A trans-synaptic nanocolumn aligns neurotransmitter release to receptors

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Synaptic transmission is maintained by a delicate, sub-synaptic molecular architecture, and even mild alterations in synapse structure drive functional changes during experience-dependent plasticity and pathological disorders1,2. Key to this architecture is how the distribution of presynaptic vesicle fusion sites corresponds to the position of receptors in the postsynaptic density. However, while it has long been recognized that this spatial relationship modulates synaptic strength3, it has not been precisely described, owing in part to the limited resolution of light microscopy. Using localization microscopy, here we show that key proteins mediating vesicle priming and fusion are mutually co-enriched within nanometre-scale subregions of the presynaptic active zone. Through development of a new method to map vesicle fusion positions within single synapses in cultured rat hippocampal neurons, we find that action-potential-evoked fusion is guided by this protein gradient and occurs preferentially in confined areas with higher local density of Rab3-interacting molecule (RIM) within the active zones. These presynaptic RIM nanoclusters closely align with concentrated postsynaptic receptors and scaffolding proteins4–6, suggesting the existence of a trans-synaptic molecular ‘nanocolumn’. Thus, we propose that the nanarchitectue of the active zone directs action-potential-evoked vesicle fusion to occur preferentially at sites directly opposing postsynaptic receptor–scaffold ensembles. Remarkably, NMDA receptor activation triggered distinct phases of plasticity in which postsynaptic reorganization was followed by trans-synaptic nanoscale realignment. This architecture suggests a simple organizational principle of central nervous system synapses to maintain and modulate synaptic efficiency.

The location of vesicle fusion within an active zone is probably dictated by a few key members of the presynaptic proteome, including RIM1/2, Munc13, and bassoon (Bsn)3 (Fig. 1a). To explore the organization of these proteins, we studied their subsynaptic distribution relative to postsynaptic scaffolding protein PSD-95 in cultured hippocampal neurons using 3D-STORM5 following immunolabelling using primary antibodies and Alexa647- or Cy3-tagged secondary antibodies (Fig. 1b). Paired synaptic clusters of active zone protein and PSD-95 with clear borders were selected. As a confirmation that these pairs constituted synapses, we measured the peak-to-peak distances between pre- and postsynaptic clusters and found them to be consistent with previous measurements8 (Extended Data Fig. 1).

The distribution of RIM1/2 within the active zone, measured as 3D local density, was distinctively non-uniform with notable high-density peaks, which we characterized as nanoclusters (Fig. 1c, e). We adapted an auto-correlation function (ACF) to test whether this distribution occurs more frequently than expected by chance. The measured ACF showed significant non-uniformity compared to random ensembles (Fig. 1d). Simulations showed that the distance for which the ACF was significantly elevated provided a means to estimate the nanocluster diameter (Extended Data Fig. 2a–c). The average estimated diameter of ~80 nm for RIM1/2 nanoclusters was very close to the reported size of PSD-95 and AMPA receptor (AMPAR) nanoclusters4–6. Similar distribution and nanocluster properties were found using a different antibody targeted towards a separate epitope in RIM1 (Extended Data Fig. 2d). Isolated non-synaptic small groups of localizations showed a weaker ACF that was significant over a much smaller distance (Fig. 1d). This and other experiments suggest that the measured non-uniformity was not likely due to over-counting molecules or to potential artefacts of primary–secondary antibody labelling (Extended Data Fig. 3).

To directly compare the nanoscale organization of key active zone proteins, we developed an algorithm that identified nanoclusters based on local densities (Fig. 1e). Nanoclusters of each protein were more likely to be located near the centre of synapses than near the edge (Fig. 1f, Extended Data Fig. 2i). Compared to PSD-95 as the common control in pairwise two-colour experiments, there were similar numbers of RIM1/2, more Munc13, and fewer Bsn nanoclusters per synapse (Fig. 1h).

Comparisons between these three proteins suggested that Munc13 had a wider distribution than RIM1/2 across the active zone and the distribution of Bsn was closer to uniform throughout the synapse (Fig. 1g–i, Extended Data Fig. 2f–n). Together, these observations revealed a complex and heterogeneous molecular architecture within single synapses, typified by dense assemblies of fusion-associated proteins nearer the centre.

To examine the potential functional impact of the active zone nanoclusters on vesicle fusion10–11, we sought to directly map the distribution of vesicle fusion sites over multiple release events within individual boutons. To do so, we adapted analysis for single-molecule localization to signals from single-vesicle fusion obtained with vGlut1–pHluorin–mCherry (vGpH). Neurons were cotransfected with cyan fluorescent protein (CFP)-tagged synapsin1a (Syn1a), a vesicle-associated protein that marks boutons, and vGpH, which increases in green fluorescence intensity upon vesicle fusion12. Single electrical field stimuli evoked vesicle fusion (Fig. 2a, b, Extended Data Fig. 4a) with a release probability (P) of 0.11 ± 0.01 (mean ± s.e.m.) per bouton, comparable to previous measurements, which was also sensitive to extracellular Ca2+ (Extended Data Fig. 4b–d), as expected. In the presence of TTX, the frequency of action-potential-independent spontaneous release events detected with vGpH was similar to the frequency of NMDAR-dependent postsynaptic Ca2+ transients measured separately using the Ca2+ sensor GCaMP6f (Extended Data Fig. 5a).

To determine whether these evoked fusion events represent single- or multi-vesicular fusion, we compared them with spontaneous release under TTX conditions (Fig. 2a–c), which most likely arises from single vesicle fusion13. By fitting the photon number distributions of evoked and spontaneous events, we estimated that ~72–82% of evoked events arose from single-vesicle fusion (Fig. 2c). With the majority of evoked release stemming from single-vesicle fusion, the location of fusion may be deduced by mathematically fitting the fluorescence profile captured immediately after fusion (Fig. 2d), analogous to single-molecule

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localization techniques. For our median count of 518 photons per nanocluster, the effective localization precision was in practice limited by vesicle diameter. In individual boutons, multiple evoked or spontaneous single-vesicle fusion events were used to generate maps that defined the areas over which vesicle fusion occurred (Fig. 2e, Extended Data Fig. 6d, e). As a local density metric for the active zone. This predicts that pHuse events would be associated with 48% and occurred over a significantly smaller proportion of the bouton size defined with Syn1a (white). Scale bar, 500 nm. i, Tessler first-rank density (δ1) for RIM1 measured versus randomized distributions as a function of distance from pHuse localizations. j, Comparison within boutons of average δ1 for RIM1 localizations within 40 nm to pHuse localization versus not. k, Average nearest pHuse distance as a function of RIM1 δ1. l, j, n = 26/13, *P < 0.05, **P < 0.01, ***P < 0.001. n given in synapses/experiments. Also see Extended Data Figs 4–6.

The distribution of RIM1–mEos3 was non-uniform and contained nanoclusters with an average diameter of 80.95 ± 5.34 nm and 78.93 ± 5.85 nm using either an adapted SR-Tessler analysis or nearest neighbour distance analysis, respectively (Extended Data Fig. 6f), consistent with our 3D-STORM results (Fig. 1). We then compared δ1 as a function of distance from the nearest pHuse localization for the measured RIM1 distributions versus randomized RIM1 distributions generated from the same number of localizations over the same area. Indeed, near pHuse sites, the average RIM1 δ1 was significantly greater than chance (Fig. 2i). Furthermore, within individual boutons, RIM1 molecules within 40 nm of a pHuse location had significantly higher δ1 than those further away (Fig. 2i). Conversely, considering all individual RIM1 localizations, the distance from the nearest pHuse localization decreased as a function of RIM1 δ1 (Fig. 2k).

Figure 1 | Vesicle release proteins form subsynaptic nanoclusters.

a, Colour-coded schematic of studied synaptic proteins. AZ, active zone; PSD, postsynaptic density. b, Synapses labelled with RIM1/2 and PSD-95 imaged using 3D-STORM (10-nm pixels) compared to wide-field composite (bottom corner, 100-nm pixels). Scale bar, 2 μm. Boxed synapse enlarged in original (top) and rotated (bottom) angles. Scale bar, 200 nm. c, En face (top) and side (bottom) views of a RIM1/2 cluster showing all localizations and local density maps for a measured synaptic cluster compared to a simulated randomized cluster. Scale bar, 200 nm. d, Auto-correlation functions of measured RIM1/2 (n = 115), isolated non-synaptic small groups of localizations due to repetitive switching of fluorophores (n = 42), and simulated randomized (n = 115) distributions. e, RIM1/2 nanoclusters (red) within a synaptic cluster. f, Distribution of nanocluster distances from the centre of synapses normalized to randomized distribution. g, Molecule density inside nanoclusters (NC) normalized to synaptic average. h, Average number of protein nanoclusters per synapse. i, Cumulative distributions of nanocluster volumes. *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA on ranks with pairwise comparison procedures (Dunn’s method) for g, h and Kolmogorov–Smirnov test for i. All experiments were repeated ≥3 times. Also see Extended Data Fig. 3 and Supplementary Table 1.

Fusion site areas for spontaneous and evoked vesicle fusion tightly correlated with bouton areas measured by Syn1a (Fig. 2f), as expected. However, the slopes of the correlations differed, even though the bouton sizes were similar between groups (Extended Data Fig. 5b). In fact, evoked fusion site areas were significantly smaller (median smaller by 48%) and occurred over a significantly smaller proportion of the bouton (median smaller by 39%) than spontaneous fusion (Fig. 2g, Extended Data Fig. 5c, d, h–j).

One interpretation is that the concentration of vesicle priming proteins in nanoclusters favours evoked fusion in these subregions of the active zone. This predicts that pHuse events would be associated with higher local RIM1 density and conversely that high local density of RIM1 increases the probability of nearby fusion. To assess these predictions, we mapped vesicle fusion site relations to Eos3-tagged RIM1 using sequential PALM-pHuse imaging on the same live boutons (Fig. 2h, Extended Data Fig. 6d, e). As a local density metric for RIM1, we applied Voronoi tessellation and measured the first-rank density (δ1) for each RIM1–mEos3 localization (as described in ref. 15).
Figure 3 | Trans-synaptic nanoscale alignment of active zone and PSD proteins. a, Distributions of synaptic RIM1/2 and PSD-95 pair as the original localizations (left) and with nanoclusters highlighted (right). Scale bar, 200 nm. Filled arrows indicate aligned nanoclusters, open arrows denote non-aligned nanoclusters. b, Paired correlation function (PCF) of measured RIM1/2 and PSD-95 compared to PCF with either distribution randomized. c, PCF of simulated distributions with (cyan) and without (orange) shuffling nanocluster positions. d, Cumulative distributions of cross-correlation index (n = 143 synapses). e, RIM1/2 protein enrichment as a function of distance from translated PSD-95 nanoclusters (top, filled points) and PSD-95 enrichment relative to RIM1/2 nanoclusters (bottom, open points). Simulations with some randomizations as in d, e were performed for each synapse. f, Protein density profile for enriched versus non-enriched nanoclusters, n = 119 PSD-95 nanoclusters, 90 RIM1/2 nanoclusters. g, Enrichment indices for RIM1/2, Munc13, and Bsn relative to PSD-95 nanoclusters (filled) and for the opposite direction (open), n > 260 nanoclusters, ***P < 0.001, ANOVA on ranks with Dunn’s method. h, GluA2 enrichment with respect to RIM1/2 nanoclusters, n = 36 synapses. Scale bar, 100 nm. All experiments were repeated ≥3 times. Also see Extended Data Fig. 6 and Supplementary Table 2.

Thus, nanodistribution of RIM predicts the local probability of evoked fusion.

For the synapse as a whole, the impact of presynaptic nanoscale organization and confined vesicle sites (Figs 1 and 2) will depend strongly on whether these RIM nanoclusters align with postsynaptic receptor nanoclusters. To assess this, we compared the distribution of PSD-95 over the face of individual synapses to the corresponding distributions of RIM1/2, as the PSD-95 nanoclusters concentrate higher density of receptors. An example synapse, presented in Fig. 3a (Supplementary Video 1), shows three RIM1/2 nanoclusters and three PSD-95 nanoclusters that appear well-aligned and one pair not aligned. We used two independent approaches to assess the relationship between active zone and postsynaptic density (PSD) protein distributions. First, we adapted a paired cross-correlation function (PCF) to measure the spatial relationship between the two distributions (see Methods). The measured active zone–PSD distributions showed a significantly elevated PCF compared to simulated active zone–PSD distributions with either distribution fully randomized (Fig. 3b). We then tested the contribution of nanocluster positions to this elevated PCF (Fig. 3c). Randomizing nanocluster positions and out-of-nanocluster molecules (keeping localizations within nanocluster borders intact) abolished the PCF to chance level, while randomizing just the out-of-nanocluster molecules only modestly reduced the PCF, indicating that the precise positioning of the nanoclusters themselves dominate the overall correlation of protein distributions (Fig. 3c, d).

Second, we reasoned that if synapses were trans-synaptically aligned on the nanoscale level, the protein distribution on one side of the synapse would predict protein density in the opposing neuron. To test this, we measured RIM1/2 localization densities as a function of radial distance from the centres of PSD-95 nanoclusters as translated across the synaptic cleft (Fig. 3e). RIM1/2 localization densities within a 60 nm radius were significantly higher than the synaptic cluster average, decaying e-fold per 43.2 ± 12.1 nm away from the peak. This enrichment was again principally dependent on the relative positioning of nanoclusters within synaptic clusters (Fig. 3e). For each individual nanocluster, we defined an enrichment index as the average molecular density of the opposed protein within a 60 nm radius from the nanocluster centre (Extended Data Fig. 7a). Nanoclusters with enrichment indices significantly greater than that of the fully randomized distribution were considered enriched (Fig. 3f). We found 44.4 ± 3.0% of PSD-95 nanoclusters to be enriched (Extended Data Fig. 7b), and these nanoclusters were opposed to RIM1/2 molecule densities that were 2.0 ± 0.1 times the average RIM1/2 synaptic cluster density (Fig. 3f). A similar PSD-95 protein enrichment profile was found relative to the centres of RIM1/2 nanoclusters (Fig. 3e). Thus, this detailed metric for assessing nanoscale alignment revealed strong co-enrichment of these key proteins along narrow, transcellular columns. In comparison to RIM1/2, the enrichment of Munc13 with respect to PSD-95 nanoclusters was considerably weaker, and Bsn intermediate (Fig. 3d, g, Extended Data Fig. 7c–e, Supplementary Table 2). Together, both the PCFs and protein enrichment analyses revealed significant trans-synaptic alignment between RIM1/2 and PSD-95 distributions, largely stemming from the correlated positions of their respective nanoclusters. We likewise found quantitatively similar number, characteristics, and alignment of pre- and postsynaptic nanoclusters in acute hippocampal slices from adult rats (Extended Data Fig. 7f–h).

To determine whether evoked release aligns with postsynaptic receptors, we compared distributions of GluA2-containing AMPARs with RIM1/2 (Fig. 3h). Similar to PSD-95, GluA2 was significantly enriched relative to RIM1/2 nanoclusters, decaying e-fold per 66.9 ± 15.4 nm. This was further confirmed with a different GluA2/3 antibody (Supplementary Table 2). Importantly, given that the probability of AMPAR activation declines with distance from glutamate release sites has previously been deduced, we can predict synaptic potency by using the observed RIM1/2 and receptor distributions. To estimate the physiological impact of this trans-synaptic alignment, we calculated receptor activation in a measured synapse versus randomized distributions. Consistent with effect sizes posited by previous models, the measured distribution with trans-synaptic alignment gained 21.8 ± 0.5% in synaptic strength compared to a uniform distribution of active zone and PSD proteins (Extended Data Fig. 8), suggesting this synaptic architecture facilitates higher single-vesicle response potency. For comparison, long-term depression induces a very similar magnitude decrease in synaptic strength.

Notably, we found that trans-synaptic molecular alignment may extend deeper into the postsynaptic cell, as postsynaptic scaffold molecules farther from the plasma membrane also colocalized with PSD-95 nanoclusters (Extended Data Fig. 9a, c), and RIM1/2 was correspondingly enriched with respect to Shank nanoclusters (Extended Data Fig. 9b). 3D-OMETR imaging of RIM1/2, PSD-95, and GAKAP1 at the same synapses further confirmed their mutual co-enrichment (Extended Data Fig. 9d–f). Altogether, these results revealed an
all reduced (Fig. 4d–f, Supplementary Table 3). These effects were long-lasting, and during the subsequent 25 min, most parameters underwent only partial recovery. In contrast, presynaptic nanostructure underwent a remarkably different pattern of reorganization that was detectable only in relation to PSD-95 nanoclusters. Unlike PSD-95, RIM1/2 distributions were not affected immediately following the stimulus (Fig. 4d–f). However, following the 25-min recovery, the enrichment index of RIM1/2 with respect to PSD-95 nanoclusters increased with a corresponding increase in the percentage of enriched PSD-95 nanoclusters (Fig. 4g, h). Remarkably, while RIM1/2 nanoclusters altogether remained constant in number and enriched percentage, there was in fact an increase in the size of those RIM1/2 nanoclusters that were enriched with PSD-95, whereas the other non-enriched RIM1/2 nanoclusters remained constant (Fig. 4i). Similar results were found when we studied NMDA-induced changes on RIM1/2 and GluA2/3 alignment (Extended Data Fig. 10a–h). Note that on a traditional microscopic level, these changes to presynaptic organization were essentially undetectable: RIM1/2 staining revealed no change in synaptic cluster size or intensity at any point. Because the delayed presynaptic modification was specific to aligned nanoclusters, it may be that nanocolumns point to an alignment-specific, retrograde presynaptic compensation following postsynaptic depression (Fig. 4j), potentially relating to previous reports of presynaptic homeostatic plasticity21.

Overall, the gradients of protein density we observed suggest a nanocolumn model, in which active zone regions with the highest likelihood of release are aligned to the densest receptor areas, optimizing the potency of neurotransmission (Supplementary Video 2). This provides a simple organizational principle that may hold for many small, central nervous system synapses, and will have the largest influence at synapses that typically release only one vesicle following an action potential. The compartmentalized active zone architecture is reminiscent of protein organization in Drosophila neuromuscular junction23 and vertebrate ribbon synapses, where vesicles and priming proteins are arrayed around tight clusters of Ca2+ channels. However, observations in small central nervous system synapses of both clustered24,25 and random distributions of Ca2+ channels26, and emerging evidence for channel mobility as an equalizer of P, for vesicles independent of channel positioning27, suggest that their precise distribution may not be the sole determinant of the active zone release likelihood landscape.

The alignment of pre and postsynaptic nanocolumn subdomains4,6 suggests that even small synapses may be composed of dynamic functional modules28,29. We hypothesize that the nanocolumn represents an especially sensitive point whereby disease-associated pathways, frequently known to alter synaptic plasticity1,2, may disrupt synapse function. It will be important to identify which, if any, of the numerous cleft-spanning adhesion systems30 or retrograde signalling mechanisms mediate release-receptor alignment and permit dynamic trans-synaptic realignment.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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METHODS

All experimental protocols were approved by the University of Maryland, Baltimore School of Medicine Institutional Animal Care and Use Committee. Dissociated hippocampal neurons from E18 SD rats of both sexes were prepared as described previously\(^3\). To increase the experiment efficiency, for three-colour STORM experiments we used the ‘sandwich’ cultures with a supporting astroglial monolayer as described previously\(^3\) in which most neuronal structures were in the same focal plane. All experiments were performed on neurons 14–21 DIV and repeated in three more separate cultures unless otherwise specified.

**Immunostaining.** Cells were fixed with 4% paraformaldehyde (PFA) and 4% sucrose in PBS (pH 7.4) for 10 min at room temperature (RT), followed by washing with 50 mM glycine in PBS. Cells were then permeabilized and blocked using 3% BSA or 5–10% donkey or goat serum in PBS with 0.1% Triton X-100, followed by incubation with primary antibody (3 h RT or 4°C overnight) and secondary antibodies (1 h RT).

For comparisons of Munc13 or RIM1/2 with Bsn made using 3D-STORM, mouse anti-Bsn (1:500, Enzo) was used with either rabbit anti-RIM1/2 (1:500; Synaptic Systems No. 140203) or rabbit anti-Munc13 (1:500; Synaptic Systems No. 126103). Cy3 or Alexa-647 conjugated goat or donkey anti-rabbit or anti-mouse secondary antibodies (1:200 in PBS; JacksonImmuno) were used\(^3\). For comparisons of Munc13 and RIM1/2, staining was performed sequentially separated by additional blocking steps of incubation with rabbit serum at RT for 30 min followed by incubation with excess unconjugated anti-rabbit Fab antibody for 1 h at RT. For this set of experiments, all permutations of the order in which the primary antibody was applied and the fluorophore used to label each protein were included. For trans-synaptic measurements, rabbit anti-Munc13, anti-RIM1/2, anti-RIM1 (1:500; Synaptic Systems No.140003) or anti-Bsn (1:500; Cell Signaling), were used with mouse anti-PSD-95 (1:200; Neuromab), mouse anti-GluA2 (1:100, Millipore), or rabbit anti-GluA2 (1:100, Millipore). Unless specified otherwise, presynaptic proteins were labelled with donkey anti-rabbit IgG conjugated with Alexa-647 and postsynaptic PSD-95 were labelled with donkey anti-mouse IgG conjugated with Cy3. For comparison of directly labelled primary antibody with primary–secondary antibody labelling, we directly conjugated Alexa-647 dye to anti-PSD-95 antibody and purified antibody using illustra NAP Columns (GE Healthcare). For comparison of nanobody labelling of expressed GFP-tagged knockdown-posed pair of synaptic proteins in a 2D scatter plot of all accepted localizations from both channels. By rotating a 3D scatter plot of localizations of a selected potential synapse, we evaluated the data quality and selected only those with clear pre- and postsynaptic components (for example, no nearby third cluster which may indicate two synapses in close proximity) for further analysis. To define the border of a synaptic cluster, the nearest neighbour distances (NND) between localizations were calculated and the mean ± 2 s.d. of NND was used as a cut-off to divide the localizations into sub-clusters. All localizations outside of the primary sub-clusters were considered to be background and discarded.

**Imaging of GKAP or Shank (1:200, Neuromab) was performed as previously described.**

**Single-molecule localization and analysis.** All data analysis was performed offline using custom routines in MATLAB (Mathworks). Molecule locations were determined by fitting an elliptical 2D Gaussian function to an 11 × 11 pixel array (pixel size 100 nm) surrounding the peak\(^4\). The lateral (x, y) and axial (z) coordinates of the fluorophore were determined from the centroid position and ellipticity of the fitted peak, respectively\(^4\). Only molecules localized with an x–y precision <10 nm (ref. 37), fitting R² > 0.6, and comprising >200 photons were used for further analysis.

**Analysis of synaptic clusters.** A potential synapse could be identified by a juxtaposed pair of synaptic proteins in a 2D scatter plot of all accepted localizations from both channels. By rotating a 3D scatter plot of localizations of a selected potential synaptic, we evaluated the data quality and selected only those with clear pre- and postsynaptic components (for example, no nearby third cluster which may indicate two synapses in close proximity) for further analysis. To define the border of a synaptic cluster, the nearest neighbour distances (NND) between localizations were calculated and the mean ± 2 s.d. of NND was used as a cut-off to divide the localizations into sub-clusters. All localizations outside of the primary sub-clusters were considered to be background and discarded.

**Owing to the irregularly curved shapes of some synapses, using the convex hull to define synaptic cluster shape would overestimate the synaptic cluster volume.** Thus, we thus defined the synaptic cluster using the alpha shape of the set of 3D localizations with α = 150 nm. This value was determined based on series of tests on >100 synapses to obtain the best synaptic cluster shape while avoiding dramatic changes in volume when individual localizations near the border were added or removed. This alpha shape algorithm gave a synaptic cluster volume of 81 ± 3% of the convex hull volume (n = 156 synapses). Subsequently, this alpha shape was used as the cluster border when localizations were randomized.

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with different densities. Consequently, they may have prevented detection of small or weakly enriched nanoclusters. In principal, we cannot completely exclude the possibility of overcounting, so a certain fraction of detected nanoclusters are potentially artificial. However, we used the same standard on all data sets. Since all the trans-synaptic analyses were well controlled by randomizing simulations, this contamination is not able to produce false positives for trans-synaptic alignment analyses. On the contrary, it may attenuate the significance of the differences in trans-synaptic analyses based on nanoclusters, including cross-correlation, protein enrichment and the fraction of enriched nanoclusters.

Since the number of localizations in one nanocluster was typically small, using convex hull or alpha shape would greatly under-estimate the nanocluster volume due to the border effect. Therefore, we tessellated the synaptic cluster with polyhedrons using MATLAB function voronoi(), with each Voronoi cell containing one localization. The nanocluster volume was calculated as a summation of volumes of all polyhedrons containing the nanocluster localizations. To avoid unexpected unbounded Voronoi cells and over-estimating the volume of cells near the cluster surface, we introduced ~10% background noise by adding randomly distributed localizations around the cluster15. Polyhedron volume for each localization was averaged over about ten independent simulations.

ACF analysis. To quantify the self-clustering of synaptic proteins, we adapted an autocorrelation function49,50 for our 3D data. The autocorrelation function $g(r)$ is a measure of density correlations, which reports increased probability of finding a second localized signal a distance $r$ away from a given localized signal. It was tabulated in MATLAB using Fast Fourier Transforms (FFTs), as in equation (1).

$$FFT^{-1}[FFT(I)FFT(W)] = \rho FFT^{-1}[FFT(I)FFT(W)]$$

(1)

FFT$^{-1}$ is an inverse Fast Fourier Transform, I is the reconstructed 3D density matrix of localized fluorophores (pixel size of 5 nm), $\rho$ is the general localization density inside the synaptic cluster, and W is a shape function that has the value of 1 inside the synaptic cluster as defined above with an alpha shape and the value of 0 elsewhere. The matrix I was padded with zeros in all three directions out to a distance larger than the range of the desired correlation function (we used 200 nm) to avoid artefacts due to the periodic nature of FFT functions. W was also padded by an equal number of zeros. FFT$^{-1}$[FFT(W)] is a normalization factor accounting for the general shape of the synaptic cluster itself so that the output of the $g(r)$ represented only the internal structure of the measured synaptic cluster. $g(r)$ was symmetric to rotations around the centre of matrix C ($x_c, y_c, z_c$), and it could be averaged over angles to obtain $g_r(r)$ by converting to polar coordinates. $g_r(r)$ was then binned by radius ($r$). Correlation functions were plotted for $r > 0$, as $g_r(r = 0)$ was a trivial contribution.

For a uniform distribution, for example, when all localizations were uniformly randomized within the alpha shape, $g_r(r) = 1$ (Fig. 1d). Any heterogeneity will result in a $g_r(r) > 1$. The extent of $g_r(r)$ over 1, that is, $n_r$ for $g_r(n_r) = 1$, is related to the pattern size of the internal heterogeneity (Extended Data Fig. 2b, c)29.

Isolated, non-synaptic small groups of localizations were taken from our experimental data. These localization groups were placed in an evergreen solution representing an overestimate of a single-dye-molecule localization spread. Nevertheless, we find that they are still significantly smaller than the large majority of the nanoclusters we detected.

Imaging vesicle exocytosis. For imaging vesicle fusion, vGLUT-T-phluorom-cherry (a gift from T. Ryan)40,41, was cotransfected with Syn1a–CFP (a gift from T. Ryan)40,41. For imaging vesicle exocytosis, vGluT-pHluorin was expressed in Cos-7 cells, and pHluorin was excited by passing 1 ms current pulses yielding fields of ~100 cells at 14–20 DIV.

Photon count distributions analysis. Data analysis was performed offline using custom routines in MATLAB (Mathworks). Boundaries for individual boutons were determined using wide-field images of Syn1a–CFP centred at the focal plane of the pFUSE experiments thresholded at 50% of the peak intensity (33% and 67% thresholds were also compared and showed no significant difference on the effect of threshold of release, shown in Extended Data Fig. 5i). Binary images were created from the thresholded image, and Syn1a–CFP puncta area calculated as a measure of bouton area, which correlated with pFUSE area, as expected20. Images for each fusion event were processed using frame-by-frame subtraction followed by background subtraction to isolate fluorescence increases (Fig. 2d). Similar detection thresholds were set for spontaneous (75 ± 15) and evoked (78 ± 14, $r = 0.88$, $P = 0.40$) release, at ~3–4 times above background noise, on an individual imaging field basis. Spatial localization of the fusion events was determined by fitting an elliptical 2-dimensional Gaussian function to a 9 × 9 pixel array surrounding the peak. Only molecules localized with a precision <25 nm27,44, elliptical form $<1.3$, and comprising >100 photons were used for further analysis. An additional criterion to exclude evoked pFUSE localizations with photon counts > mean ± 2 s.d. of spontaneous photon count distribution was used in Extended Data Fig. 5d and showed no significant difference compared to the distribution lacking this criterion (Fig. 1e). Localizations from multiple fusion events over time at individual boutons were mapped. A 2D convex hull algorithm was used to calculate the minimal convex polygon that incorporated all fusion site localization points. The area of the resulting polygon was used as the fusion site (pHUSE) area.

Calcium imaging and analysis. For Ca$^{2+}$ imaging, the genetically encoded indicator GCaMP6f (ref. 45) was transfected at 14 DIV and imaged 3 days after transfection. GCaMP6f was used to detect postsynaptic miniature spontaneous Ca$^{2+}$ transients (mSCaTs) that arose in dendritic spines following NMDA receptor activation by spontaneous release46. Covariance localizations were placed in custom-made chambers in saline solution containing 1μM TTX, 10μM DNQX, 25μM picrotoxin (Sigma), and 5μM nifedipine (Sigma). Imaging was performed on a spinning disk confocal system (Andor Technology), consisting of a CSU-22 confocal (Yokagawa) with a Zyla 4.2 CCD camera detector (Andor) mounted on the side port of an Olympus IX-81 inverted microscope, using a 60 × 1.42 oil-immersion objective, yielding a final effective pixel size of 108 nm. Continuous acquisition at 20 Hz was collected for 3 min, controlled by iQ software (Andor).

Data analysis was performed offline using custom routines in Neurobeachin (Molecular Devices), Clampex (Molecular Devices), and Matlab (Mathworks). First, using Metamorph, a baseline image was created by averaging the first three and last three image frames and a maximum intensity projection was made by averaging all image frames. Image subtraction of the baseline from the maximum intensity projection revealed spines that showed an increase in GCaMP intensity. Regions of interest (ROIs) were drawn around these ‘active’ spines as well as tetrodotoxin (TTX; Enzo) was added after identifying terminals using AP-evoked fluorescence increase.

For calculating normalized changes in fluorescence ($\Delta F/F$), images were analysed in ImageJ by custom-written plugins21. Average fluorescence intensities were measured using a circular region of interest (ROI) of radius 800 nm for each bouton. Change in fluorescence ($\Delta F$) was calculated as the difference in intensity of the frame after the stimulus was delivered and the average ROI intensity of 5 baseline frames not including the first frame or the frame immediately before the stimulus ($F_{baseline}$). $\Delta F/F$ was calculated using equation (2).

$$\Delta F/F = \frac{F_{pFUSE} - F_{baseline}}{F_{baseline}}$$

Here $p$ was constrained between 0 and 1, and $a$ had a lower bound of 0. This mixture probability defined the lower estimate (72%) for the percentage of single stimulus-evoked fusion arising from single vesicles. We calculated the higher estimate (82%) by calculating the percentage of evoked fusion events with photon counts > mean ± 2 s.d. of spontaneous fusion photon counts distribution. To assess the influence of multivesicular events on evoked pHUSE area, we used this as a cut-off to exclude localizations above this photon count. We found no significant difference between evoked area with and without excluding these events (Extended Data Fig. 5d).

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as a background region and then transferred to the original timescale. For each ROI the averaged intensity was measured per frame. The average intensity of the background ROI was subtracted from the average intensity of 'active' spine ROIs.

From this, an average fluorescence intensity was calculated for every 10 frames, and within every minute interval of imaging the lowest positive value was used as the baseline fluorescence intensity for that minute ($F_{baseline,1 min}$). A normalized change in fluorescence ($\Delta F/F$) was calculated for each frame as $(F_{frame} - F_{baseline})/F_{baseline,1 min}$. The $\Delta F/F$ values were then fed into Clamplex, and mSCATs were detected using a template search that identified peaks based on a shape profile determined from mSCAT examples with near-average rise and decay time courses.

**Conical imaging of presynaptic proteins.** Neurons 14–20 DIV were cotransfected for 3 days with RIM1-mVenus (a gift from P. Kaeser) and Syn1a–CFP to assess colocalization. Neurons transfected with only RIM1-mVenus were immunostained with chicken anti-GFP (1:200, Chemicon) labelled with secondary anti-chicken-Alexa-488, rabbit anti-RIM1/2 labelled with secondary anti-rabbit-Cy3, and mouse anti-Bsn labelled with secondary anti-mouse-Alexa-647 to assess expression levels. Imaging was performed on a spinning disk confocal system as described above. Image was used to analyse fluorescence intensity of RIM1/2 and Bsn at transfected compared to neighbouring untransfected boutons.

**PALM-pHluse.** RIM1-mEos3.1 was constructed by subcloning mEos3.1 from mEos3.1-N1 (a gift from S. McKinney) into pCMV5-RIM1-mVenus (P. Kaeser) in place of mVenus at NotI-AscI. PALM was performed on RIM1, and nanoclusters identified using local density measured by nearest neighbour distance as previously described, or using an adapted form of SR-Tesseler first rank neighbour density ($b^2$), using $\gamma \times \kappa$ of the whole synapse as the threshold for identifying nanoclusters, as described in ref. 15. Nanoclusters identified by both methods were similar in size (Extended Data Fig. 6). To map vesicle fusion to active zone nanostuctures, RIM1-mEos3.1 was cotransfected with vGph at 10–14 DIV and imaged at 14 DIV. After PALM, the two matrixes were convolved with an 11 kernel (Extended Data Fig. 1g). To avoid having the correlation be dominated by local domains with high localization density, $\rho_1/4$ and $\rho_2/4$ (Extended Data Fig. 1h) so that $G = FFT^{-1}$$\left(FFT(I_1) \times \text{conj}[FFT(I_2)]\right)$ with equation (4).

$$g(r) = \frac{Re\left(FFT^{-1}\left(FFT(I_1) \times \text{conj}[FFT(I_2)]\right)\right)}{\rho_1 \rho_2 FFT^{-1}\left(FFT(W_1) \times \text{conj}[FFT(W_2)]\right)}$$

(3)

$$G = FFT^{-1}$$\left(FFT(I_1) \times \text{conj}[FFT(I_2)]\right)$$

(4)

A is the point with the peak $G$ value. Because the originally constructed matrices $I_1$ and $I_2$ were not continuous, to reduce the noise of the correlation, we first convoluted the two matrixes with an 11 $\times$ 11 kernel (Extended Data Fig. 1g). To avoid having the correlation be dominated by local domains with high localization density, we cut the peaks of the convoluted matrixes to 1/4 of the mean localization density within synaptic clusters ($\rho_1/4$ and $\rho_2/4$) (Extended Data Fig. 1h) so that $G$ only represented the relationship between the general 3D shapes of the two synaptic clusters ($G, I_2')$ without internal heterogeneity (Extended Data Fig. 1m, n). Around A, $g(r)$ is symmetric and could be angularly averaged to get $g(r)$.

Since the information of synaptic cluster shape and overall density had been normalized, $g(r)$ was fully dependent on the internal organizations of the two synaptic clusters. If localization assemblies inside the two synaptic clusters organized in a similar pattern and opposed each other, $g(r) > 1$. If either synaptic cluster had a uniform distribution of localizations (Fig. 3b) or the internal assemblies were not aligned (Fig. 3c), $g(r) = 1$. Different from the ACF, overcounting has no effect on the PCF.

**Protein enrichment analysis.** The protein enrichment profile of protein A relative to a protein-B nanocluster, $E_{A-B}(r)$, was calculated as the angularly averaged localization density of protein A around the aligned centre of a protein-B nanocluster normalized to the average localization density in synaptic cluster A. The aligned nanocluster centre was found as shown in Extended Data Fig. 1. To avoid potential problems caused by experiment conditions, we calculated the enrichment profile as equation (5).

$$E_{A-B}(r) = \frac{N_{A-B}(m)}{N_{A-B}(m) \times m}$$

(5)

$N_{A-B}(m)$ is the binned distribution of protein-A localization number to the aligned protein-B nanocluster centre. $N_{A-B}(m)$ is the distribution of localization number for a uniformly randomized synaptic cluster $A$ with $m$ times of original localization density, and $m$ is a factor set to 15 to reduce the effect of fluctuations. A protein-B nanocluster was considered to be significantly enriched with protein A if $E_{A-B}(r) > mean[E_{A-B}(r)] + 1.96 \times \text{standard deviation}[E_{A-B}(r)]$, where $E_{A-B}(r)$ represents the enrichment profile of ten simulated uniformly randomized A synaptic clusters with the original density and the same alignment to the nanocluster centre of protein-B.

**Chemical LTP and LTD.** Chemical LTP was performed using a combination of 4AP withdrawal and application of glycine as described in ref. 19. Briefly, 3–4-week-old cultures were treated with 200$\mu$M N-AP5 in culture medium for two days and then transferred to ACSF (150 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES-Na, 10 d-glucose, all in mM, pH 7.4) with 100$\mu$M picrotoxin, 1$\mu$M strychnine, 0.5$\mu$M TTX and 200$\mu$M AP5. After preincubation for 1–2 h, chemical LTP was induced with 15 min incubation in the similar solution with 200$\mu$M glycine but without Mg$^{2+}$ and AP5. Neurons were fixed directly following induction. Chemical LTD was performed using application of NMDA as described in ref. 20. Control solutions of regular saline solution or co-application with AP5 were paired with experimental conditions. Cells were fixed either immediately after plasticity induction or washed with saline and incubated for 25 min at 37$^\circ$C to allow recovery before fixing. Cells were then immunostained and imaged as described above.

**Synaptic modelling.** We used an experimentally constrained deterministic approach to study the dependence of synaptic strength on the spatial distribution of release sites and AMPARs. Central to this approach is the relationship between channel opening probability and its distance from a release site, determined previously by stochastic modelling approaches:

$$P_B(r) = 0.42 e^{-r/88}$$

(6)

where $r$ is the lateral distance between an AMPAR and a release site (in nm). In brief, the distribution of RIM1/2 proteins and GluA2/3-containing AMPA receptors measured by STORM were used to determine the spatial coordinates of release sites and AMPARs on a model synapse. Since the precise photophysics and blink distribution of dyes are complicated and the exact efficiency of antibody labelling is unknown, we calculated gradient maps of spatial coordinates to determine putative RIM1/2 protein and AMPAR locations from the single-molecule images. First, the 3D spatial coordinates were projected onto 2D planes orthogonal to the manually determined axodendritic axis. Each projected point was assigned a Gaussian function, the amplitude and width of which were determined by the normalized local density and the lateral STORM localization precision (20 nm). Overlapping Gaussian functions within the active zone or PSD convex hull were integrated to create the pre- and postsynaptic gradient maps. The sampling pixel size was 2.5 nm (the calculated synaptic response was independent of pixilation level for sampling size from 1 to 20 nm, data not shown). The pre- and postsynaptic gradient maps were separated by 20 nm, the cleft distance used to determine equation (6).

The model synaptic response for a single synapse was computed as the expected fraction of receptors that would open given a single release, averaged over all possible release locations in the active zone. For any single release event, the expected open fraction of channels at the peak of the response was calculated as follows:

$$O(i) = \frac{\sum_j P_B(r_j) \cdot LD_j}{\sum_j LD_j}$$

(7)

where $r_j$ is the lateral distance between the $i$th pixel in the presynaptic gradient map and the $j$th pixel in the postsynaptic gradient map; the expected fraction of open channels $O(i)$ from the $i$th release site is sum of channel opening probabilities at all pixels in the postsynaptic gradient map, where each $i$th pixel is weighted by
its normalized local density LD, (that is, the channel fraction is assumed to be directly events in the active zone, we used the live-cell pHuse-PALM data, which showed that release events preferentially occurred in regions with normalized RIM local density greater than 1.5, and these events occurred over 20–60% of the active zone area (spontaneous pHuse area/PALMed RIM area, and evoked pHuse area/spontaneous pHuse area). To account for these measured features, we modelled the spatial likelihood of release as a piecewise sigmoidal function dependent on the normalized local RIM density:

$$ P_i(\text{release}) = \begin{cases} 
0.5 + 0.5 \left( \frac{1 - s}{LD_{\text{max}} - LD_{\text{infect}}} \right) & \text{if } LD_i \in [LD_{\text{infect}}, LD_{\text{max}}] \\
0.5 \left( \frac{1 - s}{LD_{\text{max}} - LD_{\text{infect}}} \right) & \text{if } LD_i \in [0, LD_{\text{infect}}] 
\end{cases} $$

(8)

where $s$ is the steepness of the sigmoid transition, LD is the normalized local density of RIM at the th pixel of the presynaptic gradient map, LD is the point of inflection in the sigmoidal function, and LD is the maximum normalized local density of RIM in the STORM measured example shown in Extended Data Fig. 8b. LD and $s$ were fitted to be 1.5 and 0.959 in order to yield a fractional release area of 40%. To calculate the average peak synaptic response per release, we calculated the expected open channel fraction averaged over all possible release sites weighted by the spatial probabilities of release:

Open channels at peak response (%) = $\sum_i P_i(\text{release})$

Code availability. All code used in the paper is available upon request.

Statistical analysis. No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment. Statistical tests were performed with Sigmapstat, MATLAB, Graphpad, or R. No statistical methods were used to predetermine sample size. The sample sizes were determined based on numbers reported in previous studies. For comparison of two or more distributions, all samples were assessed for normality using Shapiro–Wilk or Kolmogorov–Smirnov tests. If samples met criteria for normality, we used a Student’s t-test for comparison of the same group before and after a treatment, or ANOVA for more than two groups. If ANOVAs were significant, we used a post hoc Tukey test to compare between groups. For groups with combinations of discrete and continuous variables, we used MANCOVAs. We only performed two-tailed tests. Homogeneity of variances was tested using MANCOVAs. If ANOVAs were significant, we used a post hoc Tukey test to compare between groups. For groups after a treatment, or ANOVA for more than two groups. If ANOVAs were significant, we used a Student’s t-test and found to be similar between compared groups. If samples did not show that release events preferentially occurred in regions with normalized RIM local density greater than 1.5, and these events occurred over 20–60% of the active zone area (spontaneous pHuse area/PALMed RIM area, and evoked pHuse area/spontaneous pHuse area). To account for these measured features, we modelled the spatial likelihood of release as a piecewise sigmoidal function dependent on the normalized local RIM density:

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Extended Data Figure 1 | Filtering of localizations and automatic algorithm to detect the synaptic axis. a, Scatter plot of fitted peak width in y ($W_y$) against that in x ($W_x$). The colour codes the position in z. All localizations away from this centre dense region arise from multiple overlapping or poorly fitted peaks and should be rejected. b, The ellipticity ($W_x/W_y$) and the width difference ($W_x - W_y$) formed an approximate linear relationship when $W_x > W_y$ (dotted box). c, We fitted the ratios between ellipticity and the width difference to the denominators with third degree polynomial functions (black line) and rejected all localizations out of 95% confidence intervals (grey lines). The same criteria was applied to the other fraction of localizations with $W_x < W_y$. d, The same scatter plot as in a after rejection of all of the diffuse localizations (about 20–25%). e, f, The filtering protocol cleared up most of the localizations from multiple overlapping peaks or poorly fitted peaks, including most of the non-relevant background localizations (e) and those localizations with poorly calibrated z positions (f). Scale bars, 2 μm (e) and 200 nm (f). The synapse in f corresponds to the boxed synapse in e. g, A 2D section through the centre of the convoluted constructed 3D distribution matrix of a synapse. h, Peak density of the matrix set to a quarter of the mean molecule density of the synaptic cluster. i, 2D section at the same position of the 3D matrix of direct cross-correlation of the two channels (equation (3) in Methods). C is the centre of matrix, and A is the peak of the cross-correlation. j–k, Best overlap of the two synaptic clusters after PSD-95 was moved in 3D space along the vector CA. l, 3D scatter plots of the synapse in two different view angles. The arrow denotes the vector and the extended line (dotted) represents the synaptic axis. m, 3D plot of detected synaptic axis when the positions of high-density peaks in RIM1/2 (nanoclusters) were randomized within the synaptic cluster. This simulation was performed 35 times, but only 10 representative results are presented here to avoid overlapping. The red denotes the synaptic axis of the original synaptic cluster. n, Averaged distance between the detected C positions from 35 simulated clusters to the C position of the original cluster. Data shown in mean ± s.d. This <6 nm distance confirms that the high-density peaks have negligible effect on the detection of the synaptic axis in this Method. o, Distribution of all localizations along the synaptic axis with bin size of 10 nm. Peak-to-peak distance between the synaptic protein pair can be measured from this distribution. p–r, Distribution of peak-to-peak distances for three pairs of synaptic proteins.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Nanocluster organization of vesicle release machinery proteins in the active zone and postsynaptic AMPA receptors. a, *En face* (top) and side (bottom) views of local density maps of a simulated synapse with artificial nanoclusters with 40-nm diameters. Scale bar, 100 nm. b, Autocorrelation function of simulated clusters with different sized nanoclusters. The points represent the radius where \( g(r) = 1 \). c, Pooled data from 15 sets of simulations showing that the radius where \( g(r) \) first crosses 1 reasonably estimates the average nanocluster diameters. d, Comparison of nanocluster number, fraction of localization in nanocluster, and nanocluster volume across different developmental stages shows no significant difference, though the young 9 days *in vitro* (DIV) culture shows a trend towards increased nanocluster numbers (one-way ANOVA on ranks for nanocluster number and volume, one-way ANOVA for percentage localization in nanocluster). Data were from 143 RIM nanoclusters and 135 PSD nanoclusters of 64 DIV 9 synapses, 63 RIM nanoclusters and 65 PSD nanoclusters of 38 DIV 14 synapses, and 44 RIM nanoclusters and 41 PSD nanoclusters from 28 DIV 21 synapses. e, Comparison of two RIM antibodies (from left to right) in whole synaptic cluster volume, number of nanoclusters, autocorrelation function estimating average nanocluster diameter, and protein density relative to PSD-95 nanocluster centres. Anti-RIM1/2 (Synaptic Systems #140-203) targets the zinc-finger domain and anti-RIM1 targets the PDZ domain of RIM1 (Synaptic Systems #140-003). These tests suggest that there is no significant difference between these two antibodies. The numbers in bars denote the group sizes. f, Local density maps of *en face* (top) and side (bottom) views of an example Munc13 cluster. Scale bar, 200 nm. g, Autocorrelation functions for Munc13 distributions compared to simulated randomized distributions. h, i, Local density maps and ACF of Bsn cluster. Scale bar, 200 nm. j, Pooled cluster volumes, normalized to PSD-95 volumes within each synapse. Each bar pair represents data from a set of RIM1/2-PSD-95, Munc13-PSD-95 or Bsn-PSD-95 staining. The numbers in bars denote the group sizes. k, Distribution of *en face* distances between nanocluster centre and synapse centre. Data were normalized to the distribution of simulated clusters with the same number of nanoclusters as the original synapse but randomized positions. l, An example synapse with RIM1/2 and Munc13 staining of the same synapse, shown in two different angles. The translucent surfaces represent the alpha shapes that define the synaptic cluster borders. m, Pooled RIM1/2 and Munc13 cluster volumes, normalized to RIM1/2 within each synapse. n, Pooled RIM1/2, Munc13 and Bsn cluster volumes from staining of RIM1/2-Bsn and Munc13-Bsn, normalized to Bsn within each synapse. \( *P < 0.05; \ ***P < 0.001 \); Wilcoxon signed-rank test. † \( P < 0.05 \), one-way ANOVA on ranks with pairwise comparison procedures (Dunn’s method). o, Local density map of a GluA2 cluster. p, Autocorrelation functions for GluA2 distributions compared to simulated randomized distributions. q, Local density map of a GluR2/3 cluster. r, Autocorrelation functions for GluR2/3 distributions compared to simulated randomized distributions. All experiments were repeated \( \geq 3 \) times.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Detected nanoclusters are unlikely a result of labelling artefacts or overcounting of molecules. a–i. Comparison of PSD-95 labelled with monoclonal primary antibodies directly conjugated to Alexa647 dye (1°-A647, red) with the same molecules labelled with primary and secondary antibodies conjugated to Cy3 (1°-2°-Cy3, blue) as represented in c. a, b. Comparison between non-synaptic small groups of localizations arising from isolated primary antibodies and secondary antibodies. Schematic shown in a. Standard deviation of localizations in both groups along different dimensions (n = 32 for A647; n = 36 for Cy3) in b. The two types of localizations groups showed similar variation in all dimensions. d. Local density maps of the same PSD-95 cluster labelled with 1°-A647 (top) and 1°-2°-Cy3 (middle) and overlapped distribution of 1°-A647 and 2°-Cy3 with detected nanoclusters highlighted in darker colours (bottom). Scale bar, 200 nm. e. Autocorrelation of synaptic clusters labelled with 1°-A647 and 1°-2°-Cy3. f. Autocorrelation of isolated small groups of localizations of A647 and Cy3 dyes. g. Comparison of the radius at which the autocorrelation function crossed with the random level (g(r) = 1). There was no difference between PSD-95 clusters with different labelling methods, but the r(0) for isolated localization groups were significantly less than r(0) for PSD-95 clusters. **P < 0.01, t-test between the filled and open bars of the same colour. h, Nanoclusters detected in both channels displayed no difference in number, volume, or the fraction of nanoclusters enriched with localizations from the other channel. i, Protein enrichment of localizations detected in each channels with those in the other channel (n = 32 synapses). These results demonstrate that the nanoclusters we detected in our study were not due to aggregation of multiple secondary antibodies to the primary antibodies. j–r, Cells transfected with knockdown-rescue-PSD-95-GFP were labelled with nanobodies against GFP conjugated at a 1:1 ratio with Atto647 (Nb-At647, red) and primary/secondary antibodies against PSD-95 (1°-2°-Cy3, blue) as depicted in l. j, k, Comparison between non-synaptic small groups of localizations arising from isolated Nb-At647 and 1°-2°-Cy3 (as depicted in j, n = 26 and 28, respectively). k, The nanobodies showed a significant smaller size than antibodies. ***P < 0.001, two-way ANOVA, †P < 0.05, ††P < 0.01, pairwise comparison (Tukey test) between nanobodies and antibodies. m–r. Similar comparison as in d–i between PSD-95 clusters labelled with Nb-At647 and 1°-2°-Cy3 (n = 13 synapses). Scale bar, 200 nm. Overall, these results demonstrated that the nanoclusters we detected in our study were unlikely a result of artefacts of antibody binding and labelling. The difference between the size of the isolated localizations groups and PSD-95 clusters calculated by autocorrelation also argues against the possibility that the nanoclusters we detected were owing to repetitive switching of one or a few fluorophores. **P < 0.01, t-test between the filled and open bars of the same colour. s. An example synapse with nanoclusters highlighted before (upper) and after (lower) removal of localizations resulting from fluorophores lasting for multiple frames. Scale bar, 100 nm. t. Paired autocorrelation function of synaptic clusters with and without multiple-frame molecules. P = 0.77, n = 25 synapses for RIM1/2; P = 0.58, n = 25 synapses for PSD-95, two-way ANOVA with repeated measures. u. The tracking removed 13 ± 8% and 17 ± 9% of the localizations for RIM1/2 and PSD-95, respectively, but had no significant effects on autocorrelation function results, nanocluster numbers, or nanocluster volumes. ***P < 0.01; ***P < 0.001; NS, P > 0.05; Wilcoxon signed-rank test. All data were pooled from ≥3 replicas.
Extended Data Figure 4 | 1AP evoked release is [Ca^{2+}] dependent and mainly univesicular. a, Example of fluorescence signals at a single bouton over repeated trials of 1 action potential stimulation. b, Single event traces of vGpH fluorescence increase following 1 action potential stimuli in standard (2 mM) or heightened extracellular [Ca^{2+}] (4 mM). c, Comparison of distributions of fluorescence changes in 2 mM (n = 233/27) and 4 mM (n = 115/12) extracellular [Ca^{2+}], relative to noise distributions obtained from the baseline frames before stimulation. d, Comparison of noise-subtracted distributions of fluorescence changes in different [Ca^{2+}]. e, Processed images of vGpH fluorescence increase following 1 action potential stimuli from three trials ten trials apart. f, Automatic detection using pHuse of events shown in e, g. Summed projection of framewise and background subtracted vGpH fluorescence increases over 60 trials. h, pHuse localizations on Syn1a (white). i–l, Same as e–h for spontaneous events in TTX over 5 min. n given in synapses/experiments.
Extended Data Figure 5 | pHuse reveals differences between evoked and spontaneous fusion site areas. a, Comparison of spontaneous frequency measured presynaptically using vGpH ($n = 77/22$) and postsynaptically using GCaMP6f (ref. 45) ($n = 61/5$, $t = 1.02$, not significant). b, Average bouton areas across groups, $t = 0.87$, not significant. c, Cumulative distributions of fusion areas for spontaneous and evoked release (Kolmogorov–Smirnov test, $*D = 0.23$). d, Cumulative distributions of normalized fusion areas for 1 AP evoked fusion excluding events with photon counts $>$ mean $+ 2$ s.d. of spontaneous events ($n = 91/27$) compared to all evoked events ($n = 104/28$, Kolmogorov–Smirnov test, $D = 0.05$, not significant) and spontaneous events ($n = 77/22$, Kolmogorov–Smirnov test, $*D = 0.251$). e, f, Notably, while evoked $P_i$ was significantly positively correlated with Syn1a area, as reported previously, spontaneous event frequency showed no relationship with Syn1a area ($R = 0.30$, not significant). On the other hand, both spontaneous event frequency and evoked $P_i$ significantly positively correlated with pHuse area ($R = 0.64$, spontaneous $R = 0.60$). This suggests that pHuse area may be a better approximation for active zone area and the functional parameters of a synapse than bouton area. g, Normalized pHuse area as a function of cell age shows no significant correlation (evoked $R = 0.03$, not significant, spontaneous $R = 0.04$, not significant). e–g, $n_{\text{evoked}} = 104/28$, $n_{\text{spont}} = 77/22$. h, Normalized pHuse area was not significantly different at room temperature ($n_{\text{evoked}} = 51/10$, $n_{\text{spont}} = 32/7$) versus physiological temperature ($n_{\text{evoked}} = 35/9$, $n_{\text{spont}} = 34/4$) within modes of release but still significantly different between modes of release. i, Normalized pHuse area was not significantly different at different thresholds for Syn1a within modes of release but still significantly different between modes of release ($n = 51/10$). j, Both numbers of events and mode of release are significant factors for pHuse area, but they do not have a significant interaction. See Supplementary Tables for statistics, $n$ given in synapses/experiments, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. © 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 6 | RIM1-mEos3.1 PALM identifies nanoclusters. 

a, Neurons co-expressing RIM1-mVenus (a gift from P. Kaesar) and Syn1α–CFP colocalize to the same boutons. Right panels show enlargement of areas within the white boxes. Scale bars, 5 μm (left) and 1 μm (right). b. Neurons expressing RIM1-mVenus immunostained for RIM1/2 and Bsn. Arrowheads point to some colocalized active zones. Scale bar, 2 μm. c, Immunofluorescence intensity of transfected cells normalized to nearby untransfected cells show 3.74 ± 0.11-fold overexpression of RIM and 1.24 ± 0.03-fold increase in Bsn (n = 262 synapses/7 cells). d, Photon count distribution of RIM1-mEos3.1 (3997 localizations). e, Same boutons shown in Fig. 2 visualized using 5 × nearest neighbour density (NND) as a measure of local density. f–h, Cumulative distributions of PALMed RIM1 nanoclusters diameter, area, and number, respectfully, identified using adapted Tesseler analysis and 5 × NND analysis (n = 65/13). i, RIM1 localization density as a function of radial distance from pHuse localizations. (See Supplementary Tables for statistics.) j, Mean distance from pHuse localizations as a function of local density measured by 5 × NND (raw data ***R = 0.23, n = 26/13). k, Proportion of pHuse localizations within 40 nm of a RIM1 localization as a function of RIM1 local density measured by 5 × NND (***R = 0.35). n given in synapses/ experiments unless otherwise specified, ***P < 0.001.
Extended Data Figure 7 | Protein enrichment within nanocolumns.

a, Enrichment index between RIM1/2 and PSD-95. The left insets are replicas of Fig. 3e, and the enrichment index is defined as the average of the first three bins in the enrichment profile (boxed), that is, normalized localization density within 60 nm from the projection centre of a given nanocluster. Filled points show RIM1/2 relative to PSD-95 nanoclusters, open points show PSD-95 relative to RIM1/2 nanoclusters. Same randomizations as in Fig. 3e and depicted again in b. **P < 0.01; ***P < 0.001, one-way ANOVA on ranks with pairwise comparison procedures (Dunn's method).

b, The fraction of enriched nanoclusters is significantly above chance level, and is also dependent on the relative position of the two sets of nanoclusters.

c, d, Side and en face views of a synaptic Munc13 and PSD-95 pair and a synaptic Bassoon and PSD-95 pair with highlighted nanoclusters. Scale bar, 200 nm.

e, Filled points show active zone proteins relative to PSD-95 nanoclusters, open points show PSD-95 relative to active zone protein nanoclusters. **P < 0.01; ***P < 0.001, one-way ANOVA on ranks with pairwise comparison procedures (Dunn's method).

f, Example of RIM1/2 and PSD-95 in adult hippocampal slices.

g, h, Auto-correlation functions of RIM1/2 and PSD-95 in adult hippocampal slices. There were, on average, 2.02 ± 0.08 and 1.32 ± 0.21 nanoclusters with a volume of (3.6 ± 0.2) and (4.2 ± 0.7) × 10^5 nm^3 for RIM1/2 and PSD-95, respectively. Except PSD nanocluster number which was significantly less than that in cultures (P = 0.03), all other parameters were similar (Wilcoxon signed-rank test).
Extended Data Figure 8 | Preferential release in nanocolumns can increase synaptic strength. 

a, Schematic of the experimentally constrained, deterministic approach used to study the dependence of synaptic strength on the spatial distribution of release sites and AMPARs. The simulated release site distribution at a synapse was drawn from its measured RIM positions and the average measured relationship between RIM density and pHuse locations (Fig. 2). 

b, Distributions of measured RIM localizations within a single active zone (active zone) boundary (grey), and the same cluster with randomized positions of the indicated subsets of molecules. 

c, Maps of RIM local density normalized to the overall densities within the active zones. 

d, Probability density maps of possible release sites given that a release occurs. 

e, Distributions of GluA2/3 locations within the PSD boundary (grey) of the same measured synapse (ellipses refer to this distribution) and randomized. 

f, Maps of fraction of open channels at peak response per average release from the respective active zones directly above them in d. 

g, Calculated open channels at peak response, n = 20 randomly generated molecular distributions. See Methods for more details.
Extended Data Figure 9 | Enrichment of other scaffolding proteins within nanocolumns. 

**a**, Enrichment of Homer1 with PSD-95 nanoclusters, \( n = 118 \) nanoclusters from 48 synapses, scale 100 nm. 

**b**, Enrichment of RIM1/2 to Shank nanoclusters, \( n = 80 \) nanoclusters from 32 synapses. Scale bar, 200 nm. \(* P < 0.05, \) ANOVA on ranks with pairwise comparison procedures (Dunn's method) in **a** and **b**. 

**c**, GKAP2 and Shank3 densities (determined with STORM, \( n = 6 \) and 12, respectively) within PSD-95 nanoclusters (determined with PALM of transfected knockdown-replacement-PSD-95-mEos2) normalized to total PSD densities. Both proteins showed significant enrichment in PSD-95 nanoclusters. \(* P < 0.05, \) paired \( t \)-tests. 

**d**, Three-colour STORM imaging of RIM1/2, GKAP1 and PSD-95 on the same synapses example (left) and protein enrichment profiles of RIM1/2 and GKAP1 with respect to PSD-95 nanoclusters (right), \( n = 32 \) nanoclusters from 17 synapses. Scale bar, 200 nm. 

**e**, Enrichment indices of RIM1/2 and GKAP1 relative to PSD-95 nanoclusters. Colour-coded bars represent the same set of randomizations as performed in Fig. 3c: orange denotes randomization of only out-of-nanocluster localizations, cyan denotes randomization of nanocluster positions within synaptic clusters and grey denotes randomization of all localizations. 

**f**, The percentage of PSD-95 nanoclusters that were enriched with GKAP1, RIM1/2 or both with colour-coded randomizations. \(* P < 0.05; \) ** \( P < 0.01, \) ANOVA on ranks with pairwise comparison procedures (Dunn's method), \( n = 32 \) nanoclusters from 17 synapses in 7 different cultures. 

**g**, Schematic summary of the distribution of synaptic proteins within nanocolumns. The distributions of colour-coded proteins are based on our results and the proteins in grey are hypothetical, some, such as \( \mathrm{Ca}^{2+} \) channels, have been suggested previously to be clustered\(^{49,50} \). All experiments were repeated \( \geq 5 \) times.
Extended Data Figure 10 | Plasticity within nanocolumns. a, Changes in the localization density within RIM1/2 (red) and PSD-95 (blue) nanoclusters under control, 5 min NMDA treatment, 25 min washout, and NMDA + AP5 treatment conditions. b–h, Reorganization of RIM1/2 and GluR2/3 under control, 5 min NMDA treatment, 25 min washout conditions examples (b), comparison of whole synaptic cluster sizes (c), nanocluster number per synapse (d), localization density within nanoclusters (e), enrichment indices (f), percentage of nanoclusters that were enriched (g), and nanocluster volumes (h). Note that similar to the results from the RIM1/2-PSD-95 analyses, only those RIM1/2 nanoclusters that were enriched with GluR2/3 (dark red) were increased in volume. *P < 0.05; **P < 0.01, ANOVA on ranks with pairwise comparison to control group (Dunn’s method), and χ² test for the proportion. Data from 62, 21 and 37 nanoclusters from 34, 18 and 24 synapses for control, NMDA, and washout, respectively. i, Colour-coded local density map of an example live-PALMed PSD-95 cluster before and after NMDA treatment. Scale bar, 100 nm. j, Changes in PSD-95 nanocluster area induced by NMDA and blocked by AP5 (n = 28 and 21, respectively). **P < 0.01, NS, not significant, paired t-test. l–n, LTP stimulation induced changes in nanocluster volumes (l), localization density within nanoclusters (m) and nanocluster numbers (n). *P < 0.05, ANOVA on ranks with pairwise comparison to control group (Dunn’s method). All experiments were repeated ≥3 times.