Synaptic nanomodules underlie the organization and plasticity of spine synapses

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Experience results in long-lasting changes in dendritic spine size, yet how the molecular architecture of the synapse responds to plasticity remains poorly understood. Here a combined approach of multicolor stimulated emission depletion microscopy (STED) and confocal imaging in rat and mouse demonstrates that structural plasticity is linked to the addition of unitary synaptic nanomodules to spines. Spine synapses in vivo and in vitro contain discrete and aligned subdiffraction modules of pre- and postsynaptic proteins whose number scales linearly with spine size. Live-cell time-lapse super-resolution imaging reveals that NMDA receptor-dependent increases in spine size are accompanied both by enhanced mobility of pre- and postsynaptic modules that remain aligned with each other and by a coordinated increase in the number of nanomodules. These findings suggest a simplified model for experience-dependent structural plasticity relying on an unexpectedly modular nanomolecular architecture of synaptic proteins.

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As expected, combined confocal and STED imaging resolved dendritic spines that contained PSD-95 clusters tightly apposed to vGlut1 clusters spaced at a distance of ~100 nm (Fig. 1a,b). The number of apposed vGlut1 and PSD-95 clusters varied among spines. Most spines contained a single synaptic profile with one PSD-95 puncta aligned with one vGlut1 puncta. However, many spines contained more than one pair of PSD-95 and vGlut1 puncta (Fig. 1a–c,f). In control experiments, the deconvolution algorithm used improved the apparent resolution of our STED images (Supplementary Fig. 2) but did not introduce or remove clusters even after multiple rounds of photobleaching (Supplementary Fig. 3). Analysis of non-deconvolved PSD-95 and vGlut1 images gave similar results as analysis of the same set of images after deconvolution, further validating the use of the algorithm (Supplementary Fig. 4a,d,g). We confirmed that multiple aligned puncta at spines represented discrete clusters using 3X gated STED confocal microscopy that enabled us to acquire super-resolved images in x (−50 nm) and z (−200 nm) of PSD-95 and vGlut1 or PSD-95 and the presynaptic active zone marker Bassoon (Supplementary Fig. 5a,b).

To explore the nano-organization of spine synapses in more detail, we collected from EGFP-filled cortical neurons three-color STED images of either the synaptic vesicle protein synaptophysin-1 (SYP-1) or Bassoon together with vGlut1 and PSD-95 (Figs. 2 and 3; Leica SP8 triple-gated STED, fluorescence width at half maximum (FWHM) ~50 nm). At EGFP+ spines SYP-1 and Bassoon were found to colocalize with vGlut1 and were apposed to PSD-95 puncta (Figs. 2a and 3a). Consistent with our results from imaging the presynaptic marker vGlut1, most spines were apposed to a single SYP-1 or Bassoon punctum, while the remainder of spines were contacted by multiple SYP-1 (Fig. 2a,b) and Bassoon puncta (Fig. 3a–d). Thus, we find that many excitatory synapses are composed of multiple aligned pre- and postsynaptic clusters of endogenous synaptic proteins.

Surprisingly, the average size of individual PSD-95, vGlut1, SYP-1 and Bassoon clusters did not vary between single and multiclusterm spines (Figs. 1d,g, 2c and 3e, and Supplementary Fig. 4e,h). Thus, as the number of puncta at a spine increases there is a net increase in the overall amount of PSD-95, vGlut1, SYP-1 and Bassoon found at spine synapses, but the average size of each punctum remains constant (Figs. 1e,b, 2d and 3f, and Supplementary Fig. 4f,i). These results suggest that individual PSD-95 and presynaptic clusters may represent modular units of synaptic organization. We termed them ‘nanomodules’.

To test the hypothesis that nanomodules are units of spine organization, we asked how the number of pre- and postsynaptic modules was related to spine size. The number of PSD-95, vGlut1, SYP-1 and Bassoon clusters scaled with spine size, exhibiting a linear relationship with spine head area (Figs. 1i,j, 2f–h and 3h, and Supplementary Fig. 7i,j). These findings suggest that the number of PSD-95 clusters increased with the number of vGlut1, SYP-1 and Bassoon nanomodules (Figs. 1k, 2i,j and 3i). These results demonstrate a relationship between pre- and postsynaptic nano-organization and suggest that the number of PSD-95 and presynaptic modules found at a single spine may scale with dendritic spine size.

Synaptic architecture exhibits modular organization in vivo.

To determine whether dendritic spines in the cortex are also composed of pre- and postsynaptic modules, we labeled neurons of the somatosensory cortex at postnatal day (P) 7 by injecting a lentivirus that transduced only EGFP. At P28–P35, when many mature dendritic spines are present, we perfused the mice, sectioned the brains and cleared the sections using the CUBIC1/2 method to minimize light scattering during imaging. 3D super-resolved images were collected with a gated 3X STED Leica confocal system to image EGFP-labeled spines in confocal resolution (~250 nm) and endogenous PSD-95 and vGlut1 clusters in super-resolution (~80 nm in x and ~200 nm in z; Fig. 4a,b and Supplementary Video 1).

Spines were selected from primary and secondary dendritic branches of the apical dendritic arbor, where experience-dependent plasticity of spine size occurs. 3D reconstruction of non-deconvolved imaged sections demonstrated that, similarly to synapses in vitro, dendritic spines in vivo contained both single and multiple aligned PSD-95 and vGlut1 pairs (Fig. 4b–d and Supplementary Video 1). Approximately 40% of spines contained a single PSD-95 cluster apposed to a single vGlut1 cluster, while spines with two, three, four and even five aligned PSD-95 and vGlut1 clusters were also observed (Fig. 4e–f). Similar results were obtained from brain sections that were not subjected to CUBIC (Supplementary Fig. 8a,b,e). Notably, in brain sections, we found fewer spines that consisted of a single module of PSD-95 and vGlut1 than seen in vitro (~40% vs. ~60%), with a concomitant increase in the proportion of spines that contained two or more modules. To test whether these differences were due simply to higher density of synaptic clusters in the brain, we conducted Monte Carlo simulations (Supplementary Fig. 9). Simulations resulted in significantly more single module spines and fewer spines containing multiple PSD-95 and vGlut1 nanomodules than found endogenously (Fig. 4e,f and Supplementary Figs. 8b,e and 9f,g). These data suggest that in brain sections nanomodules are precisely associated with spines.

A lower fraction of spines contains single nanomodules in brain sections than in vitro, therefore we asked whether the relationship between spine size and module number is still maintained in neurons of the somatosensory cortex. The average size of PSD-95 and vGlut1 nanoclusters in vivo was not significantly different between single and multiclusterm spines (Fig. 4g,h and Supplementary Fig. 8c,f). Moreover, the number of PSD-95 and vGlut1 clusters scaled with
Fig. 1 | Modular organization of dendritic spine synapses in vitro. a. Right, representative high-contrast images of PSD-95 (green) and vGlut1 (red) modules imaged with STED (~80 nm FWHM) in dendritic spines (grayscale and dashed yellow lines) imaged simultaneously in confocal mode (~300 nm FWHM) in tdTomato-transfected DIV21 cortical neurons. Scale bar, 0.8 μm. Left, schematic demonstrating the arrangement of multiple synaptic profiles in individual spines from images at right. b. Line profiles (white lines in a) of the intensity of PSD-95 and vGlut1 labeling in spines from a indicate a high degree of apposition (~100 nm) of individual pre- and postsynaptic clusters. c,f. Quantification of the percentage of spines containing single and multiple PSD-95 (c, n = 217 spines) and vGlut1 clusters (f, n = 212 spines, graphs represent mean ± s.e.m., dots show percentage of spines from three independent experiments, 9 different neurons). d,g. Quantification of the average areas of individual PSD-95 (d) and vGlut1 (g) clusters, demonstrating no significant size differences (d, P = 0.3951; g, P = 0.6114) between single and multiclus clusters (one-way ANOVA). e,h. Quantification of the total area of PSD-95 (e; *P = 0.0130, ***P < 0.0001) and vGlut1 (h; *P = 0.0035, ***P < 0.0001), one-way ANOVA with Fisher’s least significant difference test (LSD) post hoc. i, Plots of the relationship between cluster number and spine size. Positive correlation of PSD-95 (i, green line, R² = 0.4324, slope = 1.972 ± 0.1559, P < 0.0001, F-test, n = 212 spines) and vGlut1 (j, red line, R² = 0.2795, slope = 1.524 ± 0.1689, P < 0.0001, F-test, n = 212 spines) cluster number with areas of individual spines (only spines with both PSD-95 and vGlut1 clusters were included for analysis; dots represent cluster number values of individual spines). Monte Carlo simulation (black line) of the relationship between spine size (500 simulations per spine size; spines without PSD-95 and vGlut1 puncta were not included) and the number of PSD-95 (i, simulated slope = 0.1783 ± 0.0355, ANCOVA) and vGlut1 (j, simulated slope = 0.3276 ± 0.0256, ANCOVA) clusters. ***P < 0.0001. k. A plot of the relationship between pre- and postsynaptic nanomodules (R² = 0.4383, slope = 0.6366 ± 0.0497). All experiments were repeated ≥3 times. Bar graphs show mean ± s.e.m., with numbers of individual spines or clusters represented by dots. Statistics in Supplementary Table 1.
Fig. 2 | Synaptic vesicle proteins exhibit modular organization at synapses. a, Right, representative high-contrast, three-color STED images of PSD-95 nanomodules (green, continuous wave STED, FWHM ~80 nm), vGlut1 (red) and synaptophysin-1 (SYP-1, blue) nanomodules imaged using gated STED (FWHM ~50 nm) in EGFP-labeled dendritic spines (grayscale and dashed white lines) of DIV21-25 neurons imaged simultaneously in confocal mode (FWHM ~250 nm). Scale bar, 1 μm. Left, schematic demonstrating the arrangement of synaptic profiles in individual spines from the images at right. Synaptic profiles were determined as an apposition of colocalized vGlut1 and SYP-1 with PSD-95 (white arrows). b, Quantification of the percentage of spines containing single and multiple PSD-95 (n = 406 spines), vGlut1 (n = 406 spines) and SYP-1 nanomodules (n = 189 spines). Data points indicate replicates from ≥3 independent transfection experiments. c, Quantification of the average areas of individual PSD-95 (n = 648 clusters), vGlut1 (n = 728 clusters) and SYP-1 nanomodules (n = 310 clusters, one-way ANOVA); n.s., not significant (P = 0.0759, P = 0.869 and P = 0.824, respectively). d, Quantification of the total area of PSD-95, vGlut1 and SYP-1; one-way ANOVA, Tukey’s post hoc, ***P = 0.0081, **P < 0.0001, c). e, Cumulative probability plots for the data in c (Kruskal–Wallis test); res., resolution. f–h, Correlation of spine size with PSD-95 nanomodule number (f, green line, Pearson’s R² = 0.2992, slope = 1.957 ± 0.1490), vGlut1 nanomodule number (g, red line, Pearson’s R² = 0.2755, slope = 2.212 ± 0.1785) and SYP-1 nanomodule number (h, blue line, Pearson’s R² = 0.2761, slope = 1.837 ± 0.2313). Monte Carlo simulation (sim.; black line) of the relationship between spine size (spines without PSD-95, vGlut1 and SYP-1 puncta were not included) and the number of PSD-95 nanomodules (f, simulated slope = 0.081 ± 0.02, ANCOVA), vGlut1 nanomodules (g, simulated slope = 0.474 ± 0.027, ANCOVA) and SYP-1 nanomodules (h, simulated slope = 0.243 ± 0.019, ANCOVA). ***P < 0.0001, ANCOVA, i–j, A plot of the relationship between PSD-95 nanomodules and vGlut1 nanomodules (i, Pearson’s R² = 0.7945) and SYP-1 nanomodules (j, Pearson’s R² = 0.8449). Graphs in b–d represent mean ± s.e.m.; dots show percentage of spines (b), individual nanomodules (c) and areas (d) from at least three independent experiments and at least 11 different neurons. Statistics in Supplementary Table 1.
spine size (Fig. 4j and Supplementary Fig. 8h,i), with a significantly steeper slope than expected from the Monte Carlo simulation (Supplementary Fig. 9h,i, P < 0.0001, ANCOVA). Finally, the number of PSD-95 and vGlut1 nanomodules scaled tightly with each other at spines (Fig. 4k). These data indicate that both in vivo and in vitro spine synapses are composed of aligned pre- and postsynaptic nanomodules whose number scales linearly with spine size.

Structural plasticity results in spines containing multiple aligned pre- and postsynaptic nanomodules. To examine whether coordinated increases in the number of nanomodules might be linked to structural plasticity that underlies increases in dendritic spine size, we examined whether the number of endogenous PSD-95 and vGlut1 modules per spine is affected by NMDAR-dependent cLTP. We hypothesized that the increase in PSD-95 levels observed 3 h after induction of structural plasticity might be due to the addition of new postsynaptic nanomodules. We induced spine enlargement with glycine treatment (cLTP) in DIV21–25 cortical neurons transfected with cell-filling tdTomato only. This method results in NMDAR-dependent structural plasticity in approximately 40% of dendritic spines. Neurons were imaged using a spinning-disc confocal microscope once every 6 min for 3 h after the induction of cLTP (Fig. 5a–e and Supplementary Videos 2 and 3). cLTP resulted in sustained enlargement of ~42% of dendritic spines while the remainder of spines were nonresponsive, having no lasting changes in size (Fig. 5f). Increases in spine size following glycine treatment were blocked by pretreatment with the NMDAR blockers APV (50 µM) and MK-801 (10 µM) and remained unchanged in control (unstimulated) neurons imaged for 3 h (Fig. 5f and Supplementary Videos 2 and 3).

To determine the impact of cLTP on the synaptic nanoarchitecture, we fixed neurons immediately at the conclusion of live-cell imaging and stained them for endogenous PSD-95 and vGlut1. Retrospective analysis of individual live-imaged spines was conducted using STED and confocal imaging (Fig. 5a–e). In control neurons, which were not treated with glycine, the majority of spines (>65%) contained a single PSD-95 module apposed to a single vGlut1 module, while the remaining <35% of spines had multiple clusters (Fig. 5a,g–j). In contrast, spines that increased in size after cLTP contained significantly more PSD-95 modules (Fig. 5b,g).

Plasticity may drive correlated changes in pre- and postsynaptic size. Consistent with this idea, enlarged spines also had significantly more presynaptic vGlut1 modules (Fig. 5b,h), suggesting that both pre- and postsynaptic nanomodules are affected by structural plasticity. Potentiated spines had a shift in pre- and postsynaptic module number with respect to control spines, such that only ~40% contained single PSD-95 and vGlut1 nanomodules and ~60% contained multiple PSD-95 and vGlut1 modules (Fig. 5i,j). APV and MK-801 pretreatment prevented the increases in PSD-95 and vGlut1 nanomodule numbers and reduced the proportion of multimodule spines to less than 40% (Fig. 5d,g–i). Thus, the increases in the numbers of both pre- and postsynaptic nanomodules appear related to structural plasticity induced by cLTP. Consistent with this model, nonresponsive spines, which did not increase in size after cLTP, contained significantly fewer PSD-95 and vGlut1 nanomodules than potentiated spines (Fig. 5c,f–j; PSD-95: P = 0.006; vGlut1: P = 0.03, t-test). Thus, spine enlargement following cLTP leads to the increase in the number of endogenous PSD-95 and vGlut1 nanomodules resulting in a higher proportion of multimodule spines.

Under basal conditions, synaptic nanomodule size did not differ between single and multicluster spines (Figs. 1–3). Therefore, we next tested whether PSD-95 and vGlut1 nanomodules have unitary size following cLTP. In potentiated spines, the size of PSD-95 and vGlut1 nanomodules did not differ from control or nonresponsive spines (Fig. 5k,l). However, consistent with the well-established role of neuronal activity in the maintenance of synaptic PSD-95,29,30, treatment with APV and MK-801 during the 3-h imaging period significantly reduced the area of PSD-95 but not vGlut1 nanomodules (Fig. 5k,l). Notably, in spines that underwent structural plasticity neither pre- nor postsynaptic module size changed as spine size increased (Fig. 5m,n). These results suggest that the coordinated increase in modular pre- and postsynaptic protein complexes may underlie NMDAR-dependent structural plasticity.

Pre- and postsynaptic modules exhibit increased dynamics, yet remain aligned during structural plasticity. Post hoc analysis following cLTP induction does not allow us to rule out the possibility that the spines that became larger already had multiple modules. Therefore, we simultaneously visualized the dynamic remodeling of pre- and postsynaptic nanoarchitecture during structural plasticity of dendritic spines. We used live-cell STED imaging of DIV21–25 cortical neurons following cLTP induction for 3 h at ~90 nm resolution. Pre- and postsynaptic nanomodules were visualized in living neurons by separately transfecting two groups of cortical neurons in suspension at DIV0, one group with PSD-95–EGFP and cell-filling tdTomato and the other with synaptophysin-1–mTurquoise2 (SYP-mTurq2), and then mixing the two sets of neurons (Methods). SYP-mTurq2 was chosen as the presynaptic marker for these experiments because synaptophysin is organized into nanomodules (Fig. 2) and has well-characterized fluorescently tagged expression constructs.31–33

In unstimulated control neurons, the majority of spines contacted by a SYP-mTurq2* axon contained a single PSD-95–EGFP nanomodule apposed to a single SYP-mTurq2 nanomodule (Fig. 6a). The average size of control spines did not change over the course of 3 h (Supplementary Fig. 10a), and the number of PSD-95–EGFP and SYP-mTurq2 modules remained stable on average over this imaging period (Fig. 6e,f). As expected from our previous experiments, glycine treatment resulted in two groups of spines: nonresponsive spines that remained unchanged in size and potentiated spines that exhibited a significant long-lasting increase in size (Fig. 6b,c and Supplementary Fig. 10a). Similarly to control spines, nonresponsive spines failed to show changes in the number of pre- and postsynaptic nanomodules over the imaging period (Fig. 6b,c,f). Consistent with the hypothesis that structural plasticity results in an increased number of synaptic nanomodules, 3 h after glycine treatment potentiated spines contained significantly more of both PSD-95–EGFP and SYP-mTurq2 nanomodules. These increases were blocked by NMDAR antagonists (Fig. 6e,f). These findings indicate that induction of structural plasticity results in the paired increase of pre- and postsynaptic nanomodules.

Structural changes to spine size occur rapidly during the first 30 min after induction of plasticity. We next asked when changes to the molecular nanoarchitecture might occur. In potentiated spines, there was a significant increase in the number of PSD-95–EGFP modules within an hour after glycine treatment that remained significantly higher than control for the remainder of the imaging period (Fig. 6e). These findings are consistent with previous work demonstrating a significant rise in PSD-95 levels 60 min after LTP stimulation. During the imaging period the size of individual PSD-95–EGFP clusters within single potentiated spines did not change (Supplementary Fig. 10b). Presynaptic SYP-mTurq2 modules also began to increase in number soon after cLTP, and this increase reached significance within 2 h after glycine treatment (Fig. 6f). Increases in PSD-95–EGFP and SYP-mTurq2 module number were not observed in nonresponsive glycine-treated spines or control-treated spines and were blocked by NMDAR antagonists (Fig. 6a–f). Similar results were obtained when endogenous PSD-95 was labeled with EGFP-FingR PSD-95 intrabodies (Supplementary Fig. 11a,b and Supplementary Video 4). These results indicate that the number of modules begins to increase soon after induction of structural plasticity and that by 2 h after induction newly aligned pre- and postsynaptic nanomodules remain aligned during structural plasticity.
postsynaptic modules are present in potentiated spines. Notably, while the increase in average nanomodule number is gradual, the average reflects an increasing proportion of spines with multiple modules, which appear to be added in a unitary fashion. These findings highlight the importance of coordinated reorganization of pre- and postsynaptic nanoarchitecture.

Induction of structural plasticity results in changes to spine dynamics. Therefore, we next examined whether plasticity might result in changes in the organization of synaptic nanomodules. Under basal conditions both PSD-95–EGFP and SYP-mTurq2 modules were not stationary. In control spines both PSD-95–EGFP and SYP-mTurq2 modules were not stationary. In control spines both PSD-95–EGFP and SYP-mTurq2 nanomodules were not stationary. In control spines both PSD-95–EGFP and SYP-mTurq2 nanomodules were not stationary.

Fig. 3 | Synaptic vesicle and active zone markers colocalize at spines as synaptic nanomodules. a. Right, representative high-contrast three-color STED images of PSD-95 nanomodules (green, continuous wave STED, FWHM ~80 nm), vGlut1 (red) and Bassoon (blue) nanomodules imaged using gated STED (FWHM ~250 nm) in EGFP-labeled dendritic spines (grayscale and dashed white lines) of DIV21–25 neurons imaged simultaneously in confocal mode (FWHM ~250 nm). Scale bar, 1 μm. Left, schematic demonstrating the arrangement of synaptic profiles in individual spines from the images at right. Synaptic profiles were determined as an apposition of colocalized vGlut1 and Bassoon with PSD-95 (white arrows). Similar results were obtained from 3 independent transfection experiments in spines from a total of 10 neurons. b–d, Quantification of the percentage of spines containing single and multiple PSD-95 (n = 217 spines also shown as part of Fig. 2b), vGlut1 (n = 217 spines also shown as part of Fig. 2b) and Bassoon nanomodules (n = 217 spines). Data points represent percentage of spines with the indicated number of nanomodules in three independent transfection experiments. e, Quantification of the average number of individual Bassoon nanomodules (n = 379 clusters, one-way ANOVA). f, Quantification of the total Bassoon (Bass.) area at spines (one-way ANOVA, Tukey’s post hoc). Graphs in b–f represent mean ± s.e.m.; ***P < 0.0001; n.s., not significant (P = 0.0741). g, Cumulative probability plots for the data in e (Kruskal–Wallis test). h, Positive correlation of spine size with the number of Bassoon nanomodules (gray line, Pearson’s R² = 0.2524, slope = 2.065 ± 0.2430). Monte Carlo simulation (black line) of the relationship between spine size and the number of Bassoon nanomodules (spines without Bassoon puncta were not included; simulated slope = 0.2018 ± 0.018, ANCOVA, ***P ≤ 0.0001). i, A plot of the relationship between PSD-95 and Bassoon nanomodules (Pearson’s R² = 0.8549). Statistics in Supplementary Table 1.
Fig. 4 | Modular organization of dendritic spine synapses in vivo. a. The experiment. PFA, paraformaldehyde; IHC, immunohistochemistry. b. Left, 3D Imaris reconstruction of the dendritic section, spines and corresponding synaptic modules of the layer 3 neuron shown at right. PSD-95 and vGlut1 puncta not colocalized with spines were removed for clarity. Spines in 3D rendered images in c and d were made transparent to visualize PSD-95 puncta (green) inside these structures. Similar results were obtained from brain sections of 3 EGFP-injected animals. e, f, Quantification of the percentage of spines (n=3 independent injection experiments) containing single and multiple PSD-95 (e, P<0.0001) and vGlut1 (f, P<0.0001) modules (one-way ANOVA, Fisher’s LSD post hoc, graphs represent mean ± s.e.m., dots show percentage of spines from three independent experiments). g, h. Quantification of the average areas of individual PSD-95 (g, n=171 clusters) and vGlut1 (h, n=167 clusters; one-way ANOVA) nanomodules; n.s., not significant (P=0.2301 and P=0.8650, respectively). i, j. Plots of the relationship between cluster number and spine size. There was a positive correlation between the numbers of PSD-95 (i, green line, Pearson’s R²=0.4695) and vGlut1 (j, red line, Pearson’s R²=0.5680, n=84 spines, dots represent cluster number values of individual spines) nanomodules with areas of individual spines. Monte Carlo simulation (black line) of the relationship between spine size (500 simulations per spine size) and the number of PSD-95 (i, 4,897 PSD-95 simulations, simulated slope = 0.2152 ± 0.0151, measured slope = 2.385 ± 0.2766, ANCOVA, ***P<0.0001) and vGlut1 (j, 8,011 vGlut1 simulations, simulated slope = 0.5695 ± 0.019, measured slope = 2.427 ± 0.2039, ANCOVA, ***P<0.0001) clusters. Simulated spines without PSD-95 or vGlut1 clusters were not included in the analysis. k. A plot of the relationship between pre- and postsynaptic nanomodules (Pearson’s R²=0.7265, n= number of apposed vGlut1-PSD-95 pairs per spine from 84 spines). Bar graphs show mean ± s.e.m., with numbers of individual spines or clusters represented by dots. Statistics in Supplementary Table 1.
Increased mobility of synaptic modules following induction of structural plasticity has been suggested to result in changes to the trans-synaptic complex linking pre- and postsynaptic proteins. In this model, mobility of the synaptic nanoarchitecture might be due to instability in the synaptic structure. Alternatively, movement of pre- and postsynaptic structures might reflect an ordered process linked to the formation of new synaptic components. If plasticity results in increased synaptic disorder, we would expect that pre- and postsynaptic nanomodules might begin to move independently from one another. To test this, we asked whether plasticity changed the total distance that pre- and postsynaptic modules moved. Rather than reflecting disorganization, the enhanced mobility of PSD-95–EGFP and SYP-mTurQ2 appears ordered, with each pair of nanomodules moving together (Fig. 6c and Supplementary...
Fig. 6 | Rapid remodeling of aligned pre- and postsynaptic modules underlies cLTP structural plasticity. a–d. Representative images of time-lapse dual-color live-cell STED of PSD-95–EGFP (green) and SYP-mTurq2 (red). Grayscale shows cell morphology with cell-filling tdTomato in confocal mode. Green and red arrows indicate the appearance of new PSD-95–EGFP and SYP-mTurq2 modules, respectively. ‘Track’ panels indicate the movement of dual-color live-cell STED of PSD-95–EGFP (green) and SYP-mTurq2 (red). Grayscale shows cell morphology with cell-filling tdTomato in confocal mode. Scale bar in a–d, 500 nm. e, f. Quantification of the number of PSD-95–EGFP (e) and SYP-mTurq2 (f) nanomodules per spine over the course of 3 h. Measurements were performed at each time point (one-way ANOVA with Fisher’s LSD post hoc; # indicates significant differences between all conditions (P ≤ 0.0004)). Graphs show mean ± s.e.m. at each time point. g, h. Quantification of the total distance moved over 3 h for PSD-95–EGFP (g, control, n = 23; potentiated (Pot), n = 34, nonresponsive (Non); n = 19; APV plus MK-801 (APV + MK), n = 20 modules, *P = 0.03, ***P = 0.0003) and SYP-mTurq2 (h, control, n = 21; potentiated, n = 18; nonresponsive, n = 14; APV + MK, n = 23 modules, *P = 0.013, ***P = 0.0017; one-way ANOVA with Fisher’s LSD post hoc). i, Schematic representation of the method used to determine the distance and alignment between PSD-95–EGFP and SYP-mTurq2 nanomodules (see Methods). j. Quantification of the average distance between the centers of PSD-95–EGFP and SYP-mTurq2 (control, n = 18; potentiated, n = 13; nonresponsive, n = 14; APV + MK, n = 20 module pairs, one-way ANOVA). k, Quantification of the relative alignment as described in j (control, n = 18; potentiated, n = 14; nonresponsive, n = 14; APV + MK, n = 20 aligned pairs; one-way ANOVA; *P = 0.027, t-test) for the indicated conditions. Treatment with APV + MK resulted in significantly better alignment between PSD-95–EGFP and SYP-mTurq2 (two-tailed Student’s t-test). All experiments were repeated ≥3 times. Bar graphs represent mean ± s.e.m. with numbers of individual pre- and postsynaptic clusters (g,h) and numbers of aligned pairs of clusters (j,k) indicated by dots; n.s., not significant (P = 0.34 and P = 0.13, respectively). Statistics in Supplementary Table 1.

Video 5) and covering similar distances during the imaging period (~1.5 µm; Fig. 6g,h).

To examine how plasticity might affect the nanoscale spatial organization of the synapse, we quantified the relationship between pre- and postsynaptic nanomodules using two metrics: distance between the centroids of pairs of modules (d), and the alignment between pairs of modules (θ; Fig. 6i and Methods). Despite the significantly higher mobility of PSD-95–EGFP and SYP-mTurq2 in potentiated spines, neither the distance between pairs of modules nor the alignment of modules varied significantly between groups (Fig. 6j,k and Supplementary Video 5). Blockade of NMDARs resulted in a significantly smaller θ, between pre- and postsynaptic modules, suggesting that neuronal activity may regulate the alignment of nanomodules. Regardless, examination of the movement of PSD-95–EGFP and SYP-mTurq2 modules revealed that these complexes move around the spine head as an aligned pair. These data suggest a model in which the pre- and postsynaptic nanoarchitecture participates in structural plasticity as a functional unit that spans the synaptic cleft.

Pre- and postsynaptic nanoarchitecture is rapidly modified following structural plasticity. Induction of NMDAR-dependent structural plasticity results in rapid changes to spine morphology.
To begin to examine when the nanoscale changes in synaptic organization occur after induction of structural plasticity, we performed live-cell STED imaging of cultures transfected as described above every 12.5 min for 1 h (Fig. 7). These experiments revealed that the number of PSD-95–EGFP nanomodules in potentiated spines began to increase within 12.5 min of cLTP induction. By 50 min after induction of cLTP, potentiated spines contained significantly more PSD-95–EGFP nanomodules than both the control and nonresponsive spines (Fig. 7a–c and Supplementary Video 6). These results suggest that changes to postsynaptic nanoarchitecture begin within minutes following the induction of NMDAR-dependent plasticity.

Unlike PSD-95, SYP-mTurq2 nanomodules showed a significant increase in both potentiated and unpotentiated spines immediately after cLTP induction (Fig. 7d and Supplementary Video 6). These early structural modifications may reflect short-term changes in presynaptic function associated with high levels of neuronal activity58. By 25 min after cLTP induction, numbers of SYP-mTurq2 nanomodules in unpotentiated spines had fallen to control levels while in potentiated spines the number of nanomodules was significantly higher (Fig. 7d). These data suggest that changes in presynaptic module number may occur before the increase in postsynaptic module number.

The number of pre- and postsynaptic nanomodules increases in spines by 1 h after induction of cLTP, but shortly after induction of structural plasticity, PSD-95 is thought to undergo a rapid and transient increase in mobility46. To examine events that occur during the first 12.5 min after induction of cLTP, we first determined the number of PSD-95–EGFP nanomodules in individual tdTomato+ spines using live-cell STED. Then we photobleached spines with one or two nanomodules and monitored fluorescence recovery after photobleaching (FRAP) of PSD-95–EGFP following cLTP46. At the conclusion of FRAP, neurons were again imaged with live-cell STED and nanomodule number determined (Fig. 7e,f).

Before cLTP the recovery of PSD-95–EGFP in bleached spines was similar in all conditions (Fig. 7g,h). Induction of cLTP resulted in a rapid and transient increase in PSD-95–EGFP mobility in only spines that were enlarged (≥10% sustained increase over baseline) after cLTP (Fig. 7i–j and Supplementary Video 7). These findings are consistent with previously published data for structural plasticity induced by photostimulation47 and suggest that increases in PSD-95 mobility occur rapidly after induction of structural plasticity, are transient, and are specific to spines undergoing structural changes. Although we were able to measure differences in PSD-95 mobility, there were no significant changes in PSD-95–EGFP nanomodule number in unenlarged or enlarged spines at this early time point (Fig. 7f), as expected from rapid live-cell STED imaging. These results suggest that the transient increase in PSD-95–EGFP mobility occurs before the increase in the number of PSD-95–EGFP nanomodules.

Discussion

Competing hypotheses have suggested that the synaptic microarchitecture is composed of either ridged structures trapped in nanodomains that form a trans-synaptic column59 or amorphous fluid-like structures with rapidly transitioning proteins in a dynamic equilibrium34. How these two models might explain the ability of the synapse to undergo long-lasting, activity-dependent structural changes has remained obscure. Here, using STED imaging, we demonstrate that spine synapses in vitro and in vivo are composed of discrete, aligned pre- and postsynaptic protein nanomodules of uniform size, whose number, not size, scales with the size of dendritic spines. Activity-dependent structural plasticity of individual spines results in long-lasting increases in the number of modules. These findings are reminiscent of previous work in the hippocampus suggesting an all-or-none model of synaptic plasticity19 and recent modeling work58, and are consistent with the observation that some dendritic spines may contain perforated PSDs22,46,41. The nano-organization of pre- and postsynaptic proteins was remarkably similar in vitro and in brain slices from cortex, suggesting that these are robust features of the synapse. Similar postsynaptic nanostructures of PSD-95 have been observed in the hippocampus62. Together, our data indicate that synaptic architecture is composed of modular pre- and postsynaptic organizational units and that the increase in the number of these modules underlies structural plasticity.

The tight correlation between the sizes of pre- and postsynaptic structures has led to the suggestion that the modifications of pre- and postsynaptic architecture following plasticity may occur in concert63,64. However, at most synapses plasticity is thought to be expressed by postsynaptic structural changes downstream of calcium influx through NMDARs45,46. Whether long-lasting changes in presynaptic molecular organization are associated with NMDAR-dependent plasticity is less clear. In piramidal neurons, induction of LTP and spine enlargement appear to result in an increase in the size of the associated presynaptic boutons82, while in the amygdala induction of fear learning may result in increased numbers of boutons64. Using live-cell dual-color STED imaging, we demonstrate that spine enlargement following cLTP leads to the formation of new, aligned nanomodules that undergo coordinated rearrangements pre- and postsynaptically. In this respect, multi-nanomodule spine synapses are unexpectedly similar to the neuromuscular junction, containing sets of presynaptic sites paired with postsynaptic proteins, which can be added in response to NMDAR-driven plasticity. Fast time-resolved live-cell STED imaging indicated that the changes in the number of SYP-1 nanomodules may precede modification to the postsynaptic PSD-95 nanoarchitecture. These data suggest that the expression of experience-dependent plasticity is not solely a postsynaptic phenomenon, but rather involves both pre- and postsynaptic mechanisms functioning in concert. It will be interesting to determine whether the mechanisms mediating the coordinated increase in pre- and postsynaptic nanomodule number and movement are the same or different and whether these events are linked to changes in the movement of glutamate receptors or spines12,13,45–47.

How might new nanomodules be generated rapidly after NMDAR-dependent plasticity? One possibility is that nanomodules are recruited as units from dendrites or axons into potentiated synapses. While attractive, this possibility seems unlikely as PSD-95 appears to move between spines by diffusion65 and we failed to detect nanomodules of PSD-95 moving into enlarged spines after cLTP. However, due to the limitations of live-cell STED microscopy, new nanomodules could have been rapidly delivered to spines. A second possibility is that new nanomodules could be generated by splitting of existing nanomodules. Consistent with this possibility, analysis of nanomodules in vitro and in brain slices indicates that both pre- and postsynaptic modules are limited in size. Results from FRAP experiments suggested that the exchange of PSD-95 within a spine increases before changes in the number of nanomodules occur. In this model increases in protein exchange could lead to increases in nanomodule size, driving the formation of new modules once the size limit is reached. Finally, new nanomodules might be linked to the addition of new anchoring proteins. These might be proteins such as AMPA receptors or AMPA-receptor-associated proteins that are added rapidly to the synapse after the induction of synaptic plasticity or trans-synaptic organizing molecules such as ephrin-B3 that interact directly with PSD-9566,67,11,13,37. More work will be required to determine whether one of these models may explain how the number of nanomodules increases in spines following NMDAR-dependent plasticity.

Increased NMDAR-dependent nanomodule movement is linked to the addition of nanomodules and synaptic plasticity. These rapid changes in the movement of synaptic nanomodules are reminiscent of the increased remodeling of the actin cytoskeleton following NMDAR activation69,90. Notably, although mobile, nanomodules
at synapses undergoing NMDAR-dependent plasticity remain in precise alignment, suggesting that trans-synaptic interactions are maintained during structural plasticity. Thus, even during plasticity, synaptic function would likely remain intact and the regions of the synapse with the highest likelihood of release would remain in precise register with regions of the highest concentration of glutamate receptors. We propose a simplified model for experience-dependent plasticity wherein induction of structural plasticity in individual spines is mediated by addition of unitary synaptic nanomodules and propose that these modules function as building blocks to enable synaptic plasticity.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at [https://doi.org/10.1038/s41593-018-0138-9](https://doi.org/10.1038/s41593-018-0138-9).
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Author contributions

M.H. designed and conducted experiments and wrote the paper, N.H. and S.J.L.M. performed additional experiments, and M.B.D designed experiments and wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Lentiviral transduction and immunohistochemistry. The EGFP lentivirus used to label neuronal morphology in vivo was generated at Penn Vector Core (University of Pennsylvania, Philadelphia) using the pFGEG plasmid, which expresses EGFP under control of the human ubiquitin promoter. Postnatal day (P) 7 CD-1 male and female pups were anesthetized with 5% isoflurane for 5 min and then maintained under 1–2% isoflurane anesthesia while performing bilateral stereotaxic injections of the EGFP lentivirus into somatosensory cortex of the brain (~30 min). Sparse labeling of cortical neurons was achieved by delivering 0.6 µL of the EGFP lentivirus (9.65 x 10^10 infective particles/mL) into each injection site. To allow for the efficient expression of EGFP, mice were used for 3 to 4 weeks after injection. At P28–35 mice were perfused transcardially with PBS followed by 4% PFA. Brains were postfixed overnight in 4% PFA at 4 °C. After washing three times for 10 min in PBS, brains were sectioned at 300 µm using a VT-1000S Vibratome (Leica). All subsequent steps were carried out with free-floating sections. Sections were mounted using the CUBIC reagent-1 (4% PFA, 0.5% Triton-X, 0.5% paraformaldehyde, 5% 2-mercaptoethanol) and in CUBIC reagent-2 (5% sucrose (BP220-1, Fisher Scientific), 25% urea, 1% triethanolamine (cat. no. T83800 Sigma) and 0.1% Triton-X100) overnight at room temperature. The cleared sections were then mounted using CUBIC reagent-2 on slides, covered with a 0.15 µm cover glass and used for imaging.

Methods

Animals. All animal studies were approved by the Institutional Animal Care and Use Committee guidelines at Thomas Jefferson University in accordance with US National Institutes of Health guidelines. Mouse pups for lentiviral transduction experiments were obtained from timed pregnant CD-1 mice purchased from Charles River Laboratories Inc. (Wilmington, MA) and housed (3–5 mice per cage) in a temperature- and humidity-controlled facility. All experiments were obtained from timed pregnant CD-1 mice purchased from Charles River Laboratories Inc. (Wilmington, MA) and used to make primary cortical neuron cultures (see below).

Primary cortical neuron culture preparation. Dissociated cortical neurons were prepared from E17–18 rat cerebral cortex as described previously37,51,52 and cultured in Neurobasal medium supplemented with B27 (Life Technologies), glutamine (Sigma, St. Louis, MO) and penicillin-streptomycin (Sigma) at a concentration of 100 U/mL and 100 µg/mL, respectively, at 37 °C in a humidified 5% CO2 incubator. Neurons were maintained in Neurobasal medium supplemented with B27, glutamine (Sigma, St. Louis, MO) and penicillin-streptomycin (Sigma) at a concentration of 100 U/mL and 100 µg/mL, respectively, at 37 °C in a humidified 5% CO2 incubator.

Neuronal transfection. To control for levels of protein expression, neurons were transfected at day in vitro 0 (DIV0) in suspension as previously described51 using Lipofectamine 2000 (Life Technologies), and fluorescently tagged proteins were under control of mammalian promoters (described above). Briefly, immediately after dissociation of E17–18 rat cortices, cortical neurons were resuspended in OptiMEM (Life Technologies) at 500,000 neurons per millilitre. Suspension (1 mL) was added to 1 mL of the Lipofectamine 2000/DNA mixture and the transfection mix was incubated at 37 °C for 45 min. Neurons were subsequently plated either in 24-well plates or in 35-mm dishes and left to adhere to coverslips for 1.5 h, after which they were washed once in cortical medium (Basal Medium Eagle, Life Technologies) supplemented with 10% heat-inactivated FBS (cat. no. 11095-065, Gibco). The coverslips were then blocked overnight at 4 °C with primary antibodies, washed three times in PBS, incubated for 2 h at room temperature with secondary antibodies, washed three times in PBS and then mounted. Delivery of EGFP by lentivirus was used only to label neuronal morphology. No manipulation of protein expression was performed, and therefore no randomization was done in animal studies.

Antibodies. All primary and secondary antibodies were profiled in our previous publications and were reported to be specific53,54,55. Primary antibodies: mouse monoclonal (IgG2A) anti-PSD-95 clone K28/43 (1:1,000 (IHC) or 1:200 (IHC), Neuromab, UC Davis, Davis, CA), mouse monoclonal (IgG1) anti-synaptophysin-1 (1:1,000, cat. no. 101 111, Synaptic Systems, Gottingen, Germany), guinea pig polyclonal anti-vesicular glutamate transporter 1 (1:10,000, IHC) (cat. no. 1:2,500 (IHC), Millipore, Temecula, CA, cat. no. AB9504), guinea pig polyclonal anti-Bassoon (1:300, Synaptic Systems, Gottingen, Germany, cat. no. 141 004), rabbit anti-Bassoon (1:300, cat. no. 141 003, Synaptic Systems), rabbit anti-RFP (1:500, Rockland, Limerick, PA, cat. no. 600-401-379), rabbit anti-GFP (1:500, Abcam, Cambridge, MA), secondary antibody: donkey anti-mouse IgG2A Atto 425 (1:250, Rockland, Inc., cat. no. 610-151-041), goat anti-mouse IgG Anti-467 (1:500, cat. no. 610-156-040, Rockland, Inc., goat anti-rabbit AlexaFluor-488 (1:500, Jackson Immunoresearch, cat. no. 711-545-152), donkey anti-rabbit AlexaFluor-488 (1:500, Jackson Immunoresearch, cat. no. 711-545-152), donkey anti-mouse AlexaFluor-594 (1:500, Jackson Immunoresearch, cat. no. 715-585-150), donkey anti-guinea pig AlexaFluor-594 (1:500, Jackson Immunoresearch, cat. no. 706-586-148), donkey anti-guinea pig AlexaFluor-647 (1:500, Jackson Immunoresearch, cat. no. 706-605-148).

Chemical LTP. NMDAR-dependent cLTP was induced by treatment of DIV21–25 cortical neurons transfected with tdTomato or pFGEG-PSD-95-EGFP/hSYN-1 into CUBIC-2 reagent-2 (50% sucrose (BP220-1, Fisher Scientific), 25% urea, 1% triethanolamine (cat. no. T83800 Sigma) and 0.1% Triton-X100) overnight at room temperature. The cleared sections were then mounted using CUBIC-2 reagent on slides, covered with a 0.15 µm cover glass and used for imaging.
Imaging. STED nanoscopy. Dual-color imaging of synaptic structures in fixed and immunostained cultured cortical neurons was conducted on a Leica TCS SP5 STED CW confocal microscope (Leica Microsystems, Mannheim, Germany) containing a 592 nm CW depletion line. Images of fixed neurons were acquired as single optical sections using a resonance scanner (8,000 Hz scanning), a HyD detectors (set between 100% and 200%) and 100× oil immersion objective (Leica) with 5–10× zoom to obtain 15–30 nm pixel size. The 442-nm and 488-nm lines were used to excite the Atto-425-labeled PSD-95 and the AlexaFluor-488-labeled vGlut1, respectively. The 592-nm depletion line (at 90–100% power) was used to reduce the point spread function (PSF) for both fluorophores to ~80 nm. Images were deconvolved using the 80 nm PSF in Leica TCS SP5 software and analyzed in Imagi (NIH, Bethesda, MD).

For chemical LTP experiments, time-lapse live images were acquired using a confocal spinning-disk system equipped with a Yokogawa CSU-10 and Hamamatsu EM-CCD camera (Hamamatsu Photonics, Bridgewater, NJ) attached to an inverted microscope (Nikon, Tokyo, Japan) and imaged in immersion oil. Optical sections spaced at 0.3μm were used to acquire 1–2 μm image stacks of dendrites using a 100× oil immersion objective. Adaptive focus control (Leica) was used to minimize focus shifts during 3-h image acquisition. After completion of live imaging neurons were immediately fixed, stained and subjected to STED imaging. Single optical sections of the same dendritic spines that were imaged live were next imaged with super-resolution (~80 nm) using Leica TCS SP5 STED CW as described above.

Live-cell STED chemical LTP. In vivo experiments and three-color STED experiments of cultured cortical neurons were performed using a Leica TCS SP8 gated STED (GSTED) confocal microscope equipped with a tunable white light laser, CW 592 nm and 660 nm depletion lines and a pulsed 775 nm depletion line. Live-cell STED images of PSD-95–EGFP and mTurquoise2–synaptophysin-1 and confocal images of cell-filling tdTomato were acquired as stacks (~1 μm) with a 100x oil immersion objective using a resonant scanner (8,000 Hz) and gated HyD detectors (set at 150–200%). Both EGFP and mTurquoise2 images were acquired using Leica TCS SP8 STED CW as described above.

For analysis of PSD-95 and vGlut1 clusters in vivo, outlines of spines were determined in individual z sections of thresholded images. The spine outlines were then overlaid onto the thresholded images of the channels corresponding to PSD-95 and vGlut1 clusters. From these assignments, spine colocalization of each cluster was made independently for each z section. Orthogonal views of the overlap between stacks were analyzed to verify that individual spines were associated with individual spines in the z plane. Finally, image stacks were overlaid and filtered by an edge-preserving algorithm in Imaris software (Bitplane AG). High-contrast images of puncta within the area that corresponded to the size of the spine head and shaft (approximately 100 × 100 pixels) were projected in Imaris to generate high-contrast volume-rendered images. Volume rendering was performed for each channel separately using a two-voxel separation between thresholded objects. Thresholded clusters that did not colocalize with the area of the spine were discarded. As in the in vitro cluster analysis, the PSD-95 and vGlut1 channels were binarized separately using intensity thresholds (mean + 2 × s.d. of intensity values within an area of 400 × 400 pixels). Cluster separation was determined as described above for the in vitro cluster analysis. Due to tissue expansion following the CUBIC treatment 46, the separation between aligned pre- and postsynaptic clusters was accepted to be between 100–200 nm by measuring the intensity peaks of aligned clusters. Data for both in vitro and in vivo spine analysis of module number represent observations and were acquired and analyzed without an experimenter blinding. For the live-cell LTP and retrospective STED analysis, an experimenter was blinded to the condition and the effect of spine size change by first identifying the cluster numbers and then revealing the change in spine morphology for a given condition.

Analysis of live-cell STED. The 4D (x, y, z, t) deconvolved image stacks acquired from the live-cell STED experiments were aligned using ImageJ macros (Stack reg and Turbo reg) using a rigid body transformation based on the morphology of neuronal dendrites and then were analyzed as maximum-intensity projections. Dendritic spines were visualized using a Gaussian blur (2 pixels) to the maximum projections of the tdTomato channel. The presence of PSD-95–EGFP and mTurquoise2–synaptophysin-1 clusters was assessed for each channel separately in each spine. To identify individual clusters, intensity thresholds were generated using the mean + 2 × s.d. of a local 50 × 50 pixel area corresponding to the average size of a spine head. The appearance of new clusters was determined visually from time-lapse image series by using the manual tracking algorithm in ImageJ. The identity of vGlut1 clusters was confirmed from intensity changes by measuring the line profile intensity. New clusters were defined when there was >20% difference between peak intensities of individual clusters and the trough between the peaks of the clusters.

The alignment of pre- and postsynaptic nanomodules in live-cell STED experiments was quantified using two metrics based on 2D projections of the images (Fig. 6). First, the deviation (θ) from perfect alignment (a 90°-degree angle, θ = 0°–90°) between the centers of PSD-95–EGFP and SYP-mTurq2 modules was measured along the long axis of the SYP-mTurq2 centroid was calculated at each time point and summed. This measure reflects the relative apposition of the two modules. Second, the distance (d) between the centers of PSD-95–EGFP and SYP-mTurq2 centroids was calculated for each time point. The average angular deviation and centroid distance during the 3-h imaging period were then calculated for each condition. Centroids for SYP-mTurq2 and PSD-95–EGFP were generated using the DrawEllipse plugin in ImageJ (https://imagej.nih.gov/ij/macros/DRAWoE.jpg) by manually tracing each nanomodule at each time point.
Monte Carlo simulations. The super-resolved images of PSD-95 and vGlut1 from in vitro and in vivo experiments were thresholded as described above. For in vitro data, thresholded masks for each channel were generated using maximum intensity projection images of the entire z-stack (≤1 μm). Thresholded images for brain sections were generated from a substack corresponding to the average thickness of a spine (~1 μm). Using thresholded masks, we modeled the probability of overlap between randomly positioned spines of varying areas and actual PSD-95, vGlut1, SYP-1 and Bassoon nanomodule images (Supplementary Figs. 6, 7 and 9). Spine positions were randomized using the random number generator macro in ImageJ. Clusters were assigned as belonging to simulated spines using the same criteria that were used to manually assign postsynaptic and presynaptic nanomodules in our dataset—PSD-95 was included only when puncta were entirely within the simulated spine area and presynaptic nanomodules were included only when puncta were within or touching the simulated spine (Supplementary Fig. 6c). Simulations were performed on three randomly selected images from independent experiments. For each channel, simulated spines of a specific size were placed at 500 randomly selected locations overlaid onto each raw image selected using an ImageJ macro. Simulations were conducted in three independent runs for the total of 1,500 simulations per spine size. Only simulated spines placed randomly at locations that contacted puncta of synaptic marker proteins were included for further analysis.

Statistical analysis. Data were acquired and analyzed based on the standards in the field; however, no method of randomization was used to determine how samples were allocated to experimental groups and processed. Data are expressed as mean ± S.E.M. All data points collected were included for analysis. Statistical significance of the differences among groups were determined by one-way analysis of variance followed by post hoc tests as described in individual figure legends, or by two-tailed Student’s t-test when testing differences between two conditions. A Kruskal–Wallis test was used to test differences between cumulative probability distributions, as well as the differences between FRAP recovery distributions. P values less than 0.05 were considered statistically significant. For P values less than 0.0001, we provide a range and not the exact number. See Supplementary Table 1 for statistical details. Distribution of the data was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes, but the sample sizes are similar to those reported in previous publications. Group differences in variance were tested for each dataset and determined to be similar. Unless stated otherwise, statistical tests were conducted on a per-spine basis, from cortical neurons collected from a minimum of three independent transfection experiments or animals (Supplementary Table 1).

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All relevant data and analysis are within the paper and its Supplementary Information files. Raw image stacks are available upon request to M.B.D.

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### Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - Sample sizes were determined so that experiments be replicated to ensure confidence in the results and were based on previous studies (Dalva et al., 2000, Kayser et al., 2008, McClelland et al., 2010 and Nolt et al., 2011). For all experiments there was enough statistical power to detect the corresponding effect size. Reported in the Methods in "Statistical analysis" subsection.

2. **Data exclusions**
   - Describe any data exclusions.
   - All data acquired were included in analyses. This is reported in the Methods in "Statistical Analysis" subsection.

3. **Replication**
   - Describe the measures taken to verify the reproducibility of the experimental findings.
   - Each experiment was replicated a minimum of three times and data was reliably reproduced with each replication attempt. The number indicating how many times each experiment was replicated is indicated in corresponding figure legends.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Data were acquired and analyzed based on the standards in the field, however, no method of randomization was used to determine how sample were allocated to experimental groups and processed. This is reported in the Methods in "Statistical Analysis" subsection.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Data for both in vitro and in vivo spine analysis of module number represent observations and were acquired and analyzed without an experimenter blinding. For the live-cell cLTP and retrospective STED analysis, an experimenter was blinded to the condition and the effect of spine size change by first identifying the cluster numbers and then revealing the change in spine morphology for a given condition. This is reported in the Methods in "Image Analysis" subsection.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|----------|
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| ☐   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐   | A statement indicating how many times each experiment was replicated |
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| ☐   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ✓   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☐   | Test values indicating whether an effect is present |
| ☐   | Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted. |
| ✓   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
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7. Software

Software availability of computer code

Describe the software used to analyze the data in this study.

No programs were written for this manuscript. ImageJ macro for Monte Carlo analysis of puncta was generated using built-in random number generator plug-in. This is reported in the Methods in "Monte Carlo simulations" subsection on page 36. DrawEllipse plug-in in ImageJ (https://imagej.nih.gov/ij/macros/DrawEllipse.txt) was used to generate centroids around each nanomodule for distance and angle analysis. Described in the Methods in "Analysis of live-cell STED" section. Deconvolution of images was performed either in SP5 Leica Application Suite Advance Fluorescence Software or in Huygens deconvolution software as described in the Methods in "Image processing and deconvolution" and in Figures S1, S2 and S3.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

No unique materials were used in this study. All materials used are commercially available through vendors described in the Methods.
9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All primary and secondary antibodies were profiled in our previous publications and published work from other labs and were reported to be specific (Kayser et al., 2008, McClelland et al., 2010, Noit et al., 2011, Hruska, et al, 2015 and Hanamura et al, 2017, Tang et al, 2016). We further validated secondary antibodies used in this study for their compatibility with STED in Figure S1. Primary antibodies: mouse monoclonal (IgG2A) anti-PSD-95 clone K28/43 (1:1000 (ICC) or 1:200 (IHC), Neuromab, UC Davis, Davis, CA, clone 28/43), mouse monoclonal (IgG1) anti-Synaptophysin-1 (1:1000, cat # 101 111, Synaptic Systems, Gottingen, Germany), guinea pig polyclonal anti-vesicular glutamate transporter 1 (-vGlut1; 1:5000 (ICC) or 1:2500 (IHC), Millipore, Temecula, CA, cat#: AB5905), guinea pig polyclonal anti-Bassoon (1:300, Synaptic Systems, Gottingen, Germany), rabbit anti-Bassoon (1:300, cat #: 141 003, Synaptic Systems), rabbit anti-RFP (1:500, Rockland, Limerick, PA, cat# 600-401-379), rabbit anti-GFP (1:500, Abcam, Cambridge, MA, cat# ab290). Secondary antibodies: Goat anti mouse IgG2A Atto 425 (1:250, Rockland, Inc., cat# 610-151-041), Goat anti-mouse IgG1 Atto-647N (1:500, cat # 610-156-040, Rockland, Inc.), Goat anti-rabbit Atto-647N (1:500, cat # 611-156-122, Rockland, Inc.), Donkey anti-guinea pig AlexaFluor-488 (1:500, Jackson ImmunoResearch, West Grove, PA, cat# 706-545-148), Donkey anti-rabbit Cy3 (1:500, Jackson ImmunoResearch, cat# 711-165-152), Donkey anti-rabbit AlexaFluor-488 (1:500, Jackson ImmunoResearch, cat# 711-545-152), Donkey anti-mouse AlexaFluor-594 (1:500, Jackson ImmunoResearch, cat# 715-585-150), Donkey anti-guinea pig AlexaFluor-594 (1:500, Jackson ImmunoResearch, cat # 706-586-148), Donkey anti-guinea pig AlexaFluor-647 (1:500, Jackson ImmunoResearch, cat# 706-605-148). This is reported in the Methods in "Antibodies" sub-section.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No cell lines were used in this study.

No cell lines were used in this study.

No cell lines were used in this study.

No cell lines were used in this study.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

All animal studies were approved by the Institutional Animal Care and Use Committee guidelines at Thomas Jefferson University in accordance with US National Institutes of Health guidelines. Mouse pups (P7) for lentiviral transduction experiments were obtained from timed pregnant CD-1 mice purchased from Charles River Laboratories Inc. (Wilmington, MA) and housed (3-5 mice per cage) in Thomas Jefferson University's laboratory animal facility. Standard housing was used with normal light/dark cycle. E17-18 rat embryos from timed pregnant animals purchased from Charles River Laboratories Inc. (Wilmington, MA) were used to make primary cortical neuron cultures. Both male and female mouse and rat pups were used for the experiments. This is reported in the Methods in "Animals" and "Lentiviral transduction" subsections.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human subjects were used in this study.