Supplementary Information

Vesicle uncoating regulated by SH3-SH3 domain-mediated complex formation between endophilin and intersectin at synapses

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Supplementary methods

Antibodies.

Polyclonal anti-intersectin 1 antibodies were raised in rabbits using His_6-human intersectin 1 (amino acids 1–440) as an antigen. The antibody was affinity-purified on a Hi-Trap column (GE Healthcare) with the antigen covalently coupled. For immunoprecipitations, these antibodies targeting intersectin 1 or rabbit polyclonal anti-endophilin A1 antibodies (Synaptic Systems) were used.

For immunoblotting, the following antibodies were used: mouse monoclonal antibodies against dynamin 1–DG-1 (1), 1:20.000; clathrin–CHC TD-1, 1:1000; synaptojanin 1 (a kind gift of Pietro DeCamilli, Yale University School of Medicine), 1:1000; endophilin A1 (clone D-3, Santa Cruz Biotechnology), 1:1000; transferrin receptor (Zymed), 1:2000; and beta-actin (Sigma), 1:1000; rabbit polyclonal antibodies against GST (Mutsu), 1:1000; endophilin A1/A3 (Nuts; a kind gift of Pietro DeCamilli, Yale University School of Medicine), 1:1000; and intersectin 1, 1:500. HRP-coupled goat anti mouse and goat anti rabbit antibodies were used as secondary antibodies, 1:10.000. For immunofluorescence stainings, we used mouse monoclonal antibodies against AP-2 α-adaptin (clone AP6), 1:200; and rabbit polyclonal antibodies against endophilin A1

(Synaptic Systems), 1:600. Goat anti mouse Alexa488-coupled and goat anti rabbit Alexa568-coupled secondary antibodies were used (Jackson ImmunoResearch Laboratories), 1:200.

Plasmids.

cDNA encoding human HA-intersectin 1L was kindly provided by Dr. Y. Groemping (Max-Planck-Institute for Developmental Biology, Tübingen, Germany). The sequences encoding the human intersectin 1 SH3A (aa 738–806), SH3B (aa 914-970), SH3C (aa 1000-1066), SH3D (aa 1070-1147), and SH3E (aa 1152-1214) domains were amplified and subcloned into pGEX4T-1 (Amersham Biosciences) or pET28a. Recombinant rat endophilin A1 FL or SH3 (aa 258-352) were cloned in pGEX4T-1 (Amersham Biosciences) or pET28a. All GST-fusion proteins and His_6-tagged proteins were expressed in *E. coli* and purified using GST-Bind resin (Novagen) or His-Select Nickel Affinity Gel (Sigma), respectively, according to the manufacturer's instructions.

Primers and site-directed mutagenesis.

The intersectin-1 SH3B or rat endophilin A1 point mutants were generated by PCR and confirmed by DNA sequencing. The following primers (5'->3'; mutant triplets underlined) were used:

Intersectin 1 SH3B (W949E, Y956E) (aa 914-970), forward: GATCGAATTCGAGGGGCTACAAGCTCAAGCCCTATATCC; reverse: GATCCTCGAGTCATGAAATGAGTTTCACCTCAGACTTGGGGAACCAACCCTTCT GACCTTGAACTTCTCCAAACCACTCC.

Endophilin A1 (E329K, S366K), forward: GATCGAATTCATGTCGGTGGCAGGGCTGAAGAAGC; reverse:

GCATCTCGAGCTAATGGGGCAGAGCAACCAGAATTTCTACATAGTTGATGGGG AAAAAGCCCTTCTGGCCATGAAGCATCCCCTT.

The endophilin A1 (PxxEY->AxxKA) point mutant was generated by overlap extension PCR and confirmed by DNA sequencing. The following primers were used: terminal-forward: GATCGAATTCATGTCGGTGGCAGGGCTGAAGAAGC; terminal reverse: GCATCTCGAGCTAATGGGGCAGAGCAACCAG; internal forward: GACAAGCTTCATCTCAAGCAAGAAGGAAAGCTC; internal reverse: GAGCTTTCCTTCTTGCTTGAGATGAAGCTTGTC.

Lamprey microinjection & electron microscopy analysis.

Dissection of the trunk region of the lamprey spinal cord, microinjection into giant reticulospinal axons, stimulation and fixation were performed as described previously (1). Giant axons stimulated at 5 Hz using extracellular electrodes. Microinjections of Alexa488-labeled reagents were monitored by a CCD camera (Princeton Instruments). Spinal cords were fixed during stimulation in 3% glutaraldehyde/0.5%paraformaldehyde/4% tannic acid in 0.1 M cacodylate buffer, pH 7.4 (1h) followed by the same fixative without tannic acid for an additional 3 h. After postfixation in 1% OsO4 for 1 h and dehydration in alcohol the specimens were embedded in Durcupan ACM resin (Fluka). Serial ultrathin sections were cut with a diamond knife and viewed in a Tecnai 12 electron microscope (FEI).

Effects of microinjections were analyzed in 15 synapses cut in serial sections 200-300 μm from injection sites. All described effects were reproduced in synapses from at least three microinjected axons. The number of synaptic vesicles in clusters, clathrin-coated pits and vesicles were normalized to the length of the active zone measured in middle sections. Statistical evaluation of the data was performed using Prism 6 software.

Electron microscopy analysis of mouse spinal cords

WT (N=3) or intersectin 1 KO mice (N=3) were anesthetized and perfused transcardially with 0.1 M DPBS (Gobco), pH 7.4 followed by 3% glutaraldehyde in 0.1 M DPBS. Spinal cords were dissected and postfixed in the same fixative overnight. Segments L4 and L5 of the spinal cord were cut into 100 µm frontal sections. Sections were postfixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in Durcupan ACM resin (Fluka). Serial sections were cut with a diamond knife (Diatome), collected onto formvar-coated slot grids, counterstained in uranyl acetate and lead citrate, and viewed in a Tecnai 12 electron microscope (FEI).

Fractionation

HEK cells were transfected with full-length wild-type (WT) or mutant (E329K, S336K) endophilinA1-mRFP using calcium phosphate transfection according to standard protocols. 24 h after transfection cells were harvested in buffer A (20 mM HEPES buffer, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$) and lysed using a cell cracker and three cycles of freezing-thawing in liquid nitrogen. Debris was removed by centrifugation at 1000xg for 5 min at 4°C. The supernatant was centrifuged again at 100000xg for 30 min at 4°C. The protein concentration of the high-speed supernatant representing the soluble cytosolic fraction was measured using a Bradford assay before sample buffer was added. The pellet representing the membrane fraction was resuspended in sample buffer to the same volume as the cytosolic fraction. Equal volumes of both fractions were analyzed by SDS/PAGE and immunoblotting.

Immunostaining of hippocampal neurons

Hippocampal neurons from neonatal mice brains (p1–p4) of wild-type (WT) and intersectin 1 knock-out (KO) mice were prepared according to published protocols (ref. 19; Kononenko et al., 2014). Neurons were fixed on DIV (days in vitro) 14 in 4% paraformaldehyde for 15 min at room temperature, then washed and incubated in blocking buffer (10% goat serum in PBS containing 0.1 % Triton-X100) for 30 min at room temperature. Fixed neurons were incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. Following 3x washing with PBS neurons were incubated with secondary antibodies diluted in blocking buffer for 1 h at room temperature. Samples were washed three times with PBS before mounting with Immu-Mount.

Confocal microscopy analysis of primary cortical neurons expressing clathrin and WT or mutant endophilin A1 in culture.

Primary cultures of cortical neurons were prepared from P0 brains as previously described (Ferguson et al., 2007), plated at a density of 125,000 cells/well in 12-well plate and maintained at 37°C, 5% CO_2 in neurobasal medium enriched with B27 supplement, 0.5 mM L-glutamine and penicillin/streptomycin (all media components were purchased from Invitrogen). Before plating, neurons were transfected with EGFP-clathrin light chain and WT endophilin A1-mRFP (described in 14) or mutant endophilin A1-mRFP (described above) using Amaxa system (Lonza, Basel, Switzerland). All genes were under the control of the chicken-β-actin promoter to allow for long-term and even expression. Neurons were fixed after 14-18 days-*in-vitro* with 4% paraformaldehyde/2% sucrose in phosphate buffered saline (PBS).

Samples were imaged by a Nikon/Perkin Elmer Ultraview spinning disk confocal setup equipped with 40x and 60x CFI PlanApo objectives. Images of randomly selected endophilin A1-3 TKO and control neurons were acquired with a Hamamatsu ORCA II

digital camera under the same parameters. The images were processed with MetaMorph software version 7.2 (Molecular Devices) using the application Count Nuclei that detects fluorescent puncta over a diffuse background. For each out of four experiments, at least 15 images were analyzed from a single culture from the respective control and endophilin TKO genotypes. Counted puncta were expressed as the number of puncta per 100 μm^2 and then normalized for the value of control samples.

HEK293 cell culture.

HEK293 cells were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % glutamine and 1 % penicillin/ streptomycin and cultured at 37 °C, 5 % CO2. Cells were regularly tested to ascertain that they were free of mycoplasm contamination.

Protein expression and purification.

Proteins were expressed in 2xYT medium over night at 18°C after induction at OD_{600} 0.5-0.7 with 1 mM IPTG. Cells were then harvested by centrifugation at 6000xg and 4°C for 10 min and lysed by ultrasonic treatment. Bacterial debris was removed by centrifugation at 15000xg for 20 min at 4°C, and the supernatant was filtered (0.2 µm pore size). Proteins were then purified using IMAC or GST affinity chromatography (BioRad Profinia Affinity Chromatography Protein Purification System) and subsequent gel filtration on a superdex 75 16/60 column (GE healthcare) buffered in PBS or TBS pH7.4. Eventually, affinity tags were removed before gel filtration by incubation with 1U thrombin per mg of protein for 1 h at 37°C.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry experiments (n=2) were performed at 298.15 K using a MicroCal VP-ITC system (GE Healthcare). Proteins were dialysed against TBS pH 7.4 supplemented with 1 mM β-mercaptoethanol and degassed shortly before the titration experiment. Intersectin 1-SH3B in the measurement cell (2 ml, 76 µM) was titrated against 760 µM endophilin A1-SH3 in the syringe. Peak areas were integrated and the heat of reaction was plotted against the molar ratio and fitted with a "One Set of Sites" model. For fitting, the heat of dilution was subtracted until X^2 was minimal.

Expression of isotope labeled proteins

For NMR experiments proteins were expressed in M9 minimal medium containing 750 mg/L of ^{15}N Ammonium Chloride (Cambridge Isotope Laboratories) and eventually 2 mg/L of ^{13}C D-Glucose (CAMPRO Scientific) for uniform isotope labeling. 1 L of this defined medium was inoculated with a LB preculture to an OD_{600} of 0.1 and grown to an OD_{600} of 0.5-0.7 in shaking culture at 37°C and 180 rpm. After 15 min on ice, protein expression was induced by supplementation with 1mM IPTG and carried out over night at 18°C.

^{15}N-HSQC NMR experiments

Protein samples were prepared in PBS pH 7.4 + 10% (v/v) D2O. Two-dimensional ^{15}N-HSQC spectra were recorded on a Bruker Ultrashield 700 Plus (700 MHzspectrometer) equipped with 5 mm triple-resonance cryoprobes. Measurements of ^{15}N-labeled Intersectin 1-SH3B in the absence or presence of non-labeled endophilin A1-SH3 were performed at 298 K at protein concentrations of 100-250 µM. 1024x128 complex data points were acquired with 8 scans in each HSQC experiment. In binding experiments, labeled proteins were titrated against increasing concentrations of the unlabeled binding partner (20-650

μM for SH3 domains or 20-1250 μM of a vesicular glutamate receptor 1 (VGLUT1) derived peptide 551-PRPPPPVRDY-560. To determine the SH3 binding epitopes, chemical shift changes at ligand saturation (6.5-fold excess of protein or 5-fold excess of peptide) were calculated by applying equation 1. Signals that showed changes above a threshold set by the average shift change plus the standard deviation or that disappeared by line broadening after ligand addition were included in the epitope.

$$\Delta\delta = \sqrt{(\Delta\delta\ ^{1}H)^2 + (\Delta\delta^{15}N \times 0.15)^2} \qquad \text{Equation 1}$$

Assignment of intersectin 1-SH3B and endophilin A1-SH3

Backbone assignment of the intersectin 1-SH3B and endophilin A1-SH3 were accomplished applying a series of triple resonance spectra (HNCA, HN(CO)CA, HNCACB and HN(CO)CACB). The spectra were recorded on a Bruker DRX 600 MHz spectrometer equipped with 5 mm triple-resonance cryoprobes at 298 K. ^{15}N, ^{13}C labeled proteins were applied at concentrations of 610 μM (endophilin A1-SH3) and 480 μM (intersectin 1-SH3B) in PBS pH 7.4 + 10% (v/v) D2O + 0.01% NaN_3. 1024x96x96 complex data points were acquired with 8 (HNCA, HN(CO)CA) or 16 scans (HNCACB, HN(CO)CACB) applying bandselective excitation short transient (BEST) sequences (2). Assignments of the HSQC signals of the SH3 domains via sequential connections are obtained using CCcpNmr Analysis software (3). The sequence of the SH3 constructs was assigned to 71.2% (intersectin 1) and 83.3% (endophilin A1) with exception of prolines.

Affinity chromatography and immunoprecipitations

For immunoprecipitation experiments, antibodies were coupled to protein A/G PLUS Agarose (Santa Cruz Biotechnology) and incubated with 4 mg rat P2' or lamprey brain

Triton X-100 extract in buffer A (20 mM HEPES buffer, pH 7.4, containing 50 mM KCl, 2 mM $MgCl_2$, and 1% Triton X-100) plus protease inhibitors (Sigma) in a total volume of 1 mL for 2 h at 4 °C. Following extensive washes, samples were eluted with sample buffer and analyzed by SDS/PAGE and immunoblotting. For affinity chromatography or direct binding experiments, 100 or 50 µg GST-fusion proteins were coupled to GST-Bind resin (Novagen) and incubated with 2 mg rat brain extract or 10 µg His_6-tagged recombinant protein, respectively, in a total volume of 1 ml for 1 h at 4 °C on a rotating wheel. Following extensive washes, samples were eluted with sample buffer and analyzed by SDS/PAGE and immunoblotting or direct Coomassie staining.

Statistics.

For pairwise comparisons unpaired two-sided t-tests were used to assess statistical significance. Multiple comparisons (Fig. S2) were tested for statistical significance with a one-way ANOVA followed by pairwise comparisons with a Tukey's test. In both cases we assessed that data were normally distributed by the F-test. All statistical analysis was done using GraphPad Prism software.

Samples were allocated blindly to treatments and were analyzed blind. No samples were excluded from analysis.

Animal models and handling.

Intersectin 1 knockout mice (mus musculus; 129SV/J) are published (ref. 21) and were kindly provided by the lab of Dr. M.A. Pritchard. A colony was established at the specific pathogen free animal facility of the Max-Delbrück-Center where the animals were housed in isolated ventilated cages in mixed-genotype groups of up to six adult animals with bedding, nesting material, a hiding place and food and water ad libitum in a controlled

environment (21±1°C; 40-60% humidity; lights off from 6:00 pm to 6:00 am). Heterozygote animals were interbred to obtain WT and KO littermates for experiments. Endophilin A1-3 triple knockout mice (mus musculus) are published (ref. 14). Endophilin A1-3 triple KO mice were bred by the lab of Ira Milosevic under the same conditions indicated for intersectin 1 KO mice. For experiments both genders were used indiscriminately. Electron microscopy was performed on adult animals, while neurons were generated from p1-p4 pups.

Perfusions were performed on adult mice according to German regulations and under a permit obtained by the Berlin authorities (LaGeSo).

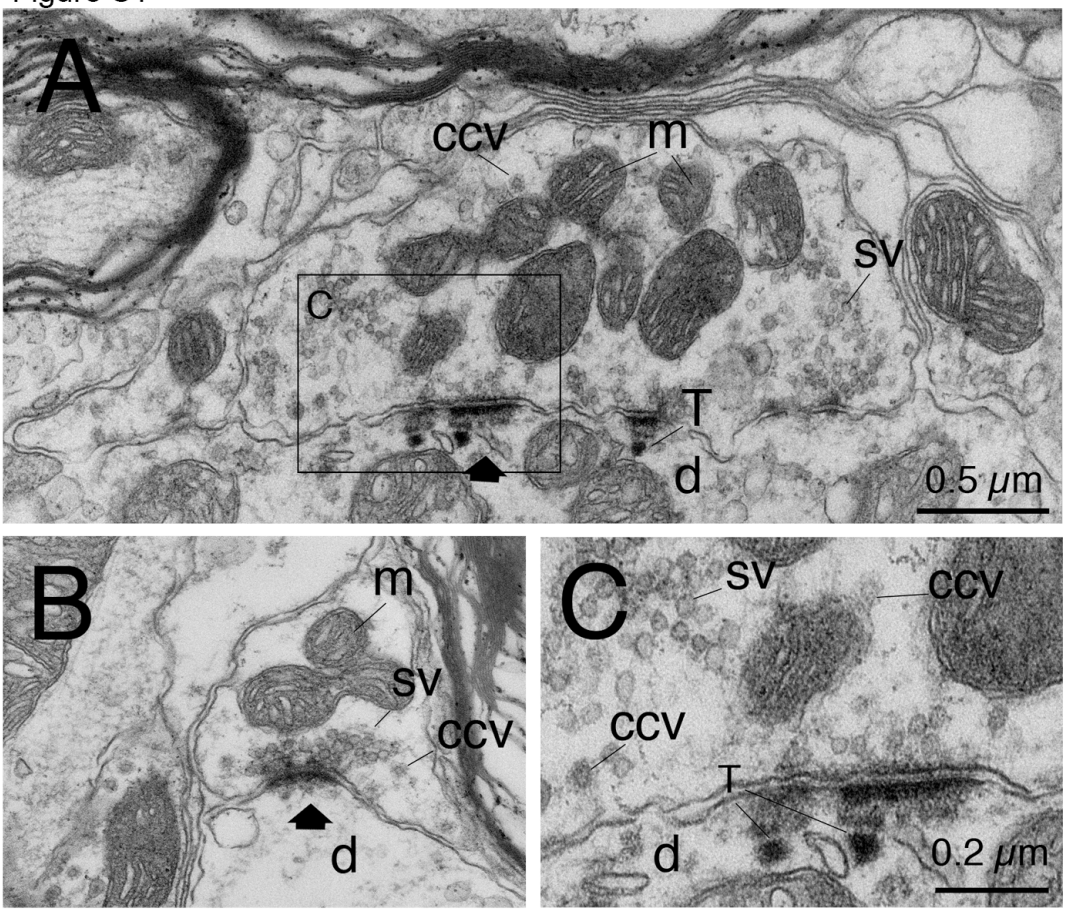
We confirm our compliance with the guidelines on animal use and reporting of animal studies.

Supplementary references

1. Gad H*, et al.* (2000) Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. *Neuron* 27(2):301-312.
2. Lescop E, Schanda P, & Brutscher B (2007) A set of BEST triple-resonance experiments for time-optimized protein resonance assignment. *J Magn Reson* 187(1):163-169.
3. Vranken WF*, et al.* (2005) The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* 59(4):687-696.

Supplementary figures

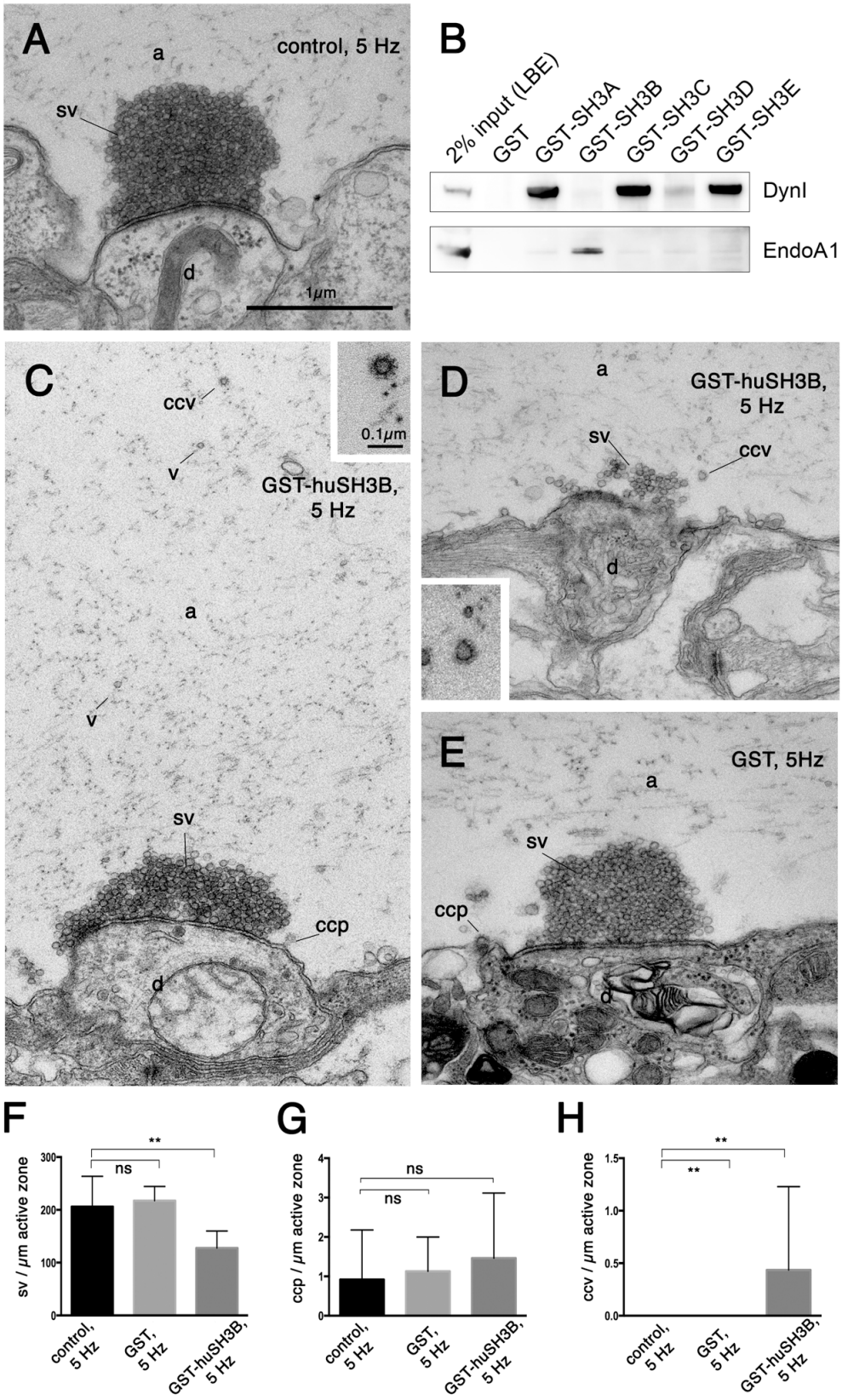
Figure S1



Supplementary figure S1: **Normal synaptic organization of M- and S-type boutons from lamina IX of intersectin 1 knockout (KO) mice.**

(A) Electron micrograph of an M-type bouton. T- designates Taxi bodies, characteristic to this population of synapses. **(B)** Electron micrograph of an S-type bouton. **(C)** shows the area marked by the rectangle in A at higher magnification. SV- synaptic vesicles; d- dendritic shafts; m- mitochondrion; thick arrows indicate active zones; CCV- clathrin-coated vesicle. Scale bars: A, B: 0.5 μm; C: 0.2 μm.

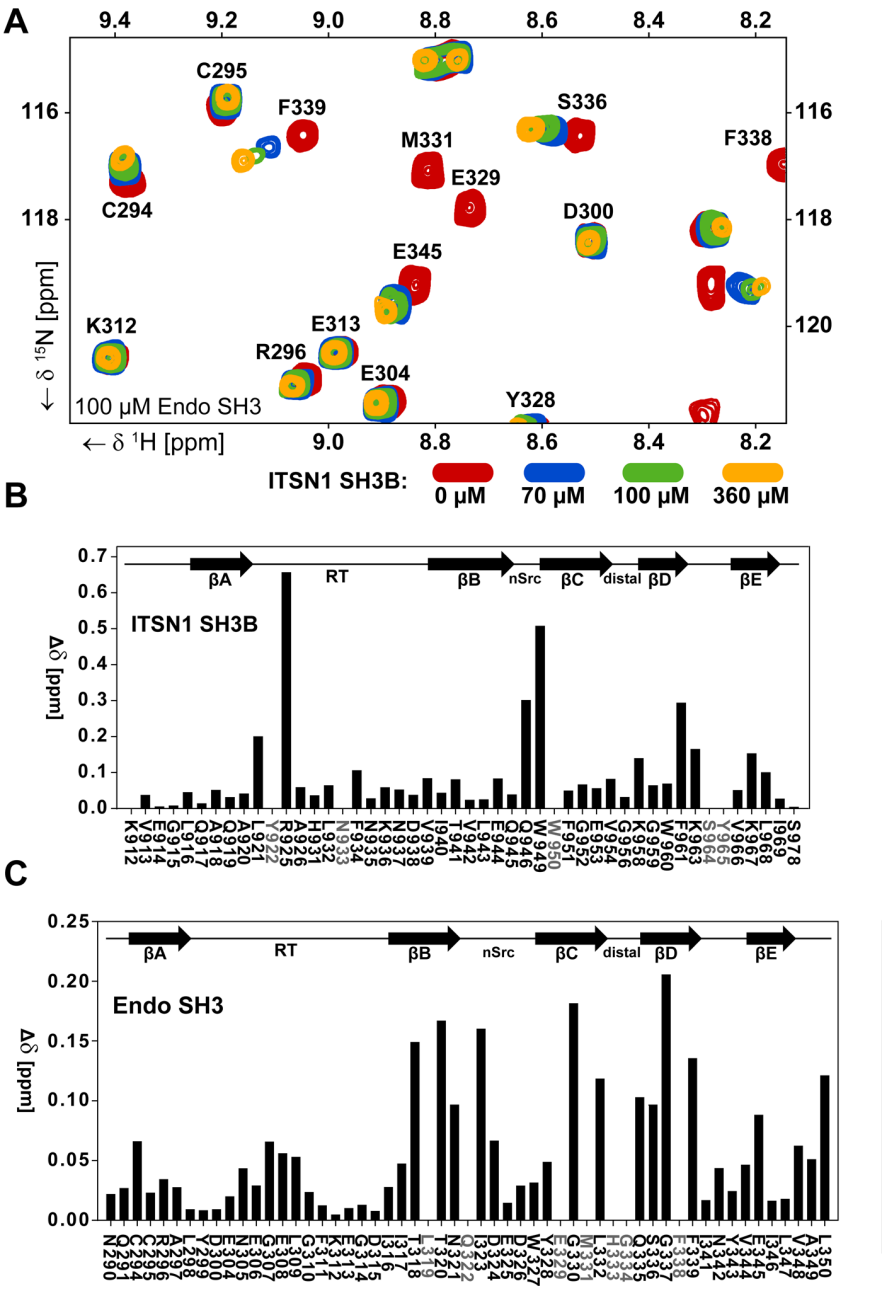
Figure S2



Supplementary figure S2: **Acute perturbation of intersectin-endophilin complex formation in the lamprey giant synapse.**

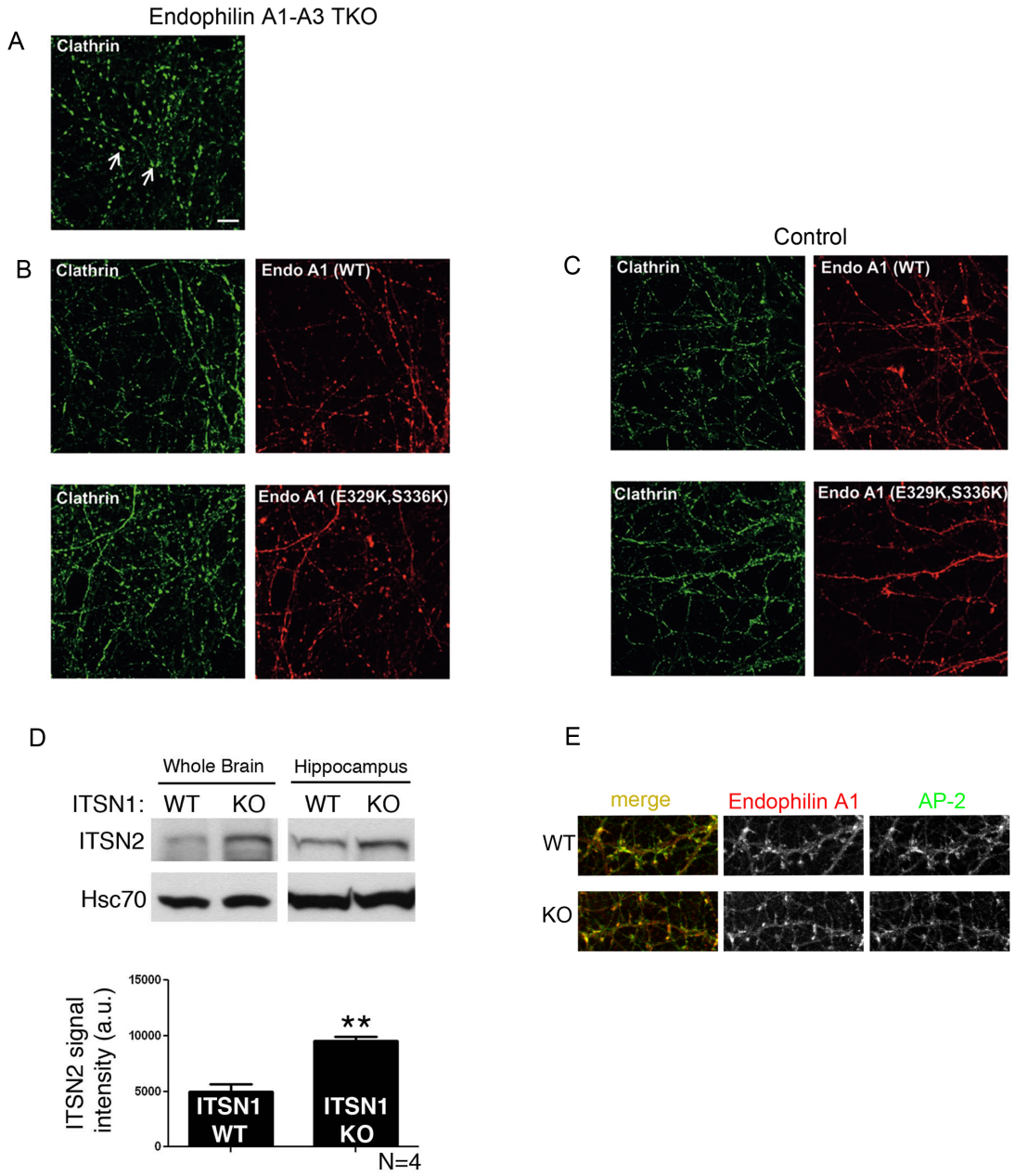
(A) Electron microscopic image of a non-injected reticulospinal synapse stimulated at 5 Hz for 30 min. **(B)** Lamprey endophilin A1 associates with lamprey intersectin 1-SH3B. GST or GST-fused intersectin 1-derived SH3 domain fusion proteins were immobilized on beads and incubated with detergent-lysed lamprey brain extract (LBE). Samples were analyzed by SDS-PAGE and immunoblotting for endophilin A1 (EndoA1) or dynamin 1 (DynI). 2% input, 2% of the total amount of lamprey brain extract (LBE) added to the assay. **(C-E)** Electron micrographs of synapses microinjected with GST-huSH3B domain (C, D) or GST taken as a control and stimulated at 5 Hz for 30 min. **(F-H)** Bar graphs illustrating the changes in number of SVs, CCPs and CCVs in synapses microinjected with huSH3B domain as compared to controls. Data are represented as mean±SD (**$p<0.01$, using ANOVA, Tukey´s post test). Scale bars: A, C-E, 1 μm; insets in C and D, 0.1 μm.

Figure S3



Supplementary figure S3: Structural basis for endophilin A1-SH3 association with intersectin 1-SH3B. (A) ^{15}N-HSQC titration experiment. Shown is an excerpt from a series of spectra recorded from 100 µM ^{15}N-labeled intersectin 1-SH3B titrated with increasing amounts of unlabeled endophilin A1-SH3. **(B)** Chemical shift changes of ^{15}N-labeled intersectin 1-SH3B upon 6.5-fold supplementation with endophilin A1 plotted for each assigned residue along the sequence. Signals that disappear upon ligand addition due to line broadening are highlighted in grey. **(C)** Corresponding chemical shift changes of ^{15}N-labeled endophilin A1-SH3 upon addition of 6.5-fold excess of intersectin 1-SH3B.

Figure S4



Supplementary figure S4: **Endophilin A1 binding to intersectin 1 regulates clathrin uncoating.**

(A-C) Exemplary fluorescence images of EGFP-clathrin light chain in cortical neuronal cultures (DIV 14-22) of **(A)** endophilin A1-3 TKO neurons, **(B)** endophilin A1-3 TKO neurons expressing wild-type (WT) or mutant (E329K,S336K) endophilin A1-mRFP, or of

(C) wild-type (WT) neurons expressing wild-type (WT) or mutant (E329K,S336K) endophilin A1-mRFP. Note that the majority of clathrin is diffusely localized in wild-type, but clustered in endophilin A1-3 TKO neurons (arrows). The presence of large clusters of clathrin in endophilin A1-3 TKO cultures was fully rescued by expression of wild-type endophilin A1-mRFP, but only to a comparably minor degree by mutant (E329K,S336K) endophilin. 15 images per condition from 4 independent experiments were analyzed for the data shown in figure 4E. Scale bar, 10 μm. **(D)** Upregulation of intersectin 2 (ITSN2) expression in the brain of intersectin 1 (ITSN1) knockout (KO) mice. Top, representative immunoblots of total brain or hippocampal lysates from wild-type (WT) or ITSN1 KO mice decorated with antibodies specific for ITSN2 or Hsc70 used as a loading control. Bottom, quantification of ITSN2 expression levels in WT and ITSN1 KO total brain and hippocampal lysates from immunoblots after normalization to loading control (N=4). Data are shown as mean +/- SEM. Statistical significance was evaluated with a two-tailed unpaired t-test (p=0.0012). **(E)** Exemplary fluorescence images of hippocampal neurons derived from wild-type (WT) or intersectin 1 knockout (KO) mice. Neurons (DIV14) were immunostained for endogenous endophilin A1 (red) and AP-2 (α) (green) and analyzed by confocal microscopy. While there is no overt difference in localization of either protein Pearson's correlation analysis reveals reduced co-localization of endophilin A1 with AP-2 in KO neurons (see Fig. 4G).