LIPRIN-α2 ORGANIZES PRESYNAPTIC COMPOSITION UPSTREAM OF RIM AND CASK TO FACILITATE SYNAPTIC TRANSMISSION

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submitted
Abstract

The presynaptic terminal regulates neurotransmitter release through fusion of synaptic vesicles at the active zone. Here, we show that in mature neurons liprin-α2 controls synaptic output by regulating synaptic vesicle pool sizes. Liprin-α2’s presence at presynaptic sites does not depend on other scaffolding proteins and is critical for organization of the presynaptic release machinery. Liprin-α2 levels are regulated by activity and therefore play an important role in modulating synaptic efficacy.
Main Text

The primary function of the presynaptic active zone (AZ) is the regulation of the release of neurotransmitter-filled synaptic vesicles (SVs) in response to action potentials entering the bouton (Sudhof, 2004). The SV cycle is tightly controlled, both temporally and spatially, and its performance is modified in response to activity (Atwood and Karunanithi, 2002). Recent studies show that regulation of presynaptic efficacy involves molecular reorganization of the release apparatus by modulating AZ protein turnover (Lazarevic et al., 2011). However, which proteins function as upstream regulators of presynaptic output remains unknown. Studies in invertebrates suggest that liprin-α family proteins dliprin-α and SYD-2 play a key role in presynaptic development (Dai et al., 2006; Kaufmann et al., 2002; Patel et al., 2006; Spangler and Hoogenraad, 2007; Zhen and Jin, 1999b). Due to the complexity of the vertebrate liprin-α family, knowledge of the presynaptic role of liprin-α in mammalian neurons is limited to its presynaptic localization by (electron) microscopy (Wyszynski et al., 2002) and its ability to bind to other AZ proteins (Spangler and Hoogenraad, 2007).

Since it is not known whether liprin-α2 proteins levels are regulated at synapses, we examined the turnover of liprin-α2. Fluorescence recovery after photobleaching (FRAP) experiments reveal that GFP-liprin-α2 fluorescence recovers to 58 ± 8% of pre-bleaching intensity within five minutes with an average recovery half-time of 52 ± 13s (Figure 2.1A-B). On this time scale the presynaptic scaffolding protein bassoon exhibits very little recovery (Tsuriel et al., 2009), indicating that liprin-α2 is a highly dynamic component of the presynaptic active zone. Because the ubiquitin-proteasome system plays an important role in synaptic protein turnover (Bingol and Sheng, 2011), we tested whether the proteasome inhibitor MG132 affects liprin-α2 expression in hippocampal neurons. MG132 (20 μM, 1 hr) caused an increase in synaptic liprin-α2, in contrast to synaptic markers PSD-95 or bassoon (Figure 2.1C-D). Human liprin-α2 contains two destruction motifs (RxLxN) that are recognized by the ubiquitin ligase anaphase-promoting complex (APC). Mutation of the first destruction box (GFP-liprin-α2ΔAPC, AAAAxN) results in a marked decrease in the turnover of presynaptic liprin-α2 (38 ± 7%, t1/2 = 88 ±
Figure 2.1 Liprin-α2 is regulated by synaptic activity and the ubiquitin-proteasome system.
(A) Quantification of fluorescence recovery after photobleaching (FRAP) of GFP-liprin-α2 and GFP-liprin-α2ΔAPC in DIV15+4 neurons. Two-way ANOVA: ***, F=4.268, p<0.0001. (B) Averages of the mobile and immobile fractions of GFP-liprin-α2 and GFP-liprin-α2ΔAPC in DIV15+4 neurons. (C) Representative images of endogenous bassoon (red), liprin-α2 (green), and PSD-95 (blue) in DIV19 hippocampal neurons treated with 20μM MG132 for 1 hour prior to fixation. (D) Quantification of (C) (liprin-α2: control: 100 ± 2%, 1h MG132: 118 ± 6%, n=5 neurons; *, t-test, p=0.02; bassoon: control: 100 ± 5%, 1h MG132: 106 ± 10%, n=5 neurons; t-test, p>0.05; PSD-95: control: 100 ± 3%, 1h MG132: 99 ± 6%, n=5 neurons; t-test, p>0.05). (E) Western blots of extracts of DIV22 hippocampal neuron cultures treated for 48h with 50μM APV and 10μM DNQX or DMSO as control. Equal volumes of extracts were loaded and immunoblotted for liprin-α2 and α-tubulin as loading control. (F) Quantification of endogenous liprin-α2 levels in cells treated with 48h APV and DNQX or DMSO (DMSO: 100%, 48h APV+DNQX: 57.6 ± 12%, n=3; *, Student’s t-test, p=0.024).
Figure 2.2 Liprin-\(\alpha\) shRNAs efficiently reduce liprin-\(\alpha\) protein levels. (A) Western blots of extracts of DIV13 hippocampal neuron culture infected at DIV7 with indicated amount of GFP-liprin-\(\alpha\) 2-shRNA expressing Lentivirus. Equal volumes of extracts were loaded and immunoblotted for GFP, liprin-\(\alpha\)1, liprin-\(\alpha\)2, and actin as a loading control. (B) Western blots of HeLa cell extracts of GFP-liprin-\(\alpha\) proteins cotransfected with either liprin-\(\alpha\)1, liprin-\(\alpha\)2, or control (CASK) shRNA constructs. (C) Quantification of knockdown of endogenous liprin-\(\alpha\)2 by liprin-\(\alpha\)2 shRNA. For Western blot (WB), DIV7 neurons were infected with indicated amounts of GFP-liprin-\(\alpha\)2-shRNA Lentivirus and harvested 6 days later (GFP: 100%, liprin-\(\alpha\)2 shRNA: 26.3%). For immunofluorescence (IF), DIV15 neurons were transfected with GFP with or without liprin-\(\alpha\)2 shRNA and fixed and stained at DIV19. Fluorescence intensity was measured in the cell body of transfected neurons (GFP: 100.0 ± 3.4%, liprin-\(\alpha\)2 shRNA: 36.3 ± 4.3%, n= 5 neurons per group; ***, t-test, p<0.0005). Intensity of liprin-\(\alpha\)2 labeling in control cells was set to 100%. (D) Representative images of liprin-\(\alpha\)2 levels in neurites of neurons infected either with GFP or GFP-liprin-\(\alpha\)2-shRNA Lentivirus (green). Cells were stained for endogenous VAMP2 (red) and liprin-\(\alpha\)2 (blue). Scale bar represents 5 \(\mu\)m.
Figure 2.3 Liprin-α2 influences synaptic vesicle release but not the number of active synapses.

(A) Quantification of the percentage of presynaptic sites transfected with liprin shRNA constructs that were loaded with FM 4-64 dye (GFP: 84.0 ± 4.3%, liprin-α2 shRNA: 83.0 ± 7.3%, liprin-α1 shRNA: 83.0 ± 5.1%; n= 5 sets of 20 synapses per group). (B) Quantification of the fluorescence intensity of FM 4-64 dye loading of presynaptic sites transfected with liprin shRNA constructs (GFP: 100.0 ± 2.3%, liprin-α2 shRNA: 83.2 ± 2.9%, liprin-α1 shRNA: 93.3 ± 4.2%; n= 100 synapses per group; ***, t-test, p<0.001). Intensity of FM4-64 labeling in control cells was set to 100%. (C) Quantification FM 4-64 destaining following unloading of presynapses transfected with liprin shRNA constructs (n=75 (GFP), 25 (liprin-α1 shRNA), or 82 (liprin-α2 shRNA) synapses; for GFP v. liprin-α2 shRNA: ANOVA Repeated Measures: ***, F=51.96, p<0.001). (D) Quantification of the difference between the first frame after unloading stimulation (t₀) and the last frame imaged (t₁₂₀) for presynapses transfected with liprin shRNA constructs (GFP: 26.8 ± 1.0 A.U., n=75 synapses, liprin-α2 shRNA: 19.4 ± 1.9 A.U., n=82 synapses, liprin-α1 shRNA: 29.9 ± 1.7 A.U., n=25 synapses; ***, t-test, p<0.001).
39s, Figure 2.1A-B), indicating that mammalian liprin-α2 is regulated by proteasome degradation via APC. We next examined whether changes in global synaptic network activity can also affect liprin-α2 levels. Blockade of synaptic activity using the NMDA antagonist APV and AMPA antagonist DNQX for 48 hours results in a 40% loss of liprin-α2 protein compared to control (Figure 2.1E-F). These data show that liprin-α2 levels are regulated both by APC and synaptic activity.

Next, we examined the role of liprin-α2 in presynaptic function by depleting the expression of liprin-α2 in mature hippocampal neurons by RNA interference. The selected liprin-α shRNAs were effective in reducing liprin-α2 protein levels by about 70%. shRNAs did only knockdown the targeted liprin-α isoform without affecting the expression of other liprin-α proteins (Figure 2.2A-D). We first investigated the effect of liprin-α2 knockdown on SV recycling using fluorescent membrane FM dyes (Fernandez-Alfonso and Ryan, 2004). We labeled the total recycling pool (TRP) of vesicles of hippocampal neuron cultures transfected with liprin-α1 or liprin-α2 shRNAs by stimulation with 70 mM KCl for 90 seconds in the presence of 10 μM FM4-64 dye (Leal-Ortiz et al., 2008). There were no significant differences in the percentage of axonal varicosities that contained FM4-64 clusters (Figure 2.3A). However, after loading, synapses deficient in liprin-α2 displayed a 17% reduction of FM4-64 fluorescence intensity compared to control while synapses lacking liprin-α1 were unchanged (Figure 2.3B). We further compared the destaining kinetics of the TRP following a second stimulation with 70 mM KCl for 60 seconds. Neurons expressing liprin-α2 shRNA exhibited decreased FM4-64 unloading both immediately following the stimulation (Figure 2.3C, second data point) and in the two minutes that followed (Figure 2.3C-D). Again, synapses in neurons expressing liprin-α1 shRNA were not different from control neurons (Figure 2.3A-D). This suggests that liprin-α2 controls TRP size and regulates the efficiency of SV release.

To understand the presynaptic function of liprin-α2 in greater detail, we performed whole-cell patch clamp recordings in cultured hippocampal autaptic neurons infected with liprin-α2 shRNA expressing Lentiviruses at DIV9 (Figure 2.5A). Though miniature EPSC (mEPSC) frequency (Figure 2.4A-B), amplitude (Figure 2.4C), rise time (Figure 2.4D) and charge
Figure 2.4 Liprin-α2 regulates evoked release.

(A) Typical examples of spontaneous vesicles release. Scale bar represents 0.1s and 25pA. (B) Spontaneous release frequency in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 3.6 ± 0.5 Hz, n=60; liprin-α2 shRNA: 2.8 ± 0.5 Hz, n=40; liprin-α1 shRNA: 4.8 ± 1.7 Hz, n=43). (C) Amplitude of spontaneous release events in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 16.9 ± 0.4 pA, n=58; liprin-α2 shRNA: 17.2 ± 0.6, n=37; liprin-α1 shRNA: 16.9 ± 0.6, n=31). (D) Rise time of spontaneous release events in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 0.91 ± 0.04 ms, n=58; liprin-α2 shRNA: 0.94 ± 0.06 ms, n=37; liprin-α1 shRNA: 1.02 ± 0.09 ms, n=31). (E) Charge of spontaneous release events in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 38.4 ± 1.2 fC, n=58; liprin-α2 shRNA: 37.4 ± 1.6 fC, n=37; liprin-α1 shRNA: 40.7 ± 2.6 fC, n=31). (F) Amplitude of excitatory synaptic response evoked by an action potential in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 1.67 ± 0.15 nA, n=73; liprin-α2 shRNA: 0.96 ± 0.16 nA, n=39; liprin-α1 shRNA: 1.70 ± 0.21 nA, n=51; ***, Kruskal-Wallis test, p<0.001). (G) Typical examples of EPSCs. Scale bar represents 20ms and 0.5nA.
Figure 2.5 Liprin-α2 does not influence neuronal morphology.

(A) Representative images of a hippocampal autaptic neuron expressing GFP stained for MAP2 (blue) and VAMP2 (red) at DIV14. Scale bar represents 30μm.

(B) Quantification of total synapse number in hippocampal autaptic neurons infected with GFP or GFP plus liprin shRNA viruses (GFP: 248.8 ± 20.8 synapses, n=70; liprin-α2 shRNA: 255.7 ± 16.6 synapses, n=70; liprin-α1 shRNA: 228.4 ± 16.6 synapses, n=33; ns., Kruskal-Wallis test, p>0.05).

(C) Quantification of total dendrite length in hippocampal autaptic neurons infected with GFP or GFP plus liprin shRNA viruses (GFP: 1112 ± 72 μm, n=70; liprin-α2 shRNA: 1142 ± 66 μm, n=70; liprin-α1 shRNA, 1194 ± 96 μm, n=33, ; ns., Kruskal-Wallis test, p>0.05).

(D) Quantification of soma area in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 214.3 ± 8.6 μm², n=70; liprin-α2 shRNA: 191.4 ± 6.9 μm², n=70; liprin-α1 shRNA: 193.2 ± 11.7 μm², n=33; ns., Kruskal-Wallis test, p>0.05).

(E) Quantification of dendritic branching by Sholl analysis in hippocampal autaptic neurons infected with liprin shRNA viruses.

(F) Quantification of number of synapses per distance from the soma (using concentric rings of Sholl analysis) in hippocampal autaptic neurons infected with liprin shRNA viruses.
Figure 2.6 Liprin-α2 regulates RRP size and synaptic release probability.

(A) RRP size as assessed with single hyperosmotic sucrose application (500 mM sucrose) in hippocampal autaptic neurons infected with liprin shRNA viruses (GFP: 1.29 ± 0.23 nC, n=19; liprin-α2 KD, 0.46 ± 0.06 nC, n=16; **, Mann-Whitney test, p=0.003). (B) Typical examples of sucrose-induced currents. Grey bars represent time of sucrose application. Scale bars represent 1s and 0.1nA. (C) Vesicular release probability (Pves) is calculated as EPSC charge divided by sucrose induced RRP charge (GFP: 6.0 ± 0.8%, n=19; liprin-α2 KD, 4.8 ± 0.8, n=16; n.s., Mann-Whitney test, p>0.05). (D) Paired-pulse ratio (ratio of the second to the first synaptic response plotted as a function of the stimulus interval) in hippocampal autaptic neurons infected with liprin shRNA viruses. (n = 37 (GFP), 21 (liprin-α2 shRNA); all Mann-Whitney test , PP(20ms): **, p=0.005; PP(50ms): *, p=0.012; PP(100ms): ***, p<0.001; PP(200ms): ns., p=0.050; PP(500ms): *, p=0.019;
(Figure 2.4E) were unchanged, evoked EPSC amplitude was decreased by ~43% in neurons lacking liprin-α2 (Figure 2.4F-G). A second shRNA against liprin-α2 showed a similar phenotype (Figure S2.1). The changes in EPSC amplitude cannot be explained by differences in neuronal morphology, as soma area, dendrite length and branching, as well as synapse number were unaffected by liprin-α2 knockdown (Figure 2.5B-F). Importantly, in all electrophysiological and morphological parameters, no significant differences were observed in neurons lacking liprin-α1 (Figure 2.4A-K and Figure 2.5B-F), further confirming that liprin-α2 is specifically important for normal presynaptic function in the hippocampus.

We investigated readily releasable pool (RRP) size in liprin-α2 knockdown cells by measuring responses to application of 500mM sucrose (Rosenmund and Stevens, 1996). Liprin-α2 deficient neurons showed an ~49% decrease in RRP size (Figure 2.6A-B) while the vesicular release probability ($P_{ves}$) was unchanged (Figure 2.6C). To test the effect of liprin-α2 deletion on synaptic release probability, cells were stimulated with paired pulses of varying interstimulus intervals. Neurons expressing liprin-α2 shRNA exhibited paired pulse facilitation, especially at low interstimulus intervals (Figure 2.6D), indicating that synaptic release probability is decreased in the absence of liprin-α2. Next, we stimulated neurons at high frequency (100AP, 40Hz), a paradigm known to deplete the RRP, followed by low frequency stimulation (0.2Hz) to allow refilling of the RRP. Normalized amplitude plots show that, RRP is depleted and refilled similarly in liprin-α2 knockdown and control cells (Figure 2.6F). In addition, the proportion of synchronous charge per total charge is similar in both genotypes. Hence, in addition to regulating the TRP, liprin-α2 is an important factor in determining RRP size but not refilling kinetics.
Figure 2.7 Liprin-α2 levels are not controlled by other active zone proteins.

(A) Table of mass spectrometry results from biotin pull downs in brain P2 fractions.

(B) Quantification of the effectiveness of shRNAs against different active zone proteins used in (C). All shRNAs efficiently knocked-down their target protein.

(C) Quantification of endogenous liprin-α2 clustering at presynaptic sites transfected with shRNAs of presynaptic proteins (n=5 sets of 20 synapses per group). None
of the shRNAs significantly affects liprin-α2 synaptic targeting. (D) Representative images of cells transfected with indicated shRNA and imaged for GFP (green) and endogenous liprin-α2 (red).

To investigate the mechanism by which liprin-α2 influences presynaptic function, we searched for liprin-α2 binding partners using pull-down assays combined with mass spectrometry. We identified several known and novel interacting proteins (Figure 2.7A). CASK, CaMKIIα, and RIM1 have previously been shown to interact with liprin-α proteins (Spangler and Hoogenraad, 2007), while others are associated with liprin-α binding partners, such as MALS and Neurexins via CASK and Rab3 via RIM1 (Jin and Garner, 2008; Schoch and Gundelfinger, 2006). To test where liprin-α2 functions in this presynaptic protein network, we first examined whether synaptic targeting of liprin-α2 is affected by depleting the expression of several prominent interacting and non-interacting presynaptic proteins in cultured hippocampal neurons. Neurons at DIV15 were transfected with shRNA constructs to CASK, RIM1, ELKS1/2, bassoon, and piccolo and immunostained for liprin-α2 after 4 days, when levels of targeted proteins decreased substantially (Figure 2.7B). Under all conditions, no significant change in liprin-α2 clustering at synapses was observed, including when both bassoon and piccolo were simultaneously depleted (Figure 2.7 C-D). Thus, synaptic targeting of liprin-α2 is not dependent on the presence of other key presynaptic scaffolding proteins.

The liprin-α2 binding proteins CASK and RIM play important roles in presynaptic function (Olsen et al., 2005; Schoch et al., 2002). Interestingly, we observed an ~35% decrease in both CASK (Figure 2.8A-B) and RIM1/2 (Figure 2.8A,C) synaptic levels upon liprin-α2 knockdown. Importantly, the postsynaptic proteins PSD-95 and Shank were unaffected (Figure 2.8D-E), indicating that the postsynapse remained structurally intact in spite of the presynaptic defects. Furthermore, in hippocampal autaptic neurons infected with liprin-α2 shRNA expressing Lentiviruses we found additional presynaptic proteins, such as bassoon, Rab3, Munc18, synapsin, and P/Q type voltage-gated Ca2+ channels (VGCC), partly depleted in synapses (Figure 2.8F-G). These data suggest that liprin-α2 knockdown causes significant presynaptic disorganization extending beyond the mere loss of liprin-α2 binding partners; thereby implying that liprin-α2 regulates
Figure 2.8 Liprin-α2 controls presynaptic protein levels.

(A) Representative images of cells transfected with GFP or GFP plus liprin-α2 shRNA and imaged for GFP (green) and endogenous CASK or RIM (red). (B) Quantification of fluorescence intensity of endogenous CASK at presynaptic sites transfected with GFP or GFP plus liprin-α2 shRNA (GFP: 100.0 ± 4.3%, liprin-α2 shRNA: 67.5 ± 3.3%; n=100 synapses per group; ***, t-test, p<0.001). Intensity of CASK labeling in control cells was set to 100%.

(C) Similar to (B) for endogenous RIM at presynaptic sites (GFP: 100.0 ± 3.8%, liprin-α2 shRNA: 67.8 ± 3.3%; n=100 synapses per group; ***, t-test, p<0.0005). Intensity of RIM labeling in control cells was set to 100%.

(D) Similar to (B) for endogenous PSD-95 at presynaptic sites (GFP: 100.0 ± 4.6%, liprin-α2 shRNA: 110.2 ± 5.5%; n=100 synapses per group). Intensity of PSD-95 labeling in control cells was set to 100%.

(E) Similar
to (B) for endogenous shank at presynaptic sites (GFP: 100.0 ± 4.5%, liprin-α2 shRNA: 103.0 ± 5.0%; n= 100 synapses per group). Intensity of shank labeling in control cells was set to 100%. (F) Quantification of fluorescence intensity of various synaptic proteins at presynaptic sites of autaptic neurons transfected with GFP or GFP plus liprin-α2 shRNA. Intensity of labeling in GFP cells was set to 100% (bassoon: WT: 100 ± 4.9%, n=35 cells, liprin-α2 shRNA: 63.7 ± 4.1%; n=37 cells, **, t-test, p=0.002; Rab3: WT: 100 ± 4.7%, n=43 cells, liprin-α2 shRNA: 62.5 ± 4.0%; n= 49 cells, ***, t-test, p<0.001; Munc18: WT:100 ± 5.1%, n=38 cells, liprin-α2 shRNA: 79.9 ± 7.5%; n= 36 cells, **, Kruskal-Wallis test, p=0.009; syntaxin1: WT:100 ± 7.9%, n=24 cells, liprin-α2 shRNA: 98.6 ± 10.8%; n= 24 cells, ns.; VGCC (Cav2.1): WT:100 ± 10.8%, n=15 cells, liprin-α2 shRNA: 59.0 ± 5.8%; n= 18 cells, **, t-test, p=0.001; synapsin: WT:100 ± 3.9%, n=69 cells, liprin-α2 shRNA: 74.3 ± 4.5%; n= 87 cells, ***, t-test, p<0.001). (G) Representative images of Rab3 levels in neurites of neurons infected either with GFP or GFP-liprin-α2-shRNA Lentivirus (green). Cells were stained for endogenous VAMP2 (red) and Rab3 (blue). Scale bar represents 5 μm.

All data are expressed as mean ± SEM.

presynaptic function by organizing the clustering of the presynaptic release machinery.

In invertebrates, loss of Liprin-α causes disorganization of synapses. Immunofluorescence analysis showed that synapses were slightly elongated in the absence of liprin-α2 (Figure 2.9A-C). To look at the ultrastructure of these synapses in more detail, we performed electron microscopy analysis on autaptic neurons infected with liprin-α2 shRNA or GFP expressing Lentiviruses. Loss of liprin-α2 reduces number of docked vesicles and shortens active zone and postsynaptic density (Figure 2.9D-H). The total number of vesicles remained unchanged (Figure 2.9I), although they were distributed more loosely as seen by an increase in cluster perimeter (Figure 2.9J). Hence, liprin-2 is an important determinant of presynapse ultrastructure since it regulates active zone length and the distribution of vesicle pools.

We next tested whether expression of the prominent binding partners of liprin-α2 could rescue the liprin-α2 knockdown phenotype. Though Lentiviral-mediated expression of both CASK and RIM resulted in increased amounts of those proteins at synapses (Figure 2.10A), neither was able to increase EPSC amplitude in liprin-α2 deficient neurons (Figure 2.10B). Together these data suggest that liprin-α2 is a critical player in the organization and maintenance of the presynapse and functions upstream of active zone components like CASK and RIM.
Figure 2.9 Liprin-α2 knockdown changes synapse ultrastructure.

(A) Representative image of hippocampal autaptic neurons infected with GFP or GFP plus liprin-α2 shRNA stained for bassoon (red) and synapsin (blue). Scale bar represents 5 μm. (B) Quantification of presynapse length in neurons transfected with mCherry and either GFP or GFP plus liprin-α2 shRNA (GFP: 1.56 ± 0.04 μm, n=87 synapses; liprin-α2 shRNA: 1.68 ± 0.05 μm, n= 90 synapses; *, t-test, p=0.04). (C) Quantification of presynapse area in hippocampal autaptic neurons infected with liprin-α2 shRNA (GFP: 1.18 ± 0.01μm², n=189 cells; liprin-α2 shRNA: 1.23 ± 0.02μm², n= 211 cells; **, Mann-Whitney test, p=0.007). (D-J) Electron microscopy analysis of rat hippocampal autaptic neurons mice infected with Lentiviruses expressing EGFP or liprin-α2 shRNA together with EGFP. N_cultures=3, EGFP: n_synapses=150; α2 KD: n_synapses=135. Graphs represent synapse averages. (D) Typical examples. Scale bar represents 100nm. (E) Mean number
of docked vesicles (EGFP: 9.85 ± 0.35; α2 KD: 8.13 ± 0.30; ***, Mann-Whitney test, p<0.001). (F) Mean active zone length (EGFP: 529.72 ± 19.77nm; α2 KD: 467.96 ± 17.67nm; *, Mann-Whitney test, p=0.014). (G) Mean postsynaptic density (PSD) length (EGFP: 526.93 ± 19.56nm; α2 KD: 460.11 ± 17.95nm; **, Mann-Whitney test, p=0.006). (H) Mean number of docked vesicles per nm active zone (EGFP: 0.0194 ± 0.0004; α2 KD: 0.0181 ± 0.0005; *, Mann-Whitney test, p=0.021). (I) Mean number of total vesicles (EGFP: 138.93 ± 6.88; α2 KD: 136.53 ± 7.14; n.s., Mann-Whitney test, p>0.05). (J) Mean cluster perimeter (EGFP: 2.76 ± 0.09μm; α2 KD: 3.17 ± 0.10 μm; **, Mann-Whitney test, p=0.002). All data are expressed as mean ± SEM.

**Figure 2.10** RIM and CASK overexpression is unable to rescue liprin-α2 phenotype.

(A) Quantification of immunofluorescence intensity of RIM and CASK at presynaptic sites infected with lentiviral particles expressing mCherry or mCherry plus RIM or CASK. Intensity of labeling in control cells was set to 100%. (B) Evoked EPSC size in hippocampal autaptic neurons infected with liprin shRNA viruses cannot be rescued by overexpression of RIM-IRES-mCherry or CASK-IRES-mCherry (GFP+mCherry: 1.99 ± 0.25 nA, n=24; liprin-α2 shRNA+Cherry: 1.18 ± 0.2 nA, n=19; liprin-α2 shRNA+RIM-IRES-mCherry: 0.72 ± 0.17 nA, n=10; liprin-α2 shRNA+CASK-IRES-Cherry: 1.14 ± 0.21 nA, n=10; **, Kruskal-Wallis test, p =0.004; GFP+mCherry vs. liprin-α2 shRNA+Cherry: p=0.016, GFP+mCherry vs. liprin-α2 shRNA+RIM-IRES-mCherry: p=0.003, GFP+mCherry vs. liprin-α2 shRNA+CASK-IRES-Cherry: p=0.046, all other combinations p>0.05).

In this study we show that liprin-α2 is an important regulator of presynaptic function and plasticity in mature hippocampal synapses. First, liprin-α2 is regulated by both synaptic activity and the ubiquitin-proteasome system via the E3-ligase APC. Second, liprin-α2 is responsible for maintenance of the total and readily releasable vesicle pools and presynaptic short-
term plasticity. Third, this effect is likely due to the disrupted localization of important presynaptic proteins in the absence of liprin-α2 and cannot be compensated for by the reintroduction of liprin-α2 binding partners like RIM and CASK. The data suggest an intriguing model wherein liprin-α2 functions as an upstream organizer of active zone composition and therewith synaptic output. Activity-dependent manipulation of liprin-α2 levels via the proteasome system may serve as a potential mechanism for fine-tuning of synaptic efficacy during presynaptic plasticity.

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Supplementary figures

Figure S2.1 Second liprin-α2 shRNA also reduces evoked release to a similar extent. (A) Amplitude of excitatory synaptic response evoked by an action potential in hippocampal autaptic neurons infected with liprin shRNA viruses (scrambled: 5.08 ± 0.62 nA, n=13; liprin-α2 shRNA #3: 3.00 ± 0.37 nA, n=14; *, Mann-Whitney test, p=0.021).

Figure S2.2 Assay for quantifying presynaptic clustering in network cultures. (A) Method of quantifying localization of various proteins at presynaptic sites. Synapses are highlighted by GFP transfection and identified as varicosities along the length of the axon. Synapses that contain clusters of the protein being analyzed are counted as positive. Scale bars, 10 μm. (B) Quantification of number of positive synapses due to labeling in non-specific axons as determined by rotation of endogenous staining (0°: 78.0 ± 6.8%, 90°: 21.3 ± 5.2%, 180°: 23.8 ± 1.3%; n=4 sets of 20 synapses per group; ***, t-test, p<0.0005).