

## Supplementary methods

More information on antibodies used can be found in the following links:

1. mouse anti-Bassoon: Enzo, clone #SAP7F407, <http://www.enzolifesciences.com/ADI-VAM-PS003/bassoon-monoclonal-antibody-sap7f407/>
2. rabbit anti-Bassoon: Cell Signaling #6897, <http://www.cellsignal.com/products/primary-antibodies/bassoon-d63b6-rabbit-mab/6897>
3. rabbit anti-RIM1/2: Synaptic Systems, Catalog No. 140203, <https://www.ssys.com/products/rim1/facts-140203.php>
4. rabbit anti-RIM1: Synaptic Systems, Catalog No. 140003, <https://www.ssys.com/products/rim1/facts-140003.php>
5. <https://www.ssys.com/products/rim1/facts-140003.php>
6. rabbit anti-Munc13: Synaptic Systems, Catalog No. 126103, <https://www.ssys.com/products/munc13/facts-126103.php>
7. mouse anti-PSD-95: NeuroMab clone K28/43, [http://neuromab.ucdavis.edu/datasheet/K28\\_43.pdf](http://neuromab.ucdavis.edu/datasheet/K28_43.pdf)
8. mouse anti-GluA2: Millipore Catalog #MAB397, [http://www.emdmillipore.com/US/en/product/,MM\\_NF-MAB397](http://www.emdmillipore.com/US/en/product/,MM_NF-MAB397)
9. rabbit anti-GluR2/3: Millipore Catalog #07-598, [http://www.emdmillipore.com/US/en/product/Anti-GluR23-Antibody,MM\\_NF-07-598](http://www.emdmillipore.com/US/en/product/Anti-GluR23-Antibody,MM_NF-07-598)
10. rabbit anti-Homer1: Synaptic Systems, Catalog No. 160003, <https://www.ssys.com/products/homer1/facts-160003.php>
11. mouse anti-GKAP: NeuroMab clone N127/31, [http://neuromab.ucdavis.edu/datasheet/N127\\_31.pdf](http://neuromab.ucdavis.edu/datasheet/N127_31.pdf)

12. mouse anti-pan-Shank: NeuroMab clone N23B/49,  
[http://neuromab.ucdavis.edu/datasheet/N23B\\_49.pdf](http://neuromab.ucdavis.edu/datasheet/N23B_49.pdf)
13. chicken anti-GFP: Chemicon Catalog #AB16901,  
[http://www.emdmillipore.com/US/en/product/,MM\\_NF-AB16901](http://www.emdmillipore.com/US/en/product/,MM_NF-AB16901)
14. GFP-booster: Chromotek gba647n, <http://www.chromotek.com/products/nano-boosters/gfp-booster/>
15. All secondary antibodies were obtained from Jackson ImmunoResearch  
(<https://www.jacksonimmuno.com/>).

**Supplementary Table 1 | Properties of nanoscale organizations in different proteins**

|   | RIM1/2          | Bassoon                    | Munc13                    | PSD-95          | GluA2               | GluR2/3             |
|---|-----------------|----------------------------|---------------------------|-----------------|---------------------|---------------------|
| <b>N (synapses)</b>   | 176             | 129                        | 150                       | 458             | 36                  | 70                  |
| <b>Number of cultures</b>   | 12              | 6                          | 8                         | 28              | 5                   | 10                  |
| <b>Cluster volume (<math>10^6 \text{ nm}^3</math>)</b>                | $6.93 \pm 0.33$ | $7.10 \pm 0.29$            | $8.20 \pm 0.37$ * †       | $6.65 \pm 0.29$ | $4.95 \pm 1.03$ * ‡ | $5.09 \pm 0.78$ * ‡ |
| <b>NC number</b>  | $2.02 \pm 0.13$ | $1.56 \pm 0.08$ *          | $2.44 \pm 0.13$ ***<br>†† | $1.86 \pm 0.07$ | $1.65 \pm 0.25$ ‡   | $1.60 \pm 0.18$ ‡   |
| <b>NC volume (<math>10^6 \text{ nm}^3</math>) -<br/>Mean</b>          | $0.36 \pm 0.04$ | $0.57 \pm 0.06$ ***<br>††† | $0.36 \pm 0.03$           | $0.31 \pm 0.03$ | $0.30 \pm 0.04$     | $0.40 \pm 0.06$     |
| <b>NC volume (<math>10^6 \text{ nm}^3</math>) -<br/>Median</b>        | 0.271           | $0.416$ *** †††            | 0.298                     | 0.269           | 0.257               | 0.245               |
| <b>Molecule density in NC<br/>normalized with cluster<br/>average</b> | $2.3 \pm 0.2$   | $1.6 \pm 0.1$ ** ††        | $2.4 \pm 0.2$             | $2.4 \pm 0.2$   | $2.4 \pm 0.4$       | $2.1 \pm 0.2$       |
| <b>%localization in NCs</b>   | $19.8 \pm 3.9$  | $35.5 \pm 9.1$ *           | $21.7 \pm 3.4$            | $16.4 \pm 2.6$  | $42.3 \pm 10.1$ ‡   | $38.6 \pm 7.5$ ‡    |

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  for comparison to PSD-95 at the same synapse, Wilcoxon signed-rank test. †  $p < 0.05$ , ††  $p < 0.01$ , †††  $p < 0.01$  for comparison to RIM1/2, one-way ANOVA on ranks with pairwise comparison procedures (Dunn's method). ‡  $p < 0.05$  for comparison between GluR2/3 and RIM1/2 at the same synapse, Wilcoxon signed-rank test. For tests on median of NC volumes, Kolmogorov–Smirnov test was used. GluR2/3 data was combined from 33 synapses with RIM-GluR staining (4 cultures) and 47 synapses with GluR-PSD-95 staining (6 cultures).

## Supplementary Table 2 | Protein enrichment results

| Enrichment index                     | Original data  | decay constant (nm) | #synapse | #NC | Rand. Fully | Rand. Out of NC molecules | Rand. NC positions | % NC with positive enrichment |
|--------------------------------------|----------------|---------------------|----------|-----|-------------|---------------------------|--------------------|-------------------------------|
| <b>RIM1/2 Enriched to PSD-95 NC</b>  | 1.32 ± 0.05**  | 43.2                | 139      | 265 | 0.99 ± 0.01 | 1.16 ± 0.04**             | 1.02 ± 0.03        | 44.4 ± 3.0 %                  |
| <b>PSD-95 Enriched to RIM1/2 NC</b>  | 1.20 ± 0.05**  | 41.6                | 139      | 272 | 1.00 ± 0.01 | 1.14 ± 0.03**             | 1.01 ± 0.03        | 33.0 ± 3.0 %                  |
| <b>Bassoon Enriched to PSD-95 NC</b> | 1.27 ± 0.03**  | 94.3                | 237      | 438 | 0.99 ± 0.01 | 1.15 ± 0.03**             | 0.98 ± 0.03        | 41.3 ± 3.8 %                  |
| <b>PSD-95 Enriched to Bassoon NC</b> | 1.13 ± 0.03**† | 46.5                | 237      | 377 | 1.02 ± 0.01 | 1.07 ± 0.03*              | 1.02 ± 0.02        | 25.1 ± 2.9 %                  |
| <b>Munc13 Enriched to PSD-95 NC</b>  | 1.16 ± 0.04*†† | 41.3                | 153      | 299 | 1.02 ± 0.02 | 1.09 ± 0.03*              | 0.99 ± 0.03        | 27.6 ± 2.9 %††                |
| <b>PSD-95 Enriched to Munc13 NC</b>  | 1.05 ± 0.04††  | N.A.                | 153      | 358 | 1.03 ± 0.02 | 1.05 ± 0.02               | 1.02 ± 0.02        | 19.4 ± 2.5 %††                |
| <b>GluA2 Enriched to RIM1/2 NC</b>   | 1.24 ± 0.07**  | 66.9                | 36       | 67  | 0.98 ± 0.03 | 1.14 ± 0.05*              | 1.01 ± 0.05        | 36.5 ± 7.6 %                  |
| <b>GluA2/3 Enriched to RIM1/2 NC</b> | 1.29 ± 0.18**  | 42.6                | 34       | 63  | 0.98 ± 0.03 | 1.17 ± 0.05*              | 1.02 ± 0.04        | 29.8 ± 5.7 %                  |
| <b>RIM1/2 Enriched to GluA2/3 NC</b> | 1.24 ± 0.11**  | 59.7                | 34       | 54  | 1.01 ± 0.04 | 1.13 ± 0.04*              | 0.98 ± 0.05        | 34.9 ± 6.5 %                  |

\*  $p < 0.05$ , \*\*  $p < 0.01$  for comparison to the enrichment index with randomized NC positions, one-way ANOVA on ranks with pairwise comparison procedures (Dunn's method). †  $p < 0.05$ , ††  $p < 0.01$  for comparison to the corresponding results of RIM1/2-PSD-95 pair, one-way ANOVA on ranks with pairwise comparison procedures (Dunn's method), and  $\chi^2$  test for the proportion.

### Supplementary Table 3 | Statistical tables for pHuse results

a.

| Mode of release × Temperature 2-Way ANOVA |     |        |         |        |            |
|---|-----|--------|---------|--------|------------|
|   | DF  | SS     | MS      | F      | p          |
| Evoked v. Spontaneous                     | 1   | 0.1144 | 0.1144  | 10.835 | 0.00125 ** |
| Temperature                               | 1   | 0.006  | 0.00597 | 0.565  | 0.45327    |
| Interaction                               | 1   | 0.0001 | 0.00006 | 0.006  | 0.93992    |
| Residuals                                 | 148 | 1.5627 | 0.01056 |        |            |

b.

| Mode of release × Syn1a Thresholding 2-Way ANOVA |     |        |         |        |              |
|--|-----|--------|---------|--------|--------------|
|  | DF  | SS     | MS      | F      | p            |
| Evoked v. Spontaneous                            | 1   | 0.2476 | 0.24764 | 21.015 | 7.42e-06 *** |
| Threshold  | 1   | 0.0196 | 0.01957 | 1.661  | 0.199        |
| Interaction                                      | 1   | 0.0129 | 0.01292 | 1.096  | 0.296        |
| Residuals  | 234 | 2.7573 | 0.01178 |        |              |

c.

| Mode of release × Number of localizations 2-Way ANOVA |     |        |        |       |              |
|---|-----|--------|--------|-------|--------------|
|   | DF  | SS     | MS     | F     | p            |
| Evoked v. Spontaneous                                 | 1   | 0.1513 | 0.1513 | 20.3  | 1.02e-05 *** |
| # of Localizations                                    | 1   | 1.7039 | 1.7039 | 228.5 | 2e-16 ***    |
| Interaction   | 1   | 0      | 0      | 0     | 0.997        |
| Residuals   | 250 | 1.8641 | 0.0075 |       |              |

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001