Supplemental Information

Capture of Dense Core Vesicles at Synapses

by JNK-Dependent Phosphorylation

of Synaptotagmin-4

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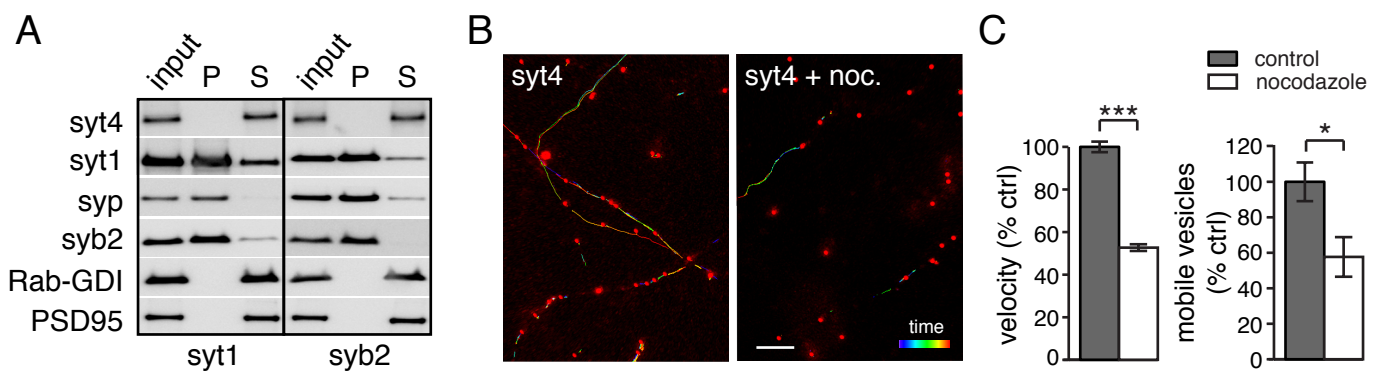
Figure S1. Syt4 is not on synaptic vesicles, and Syt4 vesicles are highly mobile on microtubules. Related to Figure 1.

(A) Western blots of immuno-organelle isolation of SVs with anti-Syt1 (left) or anti-Syb2 (right) antibodies, from brain homogenate. Input, pellet (P) containing bound proteins, and supernatant (S) containing unbound proteins, are indicated. (B) Images of mCherry-Syt4 vesicle trafficking for 5 min. in control conditions (left panel) and after nocodazole treatment (right panel); vesicle movement is indicated by color-coded tracks over time, and vesicles are indicated in red. Scale bar = 10 μm. (C) Quantitation of mCherry-Syt4 vesicle velocity and mobile vesicle % in control and nocodazole treated conditions, indicating that vesicle mobility is microtubule dependent (n= 4950 vesicles/13 videos and 4766 vesicles/13 videos for control and nocodazole-treated conditions, respectively, from 3 different cultures each; significance was determined by Student's t-test with Bonferroni correction; error = s.e.m.; * $p < 0.05$ and *** $p < 0.001$).
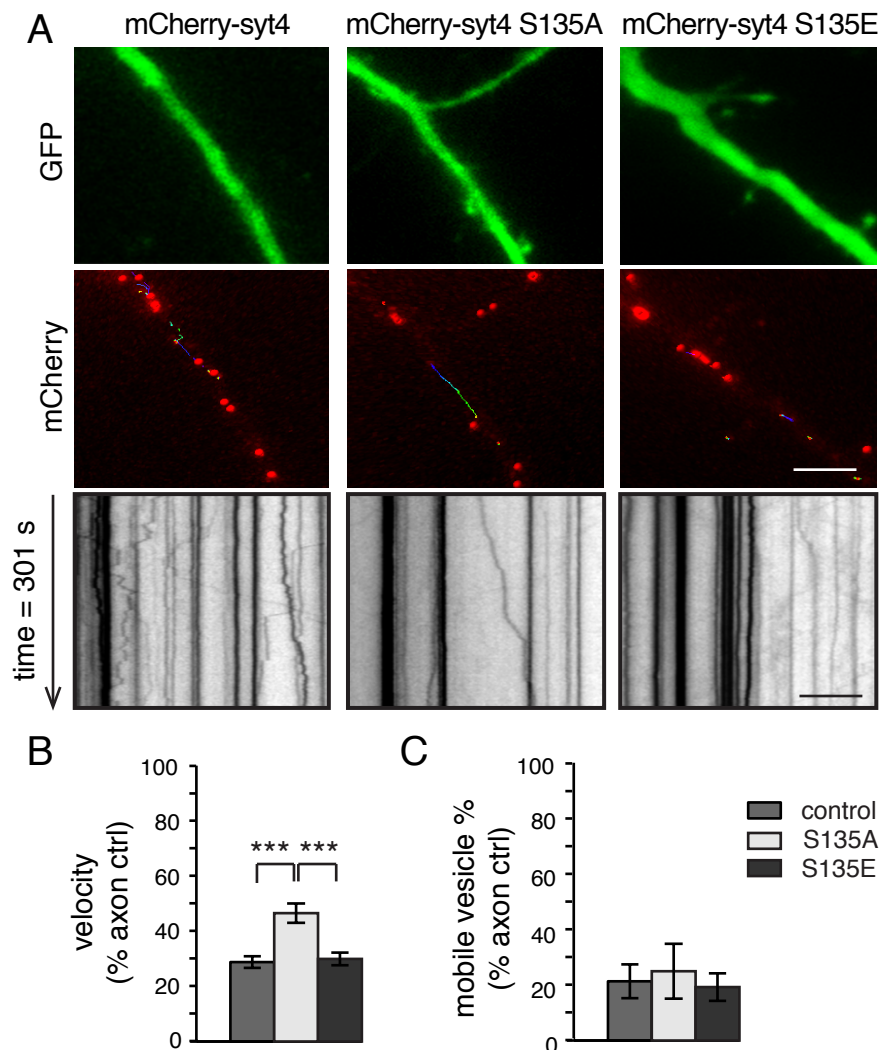
Figure S2. Syt4 vesicles are less mobile in dendrites than in axons. Related to Figure 2.

(A) Images of dendritic regions marked with EGFP (top panels) in neurons co-transfected with mCherry-Syt4, mCherry-Syt4 S135A, or mCherry-Syt4 S135E and imaged for 5 min. for vesicle trafficking indicated by color-coded mobile vesicle tracks (middle panels) and in kymographs (bottom panels). (B) Quantitation of average velocity, and mobile vesicle percentage (C) of control, S135A and S135E Syt4-harboring vesicles in dendrites, normalized to wild-type axons (100%), for comparison (n = 1319, 1489 and 1088 vesicles for average speed and velocity, and n = 13, 13 and 14 videos for mobile percentage of control, S135A and S135E vesicles, respectively, from 4 different cultures). Scale bars = 5 μm in all panels. Significance was determined by Student's t-test with Bonferroni correction; error indicates s.e.m.; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.
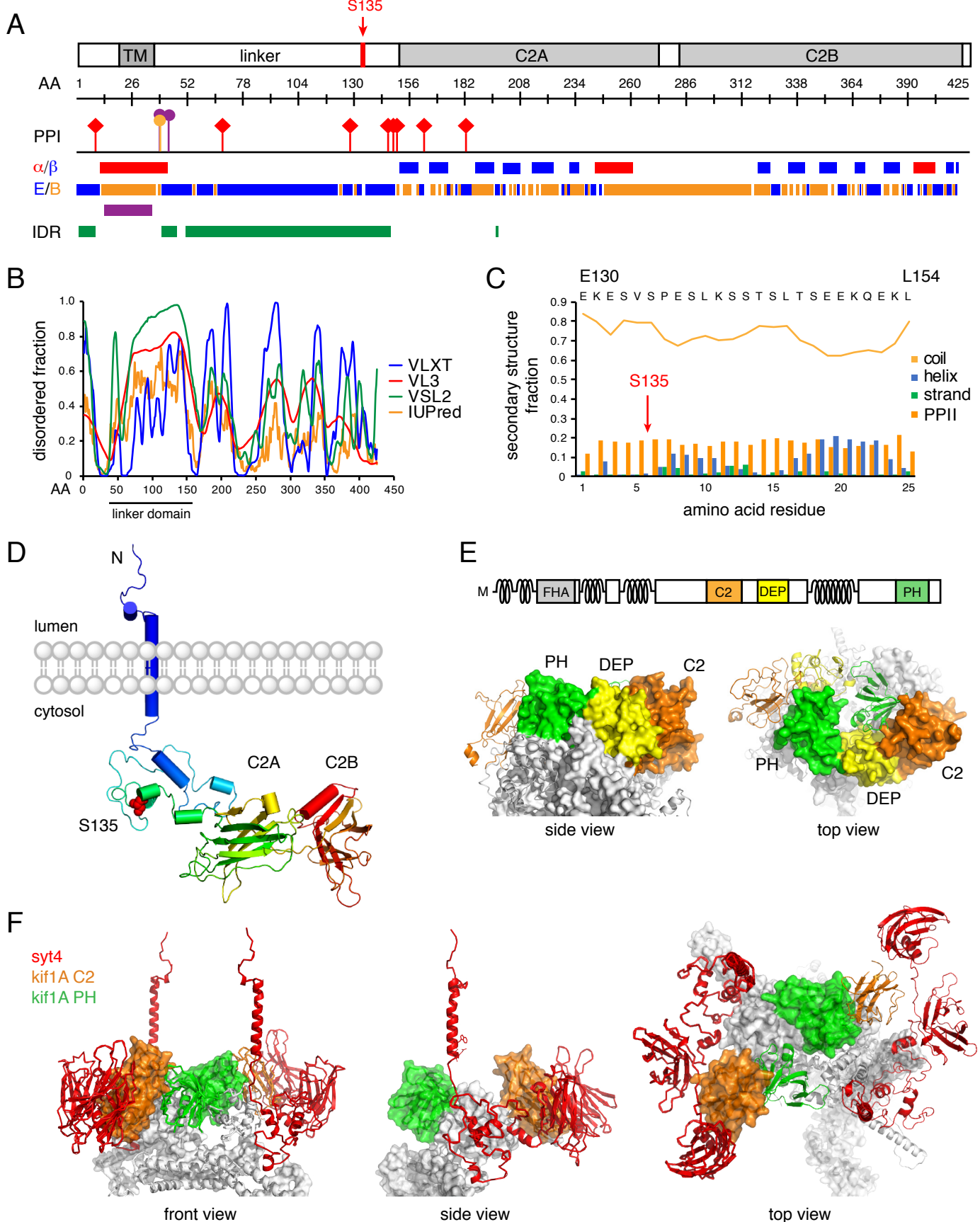
Figure S3. Domain organization of Syt4 and S135 phosphorylation-dependent interaction with Kif1A. Related to Figure 3.

(A) The 425 amino acid Syt4 protein is anchored in the membrane by a helical transmembrane region (alpha, red), followed by a 114 residue linker and two C2 domains (C2A, C2B), which contain buried (B, orange) beta-sandwich structure (beta, blue). The linker between the transmembrane and C2 domains is predicted to be exposed (E, blue) and corresponds to an intrinsically disordered region (IDR, green). Potential protein-protein interaction (PPI) sites are marked. (B) Residue-specific disorder propensity scores of Syt4 calculated by PONDR-FIT (VLXT, VL3, VSL2) and IUPred. (C) Secondary structure propensities of Syt4 residues E130-L154 according to s2d (Sormanni et al., 2015). Helical propensities are shown in blue. Residues E130-L154 have a propensity for poly-proline II structure; S135-K140 and L145-K153 have a propensity for alpha-helical structure. (D) Predicted structure of the compact conformation of Syt4. (E) Schematic of Kif1A (top); the location of the motor domain (M) and C2, DEP (dishevelled, EGL-10, pleckstrin), and PH (pleckstrin homology) domains are indicated. Side view and top view of the predicted structure of residues 849-1690 of the C-terminal membrane proximal region of Kif1A, including the C2, DEP, coiled-coil linker and PH domains (lower panels). Surface rendered structures belong to one monomer of the KIF1A dimer. (F) Predicted structural complex of Syt4 and Kif1A as viewed from the front, side and top.

A LifeAct-RFP Bsn-GFP merge

B mCherry-syt4 / PSD95-GFP

control

S135A
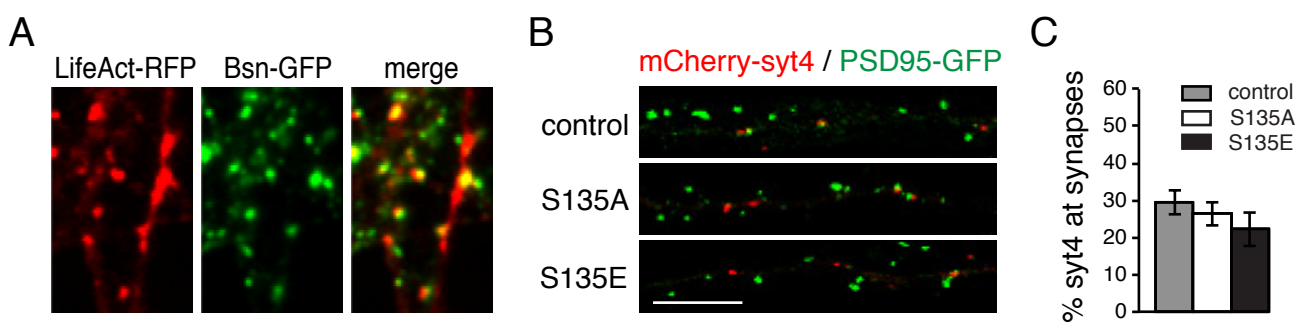
S135E

C

% syt4 at synapses

control
S135A
S135E

Figure S4. Syt4 phosphomutants show no change in localization to post-synaptic sites. Related to Figure 4.

(A) Image of axons of a hippocampal neuron transfected with LifeAct-RFP and Bassoon-GFP showing concentrated actin at presynaptic sites. (B) Cropped dendritic regions of hippocampal neurons co-transfected with PSD95-GFP and mCherry-tagged Syt4, Syt4 S135A or Syt4 S135E. (C) Quantification of percent of Syt4 colocalizing with PSD95-GFP, showing no difference in co-localization of PSD95-GFP with mCherry-Syt4, mCherry-Syt4 S135A or mCherry-Syt4 S135E (n= 23, 19 and 20 cells for PSD95-GFP co-transfected with mCherry-Syt4, mCherry-Syt4 S135A and mCherry-Syt4 S135E, respectively from 3 different cultures). Scale bar = 5 μm. Significance was determined by Student's t-test with Bonferroni correction; error indicates s.e.m.; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.
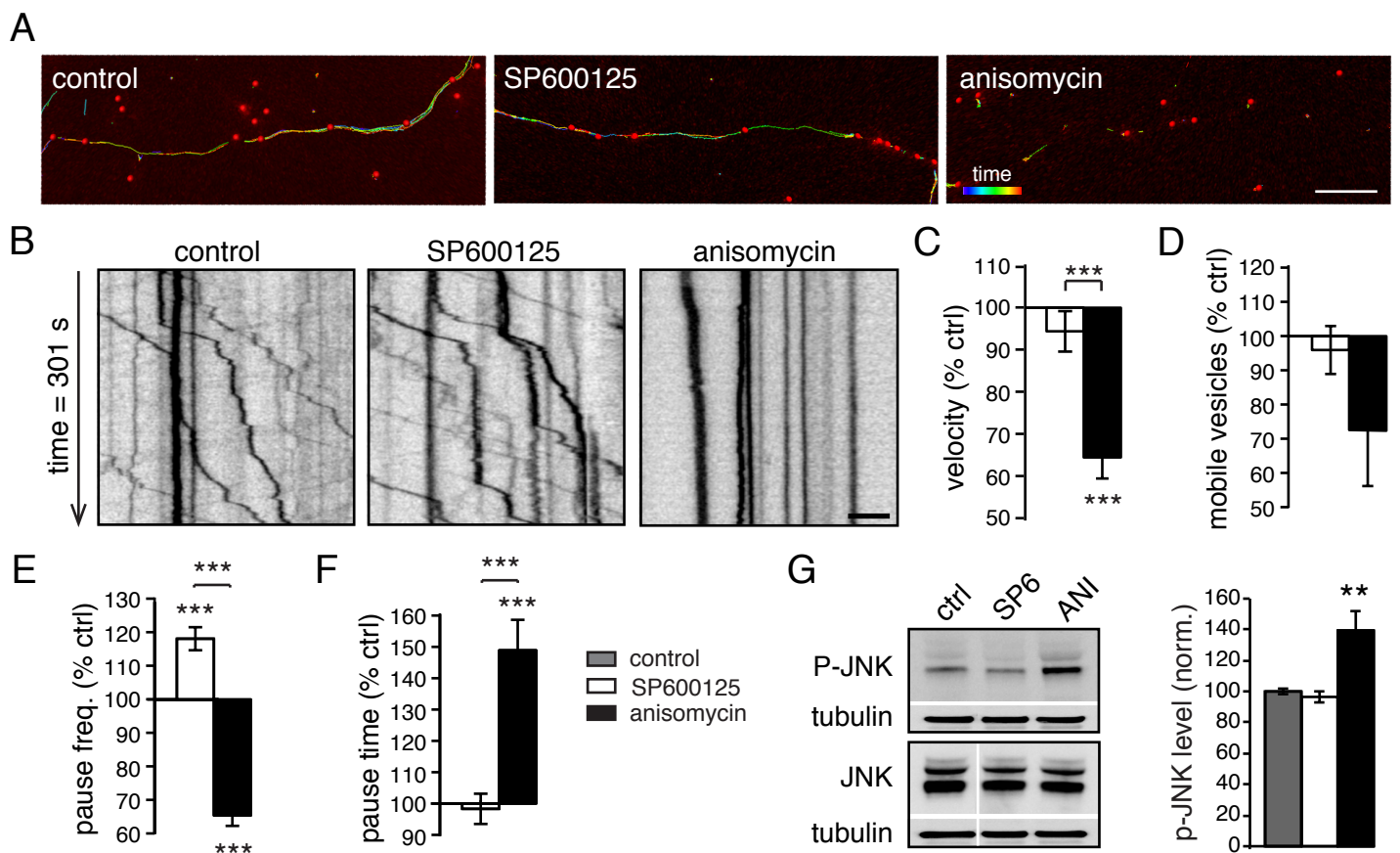
Figure S5. Pharmacological treatments show that JNK phosphorylates Syt4. Related to Figure 5.

(A) Images of vesicle trafficking of mCherry-Syt4, in control, SP600125-treated and anisomycin-treated conditions for 5 min, indicated by color-coded mobile vesicle tracks (middle panels; scale bar = 10 μm) and kymographs (B; scale bar = 5 μm). (C) Quantitation of average velocity, mobile vesicle percentage (D), vesicle pause frequency (E) and average pause time (F) shows that anisomycin-treated Syt4 vesicles are less mobile than control (n= 2643, 2362 and 2151 vesicles for average speed, velocity, pause frequency and pause time, and n = 12, 9 and 16 videos for mobile vesicle percentage for control, SP600125-treated and anisomycin-treated conditions, respectively, from 3 different cultures). Significance was determined by Student's t-tests with Bonferroni correction; error = s.e.m. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). (G) Western blots of p-JNK and JNK in hippocampal lysates of SP600125 and anisomycin-treated samples compared to control (left panel), and quantitation of p-JNK levels in all three conditions from Western blots (n = 4).
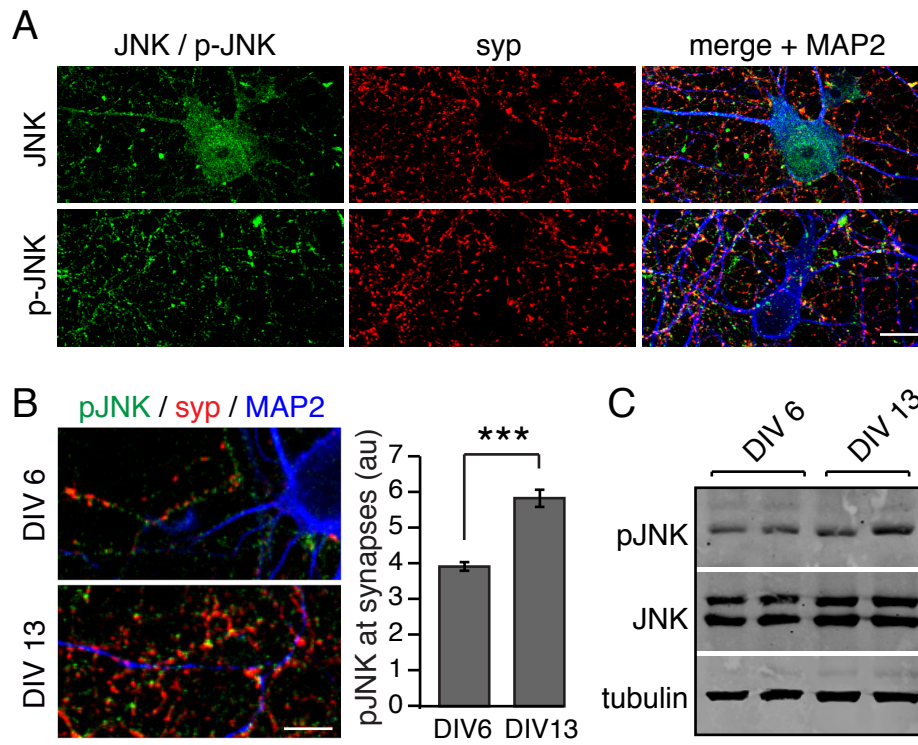
Figure S6. Localization of JNK and p-JNK in hippocampal neurons. Related to Figure 7.

(A) Representative images of hippocampal neuron cultures immunostained for JNK or p-JNK, syp (to mark synapses) and MAP2 (to mark dendrites). Scale bar = 10 μm. (B) Images of DIV6 and DIV13 hippocampal neurons immunostained for pJNK, syp, and MAP2, and quantitation of pJNK signal at synapses at DIV6 compared to DIV13. Scale bar = 5 μm. (C) Western blot for pJNK, JNK and tubulin (as a load control) from DIV6 and DIV13 hippocampal cultures.
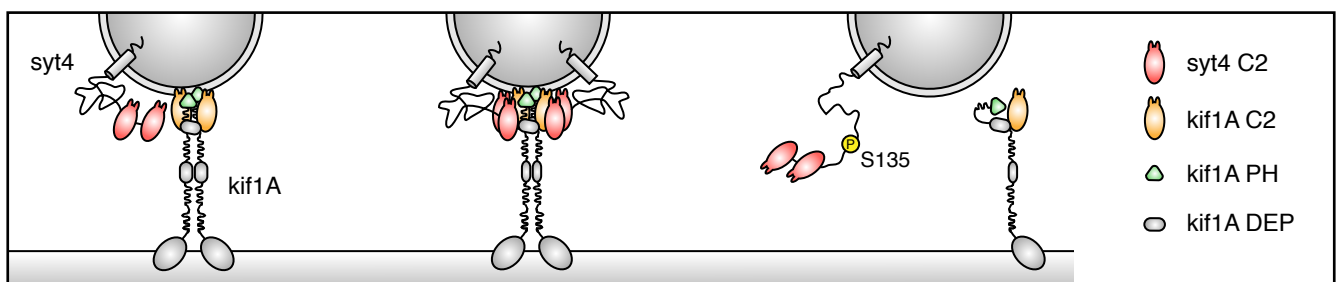
Figure S7. Model of the Syt4-Kif1A trafficking complex and how phosphorylation of Syt4 could disrupt binding to Kif1A to release vesicles. Related to Figure 3.

When Syt4 is in the non-phosphorylated compact form (middle panel), two Syt4 molecules bind to a Kif1A dimer in a 1:1 ratio via their C2 domains to clamp and stabilize Kif1A dimers in a processive conformation (middle panel). This C2-C2 interaction would also disrupt potential interaction of Kif1A DEP-PH domains, allowing the PH domain (and C2 domains of Syt4 and Kif1A) to bind vesicle membranes to stabilize the complex. The S135 site is surface accessible in this conformation. When Syt4 is phosphorylated, the linker region changes conformation such that the compact structure of Syt4 is destabilized, resulting in a loosening of the "clamp" between C2 domains, decreased interaction of C2 and PH domains with vesicle membranes, and release of vesicles (right panel).

Supplemental Experimental Procedures

Hippocampal neuron culture

Hippocampal neurons were isolated from E18-19 Wistar rats as previously described (Banker and Cowan, 1977). Extracted hippocampi were dissociated by treatment with 0.05 mg/ml trypsin for 20 min followed by trituration and filtering through a 100 μm cell strainer (BD Biosciences). Cells were plated in Neurobasal medium supplemented with B27, GlutaMAX and 100 U/ml penicillin/streptomycin (Gibco) on 12 mm glass coverslips (Carolina Biologicals) coated with 0.04% polyethyleneimine (PEI, Sigma) or 0.5 mg/ml poly-D-lysine (PDL, Sigma) in 24-well plates at a density of 80,000 cells/ well. Cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

Hippocampi from Syt4 knockout mice (Jackson Laboratory; (Ferguson et al., 2000)) were removed from P0 mouse brains in cold dissection medium (Gibco HBSS, 20 mM HEPES, 1.5 mM $CaCl_2$, 10 mM $MgCl_2$, pH adjusted to 7.4 with NaOH) and incubated in papain solution (dissection medium, L-Cysteine, NaEDTA, $CaCl_2$, NaOH, Papain equilibriated in 37° C and 5% CO_2 + DNAseI) for 30 min at 37 °C and 5% CO_2. Papain was inactivated with 5% serum medium (DMEM+glutamax, 5% BSA, Mito+ serum extender, MEM vitamins, DNAseI). Cells were triturated and then centrifuged for 5 min at 500 g at room temp. The cell pellet was resuspended in plating medium (Neurobasal, penicillin/streptomycin, B27, L-Asp acid, L-Glu acid, glutamax, 0.5% serum medium). Cells were plated at 120,000 cells/ well on 12 mm diameter acid-etched 0.04% PEI-coated glass coverslips. FUDR was added on DIV4 to block glial proliferation. 50% conditioned medium was replaced with feeding medium (Gibco Neurobasal, Glutamax, Pen/Strep, B27) on DIV7.

Mammalian expression constructs, antibodies and reagents

AAV-ESYN-mCherry-Syt4, AAV-ESYN-mCherry-Syt4(S135A) and AAV-ESYN-mCherry-Syt4(S135E) were constructed by sub-cloning WT or mutagenized rat Syt4 fused to an N-terminal mCherry into an AAV plasmid backbone with an enhanced synapsin promoter. CgA-GFP, Synaptophysin-GFP, PSD95-GFP, Bassoon-GFP, and LifeAct-RFP were provided by Thomas Dresbach (University Medical School, Goettingen, Germany), NPY-mCherry was provided by Matthijs Verhage (Vrije Universitt Amsterdam). AAV-ESYN-mCherry-Syt4-P2A-FlagJNK1a1(APF) (called JNK1(APF)), AAV-ESYN-mCherry-Syt4-P2A-FlagMKK7B2JNK1a1 (called MKK7-JNK1) and AAV-ESYN-mCherry-Syt4(S135A)-P2A-FlagMKK7B2JNK1a1 (called S135A/MKK7-JNK1) were made by GenScript Biotech Corporation (Piscataway, NJ, USA), by inserting a P2A site and FlagJNK1a1(APF) (Addgene # 13846) or FlagMKK7B2JNK1a1 (Addgene # 19726) provided by Roger Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, USA), downstream of Syt4 in AAV-ESYN-mCherry-Syt4 or AAV-ESYN-mCherry-Syt4(S135A). N-terminal His-tagged WT, S135A and S135E plasmids in pET28a were synesized by Genscript Biotech Corporation (Piscataway, NJ, USA). GST-Kif1A was provided by Geri Kreitzer (City University of New York, USA), Kif1A-GFP, and Kif1A knockdown and rescue constructs (Lo et al., 2011) were provided by Michael A. Silverman (Simon Fraser University, Canada).

Antibodies used were: from Synaptic Systems: rabbit Syt4 (cat. no.105043), guinea pig Synaptophysin (cat. no. 101004), mouse tubulin (cat. no. 302211), mouse Syb2 (clone 69.1, cat. no. 104211), mouse Syt1 (cat. no. 105101), mouse Synaptophysin (clone 7.2, cat. no. 101011), mouse Rab-GDI (cat. no. 130011), mouse PSD-95 (cat. no. 124011); from BD Transduction Laboratories: mouse JNK/SAPK1 (cat. no. 610627), and mouse Kif1A (cat. no. 612094); from Cell Signaling Technology: mouse Phospho-SAPK/JNK (cat. no. 9255), Phospho-c-Jun (cat. no. 9261), chick Map2 (Millipore, cat. no. Ab5543) and sheep CgA (AbD Serotec, cat. no. 2095-0220). Alexa 405, 488, 546 and 647 secondary antibodies from Invitrogen were used in immunocytochemistry experiments. HRP-coupled mouse and rabbit secondary antibodies from BioRad were used in Western blot experiments.

Chemicals used were nocodazole (cat. no. 1228) and latrunculin A (cat. no. 3973) from Tocris, SP600125 (cat. no. S5567), anisomycin (cat. no. A9789) and jaskplakinolide (cat. no.

J4580) from Sigma, and bicuculline (cat. no. 120108) from Abcam. NaCl, KCl, CaCl$_2$, MgCl$_2$, Tris-HCl, EDTA, NP40 and glucose were from Carl Roth, and HEPES was from Gibco.

Lipofectamine transfection
Neurons were transfected at DIV3 or DIV10 using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. For each well of a 24-well plate, 1 μl Lipofectamine 2000 was added to 50 μl Neurobasal medium in one tube, and 0.75 μg DNA was added to 50 μl Neurobasal medium in another tube, and incubated separately for 5 min The contents of these tubes were then mixed and incubated for 20 min at room temperature. Conditioned medium was removed from wells, saved, and replaced with 400 μl fresh Neurobasal medium without supplements. The Lipofectamine/DNA mixture was then added to wells, incubated for two hours at 37 °C and 5% CO$_2$, and then removed, washed once with Neurobasal medium and replaced with saved conditioned media. For co-transfections, 0.75 μg of DNA total was added per well (i.e. 0.375 μg of each construct).

Live cell imaging
For trafficking experiments, cells were imaged at room temperature on DIV13-15, with the exception of overexpressed active or dominant-negative JNK which were imaged on DIV11. Transfected coverslips were transferred to a live imaging chamber (Warner Instruments) containing 150 μl of Tyrode's solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 5.5 mM glucose, 20 mM Hepes, pH=7.3). Healthy neurons were selected by morphology for live imaging in 90 μm x 90 μm fields of view in which intact processes were visible and in which at least a few vesicles were moving. Images were acquired with 450-490ex/505-555em filters for GFP and 545-570ex/575-680em filters for mCherry on a Zeiss AxioObserver inverted microscope with a Photometrics Evolve EMCCD camera, and Lambda DG-4 high-speed wavelength switcher at 1 s intervals using Metamorph software (Molecular Devices). For pharmacological experiments, cells were treated with 10 μM nocodazole, 10 μM latrunculin, or 1 μM jasplakinolide for 30 min prior to imaging, or 10 μM SP600125 or 50 μg/ml anisomycin for 2-4 h before imaging. For activity-dependent pause experiments, high KCl solution (100 mM NaCl, 90 mM KCl, 10 mM CaCl$_2$, 2 mM MgCl$_2$, 5.5 mM glucose, 20 mM Hepes, pH=7.3) was applied by pipette for 3 minutes during time-lapse recording followed by perfusion with base Tyrode's solution.

Following acquisition, vesicles in imaged regions containing multiple contiguous processes 10-100 μm in length, were identified and their mobility analyzed using the particle-tracking module of Imaris 7.6.4 (Bitplane). For quantitation of vesicle movement and pausing, positional data from Imaris were imported into Matlab and analyzed with custom-written Matlab programs (Mathworks, Natick, MA, USA). Vesicles present for less than 3 s in the field of view were not included in analysis. Track velocity and mobile vesicle percentage (percentage of vesicles with a track duration of at least 10 s and velocity of at least 1 μm) were calculated. Pauses were defined as a drop in vesicle velocity below 0.1 μm/s (Bury and Sabo, 2011). Vesicles that were moving or paused for the entire duration of a time-lapse were excluded from average pause time analysis. Because vesicle parameters exhibited the highest variance and standard deviation (compared to neuronal processes, videos, coverslips, or cultures), vesicles were used for n number of all trafficking parameters except mobile percentage (for which video number was used). We did not consider cells as n number because fields of view often included multiple transfected axons from different cells. Kymographs were generated and analyzed using Metamorph software. For analysis of activity-dependent pauses, only mobile vesicles visible for the entire duration of high KCl stimulation were considered. Vesicles in kymographs were defined as pausing if they were immobilized during stimulation for at least 120 seconds

(two thirds of the total time of stimulation). Statistical significance was determined by Student's t test, with Bonferroni Correction for multiple comparisons.

Immunocytochemistry and fixed cell imaging and analysis
For immunocytochemistry, cultures were fixed at DIV14-16 with 4% paraformaldehyde/0.1M phosphate buffer for 20 min and then washed with PBS. Immunostains of CgA were performed following methanol fixation (15 min methanol at -20 °C). Coverslips were blocked and permeabilized in Buffer D (2% donkey serum, 0.1% Triton X-100, and 0.05% sodium azide in 2X PBS) for 20 min., and then incubated with primary antibody in Buffer D at 4 °C overnight. Coverslips were then washed 3 times for 3 min each in PBS. Coverslips were incubated with secondary antibody in buffer D at room temp for 2 h and washed again 3 times for 3 min each in PBS. Coverslips were mounted with Fluoromount Plus (Diagnostic Biosystems) sealed with nail polish and examined with a 40X 1.3 NA oil Zeiss Plan-Apochromat DIC objective on a Zeiss LSM 710 confocal microscope. For co-localization analysis, we used ImageJ and Metamorph software. Percent colocalization was determined from the percentage of the total thresholded area of one channel that overlapped with the thresholded area of the other channel for two different secondary fluorophores, using Metamorph software.

HEK cell culture and transfection
HEK cells were cultured in DMEM medium with 10% FBS and 1% penicillin/streptomycin in 10 cm culture dishes at 37 °C in a 5% CO_2 humidified incubator. Cultures at 40-50% confluence were transfected using calcium phosphate: 20 μg plasmid DNA was added to 166 μl 2 M $CaCl_2$ in a total volume of 1.3 ml dH_2O and gently mixed using a pipette. 1.3 ml transfection buffer (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM glucose, 42 mM Hepes, pH 7.09) was added dropwise under gentle vortex. This mixture was then added dropwise per 10 cm plate, and cultures incubated overnight at 37 °C in a 5% CO_2 humidified incubator. The following day, the media was removed, cultures were washed twice with pre-warmed PBS and fresh media was added.

In silico kinase prediction
GPS 3.0 software (Zhou et al., 2004) was used as a tool to predict kinases capable of phosphorylating Syt4 at S135 and c-Jun (as a control) at S63. Peptides for WT Syt4: PETEKEAVSPESLKSST, and WT c-Jun: AKNSDLLTSPDVGLLKL, were submitted to the prediction software with a medium threshold (corresponding to a false positive rate (FPR) equal to 6). All available serine/threonine kinases were selected as potential kinases. The software reported a score and a cutoff for each kinase. All kinases with Score/Cutoff > 2 were classified as significant predicted kinases for phosphorylation of S135 in Syt4 and S63 in c-Jun.

In vitro kinase assay
Peptides for WT Syt4; PETEKEAVSPESLKSST, phosphodeficient Syt4 (S135A); PETEKEAVAPESLKSST, WT c-Jun; AKNSDLLTSPDVGLLKL, and phosphodeficient c-Jun (S63A); AKNSDLLTAPDVGLLK were synthesized by GenScript Biotech Corporation (Piscataway, NJ, USA). The ADP-GloTM Kinase assay kit (Promega) was used with JNK1 (R&D Systems) according to the manufacturer's instructions. In all assays 0.2 μg/μl of each peptide and 5 μM ATP were used. Standard curves were obtained by measuring the luminescence of a series of ADP-ATP dilutions according to the kit protocol. Phosphorylation of WT Syt4 and c-Jun peptides by JNK1 was calculated based on standard curves and reported as % activity (which includes potential autophosphorylation). JNK activity of phosphodeficient Syt4 or c-Jun mutants was normalized to control for comparison.

Western blots
12-14 DIV neurons in 10 cm dishes were washed in PBS. Cells were harvested in PBS and passed through a 27-gauge needle. Lysates were centrifuged at 4000 rpm for 10 min to pellet

nuclei and cellular debris. Protein concentration of supernatants was determined by BCA assay (Novagen, cat. no. 712853) according to the manufacturer's instructions. Equal amounts of protein were then loaded per lane for comparison between conditions, resolved by SDS-PAGE, and analyzed by immunoblotting.

Co-Immunoprecipitation

For co-immunoprecipitation experiments, transfected confluent HEK293 cells growing in 10 cm dishes were harvested in IP-Lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP40, Complete protease inhibitor (Roche)). Cell lysates were then incubated with 30 µl of antibody coupled Protein A/G Dynabeads (Invitrogen) or GFP-Trap beads (Chromotech) for 2 h at 4 °C on a rotator. Uncoupled beads were used for binding controls. Supernatant was saved as the unbound fraction and bound proteins from beads were eluted by incubating beads for 10 min at 95 °C in 4X SDS sample buffer. Samples were then analyzed by SDS-PAGE and western blotting.

Pulldowns from brain lysates and direct binding assays

Recombinant His-tagged WT, S135A or S135E Syt4 was expressed in E.coli, purified, and coupled to Ni-beads for 2 h at 4 °C. After coupling, Ni-beads were incubated with solubilized mouse brain homogenate for 2 h at 4 °C. Beads were then washed three times with PBS containing 1% Triton X-100, loaded onto SDS-PAGE gels and analyzed by western blot. To test direct binding, His-tagged WT, S135A or S135E Syt4 was coupled to Ni-beads for 2h at 4°C. After coupling, purified GST-tagged full-length Kif1A was added to Syt4 WT and phosphomutant-coupled Ni-beads and incubated for 2 h at 4°C. Beads were then washed three times with PBS containing 1% Triton X-100, loaded onto SDS-PAGE gels and analyzed by Western blot.

Immuno-organelle isolation of synaptic vesicles

Mouse monoclonal antibody directed against Syt1 and Syb2 were coupled to Protein A Dynabeads (Invitrogen) in 1 mM PBS-EDTA for 1 h at 4 °C. Lysate was then added to antibody-coupled beads and incubated for 2 h at 4 °C on a rotator. Magnetic Dynabeads were then separated from the non-bound fraction (supernatant) and washed 3 times with PBS. Beads were resuspended in sample buffer (bound fraction). The input, bound fraction and unbound fraction were loaded onto and separated by a 12.5% SDS-PAGE gel and analyzed by western blot using antibodies directed against Syt4, Syt1, syp, Syb2, Rab-GDI and PSD-95.

Sequence and analysis and structural modeling

Sequence analysis was performed using PredictProtein (Yachdav et al., 2014). The intrinsic disorder predisposition of human Syt4 (UniProt ID: Q9H2B2) was evaluated by three algorithms from the PONDR family, PONDR® VLXT (Romero et al., 2001), PONDR® VSL2 (Peng et al., 2006), and PONDR® VL3 (Peng et al., 2005), as well as by the IUPred web server (Dosztanyi et al., 2005).

Swiss-Prot entries Syt4_Rat (P50232), and Kif1A_Rat (F1M4A4) were used to generate structural models. Coordinates of human C2A (PDB_ID: 1UGK, 90% identity to rat) and rat C2B (PDB_ID: 1W15) from Syt4 were arranged to generate a C2A-C2B tandem in analogy to Syt1 (PDB_ID: 5CCG). The compact N-terminal domain of Syt4 was modelled *ab inito* by threading using PHYRE2 (Kelley et al., 2015). For Kif1A, PHYRE2 generated a PH domain with 99% confidence (PDB_ID:2COA). Two PH domains were dimerized in analogy to ELMO1 (PDB_ID: 2vsz). Threading identified a C2 domain (res. 1007-1154) with 98% confidence using a template from PI-PLC delta1 (PDB_ID: 1DJG) and high fold similarity to Syt4, and a positional psi/phi blast identified a DEP-like fold (res. 1165-1265, 23% ID, 40% sim., PDB_ID: 1UHW) in the Kif1A undefined region (res. 849-1592, UDR), similar to that of pleckstrin. For complex presentation, Syt4 was manually docked to the Kif1A structure. Missing

loops in structures were refined using FOLDIT (Kleffner et al., 2017). All structures were rendered and visualized using PyMOL.