt the endophilin 3 gene ubiquitously, so that a constitutive endophilin 3 KO could be generated. The genotyping of ES cell clones and mice lines was performed by PCR.

Plasmids

A rat endophilin 2-Ruby construct was generated by replacing EGFP in pEGFP-endophilin 2 (Perera et al., 2006) with Ruby (Kredel et al., 2009) using BamHI and NotI restriction sites. A rat endophilin 2-BAR (1-247)-EGFP construct was generated by creating a PCR fragment encoding amino acids 1-247 with BglII and HindIII overhangs and then ligating it into the pEGFP-N1 vector (Clontech).

The hybrid CMV/chicken-β-actin (CAG) promoter (Niwa et al., 1991) was used to achieve reliable long term gene expression in our primary neuron culture experiments. For this purpose, an NdeI to NheI fragment of the promoter from the pCAGGS plasmid was transferred into corresponding sites in either pEGFP-N1 or pEGFP-C1 (Clontech) to modify the existing CMV promoter in these plasmids, thus generating pCAG-EGFP-N1 or pCAG-EGFP-C1.

An EGFP-clathrin LCa construct under control of the CAG promoter was generated in the same manner from the pEGFP-C1-clathrin LCa plasmid (a gift from James Keen, Thomas Jefferson University, Philadelphia, PA).

A pCherry-N1 vector was generated by replacing EGFP in pEGFP-N1 vector with mCherry. mCherry was first amplified by PCR with flanking AgeI and NotI sites, digested with these enzymes and then ligated into pEGFP-N1.

Rat endophilin 1 full length (FL)-Cherry and rat endophilin 1-BAR (1-290)-Cherry under the CAG promoters were generated in two steps. First, endophilin 1 FL or endophilin 1-BAR (1-290) flanked by BamHI and AgeI sites were generated by PCR, digested and ligated into BamHI/AgeI digested pCherry N1 vector. Second, the CMV promoters of these newly constructed plasmids were exchanged with the CAG promoters (from pCAG-EGFP-N1) using NdeI/XhoI restriction enzymes.

A rat endophilin 1 FL-mRFP under control of the CAG promoter was generated by exchanging the CMV promoter of endophilin 1 FL-mRFP vector (Perera et al., 2006) with the CAG promoter (from pCAG-EGFP-N1) using the NdeI/KpnI restriction sites. A rat endophilin 1 BAR (1-290)-mRFP under the CAG promoter construct was created by exchanging the CMV promoter of rat endophilin 1 BAR-mRFP with the CAG promoter (from pCAG-EGFP-N1) using the NdeI/EcoRI restriction sites.

A CAG promoter driven synaptopHluorin plasmid was created by cutting synaptopHluorin from the original vector (Miesenbock et al., 1998) with XhoI/NotI restriction enzymes and inserting it into pCAG-EGFP vector cut with the same enzymes.

All constructs have been verified by sequencing and restriction enzyme digestions. The proper localization of all constructs with the CAG promoter was verified in cultured fibroblasts before their use in neurons.

Endophilin knock-down experiments

RNAi-based knock-down of endophilin 2 in dynamin KO cells was achieved as described (Ferguson et al., 2009). siRNA sequences (Integrated DNA Technologies) were as follows, control: 5′-CUUCCUCUCUUUCUCUCCCUUGGA-3′ annealed to 5′-UCACAAGGGAGAGAAAGAGAGGAAGGA-3′, endophilin 2: 5′-GAGCACCUUGGCAUUAGACUGCUGCUG-3′ annealed to 5′-

GCAGCAGUCUAAUGCCAAGGUGCTC-3′. Dynamin DKO cells and dynamin DKO/endophilin 2 KD cells were transfected with EGFP-synaptojanin 1-145 and mRFP-clathrin LC, and imaged by spinning disk confocal microscopy. For the quantification of synaptojanin spots, 150 x 150 pixel regions of 8 transfected cells per condition were randomly selected, thresholded and automatically counted using ImageJ software (1.43u; National Institutes of Health, Bethesda, MD).

Electron microscopy and tomography

In experiments aimed at assessing the possible connection of clathrin-coated structures with the plasma membrane, neurons were incubated with 10μg/ml CT-HRP (Invitrogen) on ice, rinsed briefly and fixed immediately (no HRP uptake condition), or fixed after 60 min incubation at 37°C in Tyrode's buffer before processing of the peroxidase reaction.

For TEM tomography (Boulder Laboratory for 3D Electron Microscopy of Cells, University of Colorado) two orthogonal axes tilt series were recorded on a TECNAI F20 intermediate voltage electron microscope operating at 200 kV using the Serial-EM image acquisition program (Mastronarde et al, 2005). Images were captured at 1 degree intervals over a ± 60 degree range using a Gatan 2k x 2k CCD camera at a pixel size of 1 nm. STEM tomography (Italian Institute of Technology, Genova, Italy) was employed for the reconstruction of TKO synapses (figure 5 L) using a JEOL 2020 electron microscope operating at 200 kV with a spot size of 0.7 nm and the smallest condenser aperture resulting in collection angle of 3 mrad. The sample (300 nm thick sections) was mounted on a Fischione Ultra-Narrow Gap Tomography Holder and tilted over a ± 60 degree range according to a "Saxton scheme". Images were acquired using a HAADF detector at a magnification of 200.000X resulting in a pixel resolution of 1.58 nm. Tomograms reconstruction, segmentation and visualization was done using the IMOD software package (Mastronarde, 2005).

SUPPLEMENTAL REFERENCES

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