Supplementary Methods

Clustering analysis of pre- and post-synaptic appositions

To simplify analysis, reconstructed images were divided into 2x2µm ROIs around visually identified synaptic appositions. Subsequently, per ROI, pre- and post-synaptic appositions were identified visually based on pre- and post-synaptic localization clusters. In all cases, the experimenter performing the analysis was blind to the experimental conditions. Clustering was performed automatically through custom R[1] scripts using a modified OPTICS algorithm[2,3]. Briefly, ROI localizations were randomized based on a uniform distribution function. The median core-distance of this distribution after OPTICS (epsilon=500nm, minimum points=10) was used as a distance cutoff to identify staining clusters, whereby:

Cutoff = median (rcd) + *p**mad (rcd)

rcd corresponds to randomized core-distance, which indicates the core-distance of each point in the image after uniform randomization. This randomization was done so as to avoid variability caused by local assemblies. The mad, or median absolute deviation, was used as a measure of variance. *p*=5 was selected as an initial factor as the 5% significance cutoff of normal distributions is at 4.2*mad. This ensured that clusters were identified based on staining clustering rather than localization density, which may vary considerably between images. Clustering could be further refined by the experimenter by increasing *p* where clearly separate clusters were joined by several localizations.

Cluster identification was further limited for analysis by cluster volume. For Homer1 and Bassoon clusters, cluster volume was limited to a minimum of 2 000 000 nm^3, while Vamp2 clusters were limited to a minimum of 10 000 000 nm^3.

Crosstalk removal

To remove localizations that had been identified to the incorrect activator, cluster localizations were filtered based on their neighborhood density. For each localization, the 10^{th} nearest neighbor distance for each activator was calculated. Localizations whose 10^{th} nearest neighbor distance for the opposing activator was closer than for its own activator were removed.

Homer1 and Bassoon scaffolding measurements

The width, depth and length of Homer1 and Bassoon appositions were measured by fitting ellipsoids to the identified Homer1 and Bassoon clusters with the *ellipsoidhull*[4] R function (tol=1000). The fitted ellipsoid principal axes were used as width, depth and length measurements, with:

length > width > depth

and the depth axis perpendicular to the synaptic cleft. The resulting median measurements were 465.7nm (+-46.4nm) for Homer1 length, 405.4nm (+-50.5nm) for Bassoon length , 330.7nm (+-28.6nm) for Homer1 width, 319.7nm(+-22.9nm) for Bassoon width, 141.9nm (+-8.2nm) for Homer1 depth, and 168.2nm (+-8.4nm) for Bassoon width.

Distance between Homer1 and Bassoon clusters

Distance measurements between Homer1 and Bassoon were performed similarly to Dani et al.[5] Per ROI, after ellipsoid fitting, the identified depth axis of the Homer1 cluster was used as the x axis along which a Gaussian fit of both Homer1 cluster localizations and Bassoon cluster localizations was produced. Localizations within a 200nm range around the x axis were used for fitting, with a 5nm bin width along the axis. The distance between the peaks of the fitted Gaussian functions was used as the cluster separation distance.

Synaptic vesicle identification

Synaptic vesicles were identified based on VAMP2 localizations through a nested clustering algorithm similar to that described for clustering of appositions. First clustering of localizations as mentioned above was produced to identify synaptic boutons. The outer limits of the bouton were then identified using the *ashape3D* function of the alpha R package[6]. Localizations within the bouton were then put through the clustering algorithm again using a *p* of 3, determined as the best parameter cutoff based on randomized simulations. Vesicles identified through this method were taken for further analysis only if they had a minimum of 15 localizations.

Identified boutons with fewer than 12 vesicles were omitted from quantification.

Counting vesicles within the predicted AZ

The AZ prediction was produced using length, width and depth of Homer1 measurements. Briefly, to account for variability, length, width, and depth of the AZ prediction were increased by 10% of the Homer1 measurements, equivalent to a median increase of 46nm, 33nm, and 14nm respectively. A bounding box produced with these measurements and the *ashape3D* function was then projected towards the centroid of VAMP2 localizations, at 125nm, the average measured distance between Bassoon and Homer1 appositions. Identified synaptic vesicles whose centroids were contained within the prediction were counted as being docked.

Clustering of synaptic vesicles

To measure the relative clustering of synaptic vesicles, the median expected distance between vesicles was calculated based on vesicle number and bouton volume. This value was divided by the median per bouton of the median distance of each vesicle to its 12 nearest neighbors, giving a ratio of the relative clustering of vesicles per bouton normalized to bouton vesicle density.

References

1. R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL https://www.R-project.org/.
2. Ankerst, M., Breunig, M. M., Kriegel, H.-P., & Sander, J. (1999). Optics. *Proceedings of the 1999 ACM SIGMOD International Conference on Management of Data - SIGMOD '99*, 49–60. http://doi.org/10.1145/304182.304187

3. Michael Hahsler and Matthew Piekenbrock (2017). dbscan: Density Based Clustering of Applications with Noise (DBSCAN) and Related Algorithms. R package version 1.1-1. https://CRAN.R-project.org/package=dbscan
4. Maechler, M., Rousseeuw, P., Struyf, A., Hubert, M., Hornik, K.(2017). cluster: Cluster Analysis Basics and Extensions. R package version 2.0.6.
5. Dani, A., Huang, B., Bergan, J., Dulac, C., & Zhuang, X. (2010). Superresolution Imaging of Chemical Synapses in the Brain. *Neuron*, *68*(5), 843–856. http://doi.org/10.1016/j.neuron.2010.11.021
6. Thomas Lafarge and Beatriz Pateiro-Lopez (2016). alphashape3d: Implementation of the 3D Alpha-Shape for the Reconstruction of 3D Sets from a Point Cloud. R package version 1.2. https://CRAN.R-project.org/package=alphashape3d