

SUPPLEMENTARY INFORMATION

Synaptic proteome diversity is shaped by the levels of glutamate receptors and their regulatory proteins

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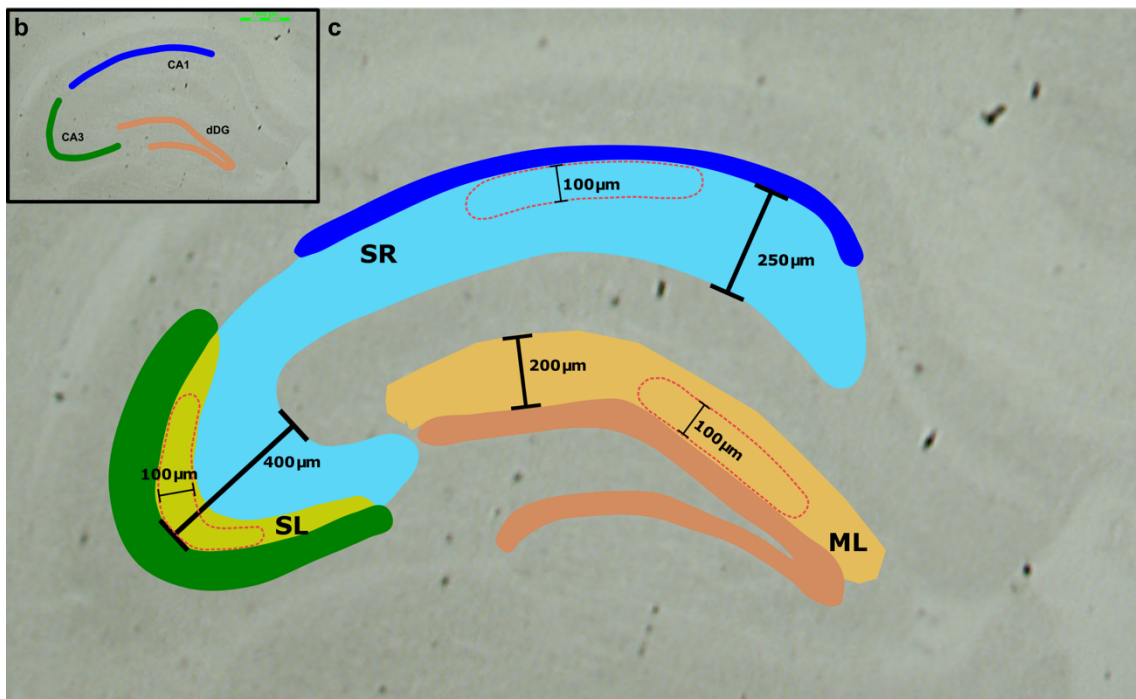
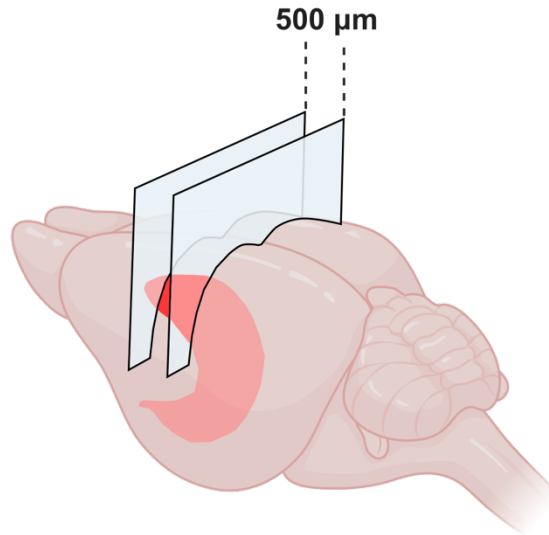
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Supplementary Figures

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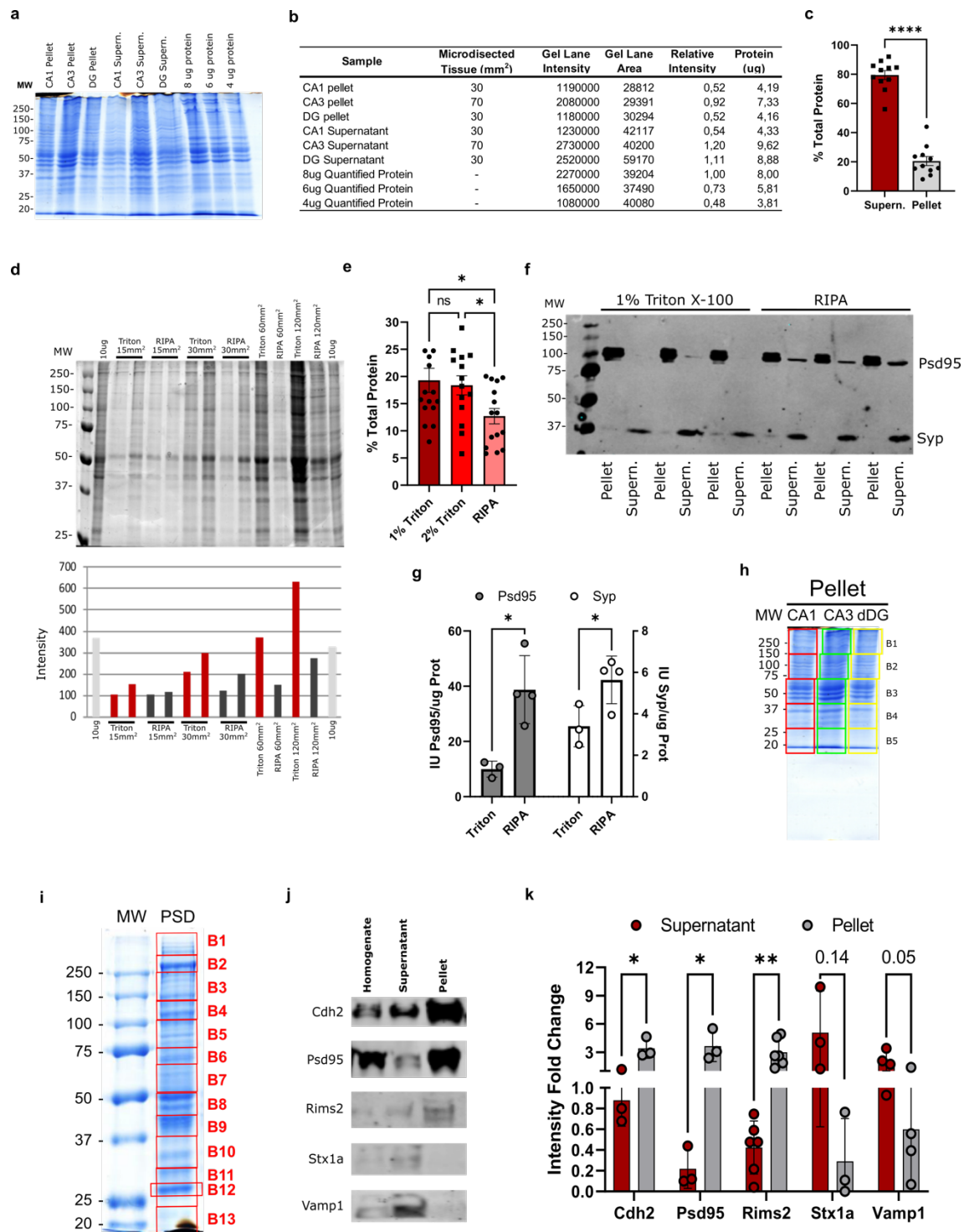


Supplementary Figure 1. Strategy used to microdissect the hippocampal layers containing the synapses of the trisynaptic circuit.

- Drawing of a mouse brain showing the localization of the hippocampus, in red. The portion of the dorsal hippocampus that was analysed in this work is shown in dark red. This covered approximately 500 μm in the longitudinal axis of the brain.

- b.** Brightfield image showing the hippocampus in a coronal section of the dorsal mouse brain. The 3 subfields, CA1, CA3 and dDG, investigated in this study are indicated. CA1 and CA3 pyramidal layers and DG granular layer are shown differently coloured. Scale bar 1000 μ m.
- c.** Anatomical localization and dimensions, particularly width, of the different hippocampal layers from which we collected microdissected neuropile. Pyramidal and granular layers coloured as in (a). Shapes delimited by red dashed lines represent examples of microdissected neuropile fragments, where their width is also shown. Collected fragments in all subfields had 100 μ m in width, approximately. Neuropile fragments were collected from the following layers: i) Stratum Radiatum (SR, in blue) in the CA1 subfield, ii) Stratum Lucidum (SL, in pale green) in the CA3 subfield and iii) Molecular Layer (ML) at the dorsal Dentate Gyrus.

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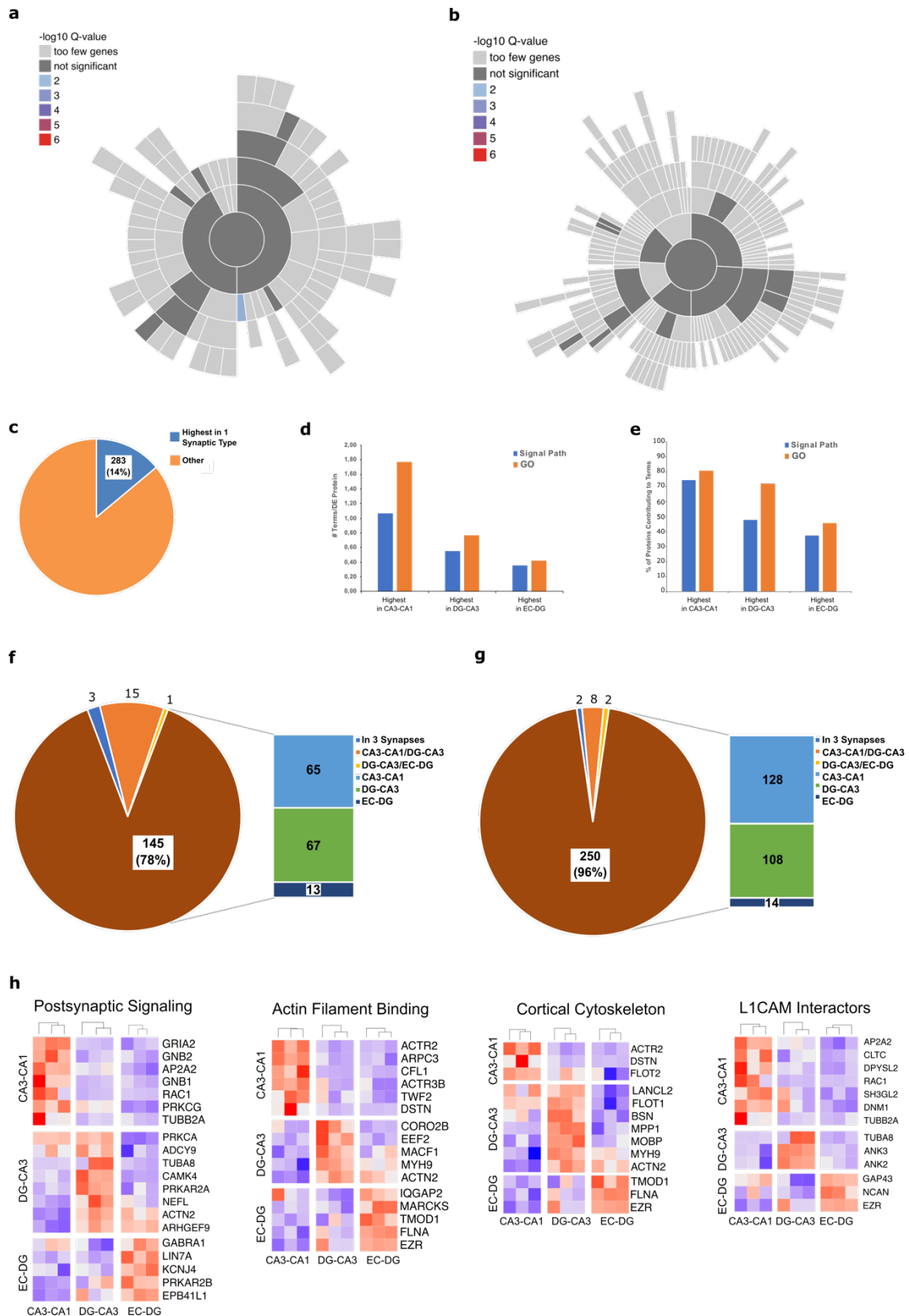


Supplementary Figure 2. Optimization and validation of the biochemical procedure used to obtain preparations enriched in synaptic structures and proteins.

- Protein gel electrophoresis stained with Coomassie Brilliant Blue with 1% Triton X-100 insoluble (Pellet) and soluble (Supern.) fractions obtained from microdissected neuropile of the three hippocampal subfields. 8, 6 and 4 μ g of precisely quantified protein from hippocampal synaptic fractions isolated by density gradient ultracentrifugation were used to estimate protein abundance of fractions derived from microdissected neuropile.
- Quantification of signal intensity of gel lanes in (a) and estimation of protein abundance in biochemical fractions from microdissected neuropile.

- c. Percentage of total protein from microdissected neuropile recovered in 1% Triton X-100 soluble (Supern.) and insoluble (Pellet) fractions. Bars = mean $\pm$ SEM. Statistics, unpaired two-sided Student's T-Test, sample size (n, biological replicates) = 11. **** $p < 0.0001$.
- d. **Top:** Silver-stained protein gel with biochemical fractions insoluble to 1% Triton X-100 or RIPA buffer obtained from increasing areas (mm²) of microdissected neuropile. 10 μ g of quantified protein from hippocampal synaptic fractions isolated by density gradient ultracentrifugation were added to the gel for reference. **Bottom:** Bar chart with signal intensity from gel lanes in above image.
- e. Percentage of protein recovered in the pellet fractions of microdissected tissue treated with a buffer containing 1% Triton X-100 (dark red column), 2% Triton X-100 (red column) or RIPA buffer (light red column). Bars = mean $\pm$ SEM. Statistics, One-way ANOVA and Fisher's LSD post-hoc test, sample size (n, biological replicates) = 14. * $p < 0.05$.
- f. Immunoblot of Triton and RIPA insoluble (Pellet) and soluble (Supern.) fractions obtained from hippocampal microdissected neuropile. Proteins investigated are Psd95, mostly insoluble to triton and Synaptophysin (Syp) a synaptic vesicle protein, mostly soluble to triton.
- g. Bar plot of Psd95 (grey bars) and Synaptophysin (Syp, white bars) abundance as determined by immunoblot of 1% Triton X-100 soluble fractions from microdissected hippocampal neuropile. Bars = mean $\pm$ SEM. Statistics, unpaired two-sided Student's T-test, sample size (n, biological replicates) = 4. * $p < 0.05$.
- h. Protein gel electrophoresis of 1% Triton X-100 insoluble pellets stained with Coomassie Brilliant Blue. 5 gel bands (B1-B5) were collected and processed independently in the proteomics workflow.
- i. Protein gel electrophoresis of synaptic preparations isolated by density gradient ultracentrifugation from mouse hippocampi stained with Coomassie Brilliant Blue. 13 bands (B1-B13) were collected and processed independently in the proteomics workflow to generate a synaptic reference proteome.
- j. Immunoblots of a transsynaptic protein involved in cell adhesion (Cdh2, cadherin 2), a postsynaptic scaffolding molecule (Psd95), and three presynaptic proteins located at: i) active zone (Rims2), SNARE complex (Stx1a) and synaptic vesicles (Vamp1). Samples analysed are whole extract (Homogenate) and triton soluble (Supernatant) and insoluble (Pellet) fractions from microdissected hippocampal neuropile.
- k. Abundance of proteins in (j) relative to their abundance in the homogenate fraction. Statistically significant difference in protein abundance between the supernatant and pellet fractions is indicated. Bars = mean $\pm$ SEM. Statistics, unpaired two-sided Student's T-test, sample size (n, biological replicates) = Cdh2,3; Psd95, 3; Rims2, 6; Stx1a 3 and Vamp1, 4. * $p < 0.05$, ** $p < 0.01$.

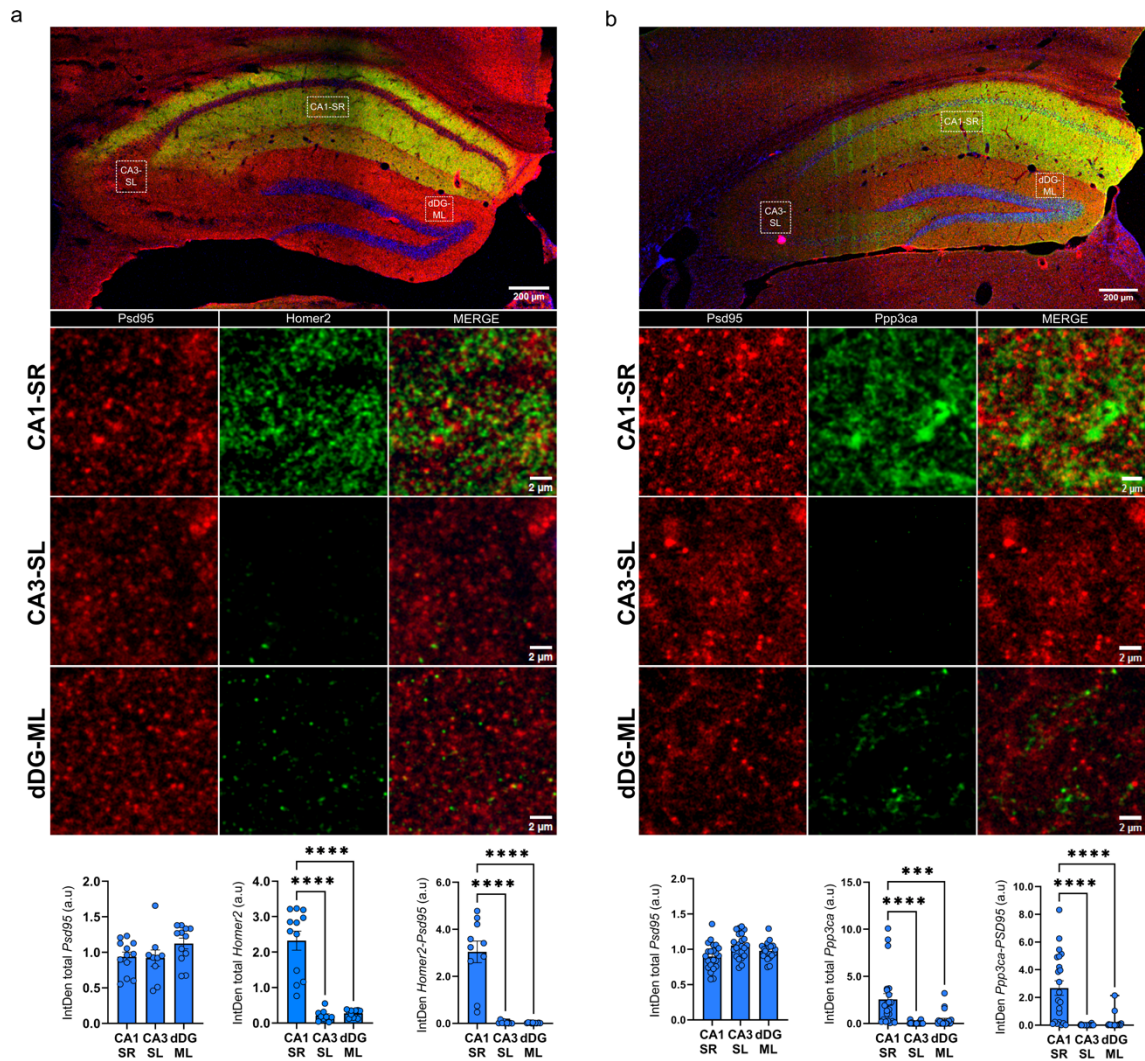
Source data are provided as a Source Data file.



Supplementary Figure 3. Most Signalling Pathways and Gene Ontology (GO) terms found in proteins differentially expressed between synaptic types are synapse-specific

- a. Sunburst plot showing SynGO Cellular Component terms enriched among proteins removed after benchmarking with the reference synaptic proteome. Note that not only one term (postsynaptic ribosome, adj. p-value 4.4e-5) is significantly overrepresented among this protein set.

- b.** Sunburst plot showing SynGO Biological Process terms enriched among proteins removed after benchmarking with the reference synaptic proteome. No terms appear significantly overrepresented.
- c.** Proportion of proteins identified by proteomics with a statistically significant highest expression in one of the three synaptic types investigated.
- d.** Ratio of significantly overrepresented terms per differentially expressed protein in each sample investigated. Signalling pathways (blue bars) investigated are from the databases Reactome, KEGG and Wikipathways. Gene ontology (GO, orange bars) terms investigated belong to the domains Cellular Component, Biological Process and Molecular Function.
- e.** Percentage of differentially expressed proteins contributing to signalling pathways (blue bars) and GO terms (orange bars).
- f.** Number of signalling pathways significantly overrepresented in 3, 2 or 1 hippocampal layers.
- g.** Number of significantly overrepresented GO terms among proteins with highest expression in 3, 2 or 1 synapses.
- h.** Heatmaps showing relative protein abundance in the 9 samples analysed by proteomics, three biological replicates per hippocampal layer. High abundance shown in red and low in blue. Abundance of proteins in four pathways/terms found significantly enriched in all three synapses is presented.

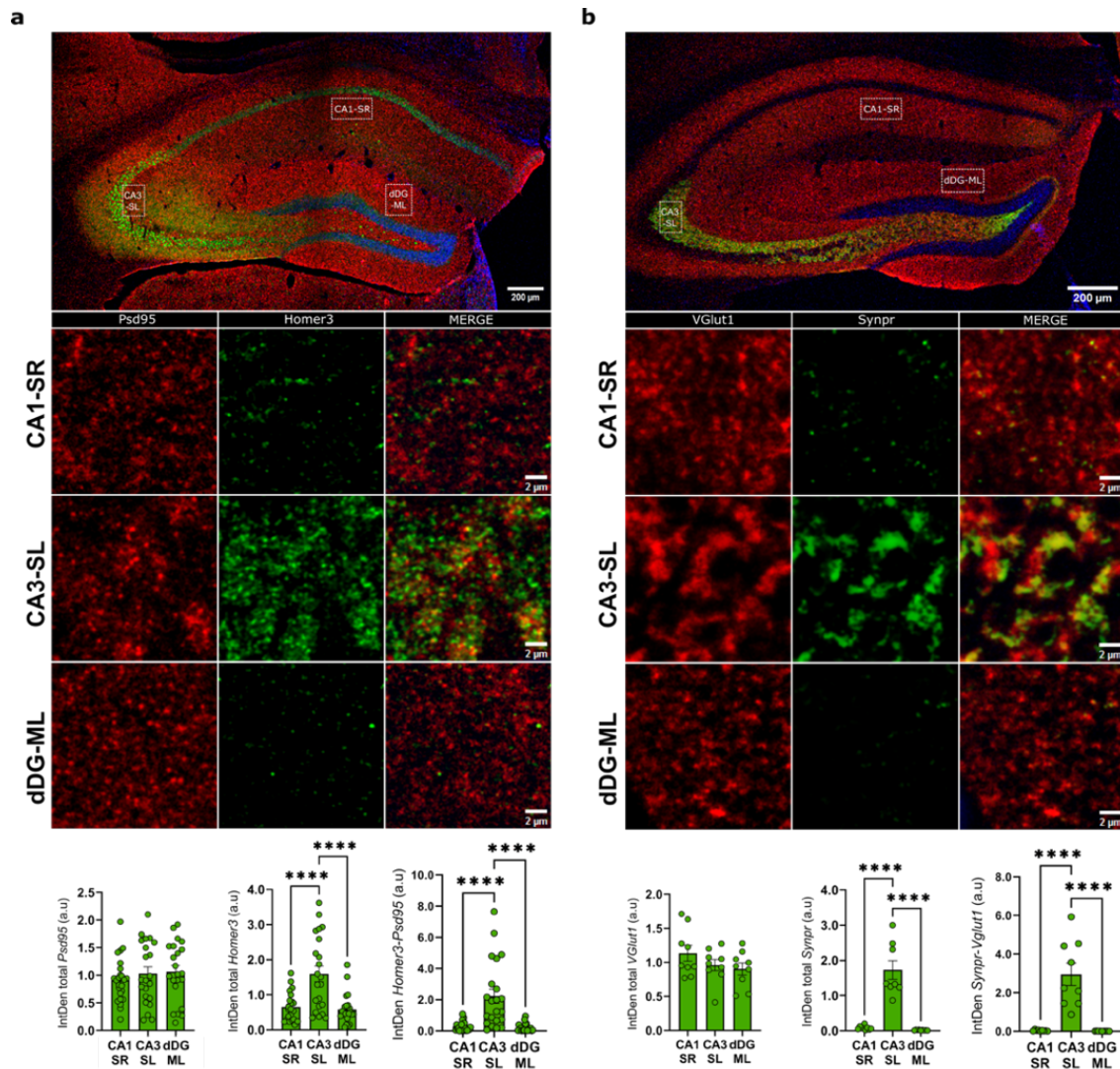


Supplementary Figure 4. Validation by double immunofluorescence of proteins found differentially expressed at CA3-CA1 synapses.

- Increased expression of Homer2 in CA3-CA1 synapses at the stratum radiatum.
Top image: Complete hippocampus. Red signal corresponds to Psd95 and green signal to Homer2. White dotted shapes denote locations where high-magnification images were taken in CA1 - stratum radiatum (CA1-SR), in CA3 - stratum lucidum (CA3-SL) and in Dentate Gyrus - dorsal molecular layer (dDG-ML). Scale bar 200 μ m.
High magnification images: columns correspond to Psd95, Homer2 and the merged signal. Rows correspond to images from CA1-SR, CA3-SL and dDG-ML. Scale bars 2 μ m.
Bottom: Bar and dots plots show the quantification of total mean fluorescence intensity of Psd95 (left), total mean fluorescence intensity of Homer2 (middle) and mean fluorescence intensity of the overlapping signal (right), error bars show SEM. Statistics, one-way ANOVA and Fisher's LSD post-hoc test, sample size (n, biological replicates) = 12. *** p < 0.001, **** p < 0.0001.
- Increased expression of Calcineurin (Ppp3ca) in CA3-CA1 synapses at the stratum radiatum.
Top image: Complete hippocampus. Red signal corresponds to Psd95 and green signal to Ppp3ca. White dotted shapes denote locations where high-magnification images were taken in CA1 - stratum radiatum (CA1-SR), in CA3 - stratum lucidum (CA3-SL) and in Dentate Gyrus - dorsal molecular layer (dDG-ML). Scale bar 200 μ m.
High magnification images: columns correspond to Psd95, Ppp3ca and the merged signal. Rows correspond to images from CA1-SR, CA3-SL and dDG-ML. Scale bars 2 μ m.

Bottom: Bar and dots plots show the quantification of total mean fluorescence intensity of Psd95 (left), total mean fluorescence intensity of Ppp3ca (middle) and mean fluorescence intensity of the overlapping signal (right), error bars show SEM. Statistics, one-way ANOVA and Fisher's LSD post-hoc test, sample size (n, biological replicates) = 22. **** p < 0.0001.

Source data are provided as a Source Data file.

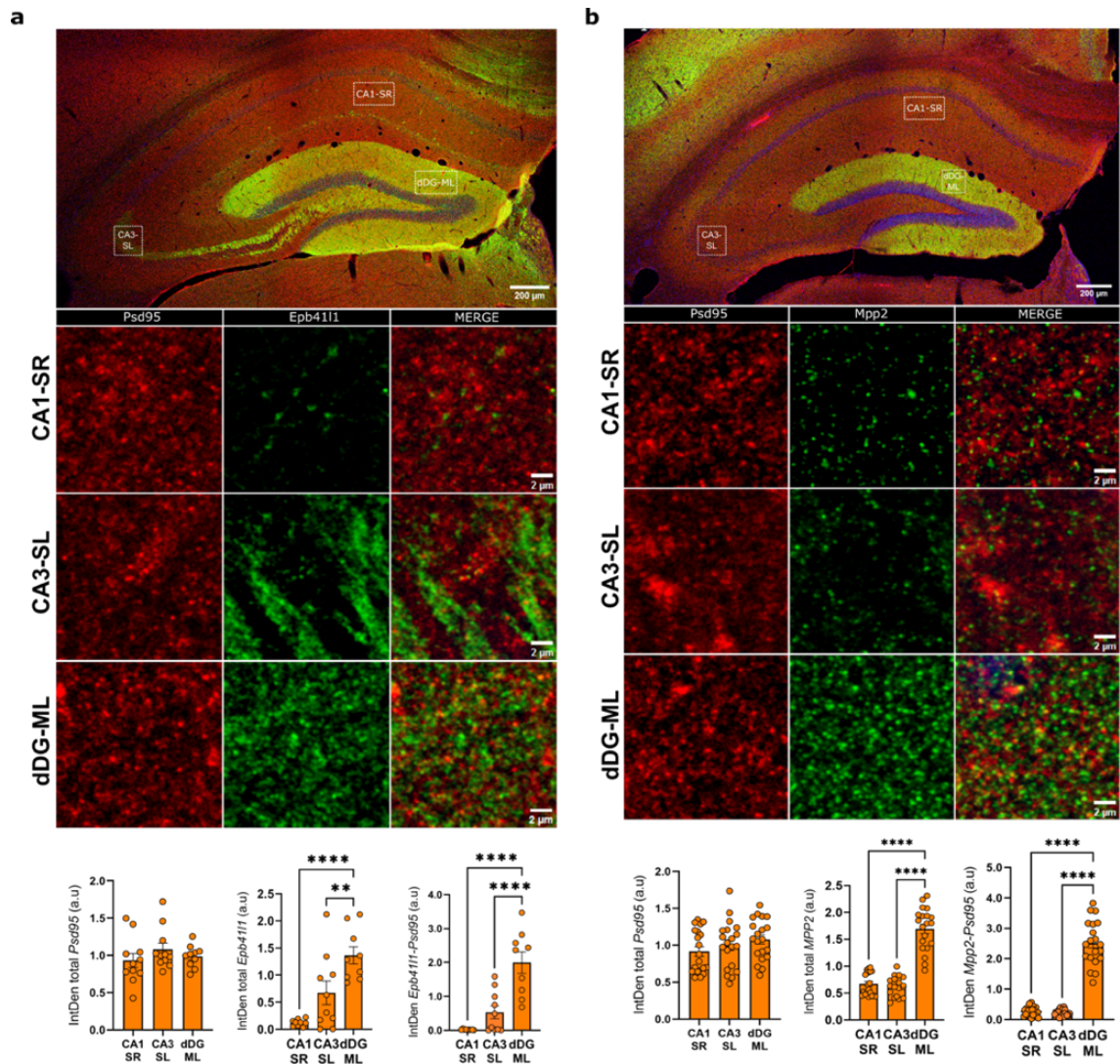


Supplementary Figure 5. Validation by double immunofluorescence of proteins found differentially expressed at DG-CA3 synapses.

- Increased expression of Homer3 in DG-CA3 synapses at the stratum lucidum.
Top image: Complete hippocampus. Red signal corresponds to Psd95 and green signal to Homer3. White dotted shapes denote locations where high-magnification images were taken in CA1 - stratum radiatum (CA1-SR), in CA3 - stratum lucidum (CA3-SL) and in Dentate Gyrus - dorsal molecular layer (dDG-ML). Scale bar 200 μ m.
High magnification images: columns correspond to Psd95, Homer3 and the merged signal. Rows correspond to images from CA1-SR, CA3-SL and dDG-ML. Scale bars 2 μ m.
Bottom: Bar and dots plots show the quantification of total mean fluorescence intensity of Psd95 (left), total mean fluorescence intensity of Homer3 (middle) and mean fluorescence intensity of the overlapping signal (right), error bars show SEM. Statistics, one-way ANOVA and Fisher's LSD post-hoc test, sample size (n, biological replicates) = 24. **** $p < 0.0001$.
- Increased expression of Synaptoporin (Synpr) in DG-CA3 synapses at the stratum lucidum.
Top image: Complete hippocampus. Red signal corresponds to vGlut1 and green signal to Synaptoporin (Synpr). White dotted shapes denote locations where high-magnification images were taken in CA1 - stratum radiatum (CA1-SR), in CA3 - stratum lucidum (CA3-SL) and in Dentate Gyrus - dorsal molecular layer (dDG-ML). Scale bar 200 μ m.
High magnification images: columns correspond to vGlut1, Synaptoporin (Synpr) and the merged signal. Rows correspond to signal from CA1-SR, CA3-SL and dDG-ML. Scale bars 2 μ m.

Bottom: Bar and dots plots show the quantification of total mean fluorescence intensity of vGlut1 (left), total mean fluorescence intensity of Synpr (middle) and mean fluorescence intensity of the overlapping signal (right), error bars show SEM. Statistics, one-way ANOVA and Fisher's LSD post-hoc test, sample size (n, biological replicates) = 9. **** p < 0.0001.

Source data are provided as a Source Data file.



Supplementary Figure 6. Validation by double immunofluorescence of proteins found differentially expressed at EC-DG synapses.

- a. Increased expression of Band 4.1-like protein 1 (Epb4111) in EC-DG synapses at the dorsal molecular layer.

Top image: Complete hippocampus. Red signal corresponds to Psd95 and green signal to Epb4111. White dotted shapes denote locations where high-magnification images were taken in CA1 - stratum radiatum (CA1-SR), in CA3 - stratum lucidum (CA3-SL) and in Dentate Gyrus - dorsal molecular layer (dDG-ML). Scale bar 200 μ m.

High magnification images: columns correspond to Psd95, Epb4111 and the merged signal. Rows correspond to images from CA1-SR, CA3-SL and dDG-ML. Scale bars 2 μ m.

Bottom: Bar and dots plots show the quantification of total mean fluorescence intensity of Psd95 (left), total mean fluorescence intensity of Epb4111 (middle) and mean fluorescence intensity of the overlapping signal (right), error bars show SEM. Statistics, one-way ANOVA and Fisher's LSD post-hoc test sample size (n, biological replicates) = 11. ** p < 0.01, **** p < 0.0001.

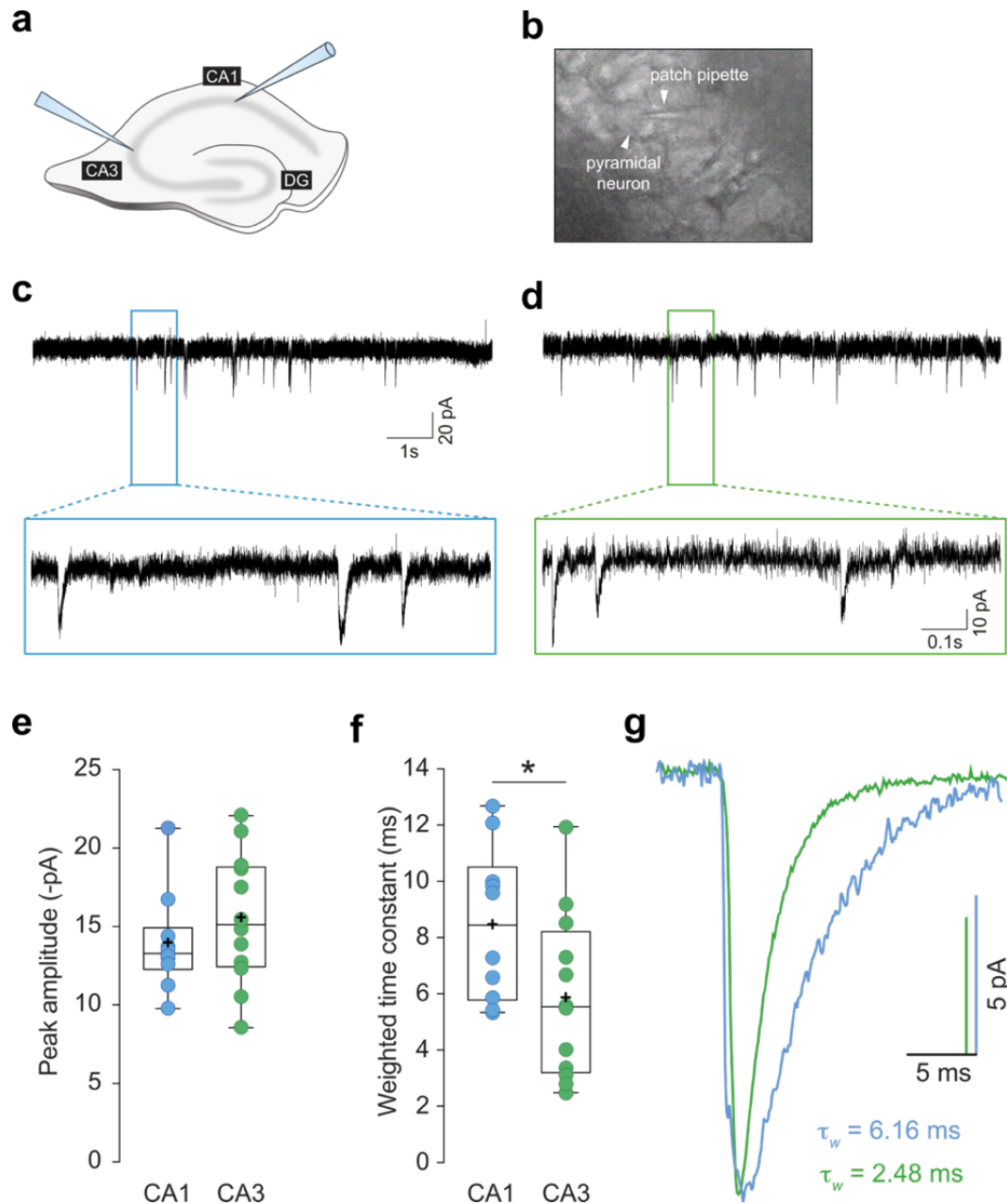
- b. Increased expression of Mpp2 in EC-DG synapses at the dorsal molecular layer.

Top image: Complete hippocampus. Red signal corresponds to Psd95 and green signal to Mpp2. White dotted shapes denote locations where high-magnification images were taken in CA1 - stratum radiatum (CA1-SR), in CA3 - stratum lucidum (CA3-SL) and in Dentate Gyrus - dorsal molecular layer (dDG-ML). Scale bar 200 μ m.

High magnification images: columns correspond to Psd95, Mpp2 and the merged signal. Rows correspond to images from CA1-SR, CA3-SL and dDG-ML. Scale bars 2 μ m.

Bottom: Bar and dots plots show the quantification of total mean fluorescence intensity of Psd95 (left), total mean fluorescence intensity of Mpp2 (middle) and mean fluorescence intensity of the overlapping signal (right), error bars show SEM. Statistics, one-way ANOVA and Fisher's LSD post-hoc test, sample size (n, biological replicates) = 20. **** $p < 0.0001$.

Source data are provided as a Source Data file.



Supplementary Figure 7. Different kinetics of AMPAR-mediated mEPSCs in CA1 and CA3 subfields indicate higher proportion of GluA2-containing AMPARs in the CA1 subfield.

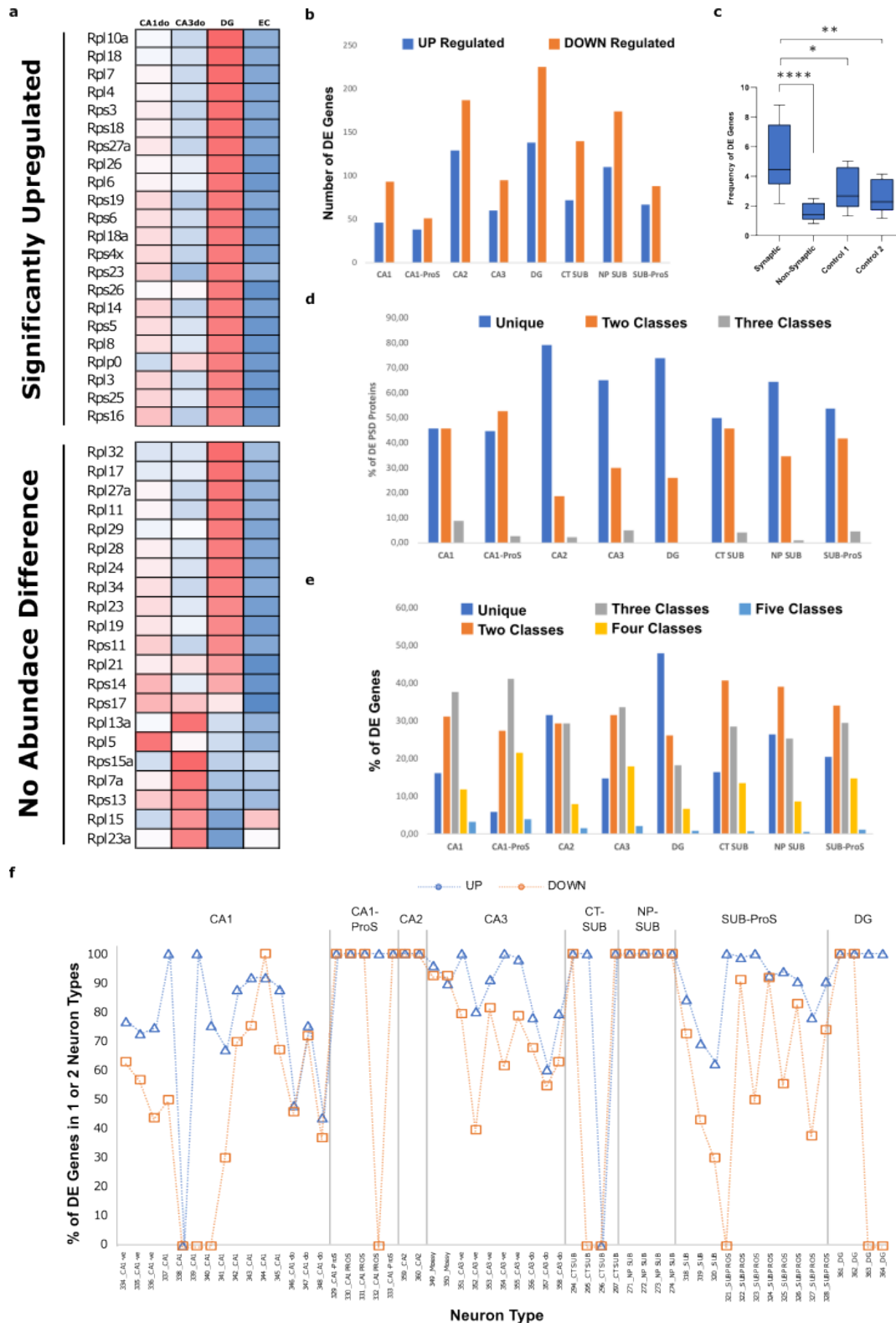
Miniature events driven by AMPAR activation were recorded from anterior coronal hippocampal slices.

- Schematic representation of the preparation of anterior coronal hippocampal slices used for the recording of CA1 and CA3 individual pyramidal neurons. Patch pipettes indicate the regions where cells were recorded.
- Example image of a pipette on a hippocampal pyramidal neuron from CA3 during a whole cell recording.
- Representative traces recorded in CA1 (c) or CA3 (d) pyramidal neurons. Bottom panels show the recording with magnification for easier comparison of individual mEPSCs.
- mEPSCs peak amplitude of CA1 (blue) and CA3 (green) pyramidal neurons. Box and Whisker plot with median. Statistics, unpaired two-sided Student's T-test, sample size (n, biological replicates) = 12. No statistical difference observed. Box and whiskers plot:

Center shows median, cross represents mean, box bounds range from 75% to 25% percentile and whiskers bounds expand from minimum to maximum values.

- f.** Weighted time constant of CA1 (blue) and CA3 (green) pyramidal neurons. Box and Whisker plot with median. Statistics, unpaired two-sided Student's T-test, sample size (n, biological replicates) = 12. * $p < 0.05$. Box and whiskers plot: Center shows median, cross represents mean, box bounds range from 75% to 25% percentile and whiskers bounds expand from minimum to maximum values.
- g.** Peak-scaled average mEPSCs from a CA1 (blue) and CA3 (green) hippocampal pyramidal neurons (average of 338 and 2735 traces for CA1 and CA3 recordings, respectively).

Source data are provided as a Source Data file.



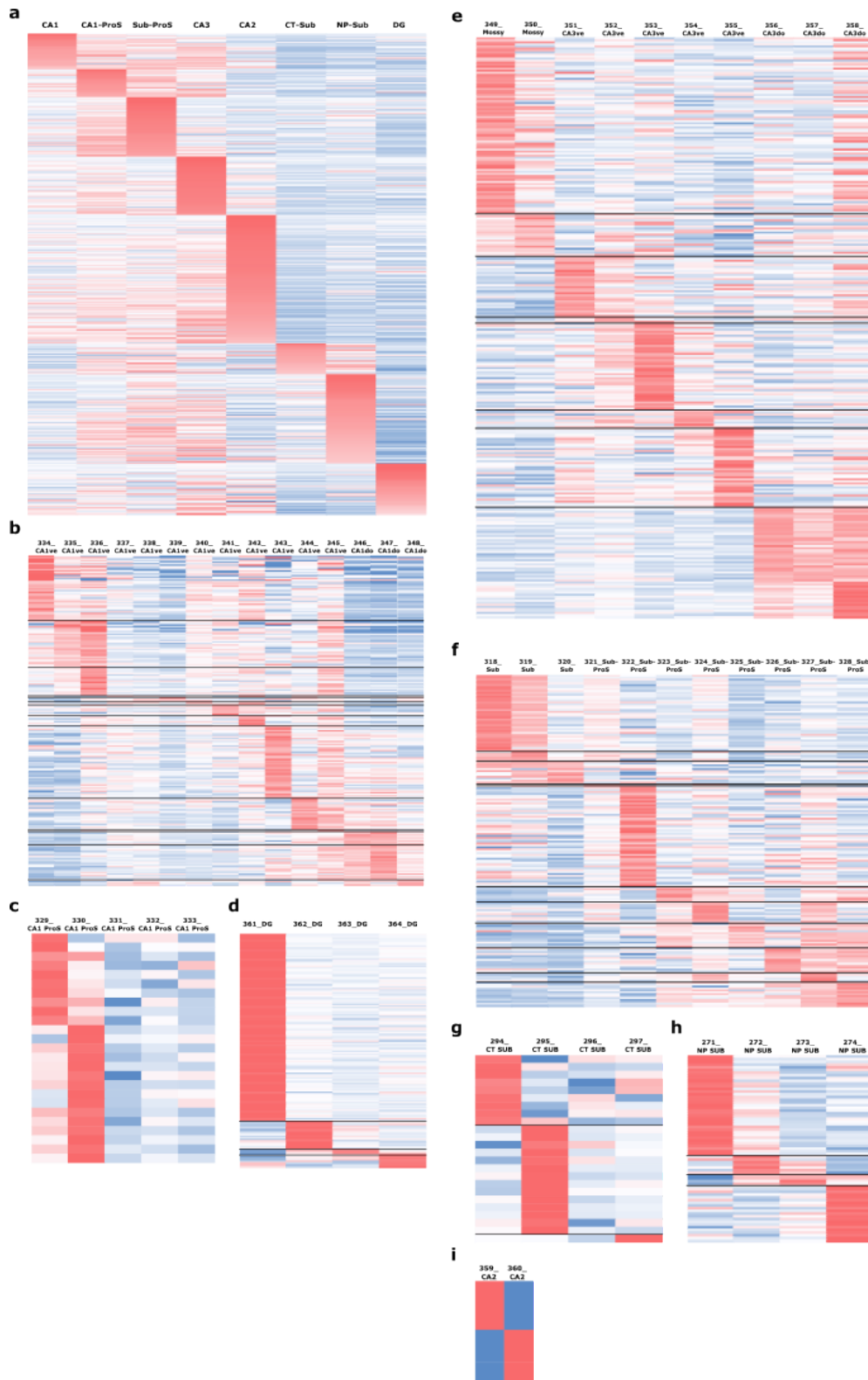
Supplementary Figure 8. Significantly up-regulated genes are more specific to neuronal classes and types than down-regulated ones.

- a. Heatmap presenting normalized (z-score) mean RNA abundance of genes coding for ribosomal proteins from the 4 classes of excitatory neurons that constitute the trisynaptic loop in the dorsal (do) hippocampus (CA1do, CA3do, dentate gyrus -DG- and entorhinal cortex -EC-). All ribosomal genes showing statistically significant RNA expression differences were upregulated in the DG (top panel). Many genes for which expression differences did not reach statistical significance also display a tendency for increased expression in the DG

(bottom panel). RNA expression data obtained from the Allen Brain Cell Atlas. Statistical analysis of RNA expression differences between neuronal classes was performed with the Seurat R package, which uses a two-sided Wilcoxon Rank Sum test, correcting for multiple testing by the Benjamini-Hochberg procedure. Abundance scale, 2 (dark red) to -2 (dark blue).

- b.** Number of genes expressed at synapses found significantly up- (blue bars) or down-regulated (orange bars) in classes of excitatory neurons from the hippocampal formation.
- c.** Frequency of differentially expressed genes among different gene sets. Including genes expressed at synapses (synaptic), genes not expressed at synapses (Non-Synaptic), a random set of all genes of the same size of the synaptic set (Control 1) and a random set of non-synaptic genes of the same size of the synaptic set (Control 2). Statistics, two-sided Chi square Test and Fisher's Exact Test, **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$. Box and whiskers plot: Center shows median, box bounds range from 75% to 25% percentile and whiskers bounds expand from minimum to maximum values.
- d.** Percentage of genes localized to synapses that are upregulated in one (blue), two (orange), or three (grey) classes of excitatory neurons.
- e.** Percentage of genes localized to synapses that are downregulated in one (blue), two (orange), three (grey), four (yellow) or five (light blue) classes of excitatory neurons.
- f.** Percentage of genes expressed at synapses being up-regulated (blue line) or down-regulated (orange line) in 1 or 2 excitatory neuron types from the hippocampal formation.

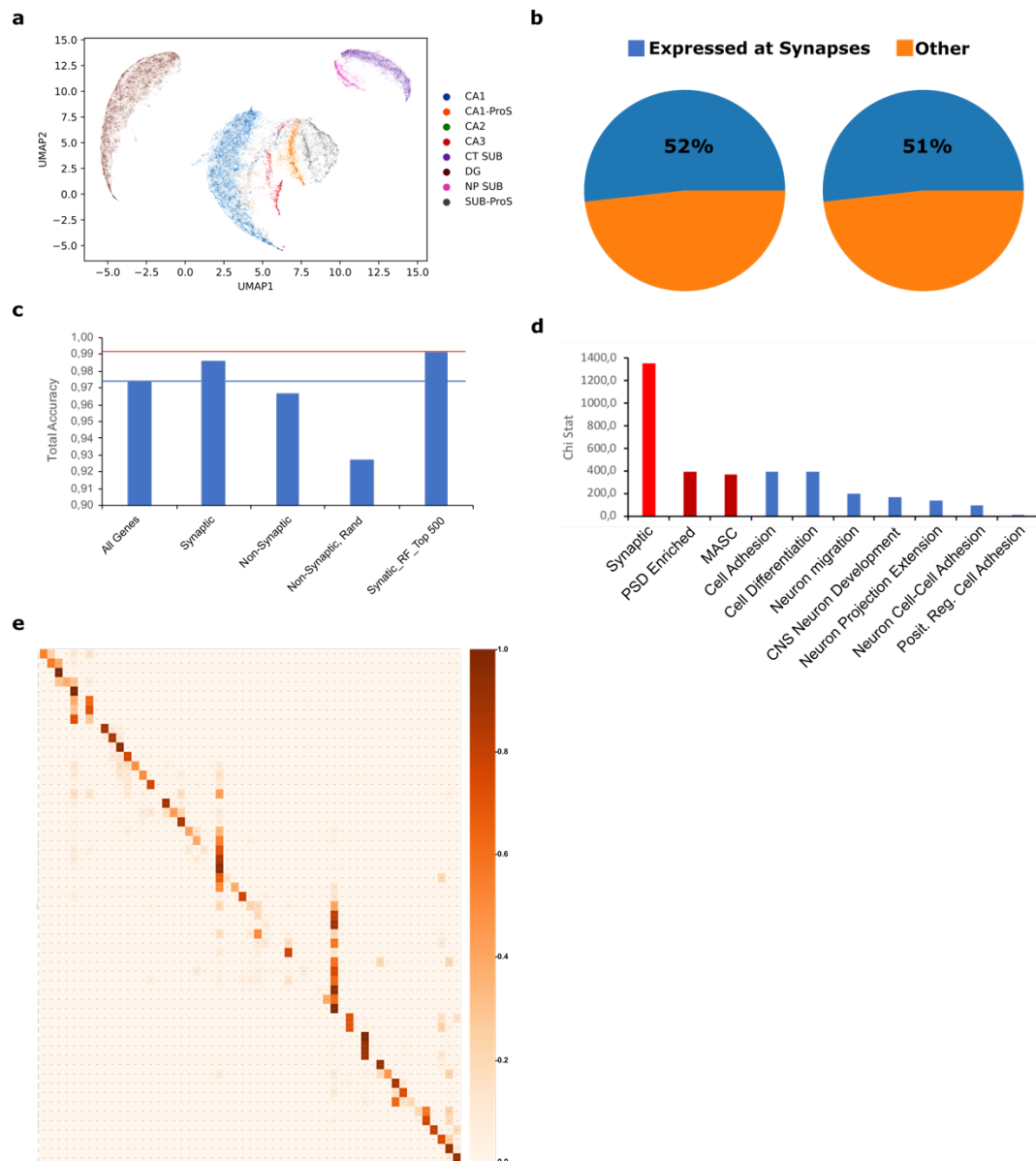
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Supplementary Figure 9. Gene expression heatmaps for synaptic genes expressed in neuronal classes and types.

- Heatmap showing relative RNA abundance data across all excitatory neuronal classes for genes found upregulated in each class.
- Heatmap showing relative RNA abundance data across types of CA1 excitatory neurons for genes found upregulated in each type.
- Heatmap showing relative RNA abundance data across types of CA1-ProS excitatory neurons for genes found upregulated in each type.

- d. Heatmap showing relative RNA abundance data across types of dentate gyrus (DG) excitatory neurons for genes found upregulated in each type.
- e. Heatmap showing relative RNA abundance data across types of CA3 excitatory neurons for genes found upregulated in each type.
- f. Heatmap showing relative RNA abundance data across types of SUB-ProS excitatory neurons for genes found upregulated in each type.
- g. Heatmap showing relative RNA abundance data across types of CT-SUB excitatory neurons for genes found upregulated in each type.
- h. Heatmap showing relative RNA abundance data across types of NP-SUB excitatory neurons for genes found upregulated in each type.
- i. Heatmap showing relative RNA abundance data across the two types of CA2 excitatory neurons for genes found upregulated in each type.



Supplementary Figure 10. Synaptic proteins are highly relevant for the classification of excitatory neurons into classes and types.

- UMAP graph generated with scRNAseq data from genes not expressed at synapses from excitatory neurons in the 8 classes from the hippocampal formation.
- Proportion of genes with synaptic (blue) or non-synaptic (orange) localization among the top 1000 genes contributing to the classification of neuronal classes (left chart) or neuronal types (right chart).
- Bar plot showing total accuracy in neuronal class prediction determined by the Random Forest machine learning method using different gene sets to train the algorithm: i) all gens in the dataset, ii) genes with a synaptic localization, iii) genes with a non-synaptic localization, iv) a set of randomly selected genes not found at the synapse and of the same size of the set of genes localized at synapses and v) the set of 520 genes expressed at synapses among the 1000 genes mostly contributing to neuronal classification.
- Bar plat with the Chi square statistic obtained from Chi square tests of overrepresentation of different gene sets among the 1000 genes mostly contributing to neuronal classification.
- Confusion or error matrix generated by the Random Forest algorithm, showing the success rates in assigning a neuronal type to each neuron. Colour legend correspond with the accuracy of the prediction, 1 being maximum accuracy.

References

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