Supporting Information

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SI Methods

Mouse Lines. The conditional RIM1/2 × ELKS1/2 quadruple homozygous KO mice were previously characterized (1) and were generated by crossing conditional RIM1 (2) (RRID:IMSR_JAX:015832), RIM2 (3) (RRID:IMSR_JAX:015833), ELKS1 (4) (RRID:IMSR_JAX:015830), and ELKS2 (5) (RRID:IMSR_JAX:015831)-KO mice. The constitutive Liprin-α3–KO mice were generated as described here later. All animal experiments were performed according to institutional regulations at Harvard University.

Generation of Liprin-α3–KO Mice. Liprin-α3–KO mice were generated for this study by CRISPR/Cas9 gene editing in one-cell zygotes (6). We first identified several 20-bp-long potential targeting sequences followed by PAM sequences in multiple exons of the *Ppfia3* gene, which encodes Liprin-α3, and tested their efficiency in mouse 3T3 cells by using transfection followed by surveyor assays. We selected a targeting sequence in exon 18 based on its efficiency in disrupting the Liprin-α3 gene in cultured cells. Cas9 mRNA, single guide RNA (sgRNA), and donor oligos were then prepared for zygote injections. The bicistronic expression vector expressing Cas9 and an sgRNA (pX330-U6-Chimeric_BB-CBh-hSpCas9) was obtained from Addgene (plasmid no. 42230). Targeting oligos for exon 18 of the mouse *Ppfia3* gene encoding Liprin-α3 (5′-CACCGAAGAGCATCAAGTCGTCCAT-3′and 5′- AAACATGGACGACTTGATGCTCTTC-3′) were annealed, phosphorylated, and ligated into the linearized pX330 vector. PCR amplification was performed to add a T7 promoter to the Cas9 coding region with primers 5′-TAATACGACTCACTATAGGGagaATGTACCCATACGATGTTCC-3′ and 5′-GCGAGCTCTAGGAATTCTTAC-3′. The T7 promoter was added to the sgRNA by using PCR with primers 5′TAATACGACTCACTATAGGAAGAGCATCAAGTCGTCCAT-3′ and 5′-AAAAGCACCGACTCGGTGCC-3′. T7-Cas9 and T7-sgRNA PCR products were gel-purified and used as the template for in vitro transcription by using the mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies) and the MEGAshortscript T7 kit (Life Technologies), respectively. Cas9 mRNA and sgRNAs were purified by using the MEGAclear kit (Life Technologies), eluted with nuclease-free water, and size-validated by denaturing agarose gel and urea Tris-boric acid-EDTA polyacrylamide gel electrophoresis, respectively. Cas9 mRNAs (5 ng/μL) and sgRNA (2.5 ng/μL) and a donor oligo (10 ng/μL) were freshly mixed in nuclease-free water and injected directly into the pronucleus of one-cell zygotes of FVB/NCrl mice. Injections were carried out in the Brigham & Women's Hospital Transgenic Mouse Core Facility at Harvard Medical School. The zygotes were immediately transplanted into foster mothers. The offspring of the zygote injections were genotyped by a PCR-based Surveyor assay (Transgenomic) with primers 5′- TGCCCGTCTTGAGAGGATGG-3′ and 5′- AAAGAGAATCCTGGGACAGC-3′ to screen for founder lines that carry insertions or deletions (indels) at the targeting locus. We identified three founders in 142 offspring by PCR and Surveyor assay. Exon 18 of indel-carrying founders was amplified by PCR, subcloned, and sequenced to identify specific mutations. In each founder, we cloned PCR fragments of the targeted gene locus and sequenced them. We detected as many as three different alleles in each founder, suggesting that gene editing continued to occur beyond the single-cell stage. A frame-shift mutation deleting 8 bp was selected, removal of Liprin-α3 was confirmed by Western blot, and the allele was then outbred to 129S2/SvPasCrl mice for five generations and maintained as heterozygous line. The mice were genotyped by using oligos 5′-GATCTTCAAGCCAGTTCTGC-3′ and 5′-AGAGCCGGCCTATGGACGAC-3′ for the WT allele (resulting in a 365-bp band) and oligos 5′-GATCTTCAAGCCAGTTCTGC-3′ and 5′-GAGCCGGCCTATGGATGCTC-3′ for the mutant allele (resulting in a 356-bp band). All experiments were performed on littermate pairs of WT Liprin-α3$^{+/+}$ and Liprin-α3$^{-/-}$ mice.

Cell Cultures and Lentiviral Infection. Primary mouse hippocampal cultures were generated from newborns within 48 h after birth as previously described (1, 4). Unless otherwise stated, neurons were plated on 0.15-mm-thick chemically stripped glass coverslips. Neurons from conditional RIM1/2 × ELKS1/2 quadruple homozygous floxed mice were infected at 5 DIV with viruses expressing a cre recombinase (to generate cKO^{R+E} neurons) or an inactive cre truncation mutant (to generate control^{R+E} neurons) under the human synapsin promoter (1). Lentiviruses were generated in HEK293T cells by Ca^{2+} phosphate transfection. HEK cell supernatants containing cre-expressing and control viruses were collected 2 d after transfection and applied to cultures at 5 DIV. Liprin-α3$^{-/-}$ and Liprin-α3$^{+/+}$ cultures were generated from littermates of heterozygote breeding pairs that were genotyped before culturing. For sypHy imaging, a sypHyA4-expressing lentivirus (1, 7) and an SV2-tdTomato–expressing lentivirus were applied to cultures at DIV 3. Lentiviruses expressing a synapsin promoter-driven, N-terminally HA-tagged rat Liprin-α3 were applied to cultures at DIV 1 (Fig. 3 and Figs. S4 and S5) or at DIV 5 (Fig. S6) for rescue experiments.

Western Blotting. Fluorescent Western blotting was used for quantitative protein-expression analyses in hippocampal lysates. Hippocampi were collected from 3–4-wk-old littermate Liprin-α3$^{+/+}$ and Liprin-α3$^{-/-}$ mice, flash-frozen, and homogenized by using a motorized glass–Teflon homogenizer in a buffer containing 150 mM NaCl, 25 mM Hepes, pH 7.5, 4 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/mL Leupeptin, Pepstatin, 2 μg/mL Aprotinin, and 1× PhosStop (Roche), and extracted in 1% Triton for 1 h at 4 °C. Protein concentration was measured by a bicinchoninic acid assay. Protein (20 μg per sample) was run on SDS/PAGE gels and transferred onto nitrocellulose membranes. Blots were washed with Tris-buffered saline (TBS) solution, blocked in TBS solution containing 5% milk, 5% goat serum, and then incubated with primary antibodies overnight at 4 °C in TBS solution containing 5% BSA and 0.1% Tween-20. Fluorescent secondary antibody incubation was done for 1 h at room temperature. Membranes were washed in TBS solution and air-dried before imaging. Membranes were scanned by using a LI-COR Odyssey Fluorescent Scanner, and quantification was done by using ImageJ on the original 16-bit images. The fluorescent signal of the test protein band was normalized to a β-actin loading control that was run in the same lane, and protein levels were expressed normalized to the average of the three WT control samples. For display of fluorescent Western blots, the 16-bit images were converted to 8-bit images, which resulted in limited loss of background information in image areas with very low background, but reflects the overall relationship between background and signal well. All analyses were done in at least three independent littermate pairs.

For nonquantitative assessment of rescue construct expression in cultured hippocampal neurons, chemiluminescent Western blotting was used to detect Liprin-α3. Cultured neurons from one coverslip were harvested in 25 μL 1× SDS sample buffer and run on 7.5% SDS gels followed by transfer to nitrocellulose membranes. Membranes were blocked in TBS solution containing 10% nonfat milk with 5% goat serum and 0.1% Tween-20 for 1 h at room temperature.

Membranes were incubated overnight at 4 °C in primary antibodies in TBS solution with 5% nonfat milk, 2.5% goat serum, and 0.1% Tween-20. Membranes were incubated with HRP-conjugated secondary antibodies (1:10,000) at room temperature for 2 h. Membranes were sprayed with a chemiluminescent reagent and exposed to film.

Primary antibodies used for Western blotting were as follows: rabbit anti–Liprin-α3-1 (1:1,000; RRID: AB_887762), rabbit anti–Liprin-α3-2 (1:200; gift from S. Schoch, Institute for Neuropathology, University of Bonn, Bonn, Germany; described in ref. 8), rabbit anti–Liprin-α3-3 (serum 4396, 1:5,000; described in ref. 9; gift from T. Südhof, Department of Molecular and Cellular Physiology and HHMI, Stanford University, Stanford, CA; RRID: AB_2617056), rabbit anti–Liprin-α1 (1:200; described in ref. 8; gift from S. Schoch), rabbit anti–Liprin-α1 (1:200; described in ref. 8; gift from S. Schoch), rabbit anti-Liprin-α2 (1:200; described in ref. 8; gift from S. Schoch); rabbit anti–Liprin-α4 (1:200; described in ref. 8; gift from S. Schoch), mouse anti-LAR (1:100; RRID: AB_10672300), mouse anti-PSD-95 (1:500; RRID: AB_10698024), mouse anti–β-actin (1:2,000; RRID: AB_476692), mouse anti–GIT-1 (1:500; RRID: AB_10672300), mouse anti-ELKS1/2α (1:1,000; RRID: AB_869944), rabbit anti-RIM1 (1:2,000; RRID: AB_2617051; gift from T. Südhof), rabbit anti-RIM1(1:1,000; RRID: AB_887774), rabbit anti–RIM-BP2 (1:500; RRID: AB_2619739), mouse anti-panMunc13 (1:100; RRID: AB_398312), rabbit anti-Munc18-1 (1:1,000; gift from T. Südhof), mouse anti-Synapsin (1:4,000; RRID: AB_2617071), rabbit anti–Synaptobrevin-2 (1:4,000; gift from T. Südhof), rabbit anti–SNAP-25 (1:2,000; gift from T. Südhof), mouse anti-Syntaxin1 (1:500; RRID: AB_528484), mouse anti-Synaptophysin (1:5,000; RRID: AB_887824), and mouse anti-Synaptotagmin1 (1:500; RRID: AB_2199314). IRDye 800CW-conjugated or IRDye 680RD-conjugated anti-mouse IgG (RRIDs: AB_621847 or AB_621848) or anti-rabbit IgG (RRID: AB_10953628 or AB_10954442) secondary antibodies were used at 1:10,000.

Immunofluorescent Staining of Cultured Neurons. DIV 14–17 neuronal cultures grown on 0.15-mm-thick chemically stripped glass coverslips were washed with PBS solution and fixed in 4% paraformaldehyde (PFA) in PBS solution at room temperature for 10 min. After three rinses in PBS solution, the neurons were blocked and permeabilized in 3% BSA/0.1% Triton X-100/PBS solution for 1 h, incubated in primary antibodies (in blocking solution) at 4 °C for 24–48 h, washed three times in PBS solution, and incubated in Oregon green 488, Alexa 555, and Alexa 633 secondary antibodies (1:200) at 4 °C for 24–48 h. Coverslips were air-dried at room temperature, mounted onto glass slides in mounting medium, and stored at 4 °C until imaging.

Primary antibodies used were as follows: monoclonal mouse anti-Bassoon^{N} (1:1,000; RRID: AB_11181058), monoclonal guinea pig anti-Bassoon^{C} (1:500; RRID: AB_2290619), rabbit anti–Liprin-α3 (serum 4396, 1:5,000; described in ref. 9; gift from T. Südhof), rabbit anti–Liprin-α2 [1:250; gift from S. Schoch (8)], anti-ELKS2α [serum E3-1029, 1:1,000; custom made (10)], rabbit anti-RIM1 (1:1,000; AB_887774), monoclonal mouse anti–PSD-95 (1:200; RRID: AB_10698024), guinea pig anti-vGlut1 (1:500; AB_887878), rabbit anti-Synapsin 1 (serum E028, 1:1,000; gift from T. Südhof), rabbit anti–RIM-BP2 (1:500; RRID: AB_2619739), and rabbit anti–Munc13-1 (1:200; RRID: AB_887735).

Confocal and STED Imaging and Analysis. Confocal and STED images were acquired with a Leica SP8 Confocal/STED 3× microscope with an oil-immersion 100×, 1.44-N.A. objective at the Harvard NeuroDiscovery Center Enhanced Imaging Core. Synapse-rich areas (23.3 × 23.3 μm^2) were selected as regions of interest (ROIs) and were scanned at a sampling frequency of ∼10 nm per pixel. Triple-color sequential confocal scans were followed by dual-color sequential STED scans by using a STED microscope with gated detectors and 40 nm *x/y* resolution and a 70-nm full width at half maximum measured with 40-nm beads. Alexa 633, Alexa Fluor 555, and Oregon green 488 were excited with 633-nm, 555-nm, and 488-nm white light lasers, respectively, at 2–5% of 1.5 mW laser power in this particular order. During STED scanning, Oregon green 488 and Alexa Fluor 555 signals were depleted with 592 nm (75% of maximum power) and 660 nm (25% of maximum power) time-gated depletion lasers. Four-times line accumulation and three-times frame averaging were applied during STED scanning. Identical settings were applied to all samples within an experiment. Images were analyzed in ImageJ with background subtraction by using a rolling ball of 0.5 μm (11). For all quantitative line-scan analyses of STED images, synapses in side view were defined as synapses that contained a synaptic vesicle cluster with an elongated Bassoon structure along the edge of the vesicle cluster. For line profile analysis, a 1-μm-long, 250-nm-wide profile was selected perpendicular to the elongated Bassoon structure across its center. An intensity profile was obtained for the synaptic vesicle cluster (imaged with confocal microscopy), the Bassoon staining (imaged by STED), and the test protein (imaged by STED). The axial position of a test protein was the *x* coordinate relative to the Bassoon maximum at which the fluorescent intensity was at maximum. A custom MATLAB program was used to analyze the distribution of the synaptic protein clusters, and all synapses were included in this analysis. In each ROI (defined as the outline of the vGluT1 vesicle cluster), Bassoon and test protein clusters (500 nm^2 < size of the detected cluster < 0.4 μm^2) were identified within the mask of the vesicle marker vGlut1. Active zone-associated components were identified as test protein clusters with ≥10% overlap with Bassoon clusters. All other synaptic protein clusters (<10% overlap with Bassoon but >10% overlap with the vesicle marker vGlut1) were classified as non–active zone-associated synaptic components. The distribution of synaptic components was calculated from individual ROIs. Protein levels in STED or confocal images were measured as the mean fluorescent intensity within an ROI. Representative images were contrast-adjusted, but all quantitative analyses were performed on original images without adjustments and were done identically for all experimental conditions. For all image acquisition and analyses comparing two or more conditions, the experimenter was blind to the condition.

Histological Analyses of Brain Sections. For histological analyses of brain sections, brains from 2–3-wk-old mice were perfusion-fixed in 0.1 M Sorensen's phosphate buffer containing 4% PFA. Cryoprotection was done in 30% sucrose overnight, and the brains were frozen in O.C.T. (Sakura Finetek) and sagittally sectioned at 40 μm in a cryostat. Brain slices mounted on glass slides were stained with 0.1% cresyl violet (Sigma Aldrich) following standard protocols, and immunostainings were performed as described for cultured cells. Slices were imaged with a slide scanner (VS120; Olympus) at the Neurobiology Imaging Facility of Harvard Medical School.

Force-Plate Analysis. Locomotor activity was assessed on two force-plate actometers (40 cm × 40 cm) as previously described (5, 12, 13). In each experiment, the center of force of a mouse was monitored for 30 min as it explored, for the first time, the open arena of the actometer in a well-lit room. Liprin-α3$^{+/+}$ and Liprin-α3$^{-/-}$ littermate mice (12 to 16 wk old) were run on the same day. The trajectory of the center of force was analyzed in three 10-min bins. The spatial confinement index and low-mobility bouts were calculated as described previously (12). Low-mobility bouts were defined as periods during which the center of force was confined to a circle of 15-mm radius for more than 10 s. For all behavioral analyses, the experimenter was blind to the genotype.

Electrophysiology. Electrophysiological recordings in cultured hippocampal neurons were performed as previously described (10). The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes·NaOH, pH 7.4, 10 glucose (∼310 mOsm), 1 μM tetrodotoxin to block action potentials,

100 μM picrotoxin to block GABA receptors, and 50 μM D-(–)-2-Amino-5-phosphonopentanoic acid (AP5) to block NMDA receptors. All recordings were performed in whole-cell patch-clamp configuration at 20–23 °C at DIV 14–17. Whole-cell glass pipettes were pulled at 2–4 MΩ and filled with intracellular solution containing (in mM): 120 Cs-methanesulfonate, 10 EGTA, 2 $MgCl_2$, 10 Hepes·CsOH (pH 7.4), 4 Na_2-ATP, 1 Na-GTP, and 4 *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride (~300 mOsm). Cells were held at −70 mV, and access resistance was monitored between sweeps. Cells in which access resistance exceeded 20 MΩ or in which the holding current exceeded −200 pA were excluded from the analysis. mEPSC data were acquired with an Axon 700B MultiClamp amplifier, digitized with a Digidata 1440A digitizer at 10 kHz, and analyzed by using a template search with visual inspection of each event in pClamp.

For recordings in acute hippocampal slices, 3–4-wk-old mice were deeply anesthetized by isoflurane and decapitated. Sagittal brain slices containing hippocampus (300-μm thick) were cut by using a vibratome (VT1200s; Leica) in ice-cold cutting solution containing (in mM): 75 NaCl, 2.5 KCl, 7.5 $MgSO_4$, 75 sucrose, 1 NaH_2PO_4, 12 glucose, 26.2 $NaHCO_3$, 1 Myo-inositol, 3 pyruvic acid, and 1 ascorbic acid. After cutting, slices were incubated at room temperature in incubation solution containing (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 12 glucose, 26.2 $NaHCO_3$, 1 Myo-inositol, 3 pyruvic acid, and 1 ascorbic acid for at least 1 h before use. For the field recordings, slices were placed in a recording chamber continuously perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 12 glucose, and 26.2 $NaHCO_3$ at 2.5–3 mL/min (heated to 30–32 °C). All solutions were constantly bubbled with 95% O_2 and 5% CO_2. All recordings were completed within 4 h of slicing. For recording field excitatory postsynaptic potentials (EPSPs), 50 μM picrotoxin and 50 μM AP5 were added. The recording electrode was filled with ACSF with a tip resistance of 3–4 MΩ and placed ~20 μm below the slice surface in the stratum radiatum of area CA1. A concentric stimulation electrode was used for electrical stimulation and was placed 500–600 μm away from the recording site in the Schaffer collateral pathway. Bipolar stimuli (0.5 ms total) were applied to the slice every 5 s. The stimulation intensity was gradually increased from 25 to 500 μA with steps of 25 μA. Each stimulation intensity was repeated five times, and the average response was used for quantification. Data were acquired with an Axon 700B MultiClamp amplifier, digitized with a Digidata 1440A digitizer, and analyzed using pClamp.

Data acquisition and analyses for electrophysiological experiments in cultured neurons and acute slices were done by an experimenter blind to the genotype.

SypHy Imaging. SypHy and SV2-tdTomato were expressed from lentiviruses in hippocampal cultures, and cultures were imaged at DIV 14–17 and quantified as previously described (1). Briefly, experiments were performed in extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Hepes·NaOH, 0.05 AP5, and 0.025 6-cyano-7-nitroquinoxaline-2,3-dione (pH 7.4, ~310 mOsm) at 20–23 °C and imaged with an upright microscope. Images were acquired with a 60×, 1.0-N.A. water-immersion objective at 0.5 Hz with 2 × 2 binning. Neurons were stimulated at 20 Hz with a focal Nichrome bipolar electrode. NH_4Cl solution (extracellular solution substituted with NH_4Cl for 50 mM NaCl, adjusted to pH 7.4) was applied at the end of each experiment to visualize all sypHy puncta. Images were analyzed in ImageJ with background subtraction by using a rolling ball of 5 μm (11). NH_4Cl-responsive puncta for which $\%(\Delta F_{NH4Cl}/F_0) > 200\%$ were included in the analysis and were used to define ROIs. Puncta that responded to action potential stimulation [40 or 200 action potentials at 20 Hz, $\%(\Delta F_{\text{during stimulation}}/\Delta F_{NH4Cl}) > 0\%$, where $\Delta F_{\text{during stimulation}}$ is defined as the mean fluorescence of $F - F_0$ during stimulation] were defined as active synapses. SypHy F_0 was defined as the mean fluorescence during 5 s before stimulation. SypHy ΔF was quantified as fluorescence intensity F at each time point minus F_0. Peak fluorescence (ΔF_{peak}) is defined as the average ΔF during the first four imaging frames immediately following the end of the stimulation. Data were normalized to the total pool as defined by sypHy response to NH_4Cl application (ΔF_{NH4Cl}). All experiments and analyses were performed by an experimenter blind to the genotype.

Transmission EM. For glutaraldehyde fixation, DIV 14 neuronal cultures were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 37 °C for 7.5 min, and were processed in the Conventional Electron Microscopy Facility at Harvard Medical School. Sample preparation was performed as described in ref. 1.

For high-pressure freezing, DIV-14 neuronal cultures plated on 6-mm-diameter, 0.12-mm-thick carbon-coated sapphire coverslips were transferred to extracellular solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Hepes·NaOH, pH 7.4, ~310 mOsm, and were frozen with a Leica EM ICE high-pressure freezer. Frozen samples were freeze substituted (in acetone containing 1% osmium tetroxide, 1% glutaraldehyde, and 1% H_2O), embedded in Epon, and sectioned at 50 nm by the Conventional Electron Microscopy Facility at Harvard Medical School. All samples were imaged by using a JEOL 1200EX transmission electron microscope with an AMT 2k CCD camera. All images were quantified as described in ref. 1 by using SynapseEM, a MATLAB macro provided by M. Verhage and J. Broeke, Center for Neurogenomics and Cognitive Research, Department of Functional Genomics, Vrije Universiteit Amsterdam, Amsterdam. Docked vesicles were defined as vesicles for which the vesicle membrane touches the presynaptic plasma membrane, and tethered vesicles were defined as vesicles within 100 nm of the active zone. Frequency distributions of the nearest distance of a vesicle to the plasma membrane opposed to the PSD were plotted in 10-nm bins. All experiments were performed by an experimenter blind to the genotype.

Resource Sharing and Statistics. Materials, code, and data may be shared with requesting investigators according to NIH Resource Sharing guidelines. Levels of statistical significance were set at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$. Student's t tests were used for pairwise genotype comparisons of means. For multiple comparisons in rescue experiments, one-way ANOVA followed by Holm–Šídák multiple-comparisons tests were used. Two-way ANOVA followed by Holm–Šídák multiple-comparisons tests were used to compare the expression levels and localization parameters of various proteins in STED imaging, in the force plate experiments, and in the electrophysiological field recordings. The number of observations and significance values for each variable of the ANOVAs are given in the figure legend, and significance values of posttests are indicated by star symbols within the figures.

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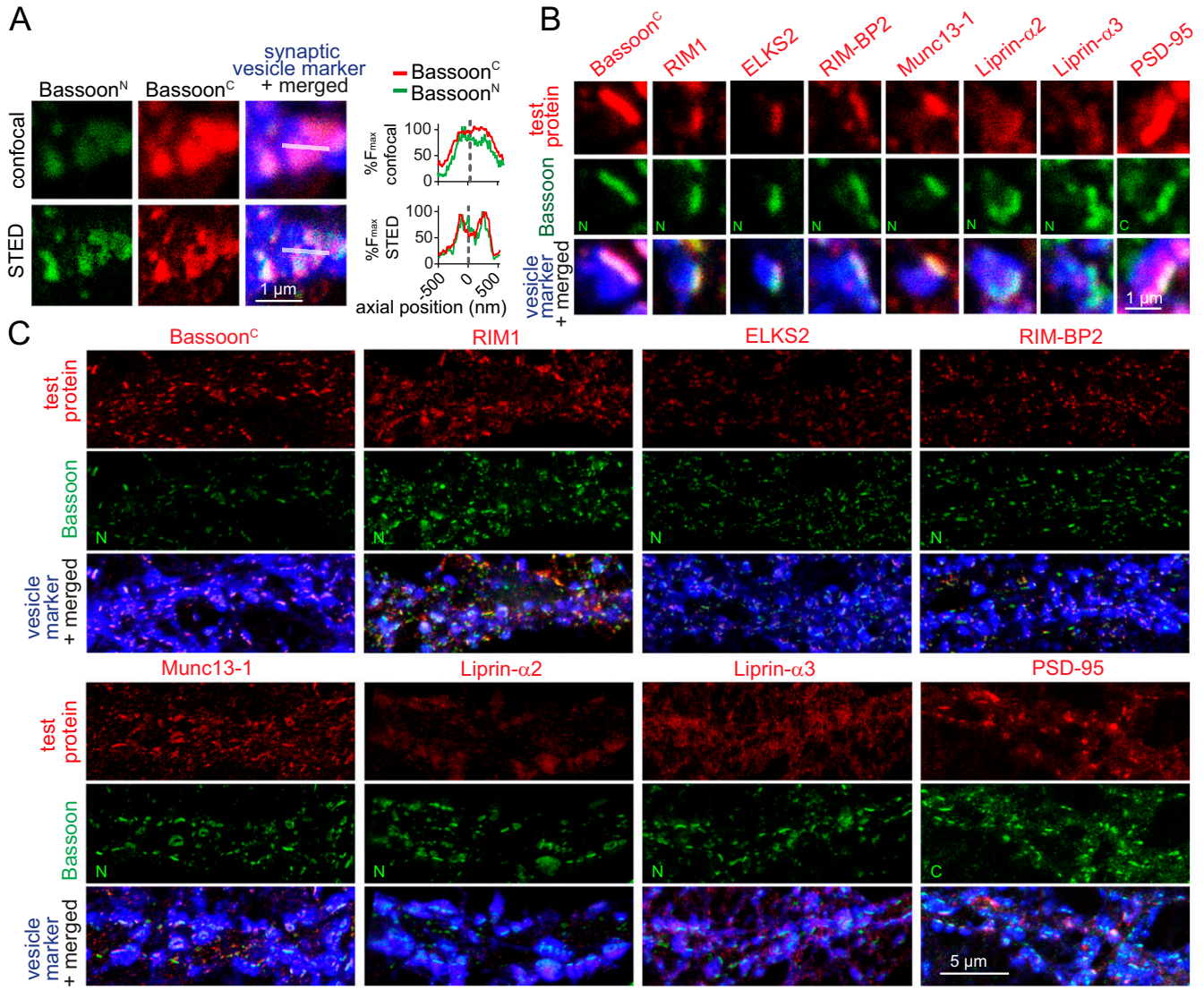

Fig. S1. Synapse in face view and additional images for Fig. 1. (*A*) Example images and line scans of a synapse stained for Bassoon^N and Bassoon^C in face view. Confocal scanning shows disk-like Bassoon structures, whereas STED images of the same synapse resolve a distinct doughnut-shaped structure of the Bassoon cluster. Synapsin was used as a marker to label the boundary of presynaptic boutons and was imaged by confocal microscopy. (*B*) Confocal scans of the STED images shown in Fig. 1*C*. (*C*) Overview STED images of cultured hippocampal neurons shown in Fig. 1.

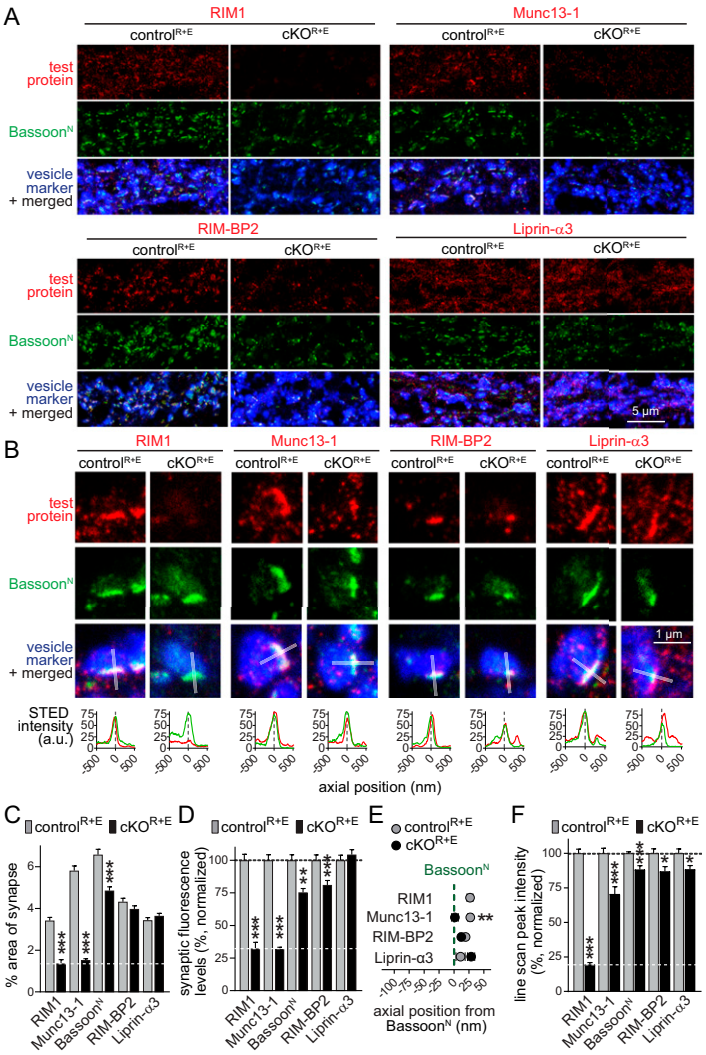

Fig. S2. Liprin-α3 localization at cKO^{R+E} synapses. (*A*) Representative overview STED images of various test proteins in control^{R+E} and cKO^{R+E} synapses (1). For generation of cKO^{R+E} synapses, neurons with floxed alleles for RIM1, RIM2, ELKS1α, and ELKS2α were infected with lentiviruses expressing cre recombinase (cKO^{R+E}) and inactive control viruses (control^{R+E}). We have previously established that this leads to strongly disrupted active zones, with almost complete loss of Munc13, ∼80% loss of Bassoon, ∼60% loss of Piccolo, and ∼50% loss of RIM-BP2 as assessed by Western blotting and confocal microscopy (1). *vGlut1* antibodies were used to label excitatory nerve terminals imaged simultaneously with confocal microscopy. (*B*) Representative side-view STED images and line profiles [as in Fig. 1, but showing arbitrary units (a.u.)] of various test proteins in control and cKO^{R+E} synapses. Dashed line at zero indicates the localization of the Bassoon^{N} peak. (*C*) Analysis of the percentage area covered by test proteins within synapses as defined by the vesicular marker vGlut1. All analyses presented here are biased toward synapses with more Bassoon because Bassoon is required for identification of side-view synapses and active-zone–like structures, but many cKO^{R+E} synapses have little Bassoon (1). Dashed white line indicates the noise level as assessed by RIM1 staining: RIM1 (control^{R+E}, $n = 462$ synapses/3 cultures; cKO^{R+E}, $n = 465/3$); Munc-13–1 (control^{R+E}, $n = 750/3$; cKO^{R+E}, $n = 607/3$); RIM-BP2 (control^{R+E}, $n = 617/3$; cKO^{R+E}, $n = 792/3$); Bassoon (control^{R+E}, $n = 519/3$; cKO^{R+E}, $n = 724/3$); and Liprin-α3 (control^{R+E}, $n = 519/3$; cKO^{R+E}, $n = 724/3$). Statistical significance was determined by two-way ANOVA (genotype, test protein, and interaction significant at $P \leq 0.001$) followed by Holm–Šídák posttests (reported in figure) for multiple comparisons. (*D*) Analysis of synaptic fluorescence levels integrated over the area labeled by the excitatory synaptic vesicle marker vGlut1, expressed normalized to the average of control^{R+E} values (dashed black line). Dashed white line indicates the noise level as assessed by RIM1 staining. Numbers of synapses/cultures are as in *C*. The reduction in Bassoon staining is somewhat milder than that observed previously (1), which is likely the result of a selection bias because here we select for synapses that contain Bassoon, and may also be influenced by the differences between STED and confocal microscopy, e.g., nonlinearities associated with STED imaging. Statistical significance was determined by two-way ANOVA (genotype, test protein, and interaction significant at $P \leq 0.001$) followed by Holm–Šídák posttests (reported in figure) for multiple comparisons. (*E*) Axial positions of the test proteins relative to Bassoon^{N} (dashed line) determined by the peak localization in intensity profiles of individual images of synapses in side view. Negative values are proximal of Bassoon^{N} inside the nerve terminal, and positive values are distal toward the postsynaptic compartment: RIM1 (control^{R+E}, $n = 83/3$); Munc13-1 (control^{R+E}, $n = 86/3$; cKO^{R+E}, $n = 77/3$); RIM-BP2 (control^{R+E}, $n = 86/3$; cKO^{R+E}, $n = 92/3$); and Liprin-α3 (control^{R+E}, $n = 88/3$; cKO^{R+E}, $n = 108/3$). RIM1 peak localization for cKO^{R+E} is not shown because it is by definition artifactual as a result of absence of the antigen. Statistical significance determined by two-way ANOVA (genotype significant at $P \leq 0.001$; test protein significant at $P \leq 0.01$; interaction significant at $P \leq 0.001$) followed by Holm–Šídák multiple-comparisons posttests (reported in figure) for peak localization of each test protein in cKO^{R+E} neurons to control^{R+E} neurons. (*F*) Peak intensity of cKO^{R+E} line scans normalized to control^{R+E} (dashed black line). Dashed white line indicates the noise level as assessed by RIM1 staining: RIM1 (control^{R+E}, $n = 83/3$; cKO^{R+E}, $n = 57/3$); Munc13-1 (control^{R+E}, $n = 86/3$; cKO^{R+E}, $n = 77/3$); Bassoon^{N} (control^{R+E}, $n = 343/12$; cKO^{R+E}, $n = 334/12$); RIM-BP2 (control^{R+E}, $n = 86/3$; cKO^{R+E}, $n = 92/3$); and Liprin-α3 (control^{R+E}, $n = 88/3$; cKO^{R+E}, $n = 108/3$). Statistical significance determined by two-way ANOVA (genotype, test protein, and interaction significant at $P \leq 0.001$) followed by Holm–Šídák multiple-comparisons posttests (reported in figure) for comparing peak intensity of each protein in cKO^{R+E} neurons to control^{R+E} neurons. All data are means ± SEM (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$). Although these data indicate that Liprin-α3 localization is not strongly impaired upon KO of RIM and ELKS, it is possible that mild impairments exist but are not detected as a result of a selection bias by only including Bassoon-positive cKO^{R+E} synapses.

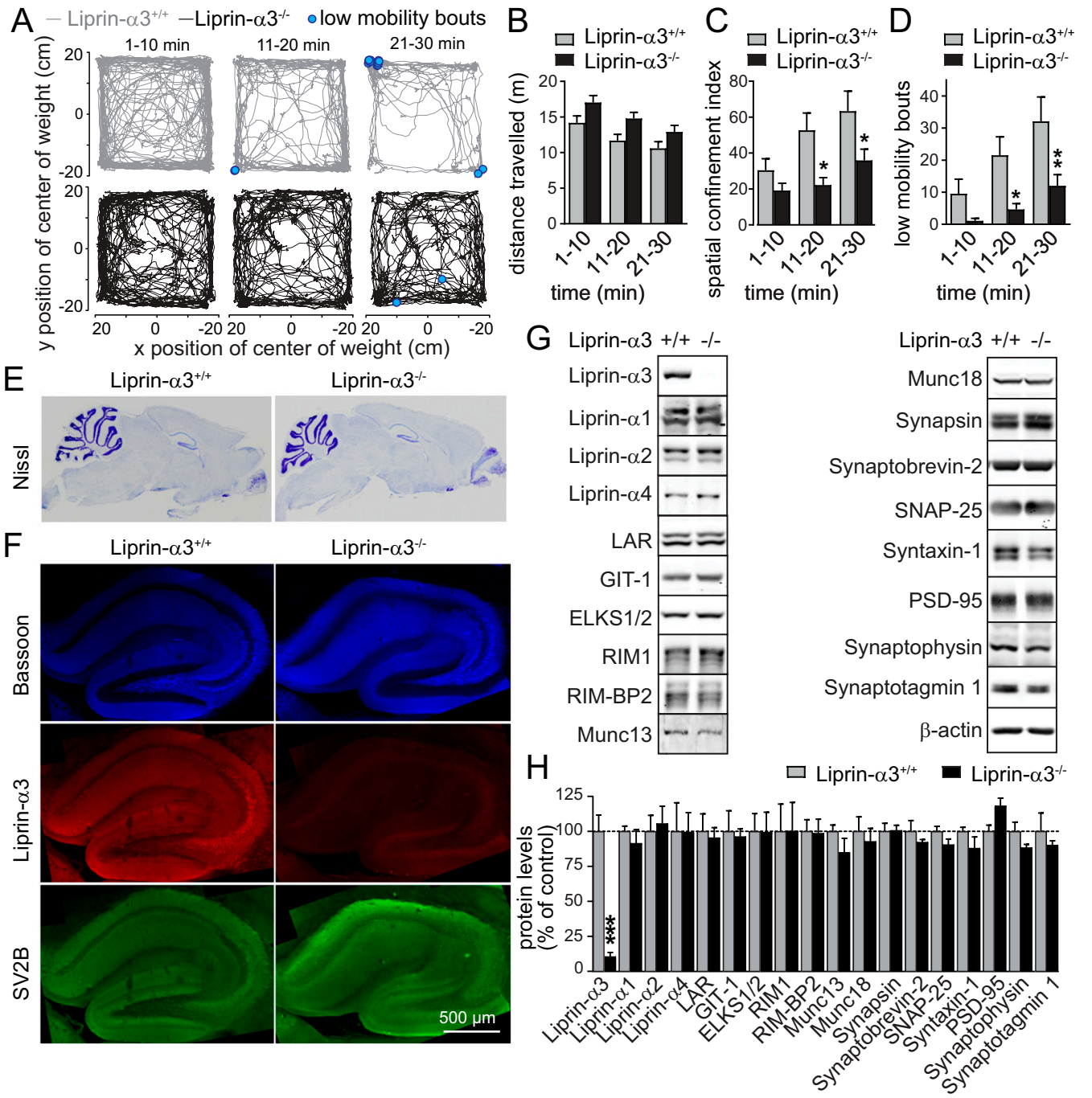

Fig. S3. Analysis of locomotion and hippocampal morphology and protein composition in Liprin-α3$^{-/-}$ mice. (*A*) Representative trajectories of a Liprin-α$^{-/-}$ mouse and a Liprin-α3$^{+/+}$ littermate control mouse from a continuous single trial recording of 30 min of open-field behavior in a 40-cm × 40-cm force-plate actometer, separated in three 10-min bins. Blue circles indicate low-mobility bouts. (*B–D*) Quantitative analysis of the distance traveled (*B*), the spatial confinement (*C*), and the number of low-mobility bouts (*D*) in trajectories (n = 9 littermate pairs). Statistical significance determined by two-way ANOVA (*B*, genotype significant at $P \leq 0.05$; time significant at $P < 0.001$; interaction n.s.; *C*, genotype significant at $P \leq 0.05$; time significant at $P < 0.001$; interaction significant at $P \leq 0.05$; *D*, genotype significant at $P \leq 0.05$; time significant at $P < 0.001$; interaction n.s.) followed by Holm–Šídák multiple-comparisons posttests (reported in figures). (*E*) Images of sagittal brain sections of 3-wk-old littermate Liprin-α3$^{+/+}$ and Liprin-α3$^{-/-}$ mice labeled by Nissl staining. (*F*) Images of hippocampi in sagittal brain sections of 3-wk-old littermate Liprin-α3$^{+/+}$ and Liprin-α3$^{-/-}$ mice labeled for Bassoon^{N}, Liprin-α3, and the synaptic vesicle protein SV2B. Note that all immunostainings were done with the Liprin-α3 antibody 3, which mildly cross-reacts with other Liprin-α proteins in Western blotting, but shows a strong decrease in immunostaining. (*G*) Representative Western blots using fluorescent secondary antibodies of homogenates from the hippocampi of a Liprin-α3$^{-/-}$ mouse and a Liprin-α3$^{+/+}$ littermate control mouse. (*H*) Quantification of fluorescent signals shown in *G*. All fluorescent signals were normalized to a β-actin loading control run in the same lane and expressed normalized to the average of Liprin-α3$^{+/+}$ mice (dashed black line; n = 3 littermate pairs). All data are means ± SEM [*$P \leq 0.05$, **$P \leq 0.01$, and ***$P < 0.001$; n.s., not significant; two-way ANOVA followed by Holm–Šídák multiple-comparisons posttests (*B–D*) or by Student's t test (*H*)].

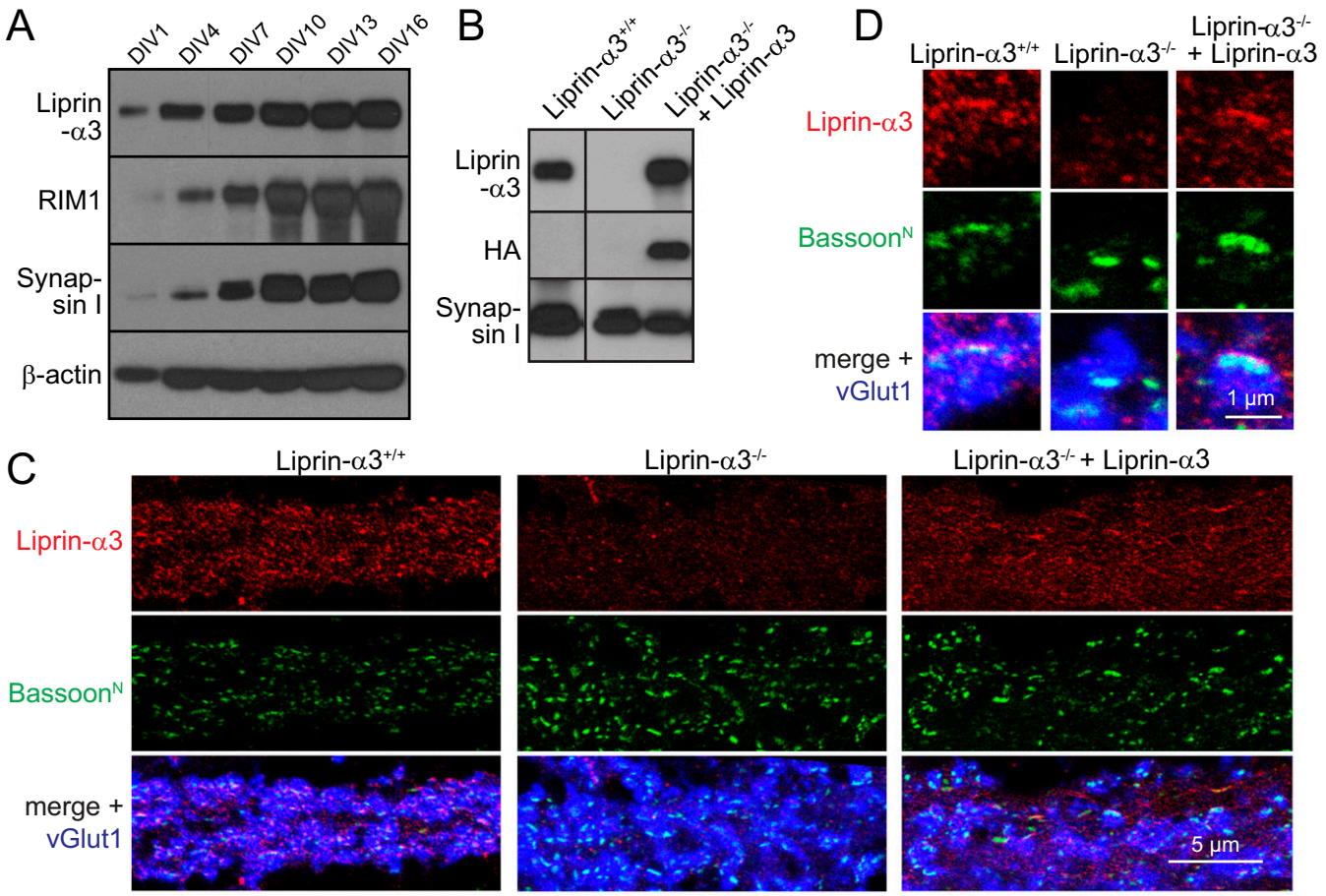

Fig. S4. Expression and localization of rescue Liprin-α3 upon lentiviral transduction at DIV 1. (*A*) Time course of expression of Liprin-α3, RIM1, and Synapsin I in cultured hippocampal neurons harvested at indicated DIVs. (*B*) Liprin-α3$^{+/+}$ neurons, Liprin-α3$^{-/-}$ neurons, and Liprin-α3$^{-/-}$ neurons rescued with lentiviral expression of Liprin-α3 were used for imaging of synaptic vesicle exocytosis. Western blots show Liprin-α3 expression in Liprin-α3$^{+/+}$ neurons, Liprin-α3$^{-/-}$ neurons, and Liprin-α3$^{-/-}$ neurons transduced with lentiviral rescue Liprin-α3 (human synapsin promoter driven, HA-tagged) at DIV 1. Neurons were harvested for Western blotting at DIV 14. All bands for a given antibody are from the same blot displayed with identical contrast and intensity settings. Images were cropped to remove additional irrelevant lanes. (*C* and *D*) Representative STED images of hippocampal neurons immunostained with Liprin-α3 and Bassoon^{N} antibodies showing localization of rescue Liprin-α3 within nerve terminals in overview images (*C*) and at high magnification (*D*). vGlut1 antibodies were used to label the synaptic vesicle cluster and the vGlut1 signal was imaged with confocal microscopy.

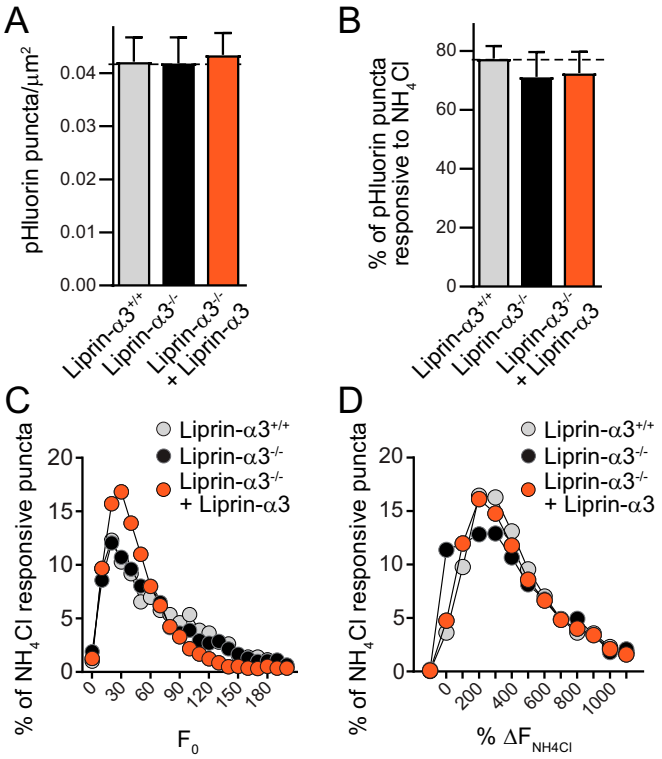

Fig. S5. Additional analyses of sypHy imaging data. (*A*) Average sypHy puncta density in cultured Liprin-α3$^{+/+}$ neurons, Liprin-α3$^{-/-}$ neurons, and Liprin-α3$^{-/-}$ neurons rescued with lentiviral expression of Liprin-α3. Numbers of synapses/cultures are as in Fig. 3. (*B*) Percentage of sypHy puncta responsive to NH_4Cl. Numbers of synapses/cultures are as in Fig. 3. (*C* and *D*) Frequency distributions of fluorescent signal of sypHy puncta at baseline (*C*; F_0 before stimulation) and upon NH_4Cl application (*D*). Numbers of synapses/cultures are as in Fig. 3 *C*–*J*.

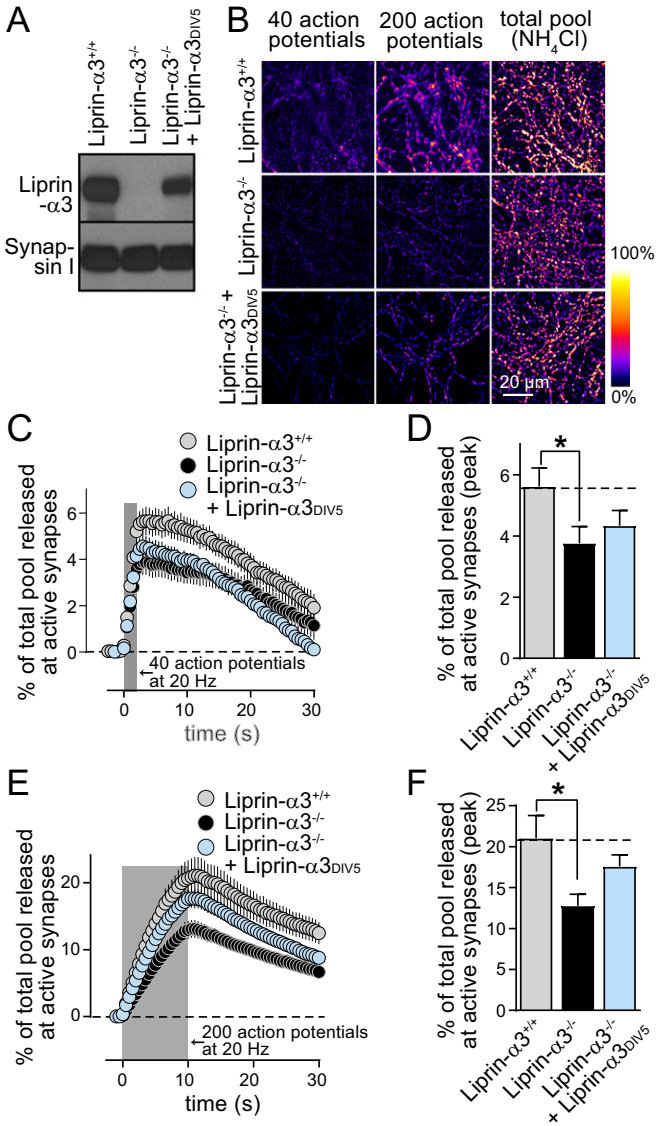

Fig. S6. DIV-5 transduction with Liprin-α3 rescue viruses impairs expression and rescue. Liprin-α3$^{+/+}$ neurons, Liprin-α3$^{-/-}$ neurons, and Liprin-α3$^{-/-}$ neurons expressing Liprin-α3 from a lentivirus were used for imaging of synaptic vesicle exocytosis. In the experiment shown in this figure, Liprin-α3$^{-/-}$ neurons were transduced with lentiviral Liprin-α3 (human synapsin promoter driven, HA-tagged) at DIV 5 (Liprin-α3$^{-/-}$ + Liprin-α3$_{DIV5}$). (*A*) Representative Western blot of expression of Liprin-α3. Note that the lentiviral rescue Liprin-α3 expression level appears lower than in neurons transduced with the same virus at DIV 1 (Fig. S4*B*). (*B*) Pseudocolored images of sypHy-expressing Liprin-α3$^{+/+}$ and Liprin-α3$^{-/-}$ neurons, and Liprin-α3$^{-/-}$ neurons rescued with lentiviral expression of Liprin-α3 at DIV 5. Neurons were stimulated with 40 or 200 action potentials and dequenched with NH_4Cl. Images represent peak fluorescence change. (*C* and *D*) Quantification of fluorescence changes in active synapses of neurons stimulated with 40 action potentials at 20 Hz (*C*, gray area) showing the time course of the mean fluorescence change in active synapses as a percentage of the fluorescence increase upon NH_4Cl application (*C*) and the peak response of active synapses (*D*). Liprin-α3$^{+/+}$, n = 1,585 NH_4Cl-responsive synapses/1,071 active synapses/9 coverslips/3 independent cultures; Liprin-α3$^{-/-}$, 1583/954/9/3; Liprin-α3$^{-/-}$ + Liprin-α3$_{DIV5}$, n = 1,709/977/9/3. (*E* and *F*) Quantification as in *C* and *D* but for neurons stimulated with 200 action potentials. Liprin-α3$^{+/+}$, n = 1,585/1,117/9/3; Liprin-α3$^{-/-}$, 1,583/1,173/9/3; Liprin-α3$^{-/-}$ + Liprin-α3, n = 1,709/1,282/9/3. All data are means ± SEM (*$P \leq 0.05$, one-way ANOVA followed by Holm–Šídák multiple-comparisons test comparing each condition vs. Liprin-α3$^{-/-}$). The number of coverslips was used as a basis for statistics in pHluorin imaging experiments. This experiment reveals that only DIV-1 transduction with lentiviruses expressing Liprin-α3 (Fig. 3 and Fig. S4) is sufficient to fully restore Liprin-α3 expression and to provide significant rescue in Liprin-α3$^{-/-}$ neurons, whereas later transduction leads to lower expression and inefficient rescue.

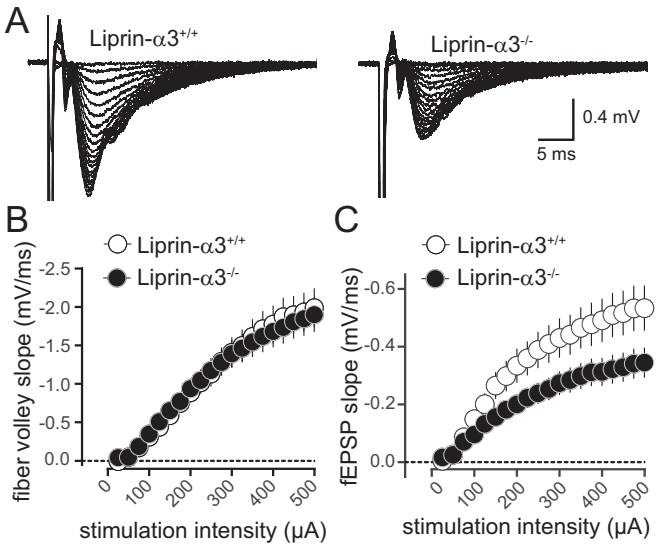

Fig. S7. Analyses of synaptic transmission in brain slices of Liprin-α3–KO mice. (*A*) Example traces of field ESPS (fEPSP) recordings in stratum radiatum of area CA1 in acute slices of the hippocampus in Liprin-$\alpha3^{+/+}$ and Liprin-$\alpha3^{-/-}$ littermate mice. Schaffer collaterals were stimulated with a concentric stimulation electrode at increasing stimulation intensities. (*B* and *C*) Quantification of the fiber volley slope (*B*) and the fEPSP slope (*C*) as a function of stimulation intensity for the experiment shown in *A* (Liprin-$\alpha3^{+/+}$, $n = 25$ slices/7 mice; and Liprin-$\alpha3^{-/-}$, $n = 29/7$). Statistical significance determined by two-way ANOVA [*B*, genotype not significant (n.s.), intensity significant at $P \leq 0.001$, interaction n.s.; *C*, genotype significant at $P \leq 0.001$, intensity significant at $P \leq 0.001$, interaction n.s.]. All data are means ± SEM.

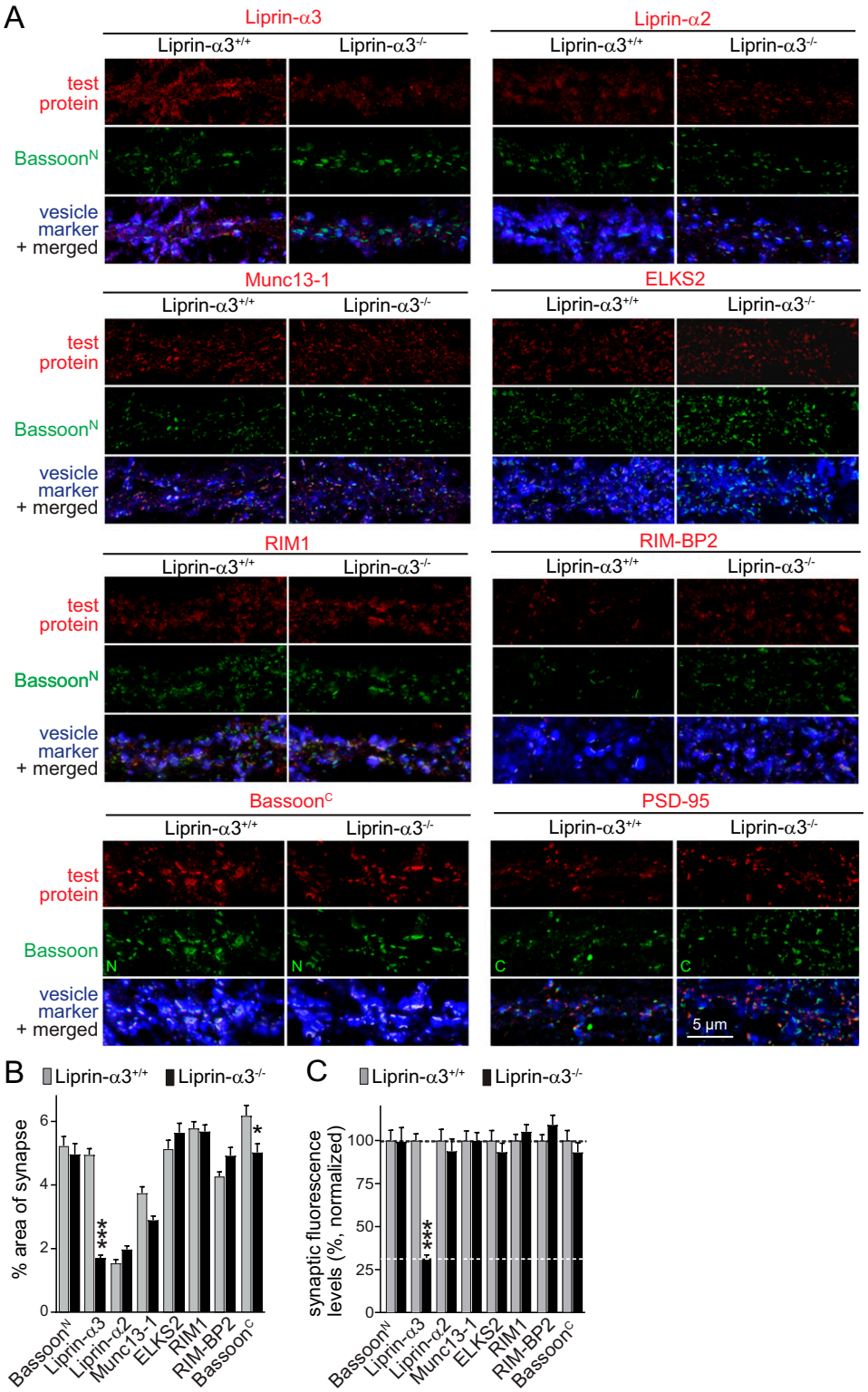

Fig. S8. Additional STED microscopy data related to Fig. 5. (*A*) Overview STED images of the experiment shown in Fig. 5. (*B*) Analysis of the percentage of the area covered by test proteins within synapses as defined by the vesicular marker vGlut1: Bassoon^{N} ($^{+/+}$, $n = 533$ synapses/3 cultures; $^{-/-}$, $n = 332/3$); Liprin-α3 ($^{+/+}$, $n = 485/3$; $^{-/-}$, $n = 550/3$); Liprin-α2 ($^{+/+}$, $n = 546/3$; $^{-/-}$, $n = 729/3$); Munc13-1 ($^{+/+}$, $n = 599/3$; $^{-/-}$, $n = 666/3$); ELKS2 ($^{+/+}$, $n = 697/3$; $^{-/-}$, $n = 589/3$); RIM1 ($^{+/+}$, $n = 642/3$; $^{-/-}$; $n = 360/3$); RIM-BP2 ($^{+/+}$, $n = 744/3$; $^{-/-}$, $n = 730/3$); and Bassoon^{C} ($^{+/+}$, $n = 533/3$; $^{-/-}$, $n = 332/3$). Statistical significance was determined by two-way ANOVA (genotype, test protein, and interaction significant at $P \leq 0.001$) followed by Holm–Šídák posttests for multiple comparisons (reported in figure). (*C*) Analysis of fluorescence levels integrated over the synapse area normalized to the average of Liprin-α3$^{+/+}$ synapses. Numbers of synapses/cultures are as in *B*. Statistical significance was determined by two-way ANOVA (genotype significant at $P \leq 0.01$, test protein significant at $P \leq 0.001$, interaction significant at $P \leq 0.001$) followed by Holm–Šídák posttests for multiple comparisons (reported in figure). All data are means ± SEM (*$P \leq 0.05$ and ***$P \leq 0.001$).

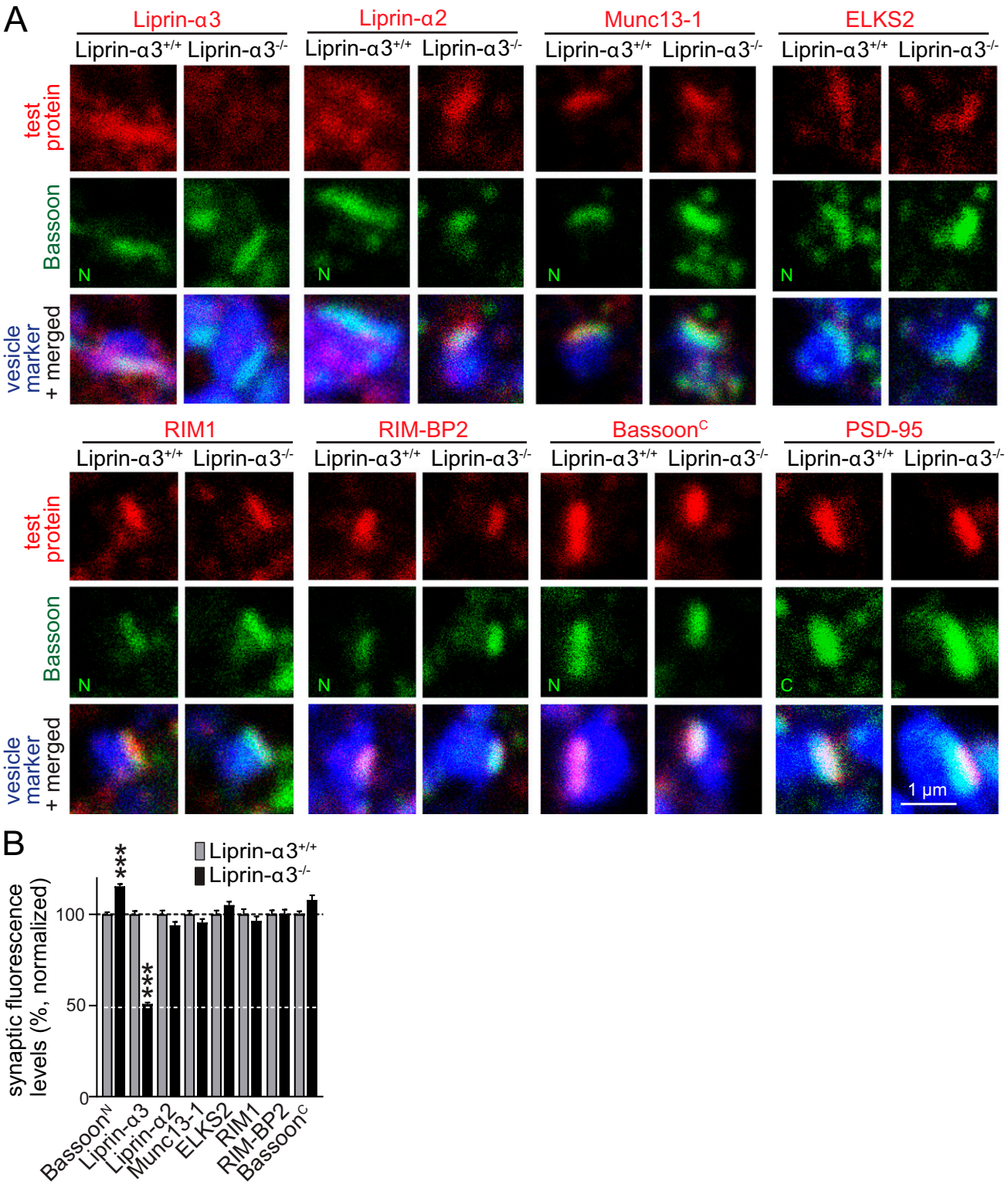

Fig. S9. Analysis of synaptic protein levels in confocal images of Liprin-α3$^{-/-}$ synapses. Nonlinearities in the signal intensity may affect protein level measurements in STED images. To circumvent this limitation, we analyzed confocal scans of the experiment shown in Fig. 5 to assess synaptic fluorescence levels. (*A*) Confocal images of the same synapses that are shown in Fig. 5*A*. (*B*) Analyses of synaptic fluorescence in confocal images, plotted as the mean fluorescence levels within the synapse area defined by the vesicle marker normalized to the average of Liprin-α3$^{+/+}$ synapses: Bassoon^{N} ($^{+/+}$, n = 3,150 synapses/8 cultures; $^{-/-}$, n = 3,330/8); Liprin-α3 ($^{+/+}$, n = 973/3; $^{-/-}$, n = 550/3); Liprin-α2 ($^{+/+}$, n = 472/3; $^{-/-}$, n = 410/3); Munc-13 ($^{+/+}$, n = 599/3; $^{-/-}$, n = 556/3); ELKS2 ($^{+/+}$, n = 559/3; $^{-/-}$, n = 605/3); RIM1 ($^{+/+}$, n =517/3; $^{-/-}$, n = 360/3); RIM-BP2 ($^{+/+}$, n = 394/3; $^{-/-}$, n = 499/3); and Bassoon^{c} ($^{+/+}$, n = 533/3; $^{-/-}$, n = 425/3). Statistical significance was determined by two-way ANOVA (genotype, test protein, and interaction significant at $P \leq 0.001$) followed by Holm–Šídák posttests for multiple comparisons (reported in figure).