Transcriptomic analysis identifies synapse-enriched lncRNAs required for glutamatergic synapse development and fear memory consolidation.

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Summary:

Characterization of brain-enriched lncRNAs have predominantly been restricted to the nuclear compartment; with limited exploration of synaptic lncRNA functions. Our RNA-seq analysis of synaptoneurosomes identify 94 synaptically-enriched lncRNAs in the adult mouse hippocampus. Among these, we characterized the roles of Pantr1, Pvt1 and 2410006H16Rik (named SynLAMP) in glutamatergic synapse development, plasticity and memory. Pvt1 regulates dendritic arborization, spine morphology and glutamatergic synapse formation *via* a molecular framework of synaptogenic genes; as detected by RNA-seq analysis of the hippocampal trancriptome following Pvt1 knockdown. SynLAMP and Pantr1 modulate mEPSC amplitude and surface AMPA receptor distribution in mature synapses. We find activity-invoked redistribution of these synaptic lncRNAs and their concommitant reversible association with RNA binding proteins. The activity-dependent, transcript-specific synaptic localization of SynLAMP and Pantr1 indicate their synapse-centric function. SynLAMP specifically regulates basal and activity-invoked nascent translation in somato-dendritic compartments and its RNAi disrupts memory consolidation, underlining its input-specific role in synaptic translation.

Introduction:

Within neurons, functional autonomy of subcellular compartments such as synapses rely on the *in-situ* availability of proteins to meet the requirements for synaptic development and plasticity. Localization of RNAs to the synapse enables neurons to rapidly fulfill such on-site demands for stimulus-specific translation in a spatiotemporal manner (Holt and Schuman, 2013; Martin and Ephrussi, 2009; Sutton and Schuman, 2006). Transcriptomic analysis of the hippocampal neuropil has identified ~2550 mRNAs in excitatory synapses (Cajigas et al., 2012). Regulatory RNAs; predominantly comprised of non-coding transcripts, such miRNAs (Kye et al., 2007; Siegel et al., 2009), circular RNAs (Piwecka et al., 2017; Rybak-Wolf et al., 2015; You et al., 2015) and diverse isoforms of 3' untranslated region (3'UTR) (Tushev et al., 2018); have been identified from the synapto-dendritic compartment. These regulatory RNAs, in association with RNA binding proteins (RBPs), determine the transport, stability, and translation of mRNAs associated with neuronal plasticity (Darnell, 2013; Holt and Schuman, 2013) and memory (Kandel, 2001; Sutton and Schuman, 2006). However, no such detailed characterization exists for long noncoding RNAs (lncRNAs).

In situ hybridization data from the Allen Brain Atlas have identified 849 lncRNAs that are expressed either ubiquitously or in specific neuroanatomical regions of the brain (Mercer et al., 2008). This, together with findings from GENCODE (Harrow et al., 2012) and NONCODE (Bu et al., 2012; Xie et al., 2014); dismissed the preexisting notion that lncRNAs are simply by-products of transcription (Briggs et al., 2015; Samaddar and Banerjee, 2021). From a functional perspective, our understanding of brain-enriched lncRNAs primarily stems from nuclear lncRNAs. Almost all the neuronal lncRNAs reported to date are restricted to the nuclear, (Clemson et al., 2009; Khalil et al., 2009; Mondal et al., 2010; Tsai et al., 2010; Wei et al., 2022; Zhao et al., 2019) with a few exceptions; the lncRNAs AK077867 (Mercer et al., 2008), and ADEPTR (Grinman et al., 2021) and select isoforms of Gas5 (Liau et al.). The number and nature of cytoplasmic lncRNAs localized at the synapse remain undocumented thus far. Here, we performed a detailed transcriptomic analysis of the synaptic compartment of the adult mouse hippocampus to

identify the full complement of lncRNAs localized at the synapse. We found 1031 lncRNAs, including a subset of 94 lncRNAs that were significantly enriched in the synaptic compartment. Amongst the synaptically enriched lncRNAs, we focused on the characterization of three evolutionarily conserved lncRNAs; 2410006H16Rik, Pvt1 and Pantr1; to define their role in functional synapse development *in vitro* and *in vivo*. We have named the hitherto uncharacterized, novel lncRNA 2410006H16Rik as Synaptic LncRNA Associated with Memory and Plasticity (SynLAMP). We have investigated the somato-dendritic distribution of lncRNAs and the dynamics of lncRNA-RBP interaction post contextual fear conditioning. Furthermore, we have explored the involvement of these lncRNAs in dendritic protein synthesis and memory.

Results:

Identification of synapse-enriched long non-coding RNAs in the hippocampus.

To identify the full repertoire of hippocampal synapse-enriched lncRNAs, we sequenced total RNA extracted from purified synaptoneurosomes. As synaptoneurosomes are highly enriched for synaptic membranes that preserve components of the translation machinery and regulatory RNAs including non-coding transcripts (Rao and Steward, 1991; Siegel et al., 2009), we reasoned that they represent an appropriate source of synapse-enriched lncRNAs. Synaptoneurosomes were purified from the mouse hippocampus (8 week old) using a Ficoll gradient (Figure 1A), with the purity of this biochemical fraction being assessed by the enrichment of synaptic proteins PSD95 and Synaptophysin, along with the depletion of nuclear protein Histone H1 and astrocytic protein GFAP (Figure 1B). Three independent sequencing runs were performed using total RNA purified from synaptoneurosomes and cell soma. GENCODE (M18) was used to identify lncRNAs from mapped reads. We have identified 563 annotated and 468

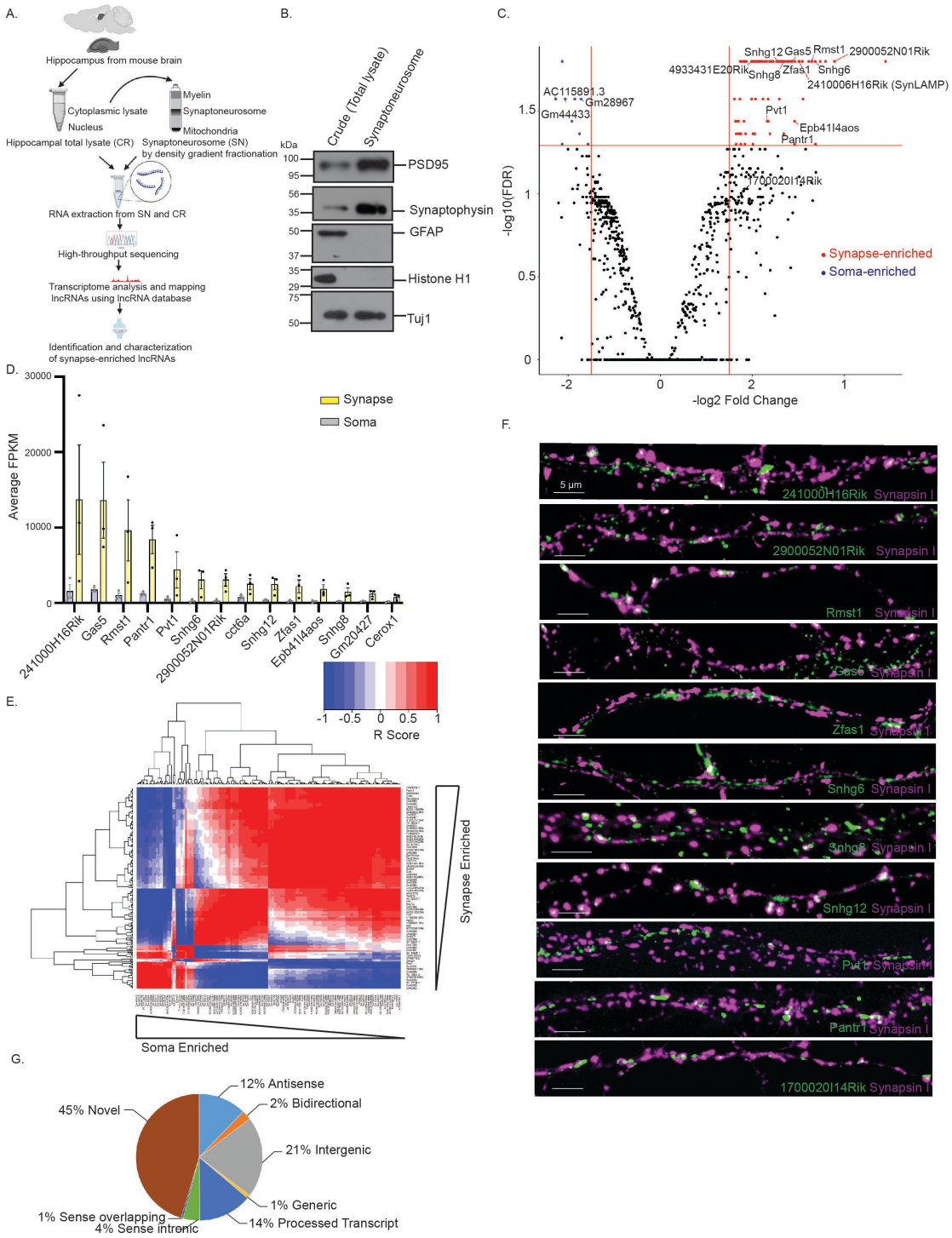

Figure 1: Identification of lncRNAs at the hippocampal synapse

(**A**) Schematic representation of synaptoneurosome preparation (SN) from mouse hippocampus and identification of synapse-enriched lncRNAs by RNA-seq analysis. (**B**) Evaluation of SN quality by western blot. (**C**) Volcano plot showing lncRNAs (FPKM>10) identified from hippocampal neurons. Highlights indicate lncRNAs significantly (FDR<0.05) enriched at the synapse (red) and soma (blue) . (**D**) Bar graph showing the expression of highly abundant and evolutionarily conserved lncRNAs in soma (black) and synapse (grey). (**E**) Heat map showing the correlation of synapse-enriched versus soma-enriched lncRNA expression. Correlated lncRNA

expressions are encoded in the heat map (low;blue to high;red). LncRNAs showing similar expression patterns are clustered together. (**F**) Dendrites from cultured hippocampal neurons showing fluorescent *in situ* hybridization of indicated lncRNAs (green puncta).Synapses marked with Synapsin I (magenta puncta) and synaptic distribution of lncRNAs confirmed by co-localization (white puncta). Scale as indicated. See Figure S1. (**G**) Categorization of lncRNAs identified from SN shown in pie chart with indicated percentage of novel and diverse types of annotated lncRNAs. The annotated lncRNAs are classified based on their genomic location.

novel lncRNAs that were expressed in the hippocampus (FPKM >10) (Table S1). Among the annotated lncRNAs, 94 were significantly enriched at the synapse (FDR <0.05) (Figure 1C and 1D). Our study also detected 45 soma-enriched lncRNAs, among which, 9 of them showed significant enrichment (FDR <0.05). The absence of correlation between the synapse- and soma-enriched lncRNAs indicated the compartmentalized localization of lncRNAs (Figure 1E). The synaptic localization of a subset of synapse-enriched lncRNAs was verified by *in situ* hybridization using anti-sense probes, and their specificity assessed with sense probes (Figures 1F and S1).

Based on their genomic location with respect to protein-coding genes; lncRNAs are classified as antisense, intergenic, sense overlapping, sense intronic, bidirectional and processed transcripts (Dunham et al., 2012; Luo et al., 2020; Mercer et al., 2008, 2009). We have identified a significant proportion of intergenic (21%, 213), processed transcripts (14%, 141) and antisense (12%, 126) lncRNAs (GENCODE M18) (Figure 1G). We selected 2410006H16Rik (SynLAMP), Pantr1 and Pvt1 for further characterization based on their abundance in the synaptic compartment (Figure 1D) and evolutionary conservation.

Functional analysis of synapse-enriched lncRNAs identifies Pvt1 as a regulator of dendrite and spine morphology.

The structural alteration of dendritic spines correlates with the maturity and strength of excitatory synapses (Matsuzaki et al., 2004; Zito et al., 2004). To investigate the importance of lncRNAs in regulating dendritic arborization and spine structure, we have inhibited the functions of SynLAMP, Pantr1 and Pvt1 in hippocampal neurons by shRNA–mediated RNA interference (RNAi). Two

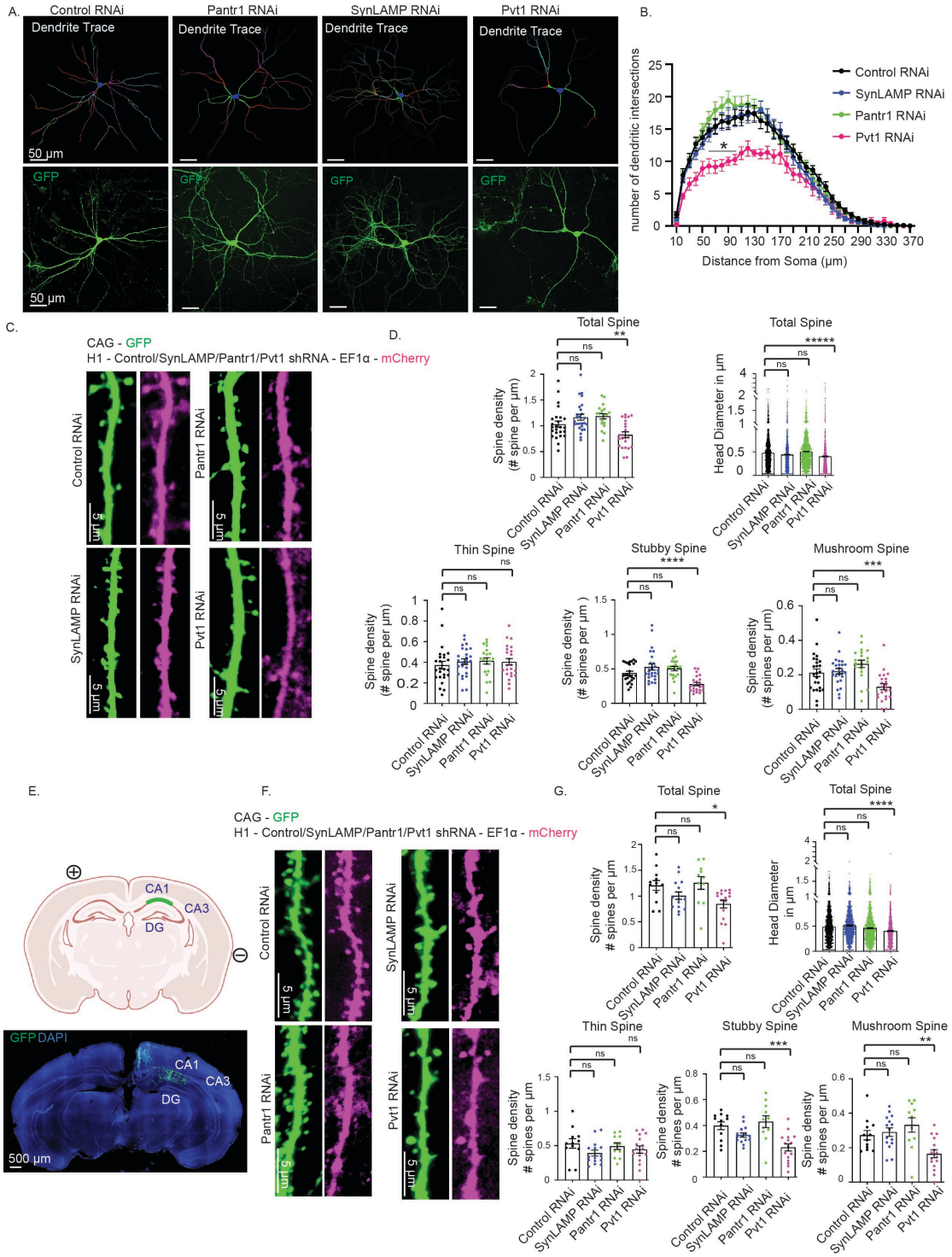

Figure 2: Pvt1 influences dendritic branching and spine growth.

(**A**) Traces of reconstructed (upper panel) and confocal images (lower panel) of cultured neurons (DIV21) expressing non-targeting control shRNA or shRNA targeting Pantr1, SynLAMP and Pvt1 respectively. Scale as indicated. (**B**) Quantification of dendritic branching. n: control RNAi (12), Pantr1 RNAi (15), SynLAMP RNAi (20), Pvt1 RNAi (8), *p< 0.03. Multiple t-test determined by Holm-Sidak method. Data shown as mean ± SEM. (**C**) Images showing dendritic spines from cultured neurons expressing control shRNA or shRNA against Pantr1/SynLAMP/Pvt1 (as indicated). (**D**) Quantification of spine density, spine head

diameter, and density of spine types. n = 1300 to 1900 spines from 8 to 12 neurons *in-vitro*, *p<0.04, **p<0.03, ***p<0.008, ****p<0.002, *****p<0.0001, ns = not significant. One way Anova and Bonferroni correction. Data shown as mean ± SEM. (**E**) Plasmids harboring control shRNA or shRNA against Pantr1/SynLAMP/Pvt1 were electroporated into E17 embryos along with GFP. Brain slices were prepared at P28 for spine analysis from CA1 neurons. (**F**) Images showing dendritic spines of *in utero* electroporated CA1 neurons expressing indicated shRNA. (**G**) Quantification of spine density, spine head diameter, and spine types. n = 1350 to 1470 spines from 5 to 8 neurons *in vivo*, *p<0.01, **p<0.02, ***p<0.0006, ****p<0.0001, ns = not significant. One way ANOVA and Bonferroni correction. Data shown as mean ± SEM.

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distinct shRNAs were designed against each transcript and their efficacy determined using qRT-PCR (for Pantr1: 55.29± 14.61% decrease, p<0.005, for shRNA#1; 65.33 ± 13.07% decrease, p<0.0001, for shRNA#2; for Pvt1: 46.71 ± 12.58% decrease, p<0.0003, for shRNA#1, and 52.73 ± 11.03% decrease, p<0.0002, for shRNA#2; for SynLAMP: 44.94 ± 8.93 % decrease, p<0.0004, for shRNA#1, 69.37 ± 10.95% decrease, p<0.0001 for shRNA#2; Figure S2). Plasmids expressing shRNA against indicated lncRNAs and EF1α-mCherry were co-transfected with EGFP (CAG-EGFP) into primary neuronal cultures (DIV 3-4). Effective RNAi was determined by mCherry expression and dendritic arborization was calculated at DIV-21 based on EGFP fluorescence intensity. Pvt1 RNAi reduced dendritic arborization (distance from the soma: 70μm, 6.292±1.609; p<0.03; 80μm, 6.792±1.481, p<0.008; 90μm, 6.000±1.435, p<0.019; 100μm, 6.500±1.588, p<0.022); whereas SynLAMP and Pantr1 had no effect (Figure 2A - 2B). The loss of Pvt1 function also led to a decrease in spine density (spine numbers per μM of dendrite; Control RNAi: 1.032 ± 0.06, Pvt1 RNAi: 0.82 ± 0.05, p < 0.03) (Figure 2C - 2D). Knockdown of Pvt1 reduced the head diameter (Control RNAi: 0.47 ± 0.008 μM, Pvt1 RNAi: 0.40 ± 0.01 μM, p < 0.0001) of dendritic spines (Figure 2C - 2D). Analysis of spine types revealed that Pvt1 knockdown reduced the density of stubby (Control RNAi: 0.43 ± 0.02, Pvt1 RNAi: 0.28 ± 0.02, p < 0.002) and mushroom spines (Control RNAi: 0.21 ± 0.02, Pvt1 RNAi: 0.129 ± 0.01, p < 0.008) without affecting thin spine density. Although Pantr1 RNAi led to a miniscule increase in head diameter, the knockdown of Pantr1 and SynLAMP did not profoundly influence spine structure or type (Figure 2D). To complement our observations from *in vitro* studies, we also determined the spine structure and type from CA1 neurons in

the hippocampus *in vivo* (P28) following *in utero* electroporation of SynLAMP, Pantr1 and Pvt1 shRNAs in CA1 neurons (E17) (Figure 2E). As in cultured neurons, knockdown of Pvt1 reduced the spine density (total spine density: Control RNAi: 1.2 ± 0.09, Pvt1 RNAi: 0.84 ± 0.07, $p < 0.018$; mushroom spine density: Control RNAi: 0.27 ± 0.02, Pvt1 RNAi: 0.16 ± 0.02, $p < 0.02$; and stubby spine density: Control RNAi: 0.398 ± 0.03, Pvt1 RNAi: 0.23 ± 0.02, $p < 0.0006$), but the density of the thin spines was not affected (Figure 2F - 2G). Pvt1 knockdown led to the reduction of head diameter (Control RNAi: 0.48 ± 0.007 μm, Pvt1 RNAi: 0.4 ± 0.006 μm, $p < 0.0001$) (Figure 2G). Consistent with our *in vitro* studies, knockdown of SynLAMP and Pantr1 had no profound effect on either spine structure or spine type *in vivo* (Figure 2F - 2G). These observations indicate that Pvt1 specifically regulates the morphology of dendritic spines that are sites for synapse formation.

Pvt1 regulates glutamatergic synapse development.

We next investigated the importance of SynLAMP, Pantr1 and Pvt1 in glutamatergic synapse development by measuring synapse density following their knockdown. Cultured hippocampal neurons (DIV 3 - 4) were transduced with lentivirus expressing each shRNA. Synapse density was measured from transduced neurons (DIV 21) on the basis of colocalization of Synapsin I and PSD95. The age of the neuron was commensurate with previous studies reporting a similar temporal window for synapse development (Fletcher et al., 1994; Paradis et al., 2007). Pvt1 knockdown led to reduced synapse density (Control RNAi: 1 ± 0.06, Pvt1 RNAi: 0.75 ± 0.07, $p < 0.03$), whereas the knockdown of Pantr1 and SynLAMP had no effect (Figure 3A-3B). To determine the involvement of Pvt1 in synapse development *in vivo*, RNAi against Pvt1 was performed in CA1 neurons *via in-utero* electroporation (E17 embryos). and synapse-density measured (P28). We observed a significant reduction in synapse density (Control RNAi: 1 ± 0.08, Pvt1 RNAi 0.68 ± 0.03, $p < 0.001$) (Figure 3C - 3D). Together, these data indicate that Pvt1 is a positive regulator of glutamatergic synapse development.

Pvt1 influences the expression of cytoskeleton effectors and synaptic proteins.

Regulators of the actin cytoskeleton determine the morphology of dendritic spines (Hotulainen and Hoogenraad, 2010; Kennedy et al., 2005). The Rho family of GTPases, such as RhoA, Rac1 and Cdc42, are key regulators of the actin cytoskeleton that profoundly influence spine morphology. Rac1 and Cdc42 promote spine growth, whereas RhoA enhances spine loss (Tolias et al., 2011). Prompted by these observations, we measured the expression of Rac1 and RhoA following the knockdown of Pantr1, SynLAMP and Pvt1. Hippocampal neurons (3-4) in culture were transduced with lentivirus expressing indicated shRNAs and Rac1 as well as RhoA protein levels were analyzed from these neurons (DIV21). We observed that Pvt1 RNAi led to the reduction of Rac1 (47.00 ± 10.44 % decrease, $p < 0.01$) (Figure 3E- 3F), whereas the level of RhoA was enhanced (106.1 ± 28.89 % increase, $p < 0.02$) (Figure 3G - 3H). However, Pantr1 and SynLAMP RNAi had no effect (Figure 3E - 3H).

To determine the Pvt1-driven molecular framework influencing synapse development, we next performed a transcriptomics analysis using total RNA from Pvt1 RNAi neurons. Cultured neurons (DIV 3-4) were transduced with lentivirus expressing shRNA against Pvt1 followed by RNA-seq analysis from these neurons (DIV 21) (Figure 3I). We found that Pvt1 RNAi predominantly reduced the expression of transcripts encoding post-synaptic proteins that included regulators of AMPA receptor activity, such as Grip2, Nptx1, Nsf and Rin1; effectors of synaptic stability, such as Efnb3 (encodes Ephrin B3) and Tamalin; and presynaptic proteins including components of the synaptic vesicle, such as VAMP1/2, Cplx1 and Sv2c. We also detected the upregulation of the actin binding protein Filip1, that negatively regulates dendritic spine morphology, as well as transcripts encoding motor protein Dnah5, necessary for retrograde transport from synapse to nucleus, and Rgs6, a regulator of G Protein Signaling 6 (Figure 3I). GO analysis of down-regulated transcripts (Table S2) revealed enrichment of synaptic GO annotations (FDR<0.05) (Figure 3J). We also observed a significant enrichment in biological processes and cellular components associated with

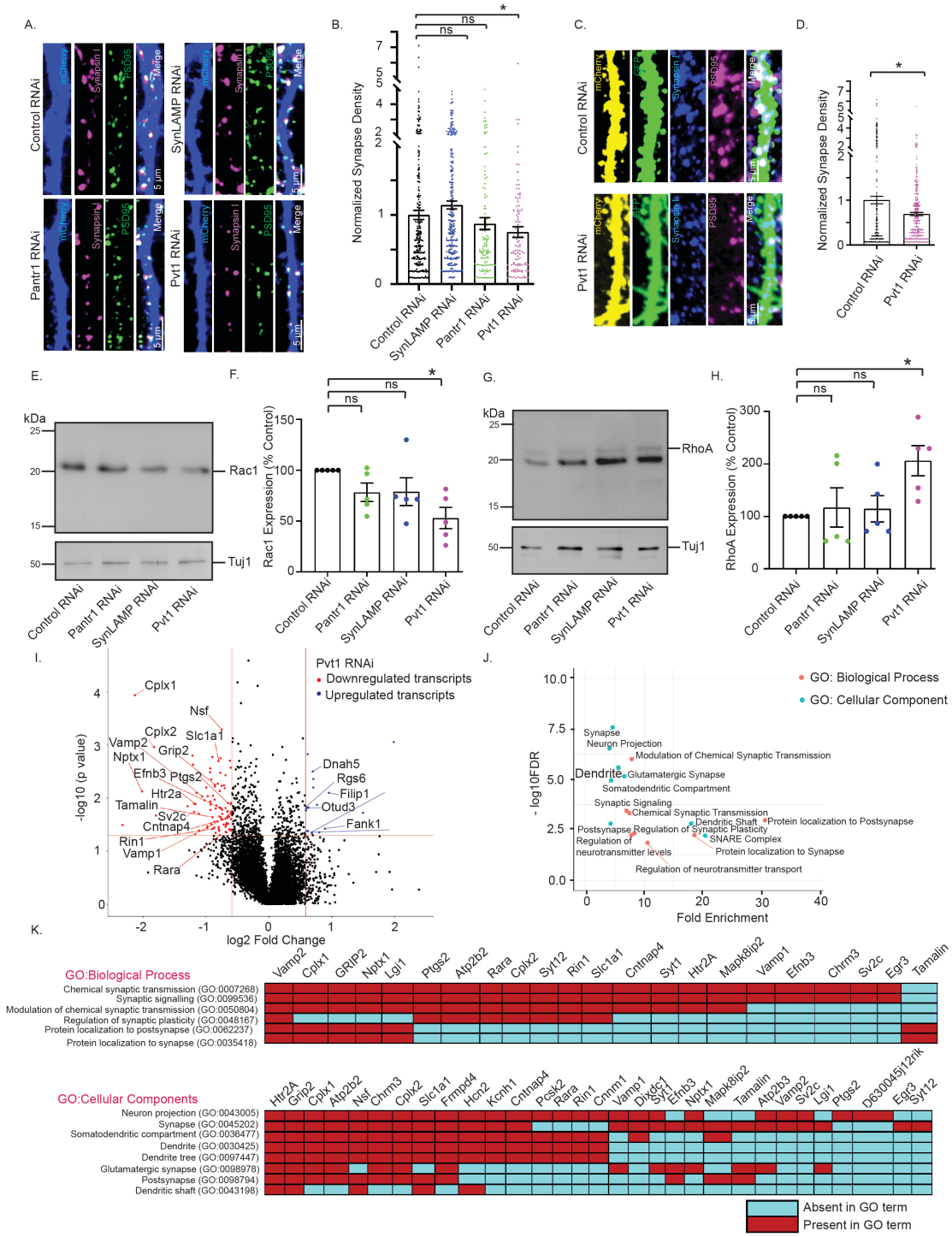

Figure 3: Pvt1 regulates excitatory synapse development.

(**A-B**) Synapse density analysis from cultured neurons expressing shRNA (as indicated). (A) Images showing immunostaining of Synapsin I (magenta) and PSD95 (green) in the dendrites expressing mCherry (blue). Glutamatergic synapses are defined by apposed Synapsin I and PSD95 puncta on dendrites (white puncta). (B) Quantification of synapse density. n= 15–26, *p<0.03, ns = not significant. One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM. (**C-D**) Synapse density analysis from *in utero* electroporated CA1 neurons expressing shRNA as indicated. (C) Image showing immunostaining of Synapsin I (blue), PSD95 (magenta),

dendrites expressing mCherry (yellow) and EGFP (green). Overlapping PSD95 and Synapsin I puncta on dendrite represents glutamatergic synapse (white). (D) Quantifiction of synapse density. n= 36 – 39. *p<0.001.One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM. (**E-H**) Expression of Rac1, RhoA and Tuj1 from cultured hippocampal neurons expressing shRNA as indicated. (E) Immunoblot showing the expression of Rac1 and Tuj1. (F) Quantification of Rac1 expression. n=5, *p< 0.01. (G) Immunoblot showing the expression of RhoA and Tuj1. (H) Quantification of RhoA expression. n=5, *p< 0.02. ns = not significant, Unpaired t-test with Welch's correction. Data shown as mean ± SEM. (**I – K**) Transcriptomics and Gene Ontology (GO) analysis following Pvt1 RNAi.(I) Volcano plot showing up- or down–regulated transcripts (FPKM>0.5) in cultured hippocampal neurons following Pvt1 RNAi. (J) GO analysis showing significant (FDR, p<0.05) enrichment of GO-terms for the function of genes encoding down-regulated mRNAs following Pvt1 RNAi. (K) Specific transcripts enriched in the indicated GO-terms. See Figures S2 and S3.

postsynaptic function. The biological processes included chemical synaptic transmission (FDR 4.72e-04), synaptic signaling (FDR 3.67e-04), modulation of chemical synaptic transmission (FDR 1.01e-06), regulation of synaptic plasticity (FDR 4.68e-03), protein localization to the post-synapse (FDR 1.06e-03), and protein localization to synapse (FDR 5.75e-03).The cellular components include neuron projection (FDR 2.94e-07), somatodendritic compartment (FDR 1.13e-05), synapse (FDR 2.72e-08), dendrite (FDR 2.77e-06), dendritic tree (FDR 2.57e-06), glutamatergic synapse (FDR 7.13e-06), postsynapse (FDR 1.55e-03) and dendritic shaft (FDR 1.54e-03) (Figure 3J - 3K). There was also a significant enrichment of GO annotations associated with presynaptic functions, such as the SNARE complex (FDR 6.11e-03), regulation of neurotransmitter transport (FDR 1.36e-02) and regulation of neurotransmitter levels (FDR 5.7e-03) (Figure S3F). This observation is consistent with our data showing that Pvt1 RNAi reduced the puncta density of both PSD95 (40.7978% decrease, p<0.03) and Synapsin I (37.6402% decrease, p<0.03) (Figures S3C - S3E).

Regulation of AMPA receptor-mediated synaptic activity by lncRNAs.

The recruitment of AMPA receptors (AMPARs) to the developing synapse drives the maturation of glutamatergic synapses. As synaptic transmission within the mature neural circuitry is predominantly governed by AMPAR activity (Hall and Ghosh, 2008; Wu et al., 1996), we used whole-cell voltage-

clamp recordings to measure the amplitude and frequency of AMPAR–mediated miniature excitatory postsynaptic currents (mEPSCs) from cultured hippocampal neurons following the knockdown of each lncRNA. Neurons (DIV 6-7) were transduced with two types of lentivirus, each expressing a distinct shRNA (as indicated), and whole-cell voltage-clamp recording was performed from these neurons (DIV 21-25). We observed a significant reduction in mEPSC amplitude following the knockdown of SynLAMP (4.36 ± 0.37 pA decrease for shRNA#1, $p<0.0001$; 5.76 ± 0.30 pA decrease for shRNA# 2, $p<0.0001$), Pantr1 (4.33 ± 0.37 pA decrease for shRNA#1, $p < 0.0001$; 5.53 ± 0.32 decrease for shRNA#2, $p<0.0001$) and Pvt1 (5.35 ± 0.40 pA decrease for shRNA#1, $p<0.0001$; 5.67 ± 0.32 pA decrease for shRNA#2, $p<0.0001$) (Figures 4A - 4B, S4A - S4B). However, we did not observe any change in mEPSC frequency following SynLAMP or Pantr1 RNAi. (Figures 4A and 4C, S4A and S4C). A small reduction in mEPSC frequency was detected upon Pvt1 knockdown (1.67± 0.61 Hz decrease, $p<0.007$ for shRNA#1, 1.08 ± 0.39 Hz decrease, $p<0.01$ for shRNA#2) (Figures 4C and S4C).

mEPSC amplitude determines synaptic strength and is a direct readout of the abundance of AMPARs on the postsynaptic membrane (O'Brien et al., 1998; Srinivasan et al., 2021). The observed reduction in mEPSC amplitude therefore prompted us to measure AMPAR levels on the postsynaptic surface (sAMPARs) following knockdown of SynLAMP, Pantr1 and Pvt1. sAMPAR distribution was analyzed by measuring the surface expression of the GluA1 and GluA2 (sGluA1/A2) subunits of AMPARs. Cultured hippocampal neurons (DIV 6-7) were transduced with lentivirus expressing the indicated shRNA. These neurons (DIV21) were live labeled with N-terminus-specific antibodies against sGluA1/A2 and synapses were marked by PSD95. We found that sGluA1/A2 expression in excitatory synapses was reduced following the knockdown of SynLAMP (33.9 ± 5.1% decrease for sGluA1, $p<0.0001$; 50.11 ± 14.05% decrease for sGluA2, $p<0.0005$), Pantr1 (33.14± 5.17% decrease for sGluA1, $p<0.0001$; 46.87±12.79% decrease for sGluA2, $p<0.0004$) and Pvt1 (27.13 ± 5.39% decrease for sGluA1, $p<0.0001$; 31.97 ± 13.66 % decrease for sGluA2, $p<0.03$) (Figure 4D - 4G and S4D - S4E). Collectively,

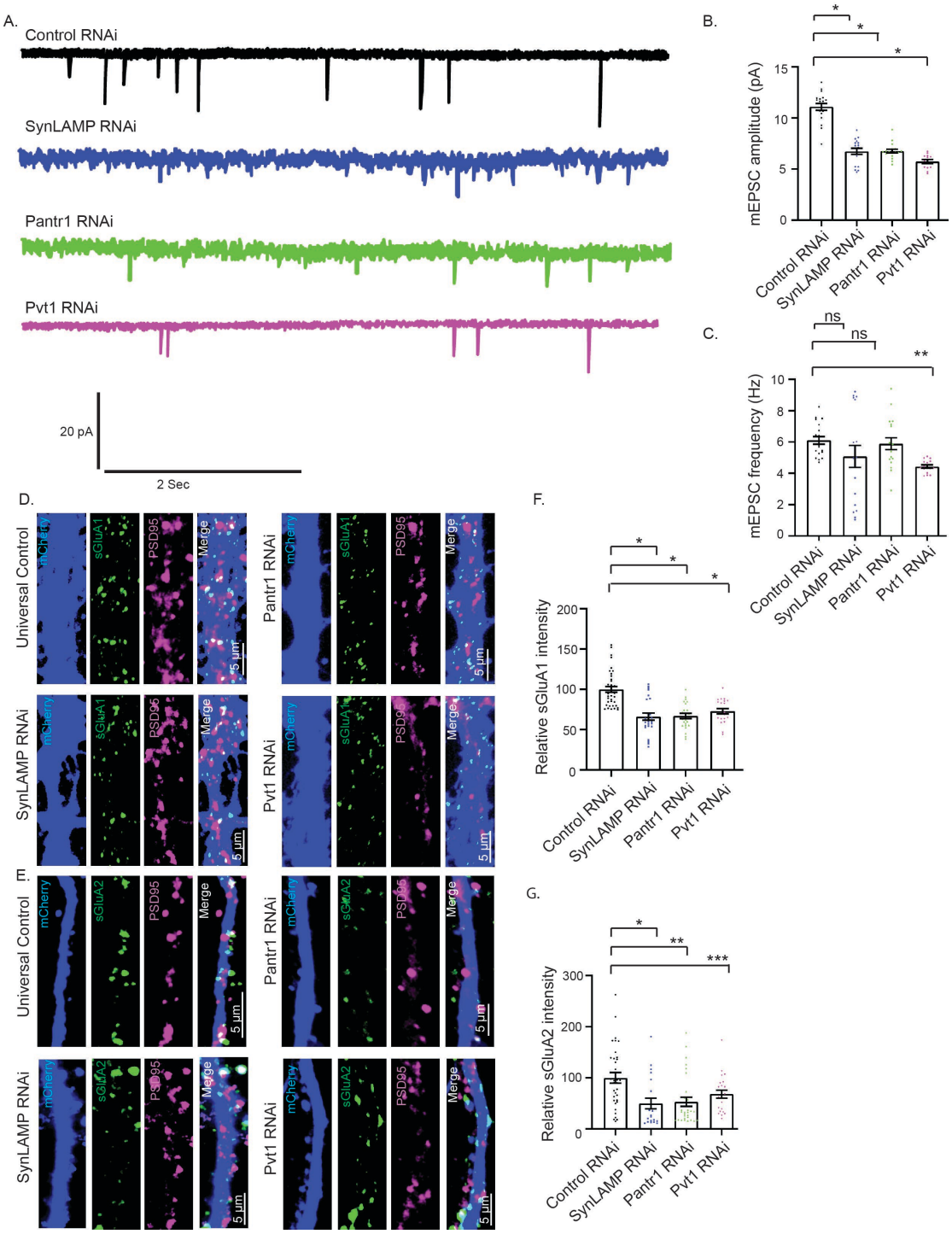

Figure 4: Loss of SynLAMP, Pantr1 or Pvt1 function affects synaptic activity *via* sAMPAR abundance.

(**A – C**) Whole-cell patch clamp recording and sAMPAR abundance analyzed from neurons transduced with shRNAs, as indicated. (A) mEPSC traces. (B) Mean mEPSC amplitude. (C) Mean mEPSC frequency. n = 16 – 19. *p <0.0001, **p<0.007. ns = not significant. One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM. (**D – G**) sAMPAR abundance following knockdown of indicated shRNAs. Image showing (D) sGluA1 (green) or (E) sGluA2 (green) on mCherry expressing dendrites (blue) from cultured neurons immunostained with PSD95 (magenta). sGluA1 or sGluA2 abundance in synapses shown in merged (sGluA1 or

sGluA2/PSD95/mCherry) images. (F) Quantification of normalized intensity of synaptic sGluA1. n = 21 – 38, *p< 0.0001. (G) Quantification of normalized intensity of synaptic sGluA2. n = 21 – 34, *p< 0.0005, **p<0.0004, ***p<0.03. ns = not significant. One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM. See Figure S4.

these findings indicate that SynLAMP, Pantr1 and Pvt1 RNAi specifically affects the activity of mature synapses. The observed reduction in synaptic strength following Pvt1 RNAi may be attributed to the formation of immature synapses (Figure 3-4).

Contextual fear conditioning influences the synaptic distribution of lncRNAs.

The localization of lncRNAs at the synapse prompted us to investigate the impact of synaptic activity on the dendritic distribution of lncRNAs. To achieve this, we used contextual fear conditioning (CFC), a hippocampus-based memory paradigm (Fanselow, 2000; Phillips and LeDoux, 1992) that relies on single-trial associative learning to assess the activity-induced distribution of lncRNAs in the dorsal hippocampus.

The local abundance of lncRNAs was analyzed from synaptoneurosomes (SN) prepared from the dorsal hippocampus after measuring the freezing response 3h post training as the activity-induced translation of plasticity related proteins occur within this timeframe (Bourtchouladze et al., 1998; O'Donnell and Sejnowski, 2014; Sutton and Schuman, 2006). qRT-PCR analysis revealed that the abundance of SynLAMP (1.83 ± 0.3 fold, p<0.0009), Pantr1 (0.49 ± 0.09 fold, p<0.002) and Pvt1 (0.88 ± 0.14 fold, p<0.001) in the synaptic compartment was enhanced following CFC. Snhg8 was depleted (0.77 ± 0.13 fold decrease, p<0.001) from the synapse; whereas the distribution of 2900052N01Rik, Snhg12, Rmst1, Snhg6, Epb41l4aos and Zfas1 was not affected (Figure 5A-B). In order to determine whether the differential abundance of lncRNA was due to altered expression of lncRNAs or due to activity-dependent altered transport between the synapse and the cell body, we assessed lncRNA expression in the whole hippocampus and in the dorsal hippocampus (comprising majorly of CA1 neurons) 3 hours post-CFC. There was a reduction in SynLAMP (0.29 ± 0.08 fold, p<0.01) and Pantr1 (0.37± 0.11, p<0.01) in the total hippocampus

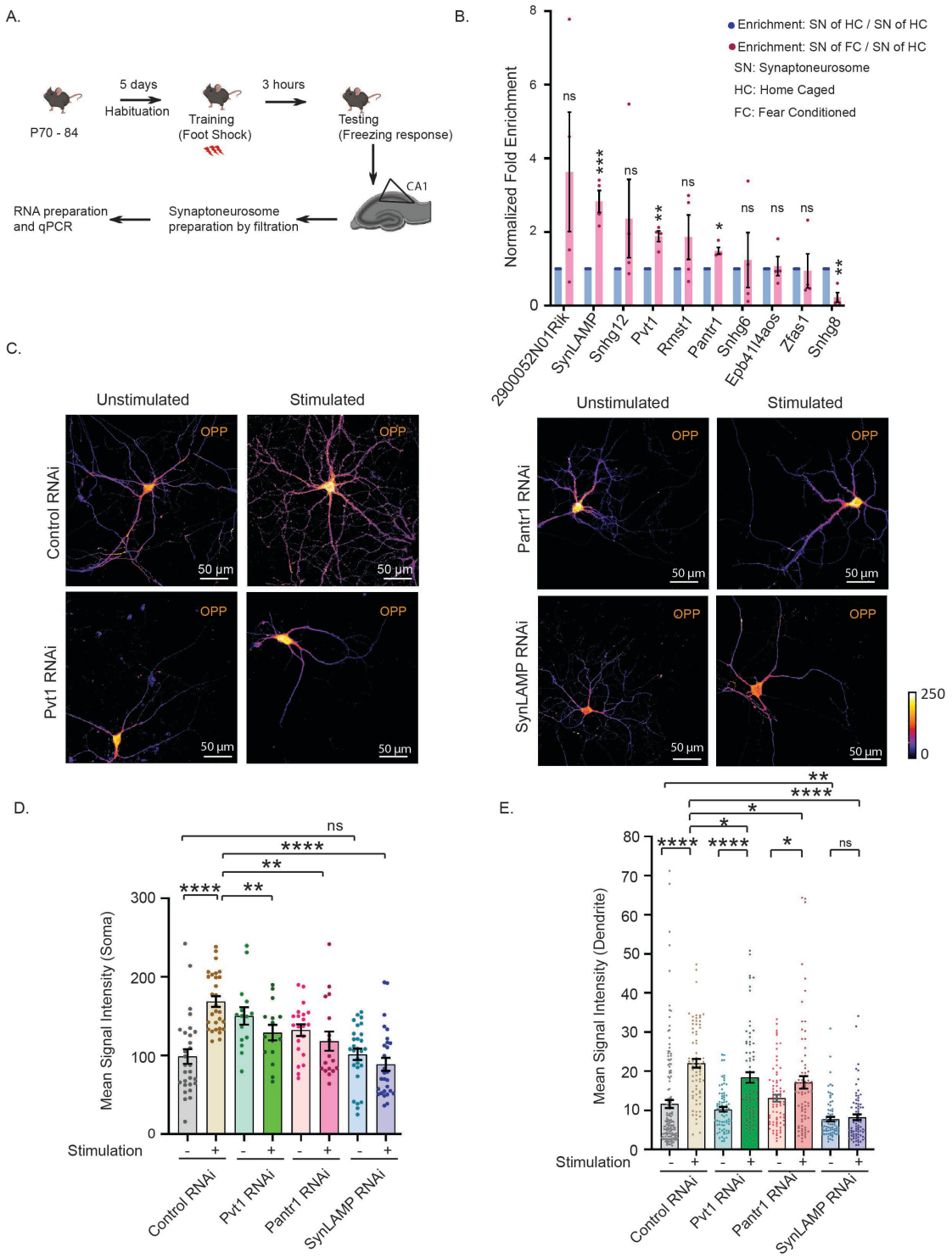

Figure 5: Activity-driven redistribution of synaptic lncRNAs and their role in regulating translation.

(**A**) Schematic representation of contextual fear conditioning in mice and subsequent analysis of lncRNA expression from the SN fraction. (**B**) Synaptic abundance of indicated lncRNAs 3 hours post fear-conditioning. Histogram represents the relative abundance of lncRNAs. n = 5. *p< 0.002, **p<0.001, ***p<0.0009, ns = not significant. Multiple t-test determined by Holm-Sidak method. Data shown as mean ± SEM. See also Figure S5. (**C**) Images representing extent of activity-dependent

translation in cultured neurons, transduced with shRNA (as indicated). Pixel intensity represented as a heat map.Calibration bar and scale bar as indicated. (**D-E**) Quantification of activity-induced translation in (D) soma and in (E) dendrites. n=15. *p<0.05, **p<0.003, ****p<0.0001. ns= not significant. Two-tailed t-test with Welch's correction. Data shown as mean ± SEM. See also figure S6.

(Figure S5A). However, lncRNA expression levels remained unaltered in the dorsal hippocampus (Figure S5B). It is therefore possible that lncRNA expression changes occur within specific cell-types in the dorsal hippocampus, other than in CA1 neurons.

Activity-dependent dendritic protein synthesis by lncRNAs.

Enhanced abundance of SynLAMP, Pantr1 and Pvt1 in SN fraction following CFC prompted us to test their involvement in *de-novo* translation. Activity-dependent somatic and dendritic translation was scored by FUNCAT assay in glutamate-stimulated neurons that expressed shRNAs against SynLAMP /Pantr1/Pvt1 and dendrites were visualized by MAP2 immunostaining. Translation assays were performed in the presence of the transcription inhibitor (Actinomycin D, 10 µg/ml) to prevent the influence of nascent transcription. We observed that stimulation of Control RNAi neurons enhanced new translation both in the soma and the dendrites (soma: 69.87 ± 11.52 mean signal intensity (m.s.i) increase; p<0.0001; dendrites: 10.42 ± 1.52 m.s.i increase; p<0.0001; as compared to stimulated control RNAi neurons) while, SynLAMP RNAi led to reduced translation in both (soma: 79.75 ± 10.65 m.s.i decrease, p <0.0001; dendrites: 13.80 ± 1.32 m.s.i decrease, p<0.0001; as compared to stimulated control RNAi neurons) (Figures 5C-5E, S6A-S6B). The loss of Pvt1 or Pantr1 also reduced activity-regulated translation in both the soma and dendrites as compared to stimulated,control RNAi neurons (soma: 39.60 ± 11.90 m.s.i decrease in Pvt1 RNAi neurons, p<0.003; 50.40 ± 13.96 m.s.i decrease in Pantr1 RNAi neurons, p<0.003; dendrites: 3.630 ± 1.74 m.s.i decrease in Pvt1 RNAi neurons, p<0.05; 4.90 ± 1.95 m.s.i decrease in Pantr1 RNAi neurons, p<0.05); but the extent of reduction in protein synthesis following SynLAMP RNAi was more pronounced (Figures 5D-E, S6A-S6B). Interestingly, basal translation within dendritic compartments was significantly hampered upon SynLAMP's loss of function, while somatic translation remained unchanged

(soma: 2.713 ± 11.76 m.s.i decrease w.r.t unstimulated control RNAi neurons, $p<0.10$; dendrites: 3.866 ± 1.187 m.s.i decrease as compared to unstimulated Control RNAi neurons, $p<0.003$). There was no significant change to the existing translational status of SynLAMP RNAi neurons upon stimulation (Figure 5D-5E).

Synaptic activity influences the association of lncRNAs with RNA binding proteins.

De novo translation at the synapse in the context of long-term memory formation relies substantially on RNA transport from the soma to dendritic spines. Transcripts in association with RNA binding proteins (RBPs) and translation regulators are packaged into “RNA granules” that are transported along the dendrites (Kiebler and Bassell, 2006). The activity-dependent release of RNA and associated translation factors from RNA granules promotes their transition from a translationally silent to an actively translating state (Krichevsky and Kosik, 2001; Shiina et al., 2005). We investigated the association between synapse-enriched lncRNAs and RBPs in response to CFC. We have analyzed the interaction between synapse-enriched lncRNAs and a select group of neuronal RBPs in a two-step process. First, we have made an *in silico* interaction map based on the POSTAR3 (Zhao et al., 2021; Zhu et al., 2019) database that listed transcriptomics analysis from immunoprecipitated RBPs (Figures 6A and S7A).

We assessed the number of binding motifs of specific RBPs present in a subset of synapse-enriched lncRNAs and evaluated their interaction based on the PhyloP score reported in POSTAR3 (Figure 6A). The lncRNA-RBP association was tested upon CFC (Figure 6B). As a proof-of-concept, we analyzed a subset of lncRNA interaction with Fus (FUsed in Sarcoma) and CIRBP (Cold Inducible RNA Binding Protein). Fus and CIRBP were immunoprecipitated from the dorsal hippocampus 3h post fear conditioning and their interaction with respective lncRNAs was analysed. Fear conditioning led to a reduction in the association between Fus and Pvt1 (1.30±0.06 reduction in relative enrichment w.r.t IgG control, $p<0.0002$) or Snhg6 (1.54 ± 0.11 reduction in relative enrichment w.r.t IgG control, $p<0.001$). This

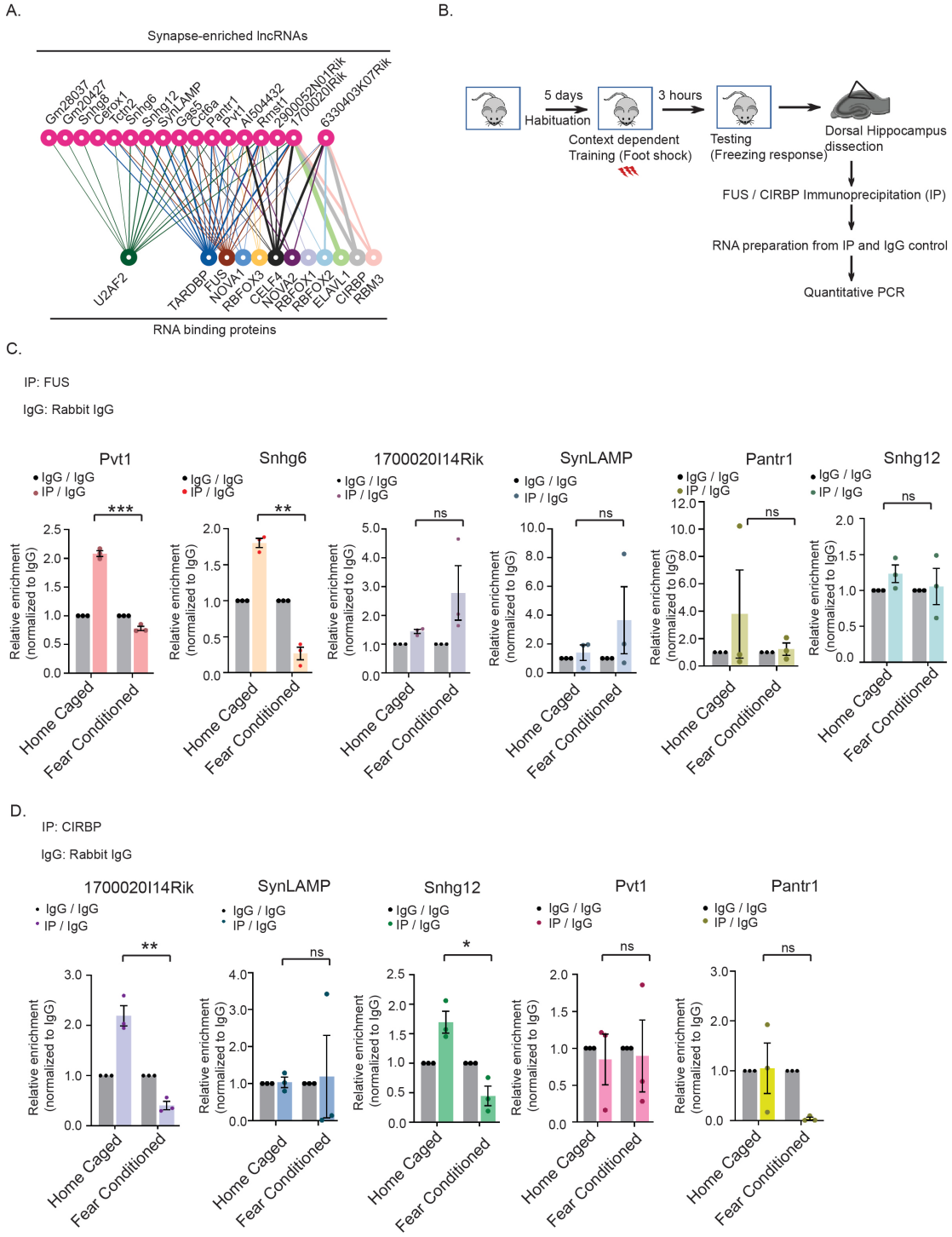

Figure 6: Activity-dependent interaction between synaptic lncRNAs and RBPs (**A**) Schematic representation of synaptic lncRNA and RBP interactions based on PhyloP score reported in POSTAR3 database. (**B**) Paradigm used to identify activity-dependent association between RBPs and lncRNAs *in-vivo*. (**C**) Association of indicated lncRNAs with FUS upon contextual fear conditioning. n=3. ***p<0.0002, **p< 0.001, ns= not significant. Multiple t-test using Bonferroni-Dunn method. Data shown as mean ± SEM.(**D**) Association of indicated lncRNAs with CIRBP upon contextual fear conditioning. n=3. **p<0.007. *p<0.04, ns= not significant. Multiple t-test using the Bonferroni-Dunn method. Data shown as mean ± SEM.

paradigm did not influence the interaction between Fus and SynLAMP, Pantr1, Sngh12 and 1700020I14Rik (Figure 6C). CFC reduced the association between CIRBP and 1700020I14Rik (1.79 ± 0.22 reduction in relative enrichment w.r.t IgG control, $p<0.007$) and Snhg12 (1.24±0.24 reduction in relative enrichment w.r.t IgG control, $p<0.04$). Although the PhyloP score of interaction between Snhg12 and CIRBP was not very high, it does have one binding site to CIRBP (Figure S7A). We did not detect any interaction between CIRBP and SynLAMP/Pantr1/Pvt1 as predicted by our *in silico* analysis (Figure 6D). Together, these findings suggest the existence of activity-dependent interactions between specific lncRNAs and RBPs.

SynLAMP (2410006H16Rik) is involved in contextual fear memory formation.

The enrichment of SynLAMP and Pantr1 in CA1 dendrites following CFC and the significant reduction of glutamate-induced dendritic translation in SynLAMP and Pantr1 RNAi neurons prompted us to investigate their role in the regulation of fear memory. Adeno-associated virus (AAV) expressing ZsGreen with indicated shRNAs were injected into the CA1 of 6 week old mice. CFC was performed at 10-12 weeks (Figure 7A-7B). We analyzed fear memory formation by measuring the freezing response 3h after training; this time window was selected on the basis of previous studies which report that application of translation inhibitor between 1h and 3h after training disrupts memory formation (Bourtchouladze et al., 1998; Sutton and Schuman, 2006). Although an expected increase in freezing between home-caged and fear-conditioned Control mice (36.88 ± 8.254 increase in % freezing, $p< 0.0001$) was observed, SynLAMP RNAi led to a reduction in freezing compared to the Control RNAi mice (difference: 21.53 ± 7.59 % freezing, $p<0.02$). In contrast, Pantr1 RNAi had no effect (Figure 7C). These data indicate that SynLAMP RNAi is specifically implicated in contextual fear memory consolidation.

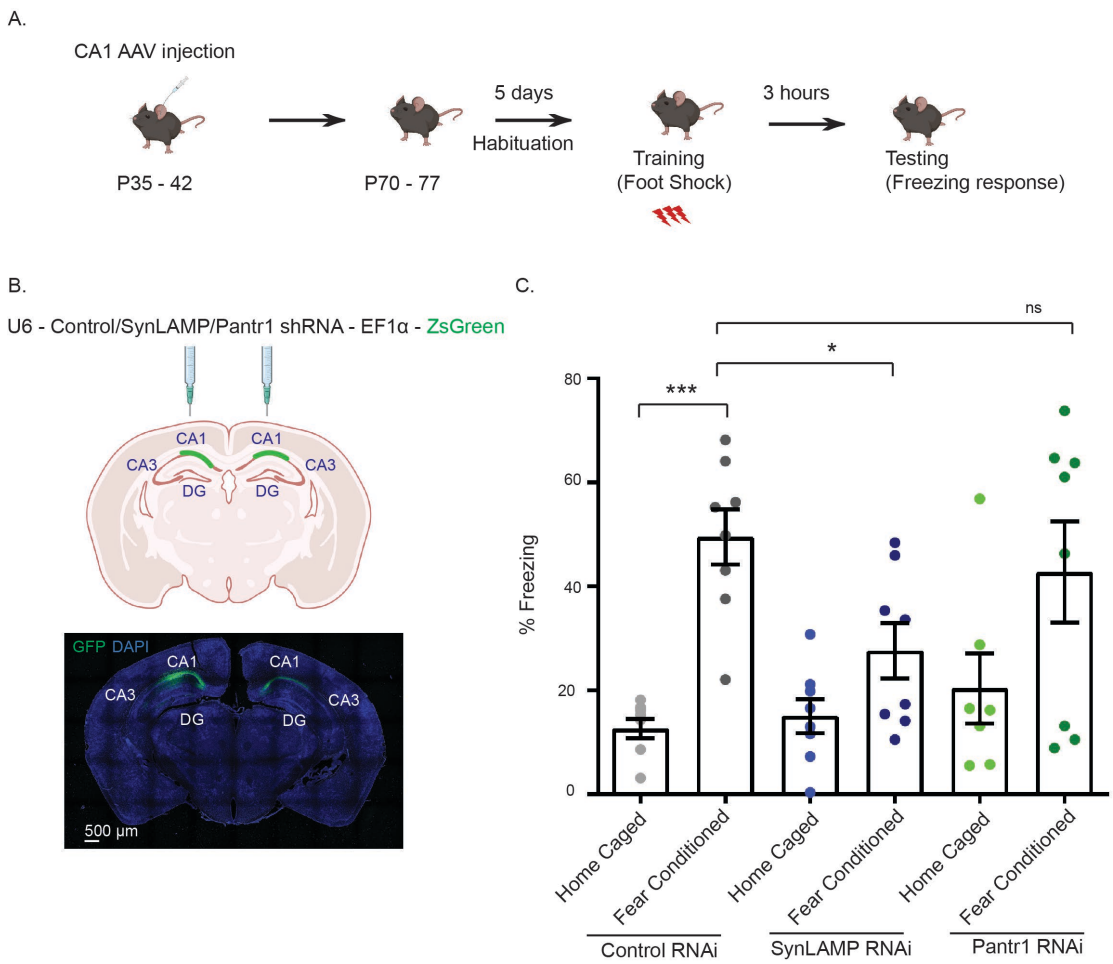

Figure 7: SynLAMP RNAi leads to deficits in contextual fear memory

(**A**) Contextual fear memory test paradigm following knockdown of SynLAMP/Pantr1 in CA1 neurons. (**B**) Schematic representation of injected AAV constructs expressing shRNAs as indicated along with ZsGreen in CA1 neurons. Co-expression of ZsGreen in CA1 indicating effective manipulation of SynLAMP or Pantr1. (**C**) Contextual freezing response during test at 3 hours after conditioning following SynLAMP/Pantr1 RNAi. n=8, ***p<0.0001, *p<0.02. ns= not significant. Two way ANOVA with Fisher's LSD. Data shown as mean ± SEM.

Discussion:

Localization of lncRNAs at the hippocampal synapse.

Almost all lncRNAs characterized in the brain thus far localize to the nucleus. For example, the expression of Malat1, a lncRNA that is highly expressed in the brain, is thought to be restricted to the nucleus (Statello et al., 2021). Malat1 functions as a RNA-scaffold (Statello et al., 2021) and regulates synapse formation by recruiting splicing factors to transcriptionally active sites (Bernard et al., 2010). *neuroLNC*; a neuron-specific, nuclear -enriched

lncRNA, has been shown to influence neuritogenesis and neuronal migration *in vivo* (Keihani et al., 2019). This lncRNA modulates synaptic vesicle release by stabilization of mRNAs that encode presynaptic proteins *via* its interaction with TDP-43 (TAR DNA binding protein 43) (Keihani et al., 2019). A long nucleolus-specific lncRNA, (LoNA) affects translation by inhibiting the ribosomal RNA transcription and ribosome biosynthesis, and is implicated in memory (Li et al., 2018), and a newly discovered nuclear lncRNA, ADRAM, epigenetically regulates NR4A2 and the formation of fear extinction memory (Wei et al., 2022).

In contrast, very few cytoplasmic lncRNAs have been characterized. ADEPTR (activity-dependent transported lncRNA) has been shown to suppress a cAMP-induced increase in spine density and spontaneous synaptic activity (Grinman et al., 2021), but little is known about the identity and function of synapse-enriched lncRNAs. The results of this study fill this void, and lay the foundation to investigate the topological diversity of lncRNAs in regulating synaptic translation and diverse cognitive functions at the resolution of the synapse.

Towards this aim, we identified 1031 lncRNAs in synaptoneurosomes derived from the hippocampus. Among these transcripts, 94 lncRNAs were significantly enriched in the synaptic compartment (Figure 1). An FPKM cut off higher than 10 was set to include lncRNAs that showed appreciable expression levels across all three independent transcriptomics experiments. The number of lncRNAs detected in the fraction was comparatively lower than that of mRNAs previously identified in the CA1 neuropil (Cajigas et al., 2012). This could be attributed to the fact that the expression levels of lncRNAs are typically lower than those of mRNAs (Mukherjee et al., 2016). Consistent with the existence of diverse classes of lncRNAs (Mattick, 2023), our annotated lncRNAs included intergenic, antisense and processed transcripts. LncRNAs identified by our study share multiple features of mRNAs. They are transcribed by RNA polymerase II, and are predominantly poly-adenylated and exported out of the nucleus. The lack of coding potential in these lncRNAs suggest that they may function as regulatory RNAs to define the proteomic composition necessary for synaptic function.

lncRNA regulates neuronal development

Nuclear-enriched lncRNA, Gm12371 (Raveendra et al., 2018) and *neuroLNC* (Keihani et al., 2019) have been shown to regulate dendritic development in cultured hippocampal neurons. Gm12371 knockdown influences the morphology of dendritic spines (Raveendra et al., 2018). However, the role of cytoplasmic lncRNAs in regulating dendritic arborization and spine morphology *in vivo* has not been investigated. We therefore selected three distinct lncRNAs, SynLAMP, Pantr1 and Pvt1, to investigate the impact of cytoplasmic lncRNAs in neuronal development (Figure1). Pvt1 knockdown, but not SynLAMP and Pantr1, led to a significant reduction in dendrite complexity (Figure 2) and spine density, together with altered spine morphology. Pvt1 RNAi reduced stubby and mushroom spines *in vitro* and *in vivo*, whereas knockdown of SynLAMP and Pantr1 had no effect on either dendritic complexity or spine morphology (Figure 2). These findings emphasize the involvement of specific dendritic lncRNAs in spine development within the developing mouse brain.

To investigate the signaling mechanisms underlying Pvt1-driven dendritic growth and spine morphology, we explored the role of Rho-related GTPases. RhoA and Rac1 are expressed in hippocampal and cortical neurons during the window of dendrite growth and spine development (Nakayama and Luo, 2000; Schubert and Dotti, 2007; Threadgill et al., 1997). Loss of Pvt1 reduces Rac1 expression in neurons (Figure 3). As there is reduction of dendritic growth (Threadgill et al., 1997) and spine density (Nakayama and Luo, 2000) in dominant negative Rac-1 phenotypes, the reduction in spine density and spine shrinkage observed in Pvt1 RNAi neurons could be mediated by Rac-1 (Figure 2). The active form of RhoA reduces dendritic branching (Nakayama and Luo, 2000) and spine number (Schubert and Dotti, 2007); we observed the enhancement of RhoA expression following Pvt1 RNAi (Figure 2). Reduced dendrite growth and altered spine morphology with a concomitant increase in RhoA expression in Pvt1 RNAi neurons indicate that Pvt1 negatively regulates active forms of RhoA. Transcriptomics analysis following Pvt1 knockdown did not show any change in RhoA and Rac1 transcripts (Figure 3); suggesting an indirect involvement of Pvt1 in the regulation of

active RhoA and Rac1. Another dendritic lncRNA, ADEPTR, has been shown to regulate spine morphology in a cyclic AMP-Protein Kinase A (cAMP-PKA) - dependent manner (Grinman et al., 2021). Pvt1-mediated regulation of dendrite growth and spine morphology *via* Rho GTPases indicate the existence of a distinct pathway other than cAMP-PKA signaling.

Excitatory synapse development by lncRNAs *in vitro* and *in-vivo*.

Our results revealed that the knockdown of Pvt1, but not SynLAMP or Pantr1, led to a reduction of excitatory synapse density in cultured hippocampal neurons (Figure 3) and this was recapitulated in CA1 neurons *in-vivo* (Figure 3). It is important to note that synapse density following Malat1 knockdown was analyzed by measuring the immunoreactivity of Synapsin I in cultured neurons. This data could be an indicator of pre-synaptic differentiation as observed in developing cerebellar synapses (Castejón et al., 2004). In contrast, our data unequivocally confirmed the necessity of Pvt1 in excitatory synapse formation as we analyzed synapse density by measuring the co-localization of both pre and post-synaptic proteins *in vitro* and *in vivo*. Although Pvt1 and Malat1 are localized in two distinct subcellular locations, both lncRNAs function as positive regulators of excitatory synapse development.

How does Pvt1 regulate excitatory synapse development? RNA-seq and GO analysis from cultured neurons identified a number of transcripts that were down-regulated due to Pvt1 loss of function (Figure 3). These transcripts encode proteins necessary for dendrite development, synapse development and stability, and neurotransmitter transport. Similar to our observation, knockdown of Malat1 in Neuro2A cells also detected the enrichment of similar GO terms, such as dendrite development, synapses and mRNA transport. However, Malat1 loss of function also showed the enrichment of GO terms associated with nuclear function, such as chromatin organization, regulation of transcription and RNA metabolic processes (Bernard et al., 2010). Knockdown of Pvt1 identified enrichment for GO annotations specifically linked with cytoplasmic function (Figure 3). It is important to note that as a transcription inhibitor was not used in both studies; our transcriptomics

analysis captured the differential regulation of mRNAs contributed by changes in transcription or mRNA stability. Our prediction is based on a recent finding that showed *neuro*LNC, a nuclear lncRNA, selectively stabilizes pre-synaptic transcripts necessary for synaptic vesicle release (Keihani et al., 2019).

Regulation of synaptic activity by lncRNAs

The knockdown of SynLAMP and Pantr1 in neurons led to a significant reduction of mEPSC amplitude whereas Pvt1 RNAi led to changes in both mEPSC amplitude and frequency (Figure 4). The results of our patch-clamp experiments indicate that while SynLAMP and Pantr1 regulate basal synaptic strength *via* the modulation of the post-synaptic compartment, Pvt1 has significant influence on both the pre- and post-synapse in excitatory neurons. This, coupled with the observed reduction in Synapsin I and PSD95 density in Pvt1 RNAi neurons (Figure S3), and the reduction in relevant GO terms associated with glutamatergic synapse development and transmission, strongly implicate Pvt1 in synapse development. In contrast to our study, it has recently been reported that knockdown of the lncRNA ADEPTR in neurons reduces the amplitude of cAMP –regulated spontaneous EPSC without affecting basal synaptic transmission (Grinman et al., 2021). These observations, together with our data, indicate a lncRNA-dependent regulation of basal or activity-induced synaptic transmission.

The redistribution of sAMPARs (mostly sGluA1/A2) resulted in changes to the mEPSC amplitude in excitatory neurons. It is pertinent to point out that none of the existing studies (Grinman et al., 2021; Raveendra et al., 2018) reported the synaptic distribution of sAMPARs. Our investigation of sGluA1/A2 intensity following the individual knockdown of SynLAMP/Pantr1/ Pvt1 in cultured neurons revealed that both subunits are significantly reduced in all RNAi conditions (Figure 4); corroborating the observed reductions in mEPSC amplitude in RNAi neurons. SynLAMP and Pantr1, therefore, are specifically required for the tuning of mature synapses. This observation was further supported by the absence of noticeable developmental defects in hippocampal neurons after SynLAMP and Pantr1 RNAi (Figure 2-4). Pvt1 RNAi also shows a decrease in sAMPARs; however, taking into consideration

multiple lines of evidence from the synapse formation assays and GO enrichment analysis, it is possible that the observed decrease in sAMPARs is due to the lesser number of synapses formed. Further research is required to determine the mechanistic details of sAMPAR distribution by lncRNAs and their implications for the regulation of synaptic strength.

Activity-dependent synaptic distribution of lncRNAs

The localization of coding and non-coding transcripts in dendrites create specialized microdomains that determine the availability of plasticity related proteins for synaptic plasticity and memory. Neuronal activity has been shown to influence the distribution of transcripts at the synapse (Ho et al., 2011; Sutton and Schuman, 2006). We therefore analyzed the subcellular abundance of lncRNAs in CA1 neurons following CFC as learning shapes the translation state of CA1 pyramidal neurons (Klann and Dever, 2004; Sutton and Schuman, 2006). CFC is a very strong learning paradigm with the ability to invoke activity-induced changes within a large number of neurons *in vivo*. CFC enhanced the synaptic distribution of SynLAMP, Pantr1 and Pvt1, but reduced the abundance of Snhg8. The synaptic localization of Snhg6, Snhg12, Zfas1, Rmst1, Epb4laos and 2900052NO1Rik were not affected (Figure 5). These data suggest an activity-invoked, state-dependent, and transcript-specific distribution of lncRNAs in the synapse. SynLAMP, Pvt1 and Pantr1 are involved in various aspects of synapse maturation and the regulation of synaptic output. It may be speculated that the selective localization of the lncRNAs to the synapse in response to neuronal activity allows them to influence the synaptic proteome by regulating the translation or post-translation modifications of localized coding transcripts. As we observed the localisation of the lncRNAs 3h after CFC; there is a high probability that they are involved in regulating protein synthesis at or near synapses immediately following a learning event.

LncRNAs regulate activity-invoked dendritic translation

Activity-dependent localization of transcripts in dendrites is essential to meet the demand for site-specific translation. Non-coding RNAs act as independent effectors that regulate translation *via* diverse mechanisms

(Mercer et al., 2007). Our understanding of non-coding RNA mediated translation in dendrites is primarily limited to miRNAs (Banerjee et al., 2009; Sambandan et al., 2017; Schratt et al., 2006). We found that there was a reduction in dendritic translation upon the individual knockdown of SynLAMP/ Pantr1/Pvt1; with SynLAMP RNAi having the most pronounced effect (Figures 6 and S6). Previously, the nuclear lncRNA LoNA had been implicated in regulating translation in Neuro2A cells by promoting ribosome biosynthesis (Li et al., 2018). Dendritic and somatic translation in neurons may therefore be controlled by distinct regulatory modules that may recruit specific lncRNAs.

Activity-regulated interaction between lncRNA and RBPs

Memory consolidation following a learning paradigm, such as CFC relies on the increased recruitment of coding/noncoding transcripts at the synapse and concomitant increase in dendritic translation following training (Bourtchouladze et al., 1998; Frey and Morris, 1997; Montarolo, 1986). Transport of SynLAMP/Pantr1/Pvt1/Snhg8 in somato-dendritic compartments within 3h post CFC (Figure 5) and their implications in dendritic translation prompted us to explore lncRNA-RBP interactions following CFC. The activity-dependent transport of regulatory RNAs is associated with changes to their metabolic state and requires the participation of select RBPs (Fernandez-Moya et al., 2014; Heraud-Farlow et al., 2013; Schieweck et al., 2020). Indeed, RBPs are key determinants of RNA transport, stability and translation (Bramham and Wells, 2007).

Here, we represent the RBP- lncRNA network as a multigraph plot; wherein line density is commensurate to the predicted lncRNA-RBP affinity (Figure 6). We tested the lncRNA association with two RBPs; FUS and CIRBP. FUS localizes to CA1 dendrites (Belly et al., 2005), forms RNA granules to regulate *de novo* translation (Yasuda et al., 2013), and affects mRNA stability (Udagawa et al., 2015). CIRBP also localizes in the dendrites of hippocampal neurons (Tong et al., 2013; Zhou et al., 2021); and has been shown to regulate RNA stability (Logan and Storey, 2020; Xia et al., 2012) and translation (De Leeuw et al., 2007) in non-neuronal cells. We found that FUS

interacts with Pvt1, Snhg6 and 1700020I14Rik but has no association with SynLAMP, Pantr1, or Snhg12, indicating the specificity of our predictive interaction. CFC reduced the association between FUS, Pvt1 and Snhg6, whereas 1700020I14Rik was unaffected. In contrast, CIRBP interacted with 1700020I14Rik and their association was reduced upon CFC (Figure 6). Of note, 1700020I14Rik was associated with both FUS and CIRBP but synaptic activity only affected its interaction with CIRBP (Figure 6). These data suggest an activity-dependent and transcript-specific interaction of lncRNAs with FUS/CIRBP. Correlating this observation with our *in silico* prediction whereby a single lncRNA can bind with multiple RBPs, we speculate that lncRNAs could function as a decoy for RBPs (Hafezqorani et al., 2019; Ray et al., 2013) and synergistically determine mRNA stability or translation through the formation of RNA granules.

Knockdown of SynLAMP affects contextual fear memory

Long-term memory formation is regulated by the activity-induced distribution of RNAs in dendrites (Swarnkar et al., 2021) and *de novo* translation from plasticity-related transcripts at the synapse (Ho et al., 2011). We observed that loss of SynLAMP in CA1 neurons disrupted contextual fear memory (Figure 7). Although Pantr1 is enriched in CA1 neurons (Goff et al., 2015) and positively regulates translation and mEPSC amplitudes in mature neurons; Pantr1 knockdown in CA1 neurons had no effect on contextual fear memory (Figure 7). We therefore speculate that Pantr1 could be involved in other forms of protein synthesis–dependent plasticity (Srinivasan et al., 2021) rather than Hebbian plasticity. As SynLAMP functions as a positive regulator of activity-dependent translation (Figure 5), and SynLAMP RNAi disrupts memory consolidation, SynLAMP–mediated dendritic protein synthesis could be a link to contextual fear memory (Figures 5 and 7). The spatiotemporal correlates of SynLAMP-driven regulation of contextual fear memory can not, however, be addressed at this point because of a lack of availability of its interacting partners.

Pvt1 is involved in both synapse development and plasticity

Pvt1 regulates dendritic complexity and synapse maturation in the early stages of neuronal development; it also undergoes activity-induced transport to regulate translation at mature synapses. These two observations can be reconciled if the temporal resolution of lncRNA function at different stages of neuronal development is considered. Spatiotemporal compartmentalization of Pvt1 function can be achieved through its diverse isoforms, the expression of which may take precedence at specific timepoints. We detected at least four isoforms of Pvt1 in our bulk RNA-seq (data not shown). However, shRNAs designed against Pvt1 were not isoform specific; thus all phenotypes resulting from Pvt1's loss of function were observed. Diverse isoforms of lncRNAs may possess distinct temporal signatures in neurons (Mercer et al., 2012) and recruit different molecular partners to regulate markedly different signaling modules during developmental decisions or synaptic plasticity. Hence the role of Pvt1 in different neuronal contexts warrants future investigation.

In summary, we provide conclusive evidence for the localization and function of lncRNAs in the synaptic compartment, *in vivo*. It is evident that lncRNAs are critically involved in synaptic plasticity and experience-dependent regulatory activities linked to diverse cellular pathways. This discovery adds a new dimension to the RNA-centric view of developmental and cognitive functions of the brain, and to their dysfunction during neurodevelopmental disorders.

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Supplementary Information:

Figure S1:

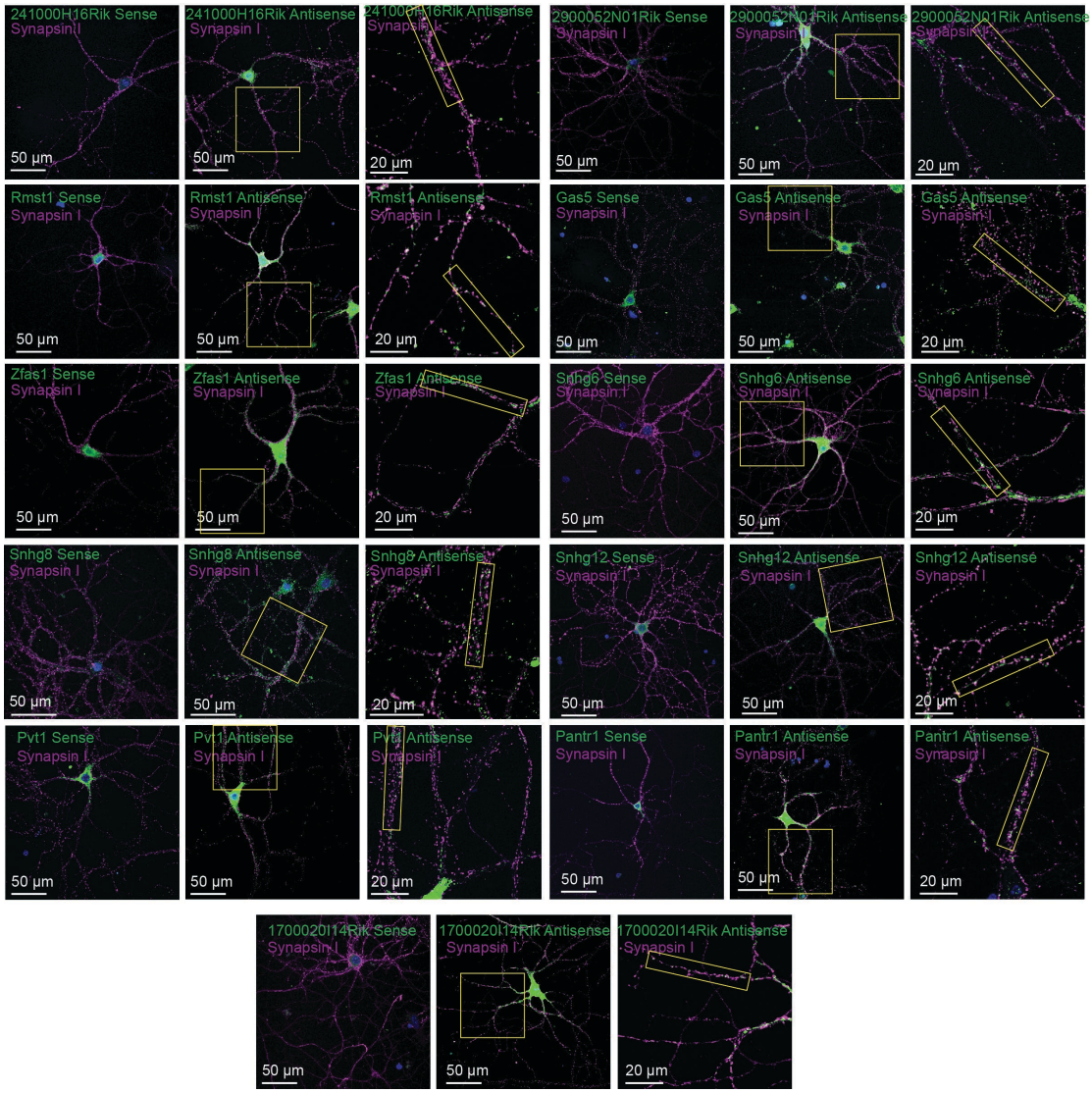


Figure S1: *In situ* hybridization of indicated synapse-enriched lncRNAs.

Cultured hippocampal neurons (DIV21) were hybridized with indicated sense and antisense probes. High magnification images of dendrites are marked in “Yellow” box. Scale as indicated. Also see Figure 1.

Figure S2:

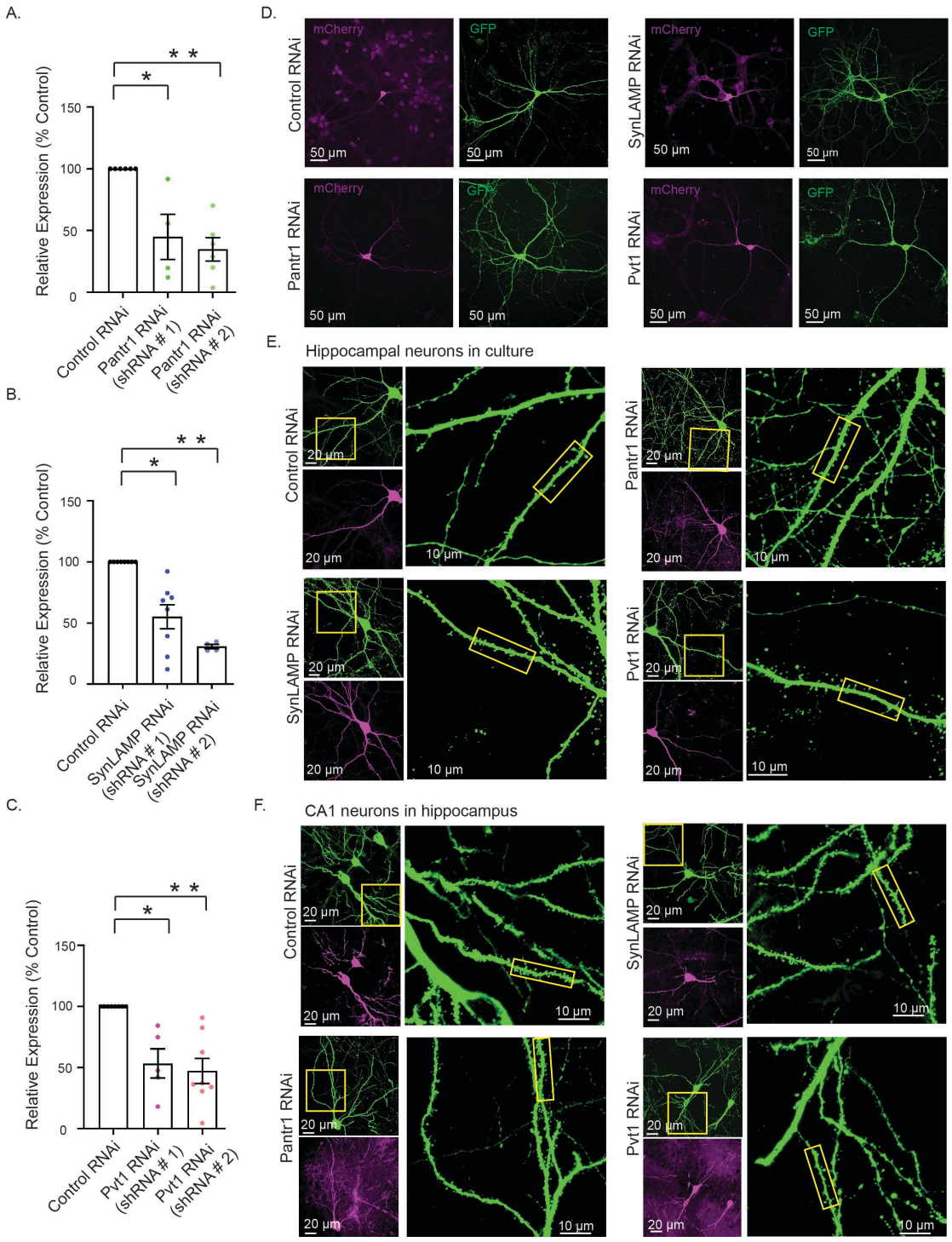


Figure S2: Spine development analysis following knockdown of Pantr1, SynLAMP and Pvt1.

(**A-B**) Cultured hippocampal neurons (DIV 5 – 7) were transduced and total RNA was purified from these neurons (DIV 21) for qPCR analysis. Tuj1 expression was used for normalization of respective lncRNA expression. (A) Knockdown of Pantr1 by two distinct shRNAs. n= 4 - 6, *p<0.005 for shRNA#1, **p< 0.0001 for shRNA#2 (B)

Knockdown of SynLAMP by two distinct shRNAs. n= 4 - 8, *p<0.0004 for shRNA#1, **p<0.0001 for shRNA#2. (C) Knockdown of Pvt1 by two distinct shRNAs. n = 5 – 8. *p<0.0003 for shRNA#1, **p<0.0002 for shRNA2. Unpaired two-tailed t- test. Data shown as mean ± SEM. (**D-E**) Cultured hippocampal neurons (DIV 3-4) were co-transfected with plasmids expressing indicated shRNAs along with mCherry and EGFP. (D) Cytosolic EGFP in neurons (DIV 21) was used to trace dendrites for Sholl analysis. (E) Cytosolic EGFP in neurons (DIV21) was used for dendritic spine analysis. High magnification images are indicated in "yellow" box. Scale as indicated. Also see Figure 2. (**F**) CA1 neurons were co-electroporated (E17 embryos) with plasmids expressing mCherry along with indicated shRNAs and EGFP. Spines were analyzed from CA1 neurons (P28). High magnification images are indicated in "yellow" box. Scale as indicated. Also see Figure 2.

Figure S3:

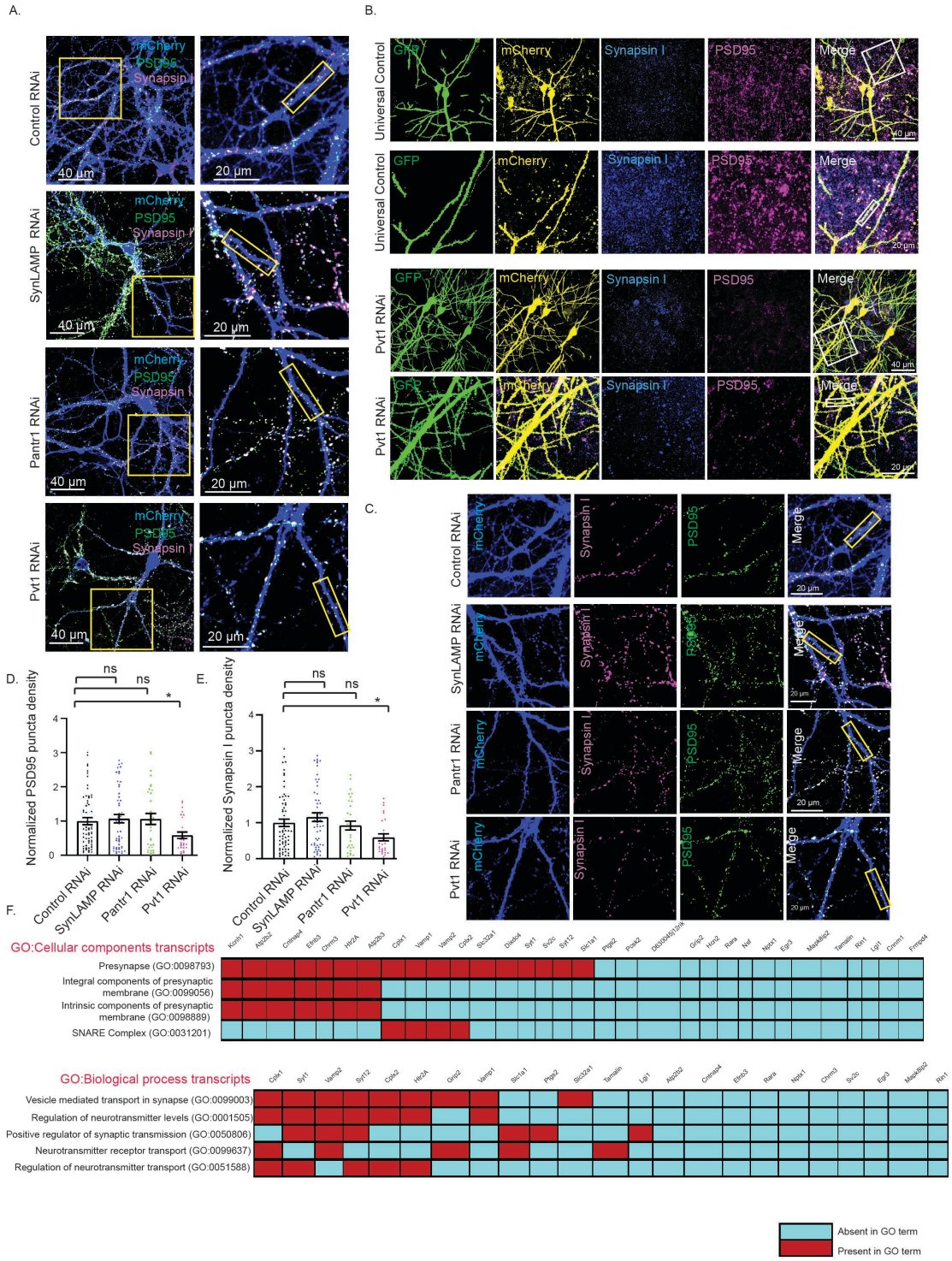


Figure S3: Effect of Pvt1 knockdown in synapse development.

(**A**) Cultured hippocampal neurons (DIV 4-5) were transduced with lentivirus expressing control shRNA or shRNA against Pantr1/SynLAMP/Pvt1 (as indicated). Images from neurons (DIV21) showing PSD95 (green), Synapsin I (magenta) and dendrites (blue). Glutamatergic synapses (white) are represented by overlapping PSD95 and Synapsin I puncta on dendrite. Scale as indicated. High magnification images were captured from dendrites (yellow).(**B**) CA1 neurons (at E17) were *in*

utero electroporated with plasmids expressing mCherry along with shRNA against Pvt1 or control shRNA and EGFP. Images of neurons (P28) showing PSD95 (magenta), Synapsin I (blue) and dendrite (mCherry and EGFP). Glutamatergic synapses (white) showing apposing PSD95 and Synapsin I puncta. High magnification images were captured from dendrites (yellow and green). Scale as indicated. White box indicates the ROI represented in Figure 3. (**C**) Representative image from cultured hippocampal neurons showing PSD95 and Synapsin I levels in neurons transduced with lentivirus expressing indicated shRNA. (**D**) Quantification of PSD95 puncta density from cultured neurons. n = 15 - 26, *p<0.03. ns = not significant. One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM.(**E**) Quantification of Synapsin I puncta density from cultured neurons. n = 15 - 26, *p<0.03. ns = not significant. One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM. (**F**) GO analysis showing significant (FDR, p<0.05) enrichment of GO-terms for the function of genes encoding down-regulated mRNAs following Pvt1 RNAi. Go-terms representing pre-synaptic function with indicated transcripts linked to pre-synaptic compartment. Also see Figure 3.

Figure S4:

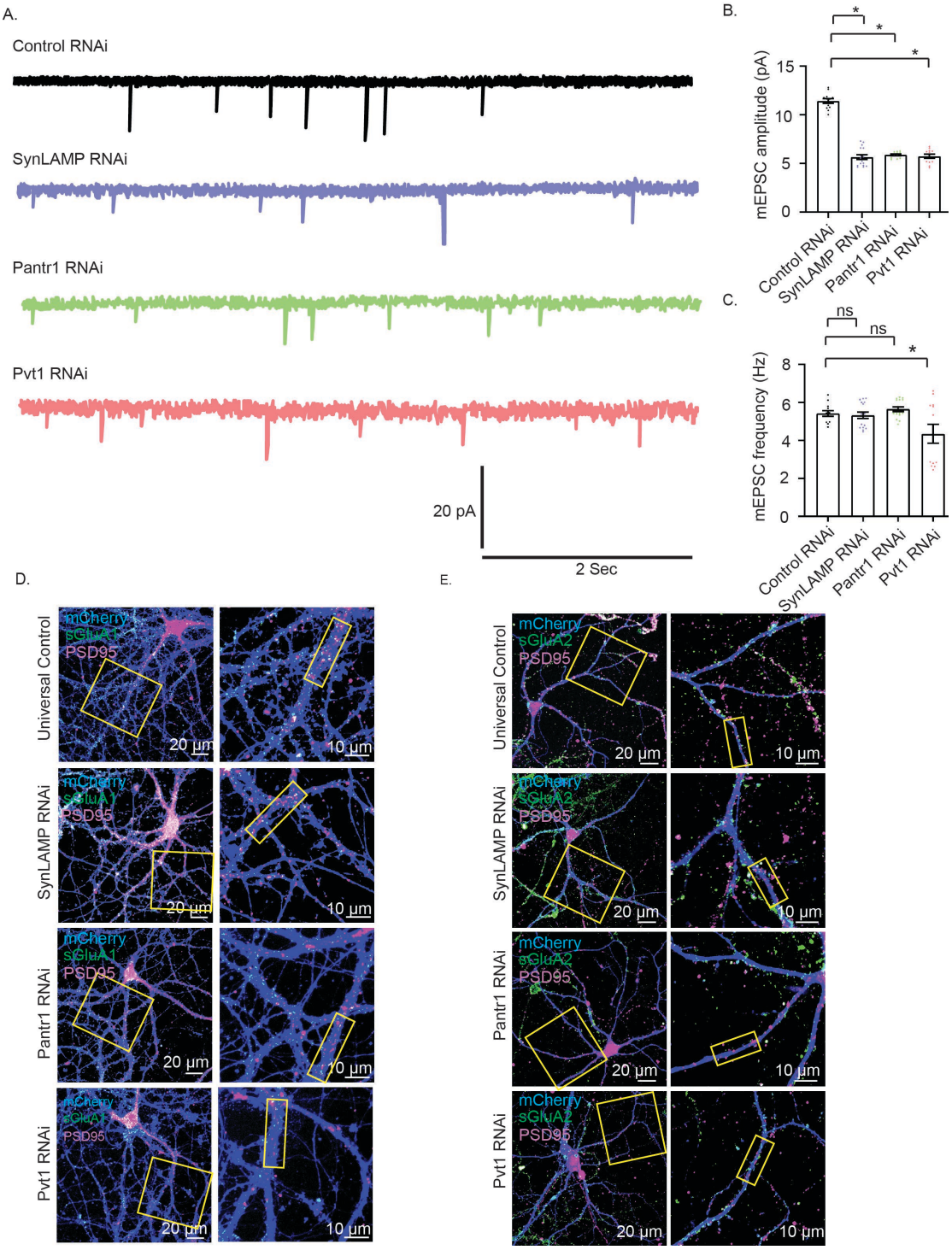


Figure S4: SynLAMP, Pantr1 and Pvt1 RNAi influence synaptic activity *via* sAMPAR abundance.

(**A – C**) Cultured hippocampal neurons (DIV 10) were transduced with lentivirus expressing shRNAs#2 as indicated. Whole-cell patch clamp recordings were performed from transduced neurons (DIV 21 - 25). (A) mEPSC traces. (B) Mean mEPSC amplitude. (C) Mean mEPSC frequency. n = 12 – 16. *p <0.01. ns = not significant. One-way ANOVA and Fisher's LSD. Data shown as mean ± SEM. See Figure 4 for shRNA#1 data point.(**D – E**) Cultured hippocampal neurons (DIV 10)

were transduced with lentivirus expressing shRNAs # 1 as indicated. sAMPAR abundances were measured by immunolableing for GluA1 or GluA2 of live transduced neurons (DI V21 - 23). Following GluA1 or GluA2 labelling neurons were fixed and immunostained with PSD95 to mark synapses. Image showing (D) sGluA1 (green) or (E) sGluA2 (green) on mCherry expressing dendrites (blue) from cultured neurons immunostained with PSD95 (magenta). sGluA1 or sGluA2 abundance in synapses shown in merged (sGluA1 or sGluA2/PSD95/mCherry) images. Yellow box indicates the ROI represented in Figure 4. See also Figure 4.

Figure S5:

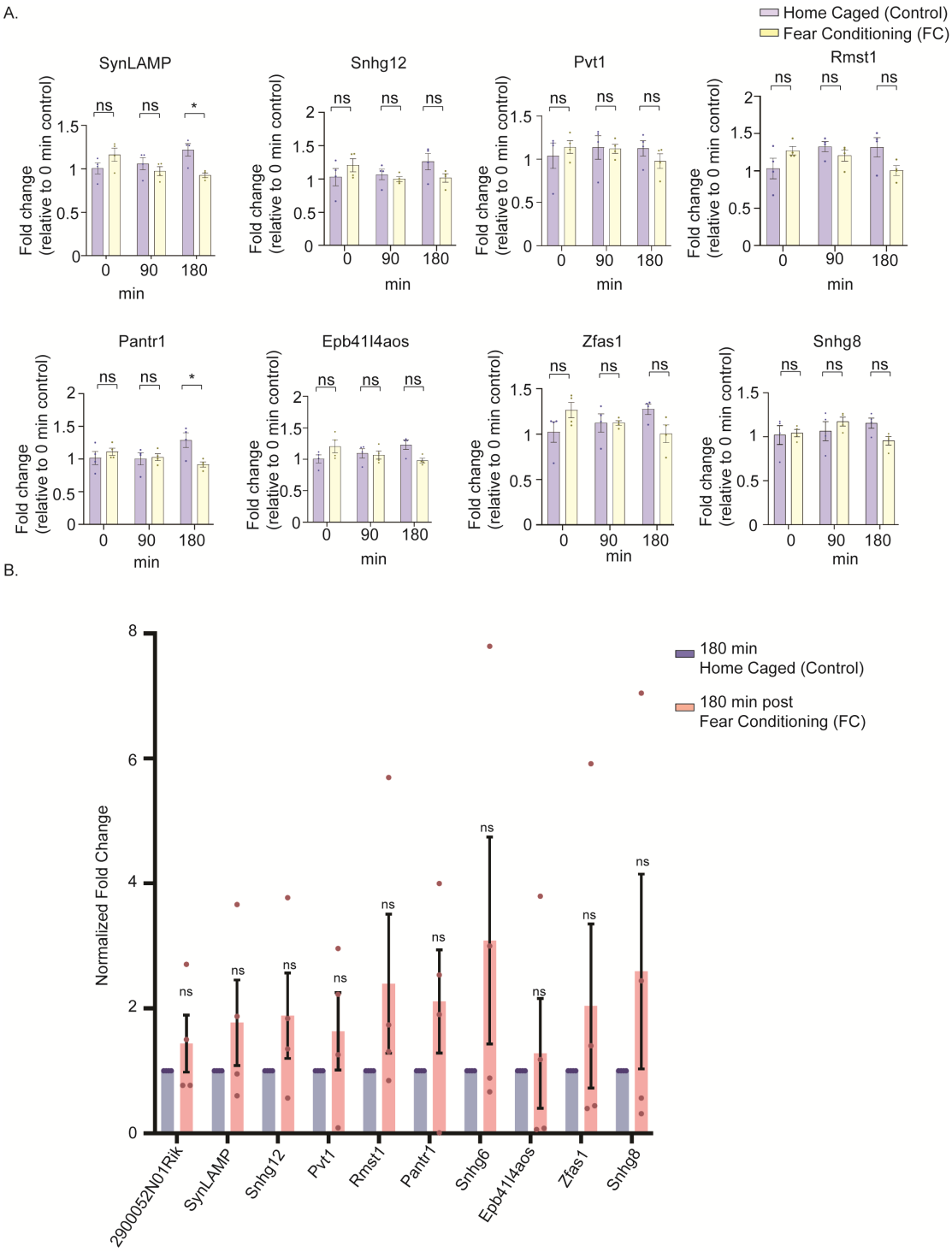


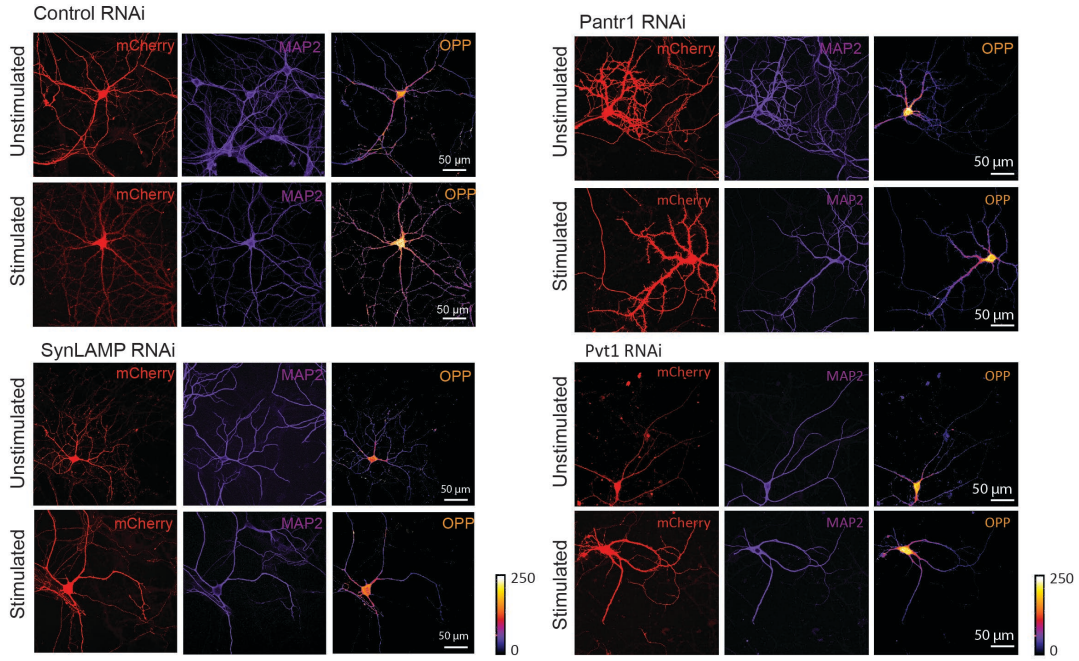
Figure S5: Expression of lncRNAs in hippocampus upon contextual fear conditioning.

(**A**)Total RNA from the whole hippocampus was purified at 0 minutes, 90 minutes and 180 minutes following contextual fear conditioning. Indicated lncRNAs were analyzed by qRT-PCR. Bar plots represent relative fold change of indicated lncRNAs.*p<0.01, ns = not significant. n = 4, 2 Way ANOVA and Bonferroni multiple

comparison test. Data shown as mean ± SEM.(**B**) The dorsal hippocampus (majorly CA1 region) was isolated from adult mice three hours (180 minutes) post contextual fear conditioning. Abundance of indicated lncRNAs was analyzed by qRT-PCR subsequent to total RNA isolation from the dorsal hippocampus. n = 4 , ns = not significant. Unpaired two tailed t-test. Data shown as mean ± SEM.

Figure S6:

A.



B.

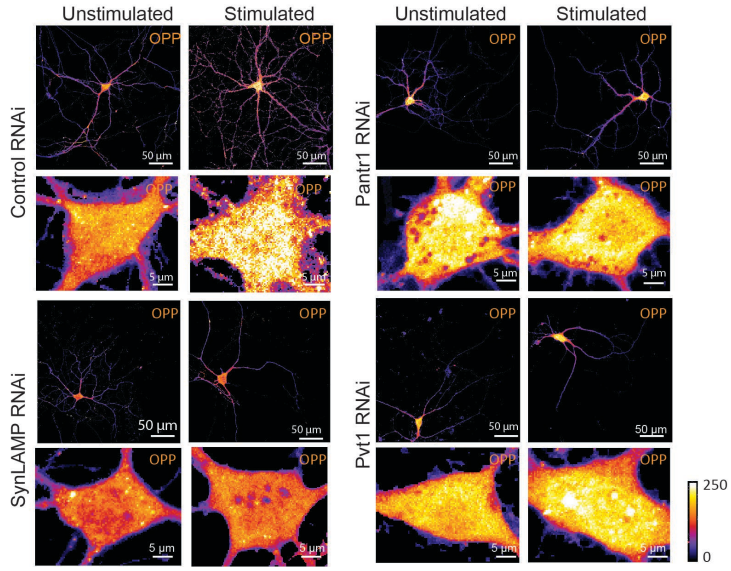


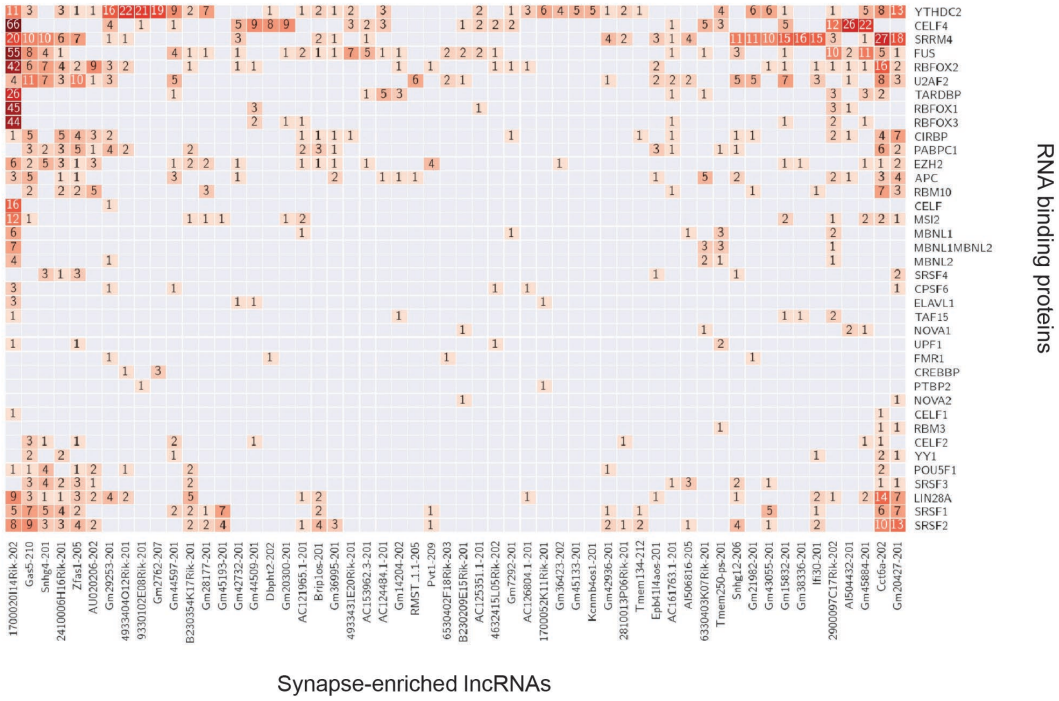
Figure S6: Activity-regulated protein synthesis in hippocampal neurons following knockdown of Pantr1, SynLAMP and Pvt1.

(**A**)Cultured hippocampal neurons (DIV 10) were transduced with lentivirus expressing shRNAs as indicated. Neurons (DIV 21 - 23) were stimulated with Glutamate in presence of Actinomycin D. Protein synthesis from the mCherry (red) expressing transduced neurons was assessed by Puromycin incorporation (O-propargyl-puromycin or OPP; an alkyne analog of puromycin) and dendrites (blue) were visualized by MAP2 immunostaining. Intensity of fluorescence observed in neurons is a direct readout of OPP incorporation due to protein synthesis. Differential pixel intensity has been represented as a heat map (low: blue to high: white).

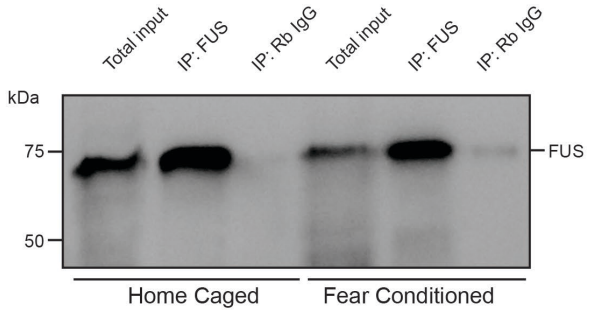
Calibration bar and scale bars as indicated. (**B**) Images showing the extent of OPP incorporation in the soma of neurons expressing shRNA as indicated. Differential pixel intensity has been represented as a heat map. Calibration bar and scale bars as indicated.

Figure S7:

A.



B.



C.

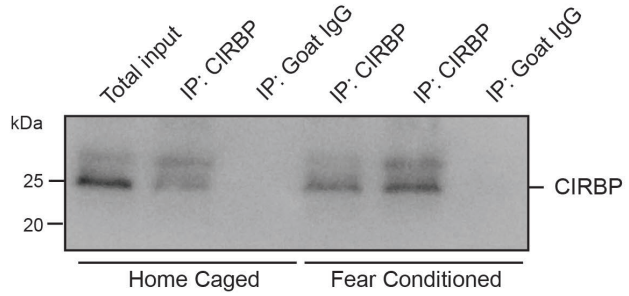


Figure S7: **Synapse-enriched lncRNA-RBP interaction following contextual fear conditioning.**

(A) RBP binding motif present in indicated synapse-enriched lncRNAs as detected by *in silico* analysis using the POSTAR3 database.(B) Representative immunoblot showing immpunoprecipitation of FUS from the dorsal hippocampus following contextual fear conditioning.(C) Representative immunoblot showing

immpunoprecipitation of CIRBP from the dorsal hippocampus following contextual fear conditioning.