PKC-phosphorylation of Liprin-α3 triggers phase separation and controls presynaptic active zone structure

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The active zone of a presynaptic nerve terminal defines sites for neurotransmitter release. Its protein machinery may be organized through liquid-liquid phase separation, a mechanism for the formation of membrane-less subcellular compartments. Here, we show that the active zone protein Liprin-α3 rapidly and reversibly undergoes phase separation in transfected HEK293T cells. Condensate formation is triggered by Liprin-α3 PKC-phosphorylation at serine-760, and RIM and Munc13 are co-recruited into membrane-attached condensates. Phospho-specific antibodies establish phosphorylation of Liprin-α3 serine-760 in transfected cells and mouse brain tissue. In primary hippocampal neurons of newly generated Liprin-α2/α3 double knockout mice, synaptic levels of RIM and Munc13 are reduced and the pool of releasable vesicles is decreased. Re-expression of Liprin-α3 restored these presynaptic defects, while mutating the Liprin-α3 phosphorylation site to abolish phase condensation prevented this rescue. Finally, PKC activation in these neurons acutely increased RIM, Munc13 and neurotransmitter release, which depended on the presence of phosphorylatable Liprin-α3. Our findings indicate that PKC-mediated phosphorylation of Liprin-α3 triggers its phase separation and modulates active zone structure and function.
Membrane-free subcellular compartments can form through liquid–liquid phase separation, a process in which multivalent, low affinity interactions enable mixing of proteins into liquid condensates. These condensates maintain high local protein concentrations and enable dynamic rearrangements and exchange of protein with the environment. Past work has established that protein complexes for many cellular processes, ranging from gene transcription to synaptic transmission, can be organized as phase condensates. It has remained challenging, however, to establish how phase separation controls intracellular functions.

Within a synapse, neurotransmitter release is restricted to specialized presynaptic structures called active zones. These membrane-attached, dense scaffolds are formed by the multi-domain proteins RIM, Munc13, RIM-BP, Piccolo/Bassoon, ELKS, and Liprin-a, and are essential for the sub-millisecond precision of synaptic vesicle exocytosis. While many release mechanisms are established, key questions on how these dense scaffolds assemble, and how they remain dynamic to support the high spatiotemporal demands of the synaptic vesicle cycle remain unanswered. Purified RIM1 and RIM-BP2 form liquid condensates in vitro, indicating that active zones may assemble following phase transition principles. Other synaptic compartments may also be organized through phase separation. Whether phase separation occurs at synapses in vivo and whether it is important for controlling synaptic release, however, remains uncertain.

Liprin-a proteins have received particular attention as assembly molecules because they control presynaptic structure of invertebrate synapses. They contain N-terminal coiled-coils with Liprin-a homology (LH) regions and three C-terminal sterile alpha motifs (SAM domains). Mammals have four genes (Ppif1-Ppif4) that encode Liprin-a1 to Liprin-a4, of which Liprin-a2 and Liprin-a3 are strongly expressed in the brain and co-localize with active zone markers, shRNA knockdown of Liprin-a2 or genetic deletion of Liprin-a3 causes loss of presynaptic proteins, similar to assembly defects after ablation of the single invertebrate gene. Liprin-a proteins are not part of the same protein complex. An upstream function aligns well with the broad interaction repertoire of Liprin-a, which includes active zone proteins, motors, cell adhesion proteins, and cytoskeletal elements. Liprin-a interactions are further regulated by phosphorylation, making these proteins candidate effectors of kinase pathways that control exocytosis, for example of protein kinase A (PKA), phospholipase C (PLC)/protein kinase C (PKC), or Ca2+/calmodulin-dependent kinase II (CaMKII) signaling. In aggregate, previous data suggest that Liprin-a may connect active zone assembly to pathways for synaptic development and plasticity.

Here, we find that PKC phosphorylation of serine-760 (S760) of Liprin-a3 rapidly triggers Liprin-a3 phase separation. PMCA and Munc13-1, two important active zone proteins, are co-recruited into plasma membrane attached phase condensates, reminiscent of active zone formation. Ablation of Liprin-a2 and Liprin-a3 using double knockout mice that were generated for this study leads to reduced levels of RIM and Munc13-1 at synapses, impaired vesicle docking and a decreased pool of readily releasable vesicles. Abolishing Liprin-a3 phosphorylation via a single point mutation prevents its phase separation and its ability to reverse defects in active zone structure and in the pool of releasable vesicles. Similarly, we discover a rapid increase of RIM and Munc13-1 at the active zone upon activation of PKC, which necessitates Liprin-a3 phosphorylation. We propose a working model in which active zone structure is dynamically modulated by Liprin-a3 phase condensation under the control of PKC.

**Results**

Liprin-a3 rapidly undergoes phase separation under the control of PLC/PKC signaling in transfected HEK293T cells. Because Liprin-a3 is regulated by phosphorylation and controls active zone assembly, we asked whether Liprin-a3 is modulated by kinase pathways to control release site structure. Prominent presynaptic pathways operate via PKA, PLC/PKC, and CaMKII signaling. We expressed mVen-tagged Liprin-a3 in HEK293T cells and investigated whether activation or inhibition of these pathways alters Liprin-a3 distribution. Under basal conditions, mVen-Liprin-a3 is predominantly soluble. Strikingly, after addition of the diacylglycerol analog phorbol 12-myristate 13-acetate (PMA), Liprin-a3 formed spherical condensates within minutes (Fig. 1a, Supplementary Fig. 1a, and Supplementary Movie 1). PMA mimics PLC-induced generation of diacylglycerol and activates PKC, suggesting that Liprin-a3 may be phosphorylated by PKC. This effect was not observed for other manipulations, including inhibition of PKC, or activation or inhibition of PKA or CaMKII (Fig. 1a and Supplementary Fig. 1b). The reorganization of Liprin-a3 into droplets occurred in all cells within minutes, was reversible upon washout, and droplet formation was independent of the mVen-tag (Fig. 1b, c and Supplementary Fig. 1c, d).

Formation of spherical droplets is indicative of liquid–liquid phase separation. Principles of liquid dynamics and phase separation predict that liquid condensates dynamically exchange molecules with the cytosol, undergo fusion and fission, relax into spherical shapes, and lack membrane boundaries between the condensed phase and the cytosol. First, to test exchange of molecules, we assessed fluorescence recovery after photobleaching (FRAP), as implemented before to study liquid phases of synaptic proteins. Individual condensates recovered to ~40% of the initial fluorescence at a fast rate (t1/2 max recovery = 16.1 ± 0.4 s), and a second bleaching of the same condensates resulted in near-complete recovery at a similar rate (t1/2 max recovery = 15.8 ± 0.5 s), establishing that the mobile fraction remains fully mobile (Fig. 1d, e). Second, we detected fusion and fission reactions of Liprin-a3 droplets (Supplementary Fig. 1e, f and Supplementary Movie 1). Droplet fusion was followed by exponential relaxation (Fig. 1f, g), as expected for liquid condensates. Third, to assess whether these fluorescent droplets are indeed membrane-free condensates, we used correlative light-electron microscopy (CLEM). Liprin-a3 condensates formed electron dense structures that were not surrounded by lipid bilayers (Fig. 1h, i). We conclude that Liprin-a3 forms liquid condensates as a function of PLC/PKC signaling.

**PKC phosphorylates Liprin-a3 at S760.** We hypothesized that PMA triggers PKC activation followed by phosphorylation of Liprin-a3 to induce phase separation. To investigate whether Liprin-a3 is a PKC substrate, we purified GST-fusion proteins covering the entire Liprin-a3 protein, and incubated them with 32P-labeled ATP and recombinant PKC (Fig. 2a, b). The linker region between the N-terminal Liprin homology regions and the C-terminal SAM domains most efficiently incorporated 32P, and mass spectrometry identified five phosphorylated serine residues (S650, S751, S760, S763, and S764, Supplementary Fig. 2a). Notably, S760, but not other residues, was surrounded by a PKC consensus sequence, and this sequence was conserved in rat, human, and mouse Liprin-a3 (Supplementary Fig. 2b). In other Liprin-a proteins, a glycine residue was present instead of serine
at the corresponding position. To determine whether any of these residues is responsible for phase transition, we engineered point mutations in mVenus-Liprin-α3 to abolish phosphorylation and expressed these constructs in HEK293T cells. S760A and S764A Liprin-α3 were incapable of PMA-induced droplet formation, while other point mutations did not impair it (Supplementary Fig. 2c).

To test whether these residues are phosphorylated, we generated anti-phospho-S760 and -S764 Liprin-α3 antibodies. The antibodies detected a band at ~150 kDa in transfected HEK293T cells. Upon PMA addition, anti-phospho-S760-Liprin-α3 antibody signals increased, and disappeared when co-incubated with PKC blockers (Fig. 2c). Anti-phospho-S764 antibody signals were unaffected by the same manipulations (Supplementary Fig. 2d). Furthermore, when we incubated a recombinant Liprin-α3 fragment with PKC, phospho-S760 antibodies detected a signal increase while phospho-S764 did not (Supplementary Fig. 2e), making it unlikely that S764 is directly phosphorylated by PKC. Phospho-S760 Liprin-α3 was...
Liprin-α3 undergoes liquid-liquid phase separation upon activation of PLC/PKC signaling pathways. a Confoal images of fixed HEK293T cells transfected with mVenus-Liprin-α3, without treatment or in the presence of forskolin (to activate PKA), PMA (to activate PLC/PKC), caffeine (to activate CamKII), H-89 (to inhibit PKA), bisindolylmaleimide-I (Bis-I, to inhibit PKC), or KN-93 (to inhibit CamKII), representative cells from two to three independent transfections. b, c Example confocal images (b) and quantification of % cells containing droplets (c) of fixed HEK293T cells transfected with mVenus-Liprin-α3 or mVenus alone. Cells were fixed 15 min after PMA addition or 6 h after washout, N = 21 images/3 independent batches of cells each for mVenus-Liprin-α3, 18/3 each for mVenus, p values: mVenus-Liprin-α3 (tested against −PMA), +PMA 8e-8 (**), washout 0.58; mVenus, 0.82. d, e Example time-lapse images (d) and quantification (e) of the fluorescence recovery after photobleaching (FRAP) of mVenus-Liprin-α3 condensates in live, transfected HEK293T cells. Two consecutive bleach steps were applied, N = 30 droplets/3 independent transfections. f, g Example live time-lapse confocal images of a Liprin-α3 condensate undergoing fusion (f) and quantification of the aspect ratio over time (g) as a measure of relaxation after fusion, N = 10 fusion events, the exponential fit (red dotted line) is described with the formula AR = 0.93 ± 1.48 ± exp(−t/7.41). h, i Correlative light-electron microscopy (CLEM) example image of a fixed HEK293T cell transfected with mVenus-Liprin-α3 and incubated with PMA showing an overview with multiple condensates (f) and individual droplets (g) magnified from the overview image (top) or independently acquired higher magnification images of the same droplets (bottom), a representative cell from a total of three cells (one transfection) that were assessed by CLEM is shown. Summary data in c, e, and g are mean ± SEM. Significance was assessed using Kruskal-Wallis tests with Holm post hoc comparison between all groups, and only results compared to the respective −PMA condition are reported in c. All tests were two-sided. For a time course of phase separation, phase separation of non-tagged Liprin-α3, and fusion and fission of Liprin-α3 condensates, see Supplementary Fig. 1 and Supplementary Movie 1.

not detected in Liprin-α3 knockout neuronal cultures (Fig. 2d), confirming antibody specificity. Together, these experiments establish that PKC phosphorylates Liprin-α3 at S760, but likely not at S764. It is possible that the S764 mutation abolishes S760 phosphorylation (and Liprin-α3 phase separation) indirectly, as the most common residue at the +4 position at PKC target sites is a serine, and this may be part of the consensus32. Alternatively, S764 may participate in molecular rearrangements that lead to phase separation of Liprin-α3. In vivo, phospho-S760 Liprin-α3 signals were detected in the frontal cortex, hippocampus, cerebellum, and brain stem with high perinatal levels that gradually decreased over time (Supplementary Fig. 2f). We conclude that PKC phosphorylates S760 of Liprin-α3 in vitro and in vivo.

S760 phosphorylation triggers liquid-liquid phase separation of Liprin-α3 in transfected HEK293T cells. We established that
PKC phosphorylates Liprin-α3 at S760. To test whether this site mediates phase separation, we generated phospho-dead (S760G Liprin-α3SG, using S→G substitution to make it similar to other Liprin-α proteins, Supplementary Fig. 2b) and phospho-mimetic (S760E, Liprin-α3SE) mutants. Liprin-α3SG abolished PKC-induced phase separation, and Liprin-α3SE formed constitutive condensates independent of PKC activation (Fig. 2e, f) that showed dynamic exchange of molecules with the cytosol (Fig. 2g, t1/2 recovery = 27.8 ± 0.9 s). We conclude that phosphorylation of S760 triggers liquid–liquid phase separation of Liprin-α3.

To evaluate whether phase separation may be shared by other vertebrate Liprin-α proteins, we first assessed whether intrinsically disordered regions (IDRs), which often drive phase condensation2,33, are detected. Mouse, rat, and human Liprin-α3 contained sequences reminiscent of IDRs at residues ~580 to ~790 (Supplementary Fig. 3a, b). Similarly, Liprin-α1, -α2, and -α4 also contained IDR sequences in the same region (Supplementary Fig. 3c). To test whether Liprin-α1, -α2, and -α4 undergo phase separation, we generated fluorophore-tagged versions of each of these proteins and assessed their distribution in transfected HEK293T cells. Liprin-α1 and -α4 appeared predominantly soluble, while Liprin-α2 had a droplet-like pattern (Supplementary Fig. 3d, e). Mimicking PLC/PKC signaling did not affect the distributions of Liprin-α1, -α2, or -α4, in line with a Liprin-α3-specific presence of S760. Furthermore, Liprin-α2 condensates did not recover after photobleaching (Supplementary Fig. 3f, g), indicating that they do not adopt liquid
Liprin-α3, Munc13, and RIM are co-recruited into phase condensates at the plasma membrane. a–c Example confocal images including line profiles of highlighted regions (a) and quantification (b) of phase condensates in fixed HEK293T cells transfected with cerulean-Liprin-α3, RIMα-mVenus, and Munc13-1-tdTomato in the absence or presence of PMA. Quantification of the number (b) and size (c) of protein condensates is shown, N = 276 (−PMA) or 405 (+PMA) objects/15 images/3 independent transfections, p values: b, 0.0011 (**); c, 2e-16 (**). d, e CLEM example images of a fixed HEK293T cell transfected with cerulean-Liprin-α3, RIMα-mVenus, and Munc13-1-tdTomato and incubated with PMA showing an overview (d) and detailed individual condensates (e) magnified from the overview image (e, top), and independently acquired images at higher magnification of the same condensates (e, bottom). Cerulean-Liprin-α3, which is consistently recruited to RIM/Munc13-containing condensates (e), is present in the transfection but not displayed because the fluorescence microscopy for CLEM lacked a laser for cerulean excitation, a representative cell from two cells (two transfections) that were assessed by CLEM is shown. f, g Example of FRAP experiment (f) and quantification (g) of droplets in live HEK293T cells transfected with cerulean-Liprin-α3 (cerulean-Liprin-α3), RIMα-mVenus and Munc13-1-tdTomato, N = 19 droplets/3 independent transfections. h Schematic of the assessment of effects of PKC activation on active zone assembly. i, j Example STED images (i) and quantification (j) of the intensity of endogenous Liprin-α3, RIM, and Munc13-1 at the active zone. Dynaspheres in side-view were identified by the active zone marker Bassoon (imaged by STED microscopy) aligned at the edge of a synaptic vesicle cluster marked by Synaptophysin (Syp, imaged by confocal microscopy). Corresponding STED intensity profiles are shown on the right of each image set. Peak intensities were measured in these intensity profiles and plotted in j, shown as arbitrary units (arbit. units). Liprin-α3: N = 71 synapses/3 independent cultures (−PMA) and 65/3 (+PMA); RIM: N = 55/3 (−PMA) and 54/3 (+PMA); Munc13-1 N = 46/3 (−PMA) and 47/3 (+PMA). p values: Liprin-α3, 0.0027 (**); RIM, 0.00017 (***); Munc13-1, 0.0022 (**). Data were shown as mean ± SEM. Significance was assessed using two-sided Mann–Whitney rank sum tests in b, c, and j. For assessment of single and double transfections, condensate formation in the presence of PKC inhibitors, and FRAP without PMA treatment see Supplementary Fig. 5. For STED analysis workflow, peak positions of each protein, and assessment of Liprin-α3 levels using an independent antibody, see Supplementary Fig. 6.

Liprin-α3, RIM1α, and Munc13-1 are co-recruited into membrane-attached liquid condensates in transfected HEK293T cells. If phase separation of Liprin-α3 participates in the control of active zone structure, active zone proteins should interact with Liprin-α3 liquid phases. Co-expression of cerulean-Liprin-α3 with either RIM1α-mVenus or Munc13-1-tdTomato in HEK293T cells resulted in recruitment of each protein into PMA-induced condensates (Supplementary Fig. 5a). Discrete, PMA-insensitive condensates were also observed when RIM1α was expressed alone (Supplementary Fig. 5b), in agreement with its intrinsic ability to phase separate.6 Munc13-1 did not form droplets on its own, but PMA-dependent membrane recruitment was observed as previously described.36–38

PLC/PKC signaling increases active zone levels of Liprin-α3, RIM, and Munc13-1 at synapses of primary mouse hippocampal neurons. Our findings suggest that activating PKC induces the formation of active zone-like, membrane-bound liquid condensates in transfected cells. If physiologically relevant, activation of this pathway should result in changes in active zone protein complexes at synapses. To test this, we assessed active zone levels of endogenous Liprin-α3, RIM, and Munc13-1 at synapses of cultured hippocampal neurons using stimulated emission depletion (STED) microscopy (Fig. 3h–j). As described previously,39–41 we analyzed synapses in side-view. These synapses were identified by the position of a bar-shaped active zone (marked by Bassoon, imaged in STED mode) relative to a synaptic vesicle cloud (identified by Synaptophysin, imaged in confocal mode), and the peak levels of proteins of interest were assessed within 100 nm of the Bassoon peak (see Supplementary Fig. 6a for an outline of synapse selection and analyses). Liprin-α3, RIM and Munc13-1 were predominantly clustered at the active zone with peak intensities falling within 50 nm from the peak of Bassoon (Supplementary Fig. 6b). Addition of PMA produced a 20–30% increase in peak active zone levels of Liprin-α3 (tested with two independent antibodies, Fig. 3i, j and Supplementary Fig. 6f–h), RIM and Munc13-1 (Fig. 3i, j) without affecting Bassoon (Supplementary Fig. 6c–e). Hence, mimicking PLC/PKC activation enhances active zone recruitment of RIM, Munc13-1, and Liprin-α3.
Fig. 4 Liprin-α2/α3 double knockout alters presynaptic composition and ultrastructure. a Schematic for simultaneous knockout of Liprin-α2 and -α3. Mice in which Liprin-α2 can be removed by cre recombination (Liprin-α2f/f) were generated and crossed to previously published constitutive Liprin-α3 knockout mice (Liprin-α3−/−, generated by CRISPR/Cas9-mediated genome editing, deleted sequence is represented with dashes)59. Cultured hippocampal neurons of Liprin-α2f/f/Liprin-α3−/− mice infected with lentivirus expressing cre recombinase (KOL23) were compared to neurons of Liprin-α2f/f x Liprin-α3+/− mice infected with lentiviruses that express an inactive cre recombinase (controlL23). b, c Example confocal images (b) and quantification (c) of neurons immunostained for Liprin-α2, Liprin-α3, RIM, Munc13-1, Munc13-2, Bassoon, CaV2.1, PSD-95, Gephyrin, or GluA1 along with Synaptophysin (for Munc13-1, RIM-BP2, and Gephyrin) or Synapsin (all others) as vesicle marker, or Synapsin-1 and MAP2. Quantification in c was performed in regions of interest (ROIs) defined by the synaptic vesicle marker and normalized to the average controlL23 levels per culture, N = 30 images/3 independent cultures per genotype per protein of interest, except for Munc13-1 (N = 15/3), RIM-BP2 (N = 14/3), and Gephyrin (N = 14/3), p values: Liprin-α2, 2e-16 (**); Liprin-α3, 2e-16 (**); RIM, 3e-11 (**); Munc13-1, 3e-05 (**); Munc13-2, 0.54; Bassoon, 0.07; PSD-95, 0.30; Gephyrin, 0.10; GluA1, 0.53; CaV2.1, 5e-10 (**); Synapsin, 7e-06 (**). d–h Example electron micrographs of synapses (d) and quantification (per section) of the number of synaptic vesicles (e), bouton size (f), PSD length (g), and number of docked vesicles (h) of neurons fixed by high-pressure freezing followed by freeze substitution, controlL23: N = 111 synapses/2 independent cultures, KO123: N = 124/2, p values: e, 0.02 (*); f, 0.51; g, 0.95; h, 0.02 (*). All data were mean ± SEM. Significance was assessed using Mann–Whitney U tests, except for PSD-95 and Gephyrin in c, for which t-tests were used. All tests were two-sided. For synaptic localization of Liprin-α1 to -α4, see Supplementary Fig. 7, for generation of Liprin-α2f/f mice and analysis of Synaptophysin levels of the experiments shown in c, see Supplementary Fig. 8.
Knockout of Liprin-a2 and Liprin-a3 alters presynaptic composition and ultrastructure in primary mouse hippocampal neurons. If Liprin-a phase separation controls active zone assembly, Liprin-a knockout should impair active zone structure and function. We generated knockout mice to simultaneously ablate Liprin-a2 and Liprin-a3, the main synaptic Liprin-a proteins (Supplementary Fig. 7)\(^1\)\(^9\)\(^,\)\(^3\(^4\). Conditional Liprin-a2 knockout mice (Liprin-a2\(^{−/−}\)) produced by homologous recombination with exon 14 flanked by loxP sites (Supplementary Fig. 8a–e), were crossed to homozygosity and subsequently bred to previously generated constitutive Liprin-a3 knockout mice (Liprin-a3\(^{−/−}\)) (Fig. 4a). We used cultured hippocampal neurons of Liprin-a2\(^{+/+}\)/Liprin-a3\(^{+/+}\) mice infected with lentivirus expressing cre recombinase (to generate KO\(^{23}\)23) neurons and neurons from Liprin-a2\(^{+/+}\)/Liprin-a3\(^{−/−}\) mice infected with lentiviruses that express truncated, inactive cre recombinase (to generate control\(^{23}\)23) neurons. First, we assessed synapse composition by measuring protein levels within synapses using confocal microscopy (Fig. 4b, c). Liprin-a2 and Liprin-a3 were efficiently removed and the remaining signals are typical for antibody background\(^22\)\(^,\)\(^40\). The levels of RIM and Munc13 were decreased by 25–35%, without significant changes in Bassoon, RIM-BP2, PSD-95, Gephyrin, and GluA1. The synaptic levels of Cave-2.1 were increased by ~50%, as were those of Synapsin-1, but Synaptophysin levels were unchanged.

High-pressure freezing followed by freeze substitution and electron microscopic analyses was next used to investigate synaptic ultrastructure. The number of synaptic vesicles per profile was decreased by ~15% in KO\(^{23}\)23 synapses, without changes in the overall bouton size or postsynaptic densities (Fig. 4d–h). A ~25% reduction of docked vesicles (identified as vesicles with no detectable space between the electron-dense vesicular and target membranes) was observed upon Liprin-a2/a3 knockout, consistent with a partial loss of the docking proteins RIM and Munc13-1. We conclude that Liprin-a2 and Liprin-a3 are involved in maintaining presynaptic ultrastructure, specifically the number of vesicles per bouton and the number of docked vesicles.

We next asked using STED side-view analyses whether the changes in synaptic protein levels observed with confocal microscopy occur at active zones of both excitatory and inhibitory synapses (marked with PSD-95 and Gephyrin, respectively). This further circumvents confounds in ROI selection in the confocal experiments that may arise from modestly increased Synapsin levels were restricted to excitatory synapses (Fig. 5). This matches with the results obtained with confocal microscopy (Fig. 4b, c) and the electrophysiologocal phenotypes described below. We conclude that Liprin-a2 and -a3 are necessary for normal active zone structure.

Synapse-specific impairments of neurotransmitter release after ablation of Liprin-a2 and -a3 in primary mouse hippocampal neurons. The altered levels of Munc13-1, RIM, and Cave-2.1 and the decreased docking predict changes in synaptic secretion. Indeed, in whole-cell electrophysiological recordings, the frequency of spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively) was decreased in KO\(^{23}\)23 neurons, but their amplitudes were unchanged (Fig. 6a–c, 6f–l), indicating presynaptic roles for Liprin-a2 and -a3.

We used electrical stimulation or stimulation by hypertonic sucrose to evoke EPSCs (Fig. 6d–i) and IPSCs (Fig. 6m–r). Release evoked by an action potential is proportional to the product of the number of vesicles that can be released (readily releasable pool, RRP) and the likelihood of a vesicle to be released (vesicular release probability, \(p\))\(^{42\)43\)44. For action-potential triggered EPSCs, NMDA-receptor currents were measured instead of AMPA-receptor currents to avoid network activity that is prominent in the cultured neurons when AMPA-receptors are not blocked. Similar to confocal and STED microscopy, we observed synapse-specific changes. At excitatory and inhibitory KO\(^{23}\)23 synapses, the RRP estimated by the application of hypertonic sucrose was decreased (Fig. 6h, i, q, r), quantitatively matching the reduction in Munc13, RIM, and docked vesicles (Figs. 4, 5). We estimated \(p\) by measuring paired pulse ratios, where the response ratio of two consecutive pulses at short interstimulus intervals is inversely correlated with \(p\). Changes at excitatory KO\(^{23}\)23 synapses were indicative for increased \(p\) (Fig. 6f, 6g), matching well with enhanced Ca\(^{2+}\) channel levels (Figs. 4, 5). Together, the reduction in RRP and the increase in \(p\) may offset one another. The evoked EPSC appeared unchanged, although there was a non-significant trend towards a decrease (Fig. 6d, e). In contrast, \(p\) was unaffected at inhibitory synapses (Fig. 6o, p), matching with normal Ca\(^{2+}\) channel levels, and leading to an overall decrease in the IPSC amplitude due to the RRP decrease (Fig. 6m, n). In summary, the electrophysiological phenotypes match with the structural active zone effects. Knockout of Liprin-a2/a3 leads to reduction in docking, protein machinery for docking and priming and the pool of releasable vesicles at excitatory and inhibitory synapses, and a select increase in Ca\(^{2+}\) channels and release probability at excitatory synapses.

S760 is necessary for presynaptic functions of Liprin-a3 in primary mouse hippocampal neurons. We asked whether reexpression of wild type Liprin-a3 reverses the presynaptic phenotypes of KO\(^{23}\)23 neurons. Lentiviral expression of Liprin-a3 in KO\(^{23}\)23 neurons restored active zone levels of Liprin-a3 (Supplementary Fig. 9a–d), the RRP (Supplementary Fig. 9e, f) and the reduced active zone levels of RIM (Supplementary Fig. 9g, h). Hence, the active zone impairments at excitatory KO\(^{23}\)23 synapses are reversible by reexpression of Liprin-a3.

PKC phosphorylation of Liprin-a3 at S760 acutely modulates active zone structure and function in primary mouse hippocampal neurons. PLC/PKC signaling acutely enhances active zone assembly (Fig. 3) and neurotransmitter release\(^44\)\(^-\)\(^46\). We
hypothesized that this enhancement may be mediated by phosphorylation and phase separation of Liprin-α3, and compared the effect of PKC activation by PMA in KO\textsuperscript{L23} neurons expressing either wild type Liprin-α3 or Liprin-α3\textsuperscript{3G}, which does not form phase condensates. We note that despite their broad use to mimic activation of the PLC/PKC pathways\textsuperscript{38,44–47}, it remains uncertain how potentiation induced by phospholipase esters relates to physiological synaptic plasticity. Neurons incubated with PMA for 20 min showed enhanced mEPSC frequencies and amplitudes (Fig. 7a–f), as observed before\textsuperscript{46}, indicating that these pathways potentiate synaptic transmission through pre- and postsynaptic effectors. The mEPSC frequency increase was impaired by 50% in Liprin-α3 knockout. 

Sample N values: RIM, 0.98; Munc13-1, 0.84; RIM-BP2, 0.81; Ca\textsubscript{v}2.1, 0.09. Data were shown as mean ± SEM. Significance was assessed by Mann–Whitney rank-sum tests (b, d, h, i, q–t) or t-tests (f, j, n, p). All tests were two-sided.

**Fig. 5** Active zone composition at excitatory and inhibitory synapses after Liprin-α2/α3 knockout. a–d Example STED images and line profiles (a, c) and quantification of peak intensities (b, d) of RIM at Synaptophysin (Syp) positive excitatory side-view synapses identified by PSD-95 (a, b) or inhibitory side-view synapses identified by Gephyrin (c, d). b: control\textsuperscript{22}; N = 96 synapses/3 independent cultures, KO\textsuperscript{L23}; N = 69/3, KO\textsuperscript{α3}; N = 87/3, p values: b, 0.0008 (**); d, 0.04 (*). e–p Same as a–d, but for Munc13-1 (e–h), RIM-BP2 (i–l), and Ca\textsubscript{v}2.1 (m–p). Munc13-1, f: control\textsuperscript{22}; N = 79/3, KO\textsuperscript{L23}; N = 99/3, h: control\textsuperscript{22}; N = 102/3, KO\textsuperscript{L23}; N = 54/3, RIM-BP2, j: control\textsuperscript{22}; N = 57/3, KO\textsuperscript{L23}; N = 66/3, l: control\textsuperscript{22}; N = 73/3, KO\textsuperscript{L23}; N = 116/3, Ca\textsubscript{v}2.1, n: control\textsuperscript{22}; N = 52/3, KO\textsuperscript{L23}; N = 72/3, p: control\textsuperscript{23}; N = 87/3, KO\textsuperscript{L23}; N = 66/3, p values: f, 0.0003 (**); h, 0.00055 (**); j, 0.49; l, 0.24; n, 0.0001 (**); p, 0.18. q Quantification of the peak intensity of PSD-95 in all line profiles, control\textsuperscript{22}; N = 286/3, KO\textsuperscript{L23}; N = 309/3, p value: 0.21. r Quantification of the average distance of peaks of RIM, Munc13, RIM-BP2, and Ca\textsubscript{v}2.1 to the peak of PSD-95, N as in b, f, j, and n, p values: RIM, 0.85; Munc13-1, 0.52; RIM-BP2, 0.86; Ca\textsubscript{v}2.1, 0.67. s Quantification of the peak intensity of Gephyrin in all line profiles, control\textsuperscript{22}; N = 353/3, KO\textsuperscript{L23}; N = 323/3, p value: 0.47. t Quantification of the average distance of peaks of RIM, Munc13, RIM-BP2, and Ca\textsubscript{v}2.1 to the peak of Gephyrin, N as in d, h, i, and p, p values: RIM, 0.98; Munc13-1, 0.84; RIM-BP2, 0.81; Ca\textsubscript{v}2.1, 0.09. Data were shown as mean ± SEM. Significance was assessed by Mann–Whitney rank-sum tests (b, d, h, i, q–t) or t-tests (f, j, n, p). All tests were two-sided.
enhancement is likely overestimated because of the robust increase in mEPSC amplitude (Fig. 7f), and as a consequence the impairment in pool enhancement of Liprin-α3 may be underestimated.

In summary, these data indicate that PKC phosphorylation and phase separation of Liprin-α3 modulate the RRP.

We finally investigated whether Liprin-α3 phosphorylation and phase separation may control active zone structure. The lack of technology to selectively photobleach active zones within synapses with the necessary resolution of tens of nanometers prevents performing FRAP experiments to directly answer this...
Fig. 6 Liprin-α2/α3 double knockout impairs neurotransmitter release. a–c Example traces (a) of spontaneous miniature excitatory postsynaptic current (mEPSC) recordings (top) and an averaged mEPSC of a single cell each (bottom), and quantification of mEPSC frequency (b) and amplitude (c), control23, N = 15 cells/3 independent cultures, KO23, N = 21/3, p values: b, 0.003 (*); c, 0.55. d, e Example traces (d) and average amplitudes (e) of single action potential-evoked NMDA receptor-mediated EPSCs, control23, N = 25/3, KO23, N = 26/3, p value: 0.63. f, g Example traces (f) and average NMDA-EPSC paired pulse ratios (g) at various interstimulus intervals, control23, N = 25/3, KO23, N = 23/3, p values: genotype 0.00002, interstimulus interval 0.009, interaction 0.007, post-tests: 20 ms 0.002 (*), 50 ms 0.11, 100 ms 0.16, 500 ms 0.18, 1 s 0.24. h, i Example traces (h) and quantification (i) of the AMPA receptor-mediated EPSC charge in response to a local 10 s puff of 500 mOsm sucrose to estimate the RRP, control23, N = 21/3, KO23, N = 25/3, p value: 0.045 (*). j Same as a–i, but for IPSCs, j, k control23, N = 18/3, KO23, N = 18/3; m, n control23, N = 23/3, KO23, N = 24/3; o, p control23, N = 23/3, KO23, N = 23/3, q, r control23, N = 22/3, KO23, N = 22/3, p values, k, 0.046 (*); f, 0.12; n, 0.002 (**), p genotype 0.37, interstimulus interval 2e–8, interaction 0.96; r, 0.011 (*). s Diagram of the rescue experiment with Liprin-α3 expression via lentiviral transduction. t, u Example traces (t) and quantification (u) of sucrrose-triggered EPSCs, KO23: N = 22/3, KO23 + Liprin-α3 (L-α3): N = 23/3, KO23 + Liprin-α3560G (L-α356): N = 23/3, p values (compared to KO23): KO23 + L-α3, 0.006 (*); KO23 + L-α356, 0.53. v, w Representative STED images (v) and quantification (w) of RIM at the active zone of side-view synapses as in Fig. 3i–j, KO23: N = 56 synapses/3 independent cultures, KO23 + L-α3: N = 43/3, KO23 + L-α356: N = 50/3, p values: KO23 + L-α3, 0.003 (**); KO23 + L-α356, 0.18. All data were mean ± SEM. Significance was assessed using Mann-Whitney U-tests (b, c, i, k, l), t-tests (e, n, r), two-way ANOVA (g, p), or Kruskal–Wallis (u, w) post hoc comparison against KO23 (comparison between all groups were performed, but only results against KO23 are reported in u and w). All tests were two-sided. For a direct comparison of control23, KO23 and KO23 + L-α3 and for STED localization of rescue Liprin-α, see Supplementary Fig. 9.

RIM, Together, these data support the model that active zone structure is rapidly modulated by PLC/PKC signaling via phosphorylation and phase separation of Liprin-α3 (Fig. 8j).

Discussion

Recruitment of proteins into liquid phases enables the formation of membrane-less intracellular compartments1,2. We investigated molecular pathways that drive and modulate assembly of the presynaptic active zone. Our experiments reveal that (1) PKC phosphorylates Liprin-α3 at S760 to drive the formation of membrane-attached liquid condensates containing RIM1a and Munc13-1, (2) genetic ablation of the prominent synaptic Liprin-α proteins (Liprin-α2 and -α3) leads to defects in active zone structure and function, including reductions in RIM and Munc13-1, and (3) PKC phosphorylation of Liprin-α3 at S760 upregulates neurotransmitter release and active zone levels of RIM and Munc13-1. These results lead to a working model in which presynaptic phase separation triggered by Liprin-α3 phosphorylation rapidly modulates active zone structure (Fig. 8j).

S760 phosphorylation may lead to the formation of liquid condensates (1) by enhancing or enabling interactions of the phosphorylated linker, (2) by recruiting adapters, or (3) by

question. We instead assessed side-view synapses of KO123 neurons, or of KO123 neurons expressing either Liprin-α3 or Liprin-α356G. In both rescue conditions, Liprin-α3, RIM, and Munc13-1 were enriched at the active zone. As observed in Fig. 3h–j, active zone levels of these proteins, but not of Bassoon, increased upon PMA addition by ~30–35% when Liprin-α3 was present (Fig. 8a–i and Supplementary Fig. 10). This increase, assessed either by quantifying raw fluorescence (Fig. 8b, e, h), or by normalization to the respective pre-PMA condition (Fig. 8c, f, i), was hampered at KO23 synapses or in those rescued with Liprin-α356G, with only ~10–15% enhancement of Munc13-1 and

Fig. 7 PKC phosphorylation of Liprin-α3 enhances synaptic vesicle release. a–c Example traces (a) and quantification of mEPSC frequencies (b, c) in KO123 neurons rescued with wild type Liprin-α3 (L-α3) or non-phosphorylatable Liprin-α3560G (L-α356G) that does not form phase condensates. The percent increase upon PMA addition over naïve conditions per culture is shown in c. KO23 + L-α3: N = 14 cells/3 independent cultures (–PMA) and 15/3 (+PMA); KO23 + L-α356G: N = 14/3 (–PMA) and 16/3 (+PMA), p values: b, KO23 + L-α3, 0.0004 (**), KO23 + L-α356G, 0.0084 (***); c, 0.03 (*). d–f Average mEPSCs from a single cell of each condition (d) and quantification of mEPSC amplitudes (e, f). N as in b, c, p values: e, KO23 + L-α3, 0.002 (**), KO23 + L-α356G, 0.001 (**); f, 0.15. g, i Example traces (g) and quantification (h, i) of the EPSC charge in response to a local 10 s puff of 500 mOsm sucrose to estimate the RRP. KO23 + L-α3: N = 15/3 (–PMA) and 17/3 (+PMA), KO23 + L-α356G: N = 17/3 (–PMA) and 17/3 (+PMA), p values: h, KO23 + L-α3, 0.00002 (**), KO23 + L-α356G, 0.0002 (**); i, 0.03 (*). All data were mean ± SEM. Significance was assessed by Mann-Whitney rank sum test (c, f, i), or by Kruskal–Wallis test with Holm post hoc tests between all groups (b, e, h), and only results compared to the corresponding −PMA control are reported in b, e, and h. All tests were two-sided.
inducing Liprin-α3 conformational changes that expose previously occluded domains. These mechanisms may cooperate and could further function in phase-separation-independent ways. The third scenario appears likely because synaptic Liprin-α functions strongly depend on N-terminal sequences that mediate Liprin-α dimerization and active zone assembly. S760 is not part of these sequences, but its phosphorylation could determine interactions and function of the N-terminus, for example through a previously proposed model in which intramolecular Liprin-α interactions mediate its functions. This possibility is further supported by the observation that no single Liprin-α3 fragment was able to form condensates when expressed alone, and sequences spanning multiple domains are necessary for Liprin-α3 phase separation.

There are notable differences in condensate formation across Liprin-α proteins. Only Liprin-α2 and Liprin-α3 form condensates in transfected cells. While Liprin-α3 condensates follow liquid dynamics, those formed by Liprin-α2 do not. Such biophysical properties may be related to different functions for these two proteins. For instance, roles in active zone remodeling may be unique to Liprin-α3 because other Liprin-α proteins are not subject to PKC-mediated triggering of phase separation. Baseline active zone assembly, however, is mediated by both Liprin-α2 and -α3 (this study and), and this role may not need the
Fig. 8 The S760 PKC phosphorylation site of Liprin-α3 acutely modulates active zone assembly. a–c Example STED images and their intensity profiles (a) and quantification (b, c) of Liprin-α3 (detected by anti-HA antibodies) in side-view synapses in the presence or absence of PMA. Quantification of levels in arbitrary units is shown in b, and the increase upon PMA addition normalized to corresponding −PMA controls is shown in c. KO123: N = 60 synapses/3 cultures (−PMA) and 71/3 (+PMA); KO123 + L-α3: N = 54/3 (−PMA) and 83/3 (+PMA); KO123 + L-α3SG: N = 63/3 (−PMA) and 68/3 (+PMA), p values: b, KO123, 0.91, KO123 + L-α3, 0.006 (**), KO123 + L-α3SG, 0.42; c (compared to KO123), KO123 + L-α3, 0.002 (**), KO123 + L-α3SG, 0.08. d Experiments as shown in c, but for RIM (d–f) and Munc13-1 (g–i). RIM (d–f): KO123: N = 81/3 (−PMA) and 61/3 (+PMA), KO123 + L-α3: N = 75/3 (−PMA) and 84/3 (+PMA); KO123 + L-α3SG, N = 65/3 (−PMA) and 59/3 (+PMA); Munc13-1 (g–i): KO123: N = 54/3 (−PMA) and 67/3 (+PMA), KO123 + L-α3: N = 55/3 (−PMA) and 67/3 (+PMA), KO123 + L-α3SG: N = 57/3 (−PMA) and 62/3 (+PMA), p values: e, KO123, 0.14, KO123 + L-α3, 0.00005 (**), KO123 + L-α3SG, 1.0; f (compared to KO123), KO123 + L-α3, 0.02 (**), KO123 + L-α3SG, 0.69; h, KO123, 0.01 (**), KO123 + L-α3, 0.00003 (**), KO123 + L-α3SG, 0.004 (**); i, KO123 + L-α3, 0.03 (**), KO123 + L-α3SG, 0.53. ** Working model for the control of active zone structure through phase separation of Liprin-α3. The formation of phase condensates is triggered by PKC phosphorylation of Liprin-α3 at S760 and Munc13-1 and RIM are recruited into these release site condensates for boosting neurotransmitter secretion. Our data indicate that Liprin-α3 phosphorylation is not required for Liprin-α3 recruitment to active zone areas, but that Liprin-α3 phosphorylation followed by phase separation enhances the levels of Munc13 and RIM and boosts neurotransmitter release. Data were shown as mean ± SEM. Significance was assessed by Kruskal–Willis and Holm post hoc tests between all groups, and only results compared to the corresponding −PMA control (b, e, and h) or versus KO123 (c, f, and i) are reported. All tests were two-sided. For assessment of Bassoon levels, see Supplementary Fig. 10.

** dynamics that result from liquid properties. Liprin-α1 and Liprin-α4 do not form condensates in transfected HEK293T cells and may operate through different mechanisms or lack important components for phase condensation in these cells. Importantly, Liprin-α1 and -α4 antibody-signals appear mostly non- synaptic and may be dendritic and, at least Liprin-α1 operates in neuronal arborization. Finally, an independent, parallel recent study found that C.elegans Liprin-α/SYD-2 also forms phase condensates that mediate aspects of presynaptic assembly. In summary, a picture emerges where phase separation and liquid properties may determine cellular functions of Liprin-α. One limitation in our gene knockout experiments is that Liprin-α1 and Liprin-α4 are present, and a full definition of vertebrate Liprin-α function will require new gene targeting experiments to remove these proteins as well.

A key question is how Liprin-α3 phases interact with other presynaptic phase condensates. RIM1α and RIM-BP2 form liquid condensates in vitro that may organize tethering of voltage-gated Ca2+ channels. It is uncertain whether Liprin-α3 is part of the same phase within a nerve terminal, or whether multiple independent phases coexist. The current evidence is most compatible with a model of multiple distinct phases. First, active zone levels of RIM and Munc13-1 decrease upon ablation of Liprin-α2 and Liprin-α3, but those of Cav2.1 increase and those of RIM-BP2 are unchanged. Similarly, ablation of RIM-BP and RIMα2, or of RIM and ELKSα2, do not lead to loss of presynaptic Liprin-α. Hence, these proteins are likely in distinct protein complexes, or phases. Second, Cav2.1 and Munc13-1 do not co-localize when assessed with immunogold electron microscopy, suggesting the presence of separate clusters. In aggregate, it appears most likely that distinct liquid assemblies exist within an active zone, one containing RIM1α and RIM-BP2 to tether Ca2+ channels and a different phase with Liprin-α1, RIM, and Munc13-1. RIM may participate in multiple distinct active zone condensates, and how these partially overlapping phases acquire their unique molecular composition is an intriguing question. Liprin-α3 may be a key determinant, as Munc13-1 is more efficiently recruited to RIM condensates when Liprin-α3 is present, and Liprin-α3 can recruit either RIM or Munc13-1 on its own. While the recruitment of RIM by Liprin-α3 can be explained by known interactions, we are not aware of direct binding between Liprin-α3 and Munc13-1, and direct or indirect interactions may be involved. Nonetheless, the observation of reduced Munc13-1 levels after Liprin-α ablation in vertebrates fits well with a previous report that Liprin-α deletion resulted in decreased levels of a specific Munc13 protein at the fly neuromuscular junction.

Phase interactions may also be at play with synaptic vesicle clusters, which are organized via phase condensation through Synapsin and Synaptophysin. Ablation of Liprin-α2 and -α3 mildly impaired vesicle clustering, and the levels of Synapsin were modestly increased, suggesting that there might be interplay between Liprin-α and vesicle phases. Release requires the transition of synaptic vesicles from the vesicle cluster to release sites. It is possible that RIM/Liprin-α/Munc13 phases embody sites that enable such transitions, as both RIM and Munc13 define secretory hotspots. Liprin-α3, Munc13, and RIM may each be involved in the recruitment of vesicles from the vesicle phase to release sites, consistent with their roles in vesicle tethering or docking and their interactions with vesicular proteins. As such, the docking reaction could be seen as the transition of a vesicle from Synapsin-phase association to active zone-phase association.

The existence of multiple phase separation-based pathways for active zone assembly may explain some of the difficulties in understanding its mechanisms. Removal of each protein family, for example RIM, RIM-BP, or Liprin-α2 and -α3 (this study), leads to at most partial assembly defects, but combinations of mutations are required to disrupt active zones. This redundancy may also be the reason why active zone deletions can lead to synapse-specific secretory deficits. In the case of Liprin-α, our work establishes that it is recruited to active zone condensates independently of phosphorylation (Fig. 3), and Liprin-α3SG, which does not undergo PKC-induced phase separation, localizes to active zones (Fig. 5). Hence, PKC-mediated phase separation of Liprin-α3 is not required for its active zone targeting. Furthermore, RIM is targeted to the active zone mostly correctly upon Liprin-α2 and -α3 ablation. We conclude that Liprin-α2 and -α3, Liprin-α3 phosphorylation and its phase separation are not required for vertebrate active zone assembly. We propose that there are constitutive mechanisms for active zone assembly that do not necessitate Liprin-α3 phase separation. Instead, phase separation of Liprin-α3, likely after it has been anchored to the active zone, may modulate and strengthen presynaptic assembly through recruitment of additional active zone proteins.

While phosphorylation-mediated phase separation of Liprin-α3 may be partially active during development, it can be acutely engaged by activating PLC/PKC signaling in established synapses. Modulating active zone assemblies through this pathway is well suited to explain rapid changes during plasticity. We propose that phosphorylation of Liprin-α3 by PKC nucleates the transition of Liprin-α3 into liquid condensates to recruit RIM, Munc13-1, and
possibly other active zone proteins. This allows addition of secretory machinery to the membrane to enhance release (Fig. 8j).

Modulation of Liprin-α phase separation by PKC complements other presynaptic mechanisms for PLC/PKC-triggered potentiation, including those mediated by Munc13-3, Munc13-1, and Synaptotagmin-1, further supporting the involvement of multiple parallel mechanisms.

Methods

Assessments of droplets in transfected HEK293T cells. HEK293T cells were plated on 0.1 mm thick coverslips and transfected with plasmids expressing proteins of interest under the CMV promoter. About 500 ng of DNA per well (1.9 cm²) were used for a single plasmid transfection. If multiple plasmids were transfected, additional DNA was used at a 1:1 molar ratio. For assessment of fixed cells, fixation was performed using 4% paraformaldehyde 10–16 h after transfection. Drugs were added 15 min before cells were fixed unless otherwise noted, at the following concentrations: forskolin (10 μM, Sigma), PMA (1 μM, Sigma), caffeine (1 mM, Sigma), H-89 (5 μM, Abcam), bisindolylmaleimide-I (Bis-1, 0.1 μM, Sigma), KN-93 (1 μM, Abcam) and cells were fixed in the presence of drugs. For experiments including only PMA, the equivalent amount of DMSO was added to control. Images were acquired with a Leica SP Confocal/STED 3X microscope, using an oil-immersion 63X objective. For single protein expression, quantification of droplet condensate diameter over time was estimated using an event in which a larger condensate generated two smaller ones. For experiments using phospho-specific antibodies, samples were gel-processed and processed for Western blotting. For phosphorylation assays in HEK293T cells, the transfected cells were treated with PMA as described with or without pre-incubation with PKC inhibitors (Ro31-8280, 1 μM) run on SDS-page gels and processed for Western blotting.

Generation of custom antibodies. Custom antibodies (A231, A232, and 247) were produced following established protocols. Phospho-specific Liprin-α antibodies were generated using keyhole limpet hemocyanin (KLH) conjugated C6APKRKS(ser)KSSIGHR or C6APKRKS(ser)KSLGR for phospho-S760 and phospho-S764, respectively. For Liprin-α3 antibodies, GST-Liprin-α3-188-576 (p588, pGEX Liprin-α3-188-576) was expressed in and purified from BL21 bacteria. Peptides and GST fusion proteins were injected into rabbits whose sera had been pre-screened to prevent nonspecific antibody signal. Rabbits were boosted every 2 weeks and bleeds were collected every 3 weeks. Sera that showed the strongest bands and specificity in western blotting were affinity-purified.

Western blotting. Western blotting was performed following established protocols. Samples were prepared in SDS sample buffer, run on SDS-PAGE gels and transferred to nitrocellulose membranes at 4 °C for 6.5 h in buffer containing (per liter) 200 mM methanol, 14 g glycine, and 6 g Tris, followed by 1 h blocking at room temperature in saline buffer with 10% nonfat milk powder and 5% normal goat serum. The membranes were incubated in primary antibodies overnight at 4 °C in saline buffer with 5% milk and 2.5% goat serum, followed by 1 h incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies preincubated in the presence of primary antibody. Antibody supershifts were performed with the following antibodies: Liprin-α2 and -α3 (A115, 1:500) were gifts from S. Schoch; rabbit anti-phospho-S760 Liprin-α3 (generated for this study; A231; 1:1000) and anti-phospho-S764 Liprin-α3 (generated for this study; A247 and A248; 1:1000); mouse anti-HA (A12, 1:500; RRID: AB_2334589, 1:10000), mouse anti-Synaptophysin (A100, 1:5000; RRID: AB_887284) and mouse anti-Synapsin-1 (A57, 1:5000; RRID: AB_2617071). Secondary antibodies used: goat anti-rabbit HRP conjugated (S53, 1:10000, RRID: AB_2334589, 1:10000), goat anti-mouse HRP conjugated (S52, 1:10000, RRID: AB_2334540). Three 5 min washes were performed between steps.

Neuronal cultures and production of lentiviruses. Primary hippocampal cultures were prepared following established protocols. Newborn (P0–P1) pups were anesthetized with isoflurane and hippocampi dissected and dissociated, and neurons were plated on glass coverslips in plating medium composed of Minimum Essential Medium (MEM) supplemented with 0.5% glucose, 0.02% NaHCO3, 0.1 mg/ml transferrin, 10% Fetal Select bovine serum, 2 mM l-glutamine, and 25 mg/ml insulin. Twenty-four hours after plating, plating medium was exchanged with growth medium composed of MEM with 0.5% glucose, 0.02% NaHCO3, 0.1 mg/ml transferrin, 5% Fetal Select bovine serum (Atlas Biologicals FS-0500-AD), 2% B-27 supplement, and 0.5 mM l-glutamine. At DIV2–3, 4 mM Cytochrome b-α-arabinofuranoside (AraC) was added. Cultures were kept in a 37 °C tissue culture incubator until DIV15–17. Lentiviruses were produced in HEK293T cells maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HEK293T cells were transfected using the Ca2+–phosphate method with the lentiviral packaging plasmids REV, RRE, and VSV-G and a separate plasmid encoding the protein of interest, at a molar ratio 1:1:1. Twenty-four hours after transfection, the medium was exchanged with neuronal growth medium composed of Neurobasal medium with B27 supplement and 1% insulin. Lentiviral particles were added to the cultures 22–24 h after attachment and cultured for a further 3 days. For experiments using lentiviral particles expressing the GPCR Cxcr2, the cells were cultured for a further 5 days. Expression and purification of GST-Liprin-α3 proteins. GST-tagged fusion proteins were generated, expressed, and purified according to standard procedures. Specifically, proteins were expressed at 20°C in E. coli BL21 cells after induction with 0.05 mM isopropyl β-D-1-thiogalactopyranoside for 20 h, and pelleted by centrifugation (45 min at 3500xg). For purification of GST fusion proteins, bacterial pellets were resuspended and lysed for 30 min in PBS buffer supplemented with 0.5 mM mg/ml lysozyme, 0.5 mM EDTA, and a protease inhibitor cocktail, followed by brief sonication and centrifugation (45 min at 11,200g). Bacterial supernatants were incubated with glutathione-Sepharose resin (GE Healthcare) for 1.5 h at 4 °C with gentle rotation, washed three times in PBS and stored until further use at 4 °C (for no more than 5 days after purification). All steps after protein induction were conducted at 4 °C and using ice-cold solutions. Protein concentrations were estimated by SDS-gel electrophoresis followed by Coomassie staining using BSA standards as a reference. The following GST-tagged proteins were produced from pGEX-KG2 constructs: pGEX Liprin-α3 (p567), pGEX Liprin-α3–577–790 (p566) and pGEX Liprin-α3–791–1192 (p570). Amino acid numbering follows NM_001270985.2.

Assessment of Liprin-α3 phosphorylation. For in vitro phosphorylation assays, 40 μg of the fusion protein bound to glutathione beads was washed 3 times with 50 mM Tris–HCl, pH 7.5, followed by 1 h PKC reaction buffer (20 mM HEPES, 10 mM MgCl2, 150 mM NaCl, 1 mM DTT) with 0.25 mg/ml PKC (Promega, V526A), 1 μM PMA, 1 μM phosphatidyl serine (Sigma, P7769), and 200 μM ATP (Sigma, A23383). For experiments in which phosphorylation was detected by autoradiography, 10 μCi γ-ATP (Perkin Elmer) were added to the PKC reaction mix and incubated for an additional 1 h at 30 °C, followed by gel electrophoresis. For mass spectrometry analyses, the phosphorylated GST-Liprin-α3–577–790 protein was isolated by SDS gel electrophoresis, Coomassie blue staining and cutting out of the protein band after the initial PKC reaction. The sample was processed by the HMS Taplin Mass Spectrometry Facility for identification of phosphorylated amino acid residues. For experiments using phospho-specific antibodies, samples were gel-processed and processed for Western blotting. For phosphorylation assays in HEK293T cells, the transfected cells were treated with PMA as described with or without pre-incubation with PKC inhibitors (Ro31-8280, 1 μM) run on SDS-page gels and processed for Western blotting.
Cre expressed under the human Synapsin promotor. For rescue, cultures were infected at DIV1–2 with a lentiviruss expressing Liprin-a2 or Liprin-a32f/f, or an empty lentivirus as control. pSFW HA-Liprin-a3 StGlu was generated for this study; pSFW control (p908) and pSFW HA-Liprin-a3 (p526) were previously described32. For PMA experiments, PMA was added 15–20 min before fixation to a final dilution of 1 μM (from a 1 mM stock diluted in DMSO), and neurons were washed and fixed in the presence of the drug. 2+PMA controls were incubated in the same amount of DMSO.

**Immunofluorescence staining and confocal microscopy of neurons.** Neurons grown on #1.5 (for STED) or #1.0 (confocal) glass coverslips were grown on DIV15 and permeabilized in blocking solution (3% BSA/0.1% Triton X-100/PBS) for 1 h, incubated overnight with primary antibodies followed by overnight incubation with AlexaFluor secondary antibodies, and mounted in glass slides. Antibodies were diluted in blocking solution. For STED imaging, coverslips were additionally post-fixed in 4% paraformaldehyde for 10 min. Three 5 min washes with PBS were performed between steps. All steps were performed at room temperature except for antibody incubations (4 °C). Primary antibodies used: mouse anti-Bassoon (A85, 1:500; RRID: AB_11810588), rabbit anti-CaV2.1 (A121, 1:100; gift from S. Schoch34, a2 (A13, 1:250; gift from S. Schoch34), a3 (A115, 1:250; gift from S. Schoch), and a4 (A2, 1:100; gift from S. Schoch34), rabbit anti-RIM (A58, 1:500; RRID: AB_887779), mouse anti-RIM1-95 (A149, 1:150; RRID: AB_1098024), mouse anti-Gephyrin (A1, 1:500; RRID: AB_2233546), mouse anti-Synaptin-3 (A57, 1:100; RRID: AB_179771), guinea pig anti-Synaptophysin (A50, 1:500; RRID: AB_1210382), rabbit anti-RIM1-PBP1-B (A126, 1:500; RRID: AB_2650066), rabbit anti-Munc13-1 (A2, 1:50; RRID: AB_887713), rabbit anti-CaV2.1 (A16, 1:50; RRID: AB_2619894), mouse anti-HA (A12, 1:50; RRID: AB_2565006), rabbit anti-MAP2 (A139, 1:500; RRID: AB_2138183), mouse anti-MAP2 (A108, 1:50; RRID: AB_2657606), rabbit anti-Bassoon (A54, 1:100; RRID: AB_2635776), and rabbit anti-Liprin-a3 (A322, 1:500, custom-made). Secondary antibodies used: goat anti-rabbit Alexa Fluor 488 (S5, 1:250; RRID: AB_2576217), goat anti-mouse IgG Alexa Fluor 488 (S7, 1:250; RRID: AB_2535764), goat anti-mouse IgG Alexa Fluor 555 (S19, 1:2500; RRID: AB_2535759), goat anti-mouse IgG2a Alexa Fluor 555 (S55, 1:250; RRID: AB_2535775), goat anti-guinea pig Alexa Fluor 633 (S34, 1:500, RRID: AB_253557), and goat anti-mouse Alexa Fluor 633 (S32, 1:500, RRID: AB_2535718). Confocal images were taken on an Olympus FV1200 confocal microscope equipped with a 63X oil immersion objective. Images of experiments with multiple groups were acquired within a single session per culture and identical settings for each condition were used within an imaging session. For quantitative analyses of synaptic protein levels, the synaptic vesicle marker signal was used to define puncta as ROIs, and the average intensity within ROIs was quantified after local background was subtracted using the “rolling average” ImageJ plugin (diameter = 1.4 μm). Data were plotted normalized to the average intensity of the control group (control23) per culture. For co-localization analyses, the “Coloc 2” ImageJ plugin was used with default thresholding. For example images in brightness and contrast were linearly adjusted equally between groups and images were interpolated to meet publication standards. All data were acquired and analyzed by an experimenter blind to genotype and/or condition.

**STED imaging of synapses.** STED microscopy was performed following established procedures19,39,41. Images were acquired with a Leica SP8 Confocal/STED 3X microscope equipped with an oil-immersion 100×1.44 NA objective, Leica SP8, STED gated detectors, and 592 and 660 nm depletion lasers. Synapse-rich areas were selected and were scanned at 22.5 nm per pixel. Triple color sequential confocal scans were acquired using a dual-color sequential STED scans. Identical settings were applied to all samples within an experiment. For quantification, side-view synapses (selected while blind to the protein of interest) were defined as synapses that contained a vesicle cluster (imaged in confocal mode, >300 nm wide) with an elongated Bassoon, Gephyrin, or P539–95 (active zone or postsynaptic density markers, respectively, imaged by STED) structure along the edge of the vesicle cluster19,39,41. A 1 μm-long, 250-nm-wide profile was selected perpendicular to the active zone/postsynaptic density marker and across its center. The intensity profile was then obtained for markers and for the protein of interest. Peak levels of the protein of interest were measured as the maximum intensity of the line profile (measured in arbitrary units). For each condition, images were acquired at the same intensity and orientation (available) for each condition, which were used for displaying the data in a suitable range. The factor varied from ~1 to ~20 and was identical within each data set of an experiment, but different for different data sets. The measured arbitrary values before division or multiplication are provided in the source data file. For PMA experiments, neurons were preincubated for a total of 20 min before fixation with PMA (for the “+” PMA condition, at a final concentration of 1 μM PMA dissolved in 1 mM stock in or in DMSO) correspon-
Electrophysiology. Electrophysiological recordings were performed following previously used procedures. DIV15–16 neurons were recorded in whole-cell patch-clamp configuration at room temperature in extracellular solution containing (in mM) 140 NaCl, 5 KCl, 2 MgCl2, 10 HEPES (pH 7.4) and 10 Glucose. Glass pipettes were pulled at 2–4 MΩ and filled with intracellular solutions containing (in mM) 120 Cs-methanesulfonate, 10 EGTA, 2 MgCl2, 10 HEPES-CaOH (pH 7.4), 4 Na2-ATP, and 1 Na-GTP for excitatory transmission; and 40 CsCl, 90 K-Gluconate, 1.8 NaCl, 1.7 MgCl2, 3.5 KCl 0.05 EGTA, 10 HEPES, 2 MgATP, 0.4 Na2-GTP, 10 phosphocreatine, Co(OH)2 (pH 7.4) for inhibitory transmission. For evoked responses, 4 mM QX314-Cl was added to the intracellular solution to block sodium channels. Neurons were clamped at −70 mV for IPSC and AMPAR-EPSC recordings, or +40 mV for NMDA-EPSCs. Series resistance was compensated to 5–5.5 MΩ. Recordings in which the series resistance increased to >15 MΩ before compensation were discarded. mEPSCs, mIPSCs, and sucrose-evoked release were measured in extracellular solution supplemented with 1 mM TTX, 50 µM D-AP, and either 50 µM picrotoxin (for EPSCs) or 20 µM CNQX (for IPSCs). 300 mM hypertonic sucrose was applied for 10 s, and the integral of the evoked release were measured in extracellular solution or in the corresponding amount of DMSO for the −PMA condition for 20 min before recording, and a maximum of two cells per coverslip were recorded within a total of 40 min after drug addition (20 min of preincubition, 20 min of recording). To calculate the % increase after PMA, the data were normalized to the average of the corresponding −PMA condition in each culture. Action potential-evoked responses were elicited by focal bipolar electrical stimulation with an electrode made from Nichrome wire and recorded in extracellular solution supplemented with 20 µM CNQX, and either 50 µM D-AP (for EPSCs) or 20 mM PTX (for NMDA-EPSCs). A Multiclamp 700B amplifier and a Digidata 1500 digitizer were used for data acquisition, sampling at 10 kHz and filtering at 2 kHz. Data were analyzed using pClamp. In all experiments, the experimenter was blind to the condition throughout data acquisition and analyses.

Statistics. Normality and homogeneity of variances were assessed using Shapiro or Levene’s tests, respectively. When test assumptions were met, parametric tests (t-test or one-way ANOVA) were used. Otherwise, the non-parametric tests (Kruskal–Wallis or Holm corrections for multiple testing) were applied for parametric and nonparametric post hoc testing. Chi-square tests were applied for categorical data. Data were analyzed using GraphPad Prism software (version 8). Data are expressed as mean ± SEM, and significance in figures is shown as *p < 0.05 (**), **p < 0.01 (***) or ***p < 0.001 (****). Exact p values are provided in each figure legend.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data sets presented in this study are included in full whenever possible, including display of individual data points. Data were available from the corresponding author upon reasonable request. Biological materials including mutant mice and custom antibodies generated for this study will be shared upon request within the limits of respective material transfer agreements for as long as they are available in the laboratory. Source data are provided with this paper.

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Competing interests
S.H.W. is currently an employee of RA Capital Management LP. M.Y.W. is currently and employee of Novartis. The remaining authors declare no competing interests.

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