Mapping the dynamics and nanoscale organization of synaptic adhesion proteins using monomeric streptavidin

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The advent of super-resolution imaging (SRI) has created a need for optimized labelling strategies. We present a new method relying on fluorophore-conjugated monomeric streptavidin (mSA) to label membrane proteins carrying a short, enzymatically biotinylated tag, compatible with SRI techniques including uPAINT, STED and dSTORM. We demonstrate efficient and specific labelling of target proteins in confined intercellular and organotypic tissues, with reduced steric hindrance and no crosslinking compared with multivalent probes. We use mSA to decipher the dynamics and nanoscale organization of the synaptic adhesion molecules neurexin-1β, neuroligin-1 (Nlg1) and leucine-rich-repeat transmembrane protein 2 (LRRTM2) in a dual-colour configuration with GFP nanobody, and show that these proteins are diffusionally trapped at synapses where they form apposed trans-synaptic adhesive structures. Furthermore, Nlg1 is dynamic, disperse and sensitive to synaptic stimulation, whereas LRRTM2 is organized in compact and stable nanodomains. Thus, mSA is a versatile tool to image membrane proteins at high resolution in complex live environments, providing novel information about the nano-organization of biological structures.

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Critical cellular functions including adhesion and signalling are performed by dynamic macromolecular platforms at the cell membrane. Synthetic neuronal contacts are examples of such complex structures, where protein concentration is extremely high and lies in a very confined and compartmentalized space. Recently developed fluorescence-based super-resolution imaging (SRI) techniques, including Stimulated Emission Depletion (STED)\(^2\), PhotoActivation Localization Microscopy (PALM)\(^3,4\), direct Stochastic Optical Reconstruction Microscopy (dSTORM)\(^5,6\) and universal Point Accumulation for Imaging in Nanoscale Topography (uPAINT)\(^7,8\), provide 20–50 nm resolution maps of single-molecule localization in biological samples, allowing a better understanding of the organization and turnover of these submicron multisubunit assemblies. To accompany such progress in imaging power, there is a pressing need for efficient labelling strategies relying on small and penetrating probes that provide high signal-to-noise ratio and minimal linkage error with respect to target proteins.\(^10,11\)

Protein labelling has relied for a long time on the use of antibodies, which have the strong advantage of targeting endogenous proteins. Yet, full-length antibodies are relatively large (10–15 nm) compared with nanoscale biological objects, thereby causing potential steric hindrance and localization bias.\(^1,12\) Moreover, because of their valence, antibodies may induce protein crosslinking at the cell surface. The recent expansion of antibody fragments such as Fab, scFv or VHH is promising improvements,\(^13,14\) however, their development including antigen preparation is time-consuming.\(^15\) Alternative labelling approaches consist of the use of recombinant proteins fused to fluorescent proteins (FPs, photoactivatable or photo-switchable proteins),\(^3,16\) reactive moieties such as modified enzymes to bind small dye-labelled synthetic molecules or small peptide tags for subsequent conjugation with exogenous fluorescent ligands.\(^17,18\)

However, FPs usually have weaker fluorescence compared to organic dyes, and the insertion of a FP or enzyme moiety may affect proper protein folding and/or function.\(^21,22\)

In this context, we searched for an efficient and orthogonal labelling approach combining minimal tag size on the protein of interest, probe penetrability in confined regions, absence of artefact due to multivalence and compatibility with multicolour imaging. We propose a new method based on streptavidin monomers (mSA) to deliver bright organic fluorophores to proteins that are enzymatically biotinylated on a 15-amino-acid acceptor peptide (AP) tag.\(^23\) This targeted biotinylation strategy was previously developed in combination with monovalent streptavidin to track membrane molecule dynamics using quantum dots.\(^24\) However, monovalent streptavidin remains a relatively large heterotetramer (molecular weight of 60 kDa and ~ 6 nm across) and is difficult to produce because of the precise 3-to-1 stoichiometry that is required among the subunits.\(^3\)

mSA is a structural monomer evolved from wild-type streptavidin tetramer with the aim of maximizing aqueous stability (denaturation temperature of 60 °C) while maintaining sufficient biotin affinity (\(K_d = 2.8 \text{ nM}\))\(^25,26\). The resulting mSA is a stable 3-nm labelling probe with a molecular weight of 12.5 kDa, which is simple to produce and to couple to various fluorophores for SRI applications.

We demonstrate that fluorescently conjugated mSA is compatible with a wide range of microscopy techniques, including uPAINT, Fluorescence Recovery After Photobleaching (FRAP), STED and dSTORM, in both live and fixed conditions. We further show that, unlike antibodies or streptavidin, mSA labels its targets without crosslinking and penetrates deep into live tissues. We applied mSA-based labelling to probe several synapticogen adhesion proteins in neurons, including the presynaptic neurexin-1β (Nrx1β), and its postsynaptic binding partners neurelin-1 (Nlg1), and leucine-rich-repeat transmembrane protein 2 (LRRTM2), against which good antibodies compatible with live cell imaging are lacking. We demonstrate differential nanoscale organization and dynamics of Nlg1 and LRRTM2, which may underlie divergent physiological roles at the synapse.

**Results**

**mSA labels biotinylated proteins at the cell surface.** Recombinant mSA was produced in bacteria, purified by affinity chromatography via a 10-His N-terminal tag and covalently conjugated to photostable organic dyes Atto 594 or Atto 647 for uPAINT and STED experiments, respectively, or to Alexa 647 for dSTORM imaging, using standard N-hydroxysuccinimide (NHS) ester coupling onto solvent-exposed primary amines (Supplementary Fig. 1). Fluorophore-conjugated mSA was used to label membrane proteins in neurons, COS-7 or HEK-293 cells that incorporate an extracellular AP tag. Biotinylation of the AP tag occurred during protein maturation by co-expressed Escherichia coli biotin ligase, BirA\(^\text{ER}\) (ref. 17). When added to the extracellular imaging solution, the mSA–dye conjugate bound specifically to cells co-expressing AP-tagged proteins and BirA\(^\text{ER}\), and not to cells expressing either haemagglutinin (HA)-tagged proteins and BirA\(^\text{ER}\), or AP-tagged proteins but lacking BirA\(^\text{ER}\) (Supplementary Fig. 2a–d). mSA labelling was efficient on all cell types and membrane proteins tested and produced images with high signal-to-noise ratio. The relatively fast dissociation kinetics of mSA (\(k_{\text{off}} = 1.05 \times 10^{-2} \text{s}^{-1}\))\(^25,26\) allow efficient release of bound mSA with excess free biotin. In this regard, addition of 200 μM biotin to the labelling solution resulted in rapid loss of the mSA label (Supplementary Fig. 2e,f). Such reversible binding may be exploited to visualize internalized protein complexes using competition with modified biotin having reduced membrane permeability\(^27\), and synthesized for this study (Supplementary Fig. 3 and Supplementary Movie 1). Covalent labelling or labelling with high-affinity streptavidin derivatives, for example, monovalent tetramer (\(k_{\text{off}} \sim 4.2 \times 10^{-6} \text{s}^{-1}\))\(^17,24,28\), would not allow such measurements. Taken together, these results demonstrate the high specificity of mSA labelling for recombinant biotinylated proteins in a cellular context.

**mSA reports on protein dynamics without crosslinking.** To evaluate the impact of probe valence on observed protein dynamics, we used uPAINT\(^8\) to measure the diffusion of biotinylated AP-tagged Nlg1 (AP-Nlg1) in neurons that were labelled with divalent biotin antibody, tetrameric streptavidin or mSA conjugated with Atto 594 (Fig. 1a and Supplementary Fig. 1). The three probes were added at a low concentration (1 nM) to isolate single Nlg1 molecules diffusing on the cell surface. Neurons electroporated with AP-Nlg1 exhibited a twofold stronger surface labelling with Nrx1β-Fc and twofold higher anti-Nlg1 signal in western blots than control neurons expressing empty vector or Homer1c-green fluorescent protein (GFP), indicating a ratio of approximately one overexpressed AP-Nlg1 molecule for one endogenous Nlg1 molecule (Supplementary Fig. 4). In immature neurons, at 7 days in vitro (7 DIV), the distribution of AP-Nlg1 diffusion coefficients after mSA labelling was centred around 0.1 \(\mu\text{m}^2 \text{s}^{-1}\), revealing fast Brownian motion (Fig. 1b,c). When the movement of AP-Nlg1 was tracked with Atto-conjugated biotin antibody or tetrameric streptavidin, the measured diffusion coefficients were shifted towards lower values (Fig. 1b,c) and there was a concomitant...
increase in the fraction of slowly diffusing molecules (Fig. 1d), defined as $D < 0.01 \text{ mm}^2 \text{s}^{-1}$ (Supplementary Fig. 5). The surface area covered by AP-Nlg1 trajectories was also higher with mSA than with biotin antibody or streptavidin (Fig. 1b). Together, these results suggest that divalent and tetravalent probes alter AP-Nlg1 distribution and dynamics through a combination of protein crosslinking and steric hindrance.

**mSA prevents aggregation compared with multivalent probes.**

To examine the effects of probe size and valence on Nlg1 distribution at the nanoscale, we used dSTORM to image Nlg1 in mature neurons (DIV 15)\textsuperscript{5,29,30}. Membrane-bound AP-Nlg1 was labelled with 100 nM mSA, biotin antibody or streptavidin conjugated with Alexa 647 for 10 min before fixation and observation. In contrast to our uPAINT experiments, which rely on sparse labelling and short acquisition sequences to focus on protein dynamics, dSTORM uses high-density labelling combined with long acquisition times (800 s) to produce a static representation of the overall protein organization. In parallel, postsynaptic densities were identified using co-expressed Homer1c-GFP\textsuperscript{31,32}. mSA labelling was fairly diffuse in the shaft and highly concentrated at synapses (Fig. 1e). In contrast, both biotin antibody and streptavidin induced the aggregation of

### Figure 1 | Super-resolution imaging of AP-Nlg1 with mSA or biotin antibody or tetrameric streptavidin.

(a) Schematic diagram of AP-Nlg1 labelled with three different probes (mSA, monoclonal biotin antibody or streptavidin), conjugated to Atto 594 for uPAINT or Alexa 647 for dSTORM. (b) Examples of DIV 7 neurons co-expressing EGFP as a volume marker, AP-Nlg1 and BirA\textsuperscript{ER}, and labelled as described above. Middle panels display AP-Nlg1 trajectories calculated from stacks of 4,000 images with 20-ms exposure time (green: fast-diffusing pool, that is, $D > 0.01 \text{ mm}^2 \text{s}^{-1}$; magenta: slow diffusing pool, that is, $D < 0.01 \text{ mm}^2 \text{s}^{-1}$). Merged images show EGFP (grey) overlaid with AP-Nlg1 trajectories. Note that mSA explores larger surface areas with faster diffusion than multivalent ligands. (c) Distribution of AP-Nlg1 diffusion coefficients in a semi-log plot, where the grey-shaded area represents slow trajectories (that is, with $D < 0.01 \text{ mm}^2 \text{s}^{-1}$). (d) Corresponding percentage of slow trajectories measured in the three different conditions ($**P < 0.0001$). Data are from three different experiments. (e) Examples of DIV 15 neurons co-expressing Homer1c-GFP, AP-Nlg1 and BirA\textsuperscript{ER}, and labelled with the three Alexa647-conjugated probes shown in a. Top panels: Homer1c-GFP signal showing mature synapses (white). Middle panels: super-resolved AP-Nlg1 detection maps generated from 40,000 frames with 20-ms integration time. Bottom panels: merged images showing AP-Nlg1 detections (green) overlaid with Homer1c-GFP (magenta). Note the presence of large AP-Nlg1 aggregates in anti-biotin and streptavidin-labelled neurons. (f) Histogram showing the number of Nlg1 clusters per $\mu$m$^2$ in the three conditions ($**P < 0.01$). (g) Histogram showing the synaptic enrichment of AP-Nlg1 compared with the shaft ($**P < 0.01$, $*P < 0.05$). Data are from two different experiments. Numbers in the bar charts represent the number of cells examined.
AP-Nlg1 into numerous small clusters on dendrites (Fig. 1e,f), which are most likely caused by ligand-mediated receptor crosslinking. The anti-biotin-labelled AP-Nlg1 was found mostly on the edge of the synapses around rather than inside, presumably because the antibody has limited accessibility to the synaptic cleft because of its large size. With its intermediate size, tetrameric streptavidin penetrated well inside synapses, but because of massive extrasynaptic clustering was less enriched than anti-biotin at synapses compared with shaft levels. Overall, the enrichment of AP-Nlg1 at Homer1c-GFP-positive puncta was significantly higher for mSA compared with streptavidin and antibody (Fig. 1g), indicating that mSA enables a more accurate visualization of receptor localization and organization at the nanometre scale.

**mSA efficiently labels Nlg1 in the confined synaptic cleft.** To assess the ability of mSA-labelled Nlg1 to dynamically access the synaptic cleft (20 nm across), we performed uPAINT experiments on live DIV 10–15 neurons expressing AP-Nlg1, and labelled using either mSA or biotin antibody conjugated to Atto594. Using mSA, we observed a gradual decrease in AP-Nlg1 diffusion across neuronal development (Supplementary Fig. 6a–c), revealing the stabilization of Nlg1 associated with an increase in synapse density and maturation33. On DIV 15, AP-Nlg1 trajectories recorded with mSA populated both the dendritic shaft and synapses and substantially overlapped with the synaptic Homer1c-GFP staining (Fig. 2a and Supplementary Movie 2), whereas AP-Nlg1 trajectories obtained with biotin antibody were mostly localized on the dendritic shaft and confined to areas around the postsynaptic density. Quantitatively, 85% of synapses contained mSA-labelled Nlg1, while only 40% contained antibody-labelled Nlg1 (Fig. 2b), and the synaptic area covered by mSA-labelled Nlg1 was fourfold higher than antibody-labelled Nlg1 (30% versus 8%, respectively; Fig. 2c). mSA-labelled Nlg1 exhibited fast diffusion in extrasynaptic compartments and slower diffusion at synapses (Fig. 2d), reflecting interactions with presynaptic axonal adhesion protein, Nrx and/or postsynaptic scaffolding proteins32. In contrast, anti-biotin labelling showed consistently slower diffusion across the entire neuronal surface, similar to the observations on DIV 7 (Fig. 1c). An additional level of immobilization of mSA-labelled AP-Nlg1 on DIV 15 was observed on incubation with soluble dimeric Nrx1β-Fc (Supplementary Fig. 6d,e), which is consistent with the preferential anchorage of Nlg1 to synaptic PSD-95 triggered by Nrx1β binding32. These observations show that mSA can be used to label both fast- and slow-moving AP-Nlg1 in developing neurons, and to visualize trapping events in mature synapses without introducing steric and valence bias compared with divalent antibodies.

**mSA labels target proteins in organotypic brain tissue.** Given the small size and high labelling efficiency of mSA, we tested its capability to label neurons in dense organotypic tissue. We co-expressed GFP, AP-Nlg1 and BirAER in individual CA1 pyramidal neurons from 150-μm-thick mouse organotypic brain tissue. Figure 2 | Different ability of mSA and biotin antibody to label AP-Nlg1 within the synaptic cleft in live conditions. (a) DIV 15 neurons expressing Homer1c-GFP, AP-Nlg1 and BirAER were labelled with mSA or anti-biotin conjugated to Atto594 to track individual AP-Nlg1 molecules by uPAINT. From top to bottom: Homer1c-GFP signal staining mature synapses; super-resolved AP-Nlg1 detection maps; merged images showing extrasynaptic AP-Nlg1 trajectories (green) and synaptic trajectories (magenta) overlaid with Homer1c-GFP signals (grey); insets show that mSA-stained Nlg1 fills the entire synaptic area, whereas antibody remains on the edge of the postsynaptic density. (b) Percentage of synapses containing AP-Nlg1 labelled with mSA or biotin antibody (***P < 0.0001). (c) Percentage of the synaptic area occupied by AP-Nlg1 when labelled with mSA or anti-biotin (**P < 0.01). (d) Semi-log distribution of synaptic (solid lines) and extrasynaptic (dashed lines) diffusion coefficients for AP-Nlg1 measured with the two different probes. Data are from two different experiments. Numbers in the bar charts represent the number of cells analysed.
hippocampal slices using single-cell electroporation. Slices were labelled by one-step incubation with 100 nM mSA-Atto 647N (for STED) or mSA-Alexa647 (for dSTORM), then rinsed and observed live (STED) or after fixation (dSTORM). The mSA-labelling of AP-Nlg1 was highly specific since it stained only GFP-co-expressing cells throughout the slice with extremely low background, and localized exclusively to somatodendritic regions (Fig. 3a,b and Supplementary Movie 3). The photostability of Atto dyes combined with the thermal stability of mSA allowed an extensive live STED imaging of optical sections at 37°C down to 40 μm deep. Zoom on dendritic portions showed specific enrichment of Nlg1 in spines compared with GFP (Fig. 3c,d and Supplementary Movie 4), which is also observed in dissociated hippocampal cultures (Fig. 1e–g).

Using conventional confocal microscopy, we evaluated how well mSA penetrates into brain slices compared with streptavidin and biotin antibodies. mSA and tetrameric streptavidin were able to label AP-Nlg1 expressed in live electroporated neurons within a few minutes, although the labelling efficiency of streptavidin decreased rapidly with depth, suggesting an impact of the probe size and/or valence in reaching deep tissues (Supplementary Fig. 7a,b). At the same sample depth (~15 μm below the surface of the slice), the anti-biotin signal was not detectable after a 10-min live labelling, but became apparent after 1 h (Supplementary Fig. 7c). Finally, we used astigmatism-based three-dimensional (3D) dSTORM to image the distribution of mSA-labelled AP-Nlg1 in small dendritic regions on the surface of a slice from Nlg1 knockout (KO) mice, revealing high enrichment of Nlg1 in spines (Fig. 3e,f and Supplementary Movie 5). These results show that mSA penetrates deep into thick tissues, where it can be used to label target proteins at high density and with specificity for immediate application in SRI. Interestingly, a similar AP-Nlg1 localization was observed in STED images made on organotypic slices from wild-type mice, and in STORM images made on organotypic slices from Nlg1 KO mice, where AP-Nlg1 expressed in CA1 cells replaces endogenous Nlg1.

**Figure 3 | Super-resolution imaging of AP-Nlg1 in organotypic hippocampal slices.** (a) Confocal (EGFP, left), STED (mSA-Atto 647N, middle) and merged (right) images acquired from live neurons expressing GFP, AP-Nlg1 and BirAER in organotypic hippocampal slices. Images are projections of a z-stack of 60 planes taken by 1-μm increments and colour-coded with respect to sample depth. (b) Linescan measurements of mSA-Atto 647N and GFP staining along the z axis. The reduction in mSA-Atto 647N intensity with the sample depth likely reflects reduced laser penetration rather than weaker staining, since the GFP signal also decreases with depth. (c) High-magnification deconvoluted STED projection of mSA-labelled Nlg1 in a hippocampal slice shows the accumulation of Nlg1 at dendritic spines. (d) Linescans of GFP and mSA-Atto 647N fluorescence intensity in the shaft membrane and in a dendritic spine normalized to the respective fluorescence of GFP and Atto 647N in the shaft. (e) Wide field image of mSA-Alexa647 selectively labelling one neuron expressing GFP, AP-Nlg1 and BirAER, a few microns deep from the surface of an organotypic brain slice. (f) Astigmatic-based 3D dSTORM imaging in an organotypic brain slice from Nlg1 KO mice: (i) wide field image of mSA-Alexa647-labelled AP-Nlg1. (ii) 3D dSTORM-reconstructed image of a dendritic segment based on 1,038,506 single-molecule localizations from 64,000 images. The image is colour-coded with respect to the z distance (~600 to +600 nm). (iii) Normalized localization detection maps integrated within z = ±400 nm. (iv) Magnified view of iii showing the enrichment of Nlg1 in dendritic spines.
mSA and GFP nanobody similarly probe protein diffusion. Monomeric VhH antibodies (nanobodies) against GFP and red fluorescent protein have been previously used for SRI, where their small size provides smaller linkage error relatively to the target structure.\textsuperscript{13,14} We thus sought to compare mSA to these well-characterized tools. To this end, we labelled the same axonal molecular target, Nrx1β, using both approaches and compared the measured diffusion dynamics and membrane organization. On the one hand, we labelled Nrx1β carrying a biotinylated N-terminal AP tag with mSA-Atto594. On the other hand, Nrx1β fused with an N-terminal GFP tag was labelled with Atto594-conjugated GFP nanobody, resulting in a specific enrichment at excitatory pre-synapses immunostained for the vesicular transporter VGlut1 (Fig. 4a). Single-molecule trajectories were then recorded for each labelling scheme using uPAINT in DIV 15 neurons (Fig. 4b,c). The distributions of diffusion coefficients were very similar for the two probes (Fig. 4d), with a major fast diffusion peak centred around $0.5 \mu m^2 s^{-1}$, and a minor slower diffusion peak around $0.005 \mu m^2 s^{-1}$, representing Nrx1β confined at presynaptic terminals.\textsuperscript{34,35} These results show that, in agreement with their small size and monomeric nature, mSA and nanobody labelling lead to similar diffusion measurements. Given their orthogonality, the two approaches may thus be combined for dual-colour SRI.

The AP tag can be inserted into small extracellular loops. An advantage of using mSA is that the short 15-amino-acid AP tag can be readily incorporated into extracellular protein domains, such as protein loops, without perturbing the native function. To demonstrate the use of the AP tag in a system that may not be amenable for GFP fusion, we inserted the AP sequence in the first extracellular loop of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor auxiliary protein stargazin (AP-Stg)\textsuperscript{36} (Fig. 4e). Using mSA-Atto594, we observed the localization and dynamics of AP-Stg in DIV 15 neurons by uPAINT (Fig. 4f). AP-stargazin localized at synapses where its diffusion was reduced, consistent with previously published work\textsuperscript{37} (Fig. 4f,g). Thus, the mSA-labelling strategy can be successfully applied to situations where the use of a GFP tag may not be allowed for structural or functional reasons. To further illustrate this point, we inserted the mCherry

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**Figure 4 | Comparison of mSA and GFP nanobody.** (a) DIV 15 axons expressing GFP-Nrx1β were live-labelled using anti-GFP nanobody-Atto647, followed by immunolabelling for VGlut1 to stain pre-synaptic glutamatergic terminals. The merged image shows the colocalization between GFP-Nrx1β (red) and VGlut1 (green). (b) Schematics showing the labelling of AP-Nrx1β with mSA-Atto594 (top) and GFP-Nrx1β with GFP nanobody-Atto594 (bottom). (c) Examples of axonal regions from DIV 15 neurons expressing EGFP, AP-Nrx1β and BirA\textsuperscript{ER} (top); or GFP-Nrx1β (bottom). From left to right: GFP signal, Nrx1 β detection maps, colour-coded Nrx1β trajectory maps (green: fast-diffusing pool, that is, $D > 0.01 \mu m^2 s^{-1}$; magenta: slow diffusing pool, that is, $D < 0.01 \mu m^2 s^{-1}$), merged image showing Nrx1β trajectories overlaid with GFP (grey). (d) Distributions of the diffusion coefficients obtained for AP-Nrx1β or GFP-Nrx1β (mSA, $n = 6$; GFP nanobody, $n = 5$ cells from two different experiments). (e) Schematics showing the structure of the AMPA receptor auxiliary protein stargazin (Stg) with the insertion of an AP tag in the first extracellular protein loop. (f) Example of DIV 15 neurons co-expressing the synaptic marker Homer1c-GFP, AP-Stg and BirA\textsuperscript{ER}. AP-Stg individual molecules were tracked using mSA-Atto594 by uPAINT. Super-resolved AP-Stg localization and trajectory maps were reconstructed from 4,000 images of 20-ms exposure time, with the same colour code as in b. (g) Diffusion coefficient distribution of AP-Stg inside and outside synapses ($n = 6$ cells from two different experiments).
calcium chelator decreased the number of synaptic Nrx1b long-emitting fluorescent signals coming from the same location. of molecules at the synapse, whose intensity compared with conditions, these domains reflect the trapping of a small number of both proteins (Supplementary Fig. 9). Both changes are likely caused by a dissociation of calcium-dependent Nrx1b/Nlg1 adhesion in response to synaptic stimulation in live conditions. We treated neurons with 20 μM N-methyl-d-aspartate (NMDA) for 10 min to chemically induce synaptic depression and monitored Nrx1b/Nlg1 dynamics by uPAINT. NMDA caused a specific and time-dependent decrease in BFP-Nrx1b and AP-Nlg1 molecules from the cell surface (Fig. 5g,h), whereas their number remained constant under control conditions. Such a loss of the Nrx1b/Nlg1 complex thus represents coordinated, activity-dependent destabilization of trans-synaptic contacts at the single-molecule level.

Differential dynamics of Nlg1 and LRRTM2 at synapses. To gain insight into the membrane dynamics of other synaptogenic proteins, we examined the behaviour of another excitatory postsynaptic adhesion molecule, LRRTM2, that competes with Nlg1 for the binding to presynaptic neurexins and LRRTM2 observed in uPAINT to estimate the diffusion coefficient of free molecules (∼0.1μm²s⁻¹) and adjusted the two other parameters to obtain the best fit. The exchange rate was approximately threefold lower for AP-LRRTM2 than for AP-Nlg1, and the trapped fraction was higher (81% versus 73%, respectively). Therefore, FRAP predicts a longer synaptic residence time for LRRTM2 than for Nlg1, consistent with the uPAINT data.

Differential nanoscale organization of Nlg1 and LRRTM2. To confirm that Nlg1 and LRRTM2 also exhibited differential dynamics at the population level, we performed FRAP experiments. We labelled AP-Nlg1 and AP-LRRTM2 at the cell surface with 100 nM mSA-Atto594 and photobleached individual synapses using a focused 561-nm laser beam. Fluorescence recovery was analysed for up to 30 min (Fig. 6d,e). Control FRAP experiments on Nlg1-GFP showed similar recovery curves (Supplementary Fig. 11). The recovery curves were fitted with a diffusion-reaction model described previously, which includes three adjustable parameters: the fraction of adhesion molecules trapped at the synapse, their steady-state exchange rate and the diffusion coefficient of free molecules.45 We used the fastest AP-Nlg1 and LRRTM2 observed in uPAINT to estimate the diffusion coefficient of free molecules (∼0.1μm²s⁻¹) and adjusted the two other parameters to obtain the best fit. The exchange rate was approximately threefold lower for AP-LRRTM2 than for AP-Nlg1, and the trapped fraction was higher (81% versus 73%, respectively). Therefore, FRAP predicts a longer synaptic residence time for LRRTM2 than for Nlg1, consistent with the uPAINT data.

Differential nanoscale organization of Nlg1 and LRRTM2. Finally, to gain insight on the nanoscale organization of Nlg1 and LRRTM2, we imaged Nlg1 and LRRTM2 by dSTORM in mature neurons (Fig. 7a–d). Unlike in uPAINT, originally diffusing molecules appear with a similar intensity to confined ones because of fixation, thus contributing to a homogenous signal in the shaft and spines. This effect was especially prominent for Nlg1, which was more diffusive than LRRTM2, resulting in a lower synaptic enrichment when compared with shaft levels (2.7 ± 0.6 versus 5.3 ± 0.8, respectively). Furthermore, the dense labelling allows the identification of many simultaneous Nlg1-trapping events in the dendritic spine, yielding a fairly disperse localization with respect to the synapse centroid (Fig. 7e), but occasionally forming one or two clusters of similar sizes as those observed with uPAINT (average diameter 98 ± 6 nm; Fig. 7fg). In contrast, LRRTM2 seemed to visit a more restricted postsynaptic area, mostly forming one compact mass comprising several small subclusters (107 ± 7 nm) and being localized at a shorter distance from the centroid (Fig. 7bd–g). This specific scattering of Nlg1 was not due to an increase in postsynaptic size compared with LRRTM2 (Fig. 7h). In summary, the two Nrx partners, Nlg1 and LRRTM2, differed considerably in their postsynaptic nano-organization and dynamics.

Finally, to rule out the possibility of a localization artefact due to AP-Nlg1 overexpression, saturating synaptic binding sites, we designed a rescue strategy by replacing endogenous Nlg1 with similar levels of exogenous AP-Nlg1. To this aim, we either co-expressed a short hairpin RNA to Nlg1 together with a resistant AP-Nlg1 construct in rat cultures, or expressed AP-Nlg1 in primary hippocampal cultures from Nlg1 KO mice. Western blots and Nrx1b-binding assays confirmed that exogenous AP-Nlg1 levels roughly reached those of endogenous Nlg1 in both approaches (Supplementary Fig. 4). STORM images performed with mSA-Alexa647 on these cultures revealed a very similar distribution of rescue AP-Nlg1 compared with what we had previously observed using AP-Nlg1 expression alone, with a
similar spine/shaft enrichment (Fig. 7i–k). Together with the STORM images obtained in organotypic hippocampal slices from Nlg1 KO mice (Fig. 3e,f), these results demonstrate that the relative scattering of AP-Nlg1 within spines most likely reflects the localization of endogenous Nlg1.

**Discussion**

In this study, we developed and extensively characterized a new labelling method compatible with SRI, based on fluorescent monomeric streptavidin (mSA), to target recombinant biotinylated proteins at the cell membrane. mSA can be produced using standard bacterial protein production, and conjugated to fluorophores of interest with established coupling protocols. This enables customization of the labelling toolbox with addition of new fluorophores, such as the recently reported silicon–rhodamine derivatives that have improved photostability and fluorogenic properties. The high thermodynamic and aqueous stability of mSA allows for its conjugation to a variety of organic dyes with different physical properties (Atto-565, -594, -647N and Alexa 647) without having an impact on its behaviour in cellular environments, even when some dyes, for example, Atto 647N and Alexa 647, are prone to incorporation in the membrane. Although mSA cannot be used to label endogenous proteins, the low abundance of naturally biotinylated proteins allowed highly specific labelling of target proteins with all mSA conjugates tested. Therefore, using a similar labelling procedure...
Figure 6 | Comparison of Nlg1 and LRRTM2 dynamics in mature hippocampal neurons. (a) DIV 15 neurons expressing AP-LRRTM2, Homer1c-GFP and BirA\textsuperscript{E. coli} were labelled using mSA-Atto 594 for uPAINT imaging of single LRRTM2 molecules. Super-resolved localization and trajectory maps are shown (green, fast-diffusing molecules, magenta, slow-moving molecules). Note the absence of diffusion on dendritic shafts. (b) Semi-log distribution of AP-LRRTM2 and AP-Nlg1 diffusion coefficients. (c) Corresponding percentage of synaptic LRRTM2 and Nlg1 detections by uPAINT (LRRTM2, n = 8; Nlg1, n = 15 cells from three different experiments). (d) FRAP experiments performed on AP-Nlg1 and AP-LRRTM2 labelled with mSA-Atto594. (e) Corresponding normalized fluorescence recovery curves. The intensity of unbleached synapses is shown as control for observational photobleaching. Solid lines represent fits of the mean data points with the diffusion-reaction equation given in the Methods. The parameters obtained for Nlg1 and LRRTM2 were the fraction of free molecules $\phi = 0.27$ and 0.19 and the turnover rate of adhesions $k_{\text{rea}}$ = $1.4 \times 10^{-2}$ and $5.0 \times 10^{-3}$ min\textsuperscript{-1}, respectively. The ratio of all synaptic molecules versus free molecules ($1/\phi$) gives 3.5 for Nlg1 and 5.1 for LRRTM2, closely corresponding to the synaptic enrichment values measured by dSTORM (Nlg1, n = 25; LRRTM2, n = 18; Nlg1 unbleached, n = 10; LRRTM2 unbleached, n = 8 cells for each condition from three different experiments).
scheme, we were able to explore real-time single-molecule dynamics at the nanoscale level (uPAINT), and to map representative protein organization at a precise time point to measure local densities within immobile structures (STED and dSTORM).

Antibodies are commonly used for labelling; however, high-quality antibodies compatible with live imaging are not always available, a critical issue for the synaptic adhesion molecules studied here. Furthermore, our comparison of monomeric (mSA), dimeric (antibody) and tetrameric (streptavidin) probes clearly

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**Figure 7 | Comparison of Nlg1 and LRRTM2 organization in mature hippocampal neurons.** (a,b) DIV 15 neurons expressing either AP-Nlg1 or AP-LRRTM2, plus Homer1c-GFP and BrA were labelled at high density with mSA-Alexa 647 for dSTORM imaging. Integrated densities over 40,000 frames are shown. (c,d) AP-Nlg1 and LRRTM2 fluorescence across linescans represented in insets show local fluorescence accumulation within synapses. (e) Dispersion of AP-Nlg1 and AP-LRRTM2 molecules within synapses represented as the distribution of distances from individual synaptic detections relatively to the centroid of the Homer1c-GFP signal (LRRTM2, n = 7; Nlg1, n = 5). (f) The median sizes of AP-Nlg1 and AP-LRRTM2 locally enriched synaptic domains (Nlg1, 87.00, IQR 68–110, n = 5; LRRTM2, 98.35, IQR 76–122, n = 7; ***P < 0.0001). (g) Distribution of the number of locally enriched AP-Nlg1 and AP-LRRTM2 domains within synapses (LRRTM2, n = 7; Nlg1, n = 5). (h) The median sizes of AP-Nlg1 and AP-LRRTM2 locally enriched synaptic domains (Nlg1, 87.00, IQR 68–110, n = 5; LRRTM2, 98.35, IQR 76–122, n = 7; ***P < 0.0001). (i) Representative STORM image of AP-Nlg1r expressed on a knockdown background in DIV 15 rat neurons, shown with the corresponding low-resolution mSA-Atto647 labelling. (j) Average intensity corresponding to the linescan in i showing local AP-Nlg1r fluorescence accumulation within a spine in a knockdown background, similar to AP-Nlg1. (k) Synaptic enrichment of AP-Nlg1, AP-Nlg1r co-expressed on a knockdown background and AP-LRRTM2 with respect to shaft levels (AP-Nlg1, 2.73 ± 0.62, n = 5; AP-Nlg1r, 2.54 ± 0.34, n = 4; AP-LRRTM2, 5.29 ± 0.81 n = 7; *P < 0.05). Data are from three different experiments for AP-Nlg1 and AP-LRRTM2, and two experiments for AP-Nlg1r.
shows that, for live SRI applications, probe multivalence affects the measurements by generating artificial nanoscale clusters and biasing protein diffusion. For single-particle-tracking applications, mSA-Atto conjugates represent a significant improvement over large and multivalent nanoparticles such as antibody- or streptavidin-coated quantum dots, which often get sequestered at the periphery of the postsynaptic density because of steric hindrance\(^\text{31,32,47}\). However, because of their resistance to photobleaching compared with organic fluorophores, nanoparticles will remain a method of choice to track molecules for long durations, even more so with the recently developed non-blinking fluorescent nanoparticles\(^\text{48,49}\). mSA labelling is in many regards comparable to that of anti-GFP nanobody labelling, an example of monovalent detection recently used in SRI\(^\text{13}\). In some cases, however, labelling with mSA may be advantageous if the use of GFP as an antigenic tag is problematic. Indeed, the 15-amino-acid AP tag, which is similar in size to other commonly used epitopes, such as FLAG or c-Myc, can be readily inserted between structural domains or within protein loops with minimal perturbation of the native function. We demonstrated this point by fusing the AP tag on a short extracellular loop of stargazin that was previously targeted with an engineered HA tag\(^\text{36}\), whereas FP insertion at this location resulted in a dramatic mislocalization of the resulting fusion construct.

A benefit of developing an orthogonal technique of comparable capability includes the potential for dual-colour SRI. To this end, we simultaneously applied mSA and GFP nanobody labelling of trans-syntaptic targets in hippocampal neurons to reveal a differential dynamic organization of the two main Nrx1β adhesion partners at excitatory synapses, namely Nlg1 and LRTM2. We showed a differential trapping of presynaptic Nrx1β and postsynaptic Nlg1, resulting in selective trans-synaptic apposition of those proteins at axon–dendrite contacts. Labelling of Nrx1β/Nlg1 complexes was rapid with both mSA and GFP nanobody, neither of which affected their intrinsic interaction. In contrast, some labelling strategies, such as GFP complementation, have intrinsically slower kinetics owing to GFP folding and can artificially increase the binding strength between Nrx and Nlg, thus enhancing synaptogenesis\(^\text{38}\). That Nrx1β escaped synapses more readily than Nlg1 on EGTA treatment suggests that the molecule may be retained at presynapses through extracellular calcium-dependent interactions, for example, with Nlgs and LRTMts. This observation is consistent with the report that the Nrx intracellular domain is dispensable for Nlg1-induced presynaptic differentiation\(^\text{50}\). Nlg1 may be anchored at the synapses not only via extracellular interactions with Nrxs but also via intracellular interactions with PSD-95, which may be promoted by Nrx1β binding\(^\text{32}\).

Whether adhesion molecules are functionally regulated by synaptic activity remains an important, unresolved issue. Nlg1 interacts directly with the extracellular domain of NMDA receptors\(^\text{51}\) and Nlg1 knockdown affects NMDAR-mediated synaptic transmission\(^\text{52,53}\). In addition, the synaptogenic effect induced by Nlg1 expression is abolished by chronic blockade of the NMDA receptor activity\(^\text{53}\), whereas synapse elimination caused by the absence of Nlgs and LRTMts is promoted by calcium-calmodulin kinase II (CamKII)-dependent synaptic activity\(^\text{54}\). Our results show that both Nlg1 and Nrx1β trans-synaptic clusters rapidly vanish on NMDA treatment, suggesting that Nlg1 plays a role in activity-dependent synaptic remodelling through NMDA receptor activity. Proteolytic shedding of Nlg1 at the cell membrane by the metalloprotease MMP9 (ref. \(\text{55}\)) or altered export of Nlg1 from the cell by phosphorylation by CamKII (ref. \(\text{56}\)) provide possible mechanisms. In contrast to Nlg1, LRTM2 is less mobile and is confined in more compact synaptic domains, suggesting that the molecule plays a strong role in maintaining trans-synaptic connectivity. This specific function of LRTM2 might be achieved through direct extracellular interactions with presynaptic Nrxs or with postsynaptic AMPA receptors\(^\text{43}\), the latter playing a retrograde role on presynaptic differentiation\(^\text{57}\). Indeed, alternative splicing of presynaptic Nrx3 decreases both postsynaptic LRTM2 and AMPA receptor levels\(^\text{58}\), and LRTM knockdown alters AMPA receptor-mediated synaptic transmission and plasticity\(^\text{59}\). Both the number and size of synaptic LRTM2 clusters match the recently identified AMPA receptor nanodomains\(^\text{58}\), suggesting that they may be structurally and/or functionally related. One interesting mechanism could be that extrasynaptic surface-diffusing AMPA receptors get dynamically trapped at Nrx1β/Nlg1 adhesions through PSD-95 scaffolds\(^\text{31,35}\), and then become stabilized at synapses by more durable interactions with LRTM2.

Obtaining a more integrated view of the molecular ultrastructure of the synapse requires multicolour SRI of adhesion molecules, scaffolding proteins and neurotransmitter receptors. The penetrability and labelling efficiency reached with mSA, together with targeted expression of adhesion proteins such as Nrx and Nlg in intact organisms\(^\text{60}\) and improved optical detection in deep tissue\(^\text{61}\), should enable further investigation of activity-dependent modulation of synaptic protein organization with high spatial and temporal resolution. In addition to addressing specific questions in neuroscience, the mSA-based labelling strategy constitutes a significant progress towards developing a robust staining technique for fluorescence-based super-resolution microscopy, which should stimulate applications to a wide range of questions in fundamental biology.

**Methods**

**DNA plasmids.** The AP-Nlg1, AP-Nrx1β, pDisplay-6His-AP-CFP and BirA\(^\text{ER}\) constructs\(^\text{21,22}\) were kind gifts from A. Ting (MIT, Boston). HA-Nlg1 and short hairpin RNA to Nlg1 (shNlg1) were obtained from P. Scheiffele (Bionzentrall, Basel). The AP-Nlg1 rescue was generated by changing the nucleotide sequence of AP-Nlg1 recognized by the shNlg1 5’-gaagtagctgaagctc-3’ to 5’-gataggacaagcttcag-3’, using silent mutations. Myc-LRTM2 (ref. \(\text{43}\)) was a gift from J. de Wit (Leuven, Belgium). Nlg1 with GFP insertion at position 728aa (Nlg1-GFP) was generously provided by T. Dresbach (Goettingen, Germany)\(^\text{62}\). AP-LRTM2 was generated using the In-Fusion HD Cloning kit (Clontech), replacing the myc-tag from myc-LRTM2 by the AP tag (amino-acid sequence GLMDPEAQKXEWHE). The AP tag sequence was amplified from pDisplay-6His-AP-CFP using the primers GCTAGCCGCCATACCCAG-3’ and GACCTTATGCTAGGCAGTCTGCTGCACTGCTCAG-5’. The AP-LRTM2 sequence was inserted by HindIII digestion and ligation of the PCR product into pBFP2-Nuc (obtained from Addgene, plasmid 14893). AP SEP-transferrin receptor was a kind gift from D. Perras (IINS, Beadoures). HA-stargazin\(^\text{59}\) was a gift from R. Nicola (UCSF, USA). AP-stargazin was obtained by inserting a synthetic fragment containing the AP tag between residues 50 and 51 (in the first extracellular loop, same position as HA-stargazin) using HindIII and BspEI restriction sites. For comparison of the impact of FP insertion, mCherry was inserted into the same stargazin construct either at the same position as the AP tag (first extracellular loop, between residues 50 and 51, and Nhel restriction sites) or at position 56 (BglII site) with respect to the C terminus (cytoplasmic tail). The plasmid for bacterial expression of the anti-GFP nanobody\(^\text{14}\) was a kind gift from Alexis Guteau (LEBS, Gif-sur-Yvette, France). mSA was subcloned from the previously described pSet-A vector\(^\text{25,26}\) into PET-IG, a homemade vector derived from pEGFP-N1 (Novagen) and engineered to incorporate after the start codon a decahistidine tag immediately followed by a Tobacco Etch Virus cleavage site (-ENLYFQK-) and no tag on the C terminus.

**Protein expression and purification.** Nrx1β-Fc was obtained as follows. Conditioned medium from a stable hygromycin-resistant HEK cell line producing Nrx1β-Fc lacking splice insert 4 was collected, and recombinant Nrx1β-Fc was purified on a protein G column (Hitrap Protein G HP, GE Healthcare) to a concentration of 0.6 to 1.8 mg ml\(^\text{-1}\) (refs 31,32). mSA was produced similarly to previously reported methods\(^\text{23,26}\), with slight modifications. Briefly, PET-IG-mSA was transformed into E. coli BL21 codon plus (DE3)-RII and expressed for 12 h at...
16°C in 300 ml using an autocloding protocol. The cells were harvested and resuspended in 9% NaCl, transferred into a conical tube and kept at −80°C until purification. The pellet was thawed and resuspended in 50 ml of freshly prepared denaturing lysis buffer (50 mM Tris acetate, pH 8.0, 8.0 M urea and 40 μl Protease Cocktail III from Calbiochem) and gently mixed for 30 min at 4°C. Cells were lysed by sonication and lysates were centrifuged at 10,000g (60 min, 4°C). His-Bust Cobalt Affinity gel (AMOCOL, 1 ml) was added and incubated with the supernatant for 2 h at 4°C. The resin was collected washed extensively with washing buffer (50 mM Tris-OAc, pH 8.0, 300 mM NaCl, 50 mM imidazole and 8 M urea, freshly prepared) and the protein was eluted with 3 × 1 ml of elution buffer (50 mM Tris-OAc, pH 8.0, 300 mM NaCl, 500 mM imidazole, 8 M urea, freshly prepared). The elution fractions were added drop by drop to 40 ml of ice-cold PBS buffer (5 mM Na2HPO4, 5 mM NaH2PO4 and 150 mM NaCl) containing 0.3 mg ml−1 d-biotin, 0.2 mg ml−1 oxidized glutathione and 1 mg ml−1 reduced glutathione under rapid stirring to refold the protein. The precipitates were removed by centrifugation. The refolded protein was concentrated to ~1 mg ml−1 using Amicon Ultra centrifugal filters with a 10-kDa cutoff. The anti-GFP nanobody was expressed in the same conditions as mSA and purified under native conditions by affinity chromatography using the His-Bust Nickel Affinity gel. The protein was dialysed in PBS buffer and concentrated to ~1 mg ml−1 using Amicon Ultra centrifugal filters with a 10-kDa cutoff. mSA and anti-GFP nanobody were kept at 4°C and used for electroporation.

Cell culture and electroporation. Gestant rat females were purchased weekly from Janvier Labs, Saint-Berthevin, France, while wild-type and Nlg1 KO mouse strains obtained from F. Varoqueaux and N. Brose (MPI Göttingen) were raised in our animal facility. Animals were handled and killed according to European ethical rules. Dissociated hippocampal neurons from E18 Sprague–Dawley rat embryos or P0 mice (from Nlg1 WT or KO background) were prepared as described, and electroporated with the Amaxa system (Lonza) using 500,000 cells per cuvette. The following plasmid combinations were used: (GFP or Homer1c-GFP) + AP-Nlg1 + BirAER (1.5:2:1), (AP-Nlg1 + BirAER) + mSA-Atto 594, mSA-Alexa 647 and the Atto 647N-GFP nanobody were further purified to homogeneity by size exclusion chromatography with a Superdex 75 HiLoad 16/60 column (GE Healthcare) on an AKTA purifier system (GE Healthcare) using PBS as a running buffer. Labelled proteins were concentrated to ~0.2 mg ml−1 using Amicon Ultra centrifugal filters with a 10-kDa cutoff. All proteins were aliquoted and flash-frozen for storage at −80°C until use.

Single Molecule Tracking (uPAINT). uPAINT experiments were carried out as previously reported. Cells were mounted in Tyrode solution (15 mM D-glucose, 108 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2 and 25 mM HEPES, pH 7.4) containing 1% globulin-free BSA (Sigma) in an open Inox observation chamber (Life Imaging Services, Basel, Switzerland). The chamber was placed on an inverted microscope (Nikon Ti-E Eclipse) equipped with an EMCCD camera (Evolve, Roper Scientific, France), a thermostated box (Life Imaging Services, Basel, Switzerland) and a Metamorph software controlled through acousto-optical tunable filters driven by the Metamorph software (Molecular Devices, USA). Atto 594 and Atto 647N were excited with the 561- and 647-nm lines of a 15 mW Argon-laser beam (488 nm, 635 nm; Semrock). Samples were imaged by oblique laser illumination, allowing the excitation of individual Atto-conjugated ligands bound to the cell surface, without illuminating ligands in solution. Fluorescence was collected using FF01-677/73 and FF01-593/40 emission filters (Semrock), respectively, placed on a filter wheel (Sutter). Stacks of 2,000–4,000 consecutive frames were obtained from each cell, with an integration time of 20–50 ms. Multicolour fluorescent 100-nm beads (Tetraspeck, Invitrogen) were used to register long-term acquisitions and correct for lateral drifts. In some experiments, EGTA (10 mM) was added directly in the bath and left for 10 min. For FMN experiments, a Gilson perfusion system was used to perfuse and change the fluorescence image with an avian Gfp-expressing cell that was 500,000 neurons in Tyrode solution containing the fluorescent labels during the whole acquisition time. Cells were recorded in control solution for 10 min, treated with 20 μM NMDA in Tyrode for 10 min and allowed to recover in control solution for 10 min. Control cells were kept in Tyrode solution the whole time. Acquisitions were steered using the Metamorph software (Molecular Devices) in a streaming mode at 50 Hz. Cycles of two consecutive series of acquisitions were made sequentially to 1 h. One series corresponds to GFP (20 frames, 50 Hz), mSA-Atto 594 (1,500 frames, 50 Hz) and Atto 647N-GFP nanobody (1,500 frames, 50 Hz).

Neuronal lysates. DIV 14 mouse or rat neuronal cultures were rinsed in ice-cold PBS, and then scrapped into 100 μl RIPA buffer (50 mM HEPES, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 1% IGEPAL CA-630 and 0.5% sodium deoxycholate, pH 7.2). Homogenates were kept on ice for 15 min to 4°C to remove cell debris. Ten microlitres per condition were loaded for western blots.

SDS-PAGE and immunoblotting. Samples were separated by TGX stain-free precast gels (4–15% gradient, Bio-Rad), ultraviolet-activated with ChemiDoc Touch system (Bio-Rad) and direct imaging of total proteins and then transferred to nitrocellulose membranes for immunoblotting analysis. After blocking with 5% non-fat dried milk in Tris-buffered saline Tween-20 (TBST; 28 mM Tris, 136.7 mM NaCl, 0.05% Tween-20, pH 7.4) for 45 min at room temperature, membranes were incubated with rabbit anti-Nlg1 (1:2000, Synaptic systems) diluted at 1:10,000 with 0.5% non-fat dried milk in TBST, followed by horse radish peroxidase conjugated anti-rabbit antibody (Jackson ImmunoResearch) for 1 h at room temperature. Target proteins were detected by chemiluminescence with Super signal West Dura (Pierce) on the ChemiDoc Touch system (Bio-Rad). The theoretical molecular weight of Nlg1 is 93 kDa, but the apparent molecular weight in immunoblots is ~130 kDa, likely due to glycosylation. For quantification, the intensity of the chemiluminescence signal of each lane was normalized by the total protein signal on the same lane, revealed by the stain-free technology.

Organotypic cultures and single-cell electroporation. Organotypic hippocampal slice cultures were prepared from either wild-type or Nlgl1 KO mice (C57B/6J strain), as described. Briefly, animals at postnatal day 4–6 were quickly decapitated and their brains placed in ice-cold Gey’s balanced salt solution (Gibco) containing 10 mM HEPES, 2 mM MgSO4 and 1 mM CaCl2. The animals were killed according to the Amaxa system (Lonza) using 500,000 cells per cuvette. The pipette containing 33 ng mSA, 1 mg ml−1 oxidized glutathione and 1 mg ml−1 reduced glutathione under rapid stirring to refold the protein. The precipitates were removed by centrifugation. The refolded protein was concentrated to ~1 mg ml−1 using Amicon Ultra centrifugal filters with a 10-kDa cutoff. The anti-GFP nanobody was expressed in the same conditions as mSA and purified under native conditions by affinity chromatography using the His-Bust Nickel Affinity gel. The protein was dialysed in PBS buffer and concentrated to ~1 mg ml−1 using Amicon Ultra centrifugal filters with a 10-kDa cutoff. mSA and anti-GFP nanobody were kept at 4°C and used for electroporation.

Trajectory analysis and image reconstruction. Image stacks were analysed using a custom programme running on Metamorph based on wavelet segmentation for localisation and simulated annealing algorithms for tracking, described earlier. The trajectories were kept for 15 min and used for the determination of the positions of localized single molecules into a single image, and tracking of localized molecules through successive images. The instantaneous
diffusion coefficient, $D$, was calculated for each trajectory from linear fits of the first 4 points of the mean square displacement (MSD) function versus time. Slow trajectories were excluded as trajectories with a MSD 10% below 0.0093 $\pm$ 0.01 mm$^2$ s$^{-1}$ (Supplementary Fig. 4). This threshold corresponds to molecules exploring a region smaller than that defined by the spatial resolution of the system ($\sim$0.054 mm, full-width at-half-maximum) during the time used to fit the initial slope of the MSD (4 points, 20 ms) and is given by $D_{\text{res}} = (0.054 \text{mm})^2/(4 \times 0.0093 \pm 0.01 \text{mm}^2 \text{s}^{-1}$, as derived from ref. 16.

Spatial resolution was determined using fixed Atto samples. Overall, 415 two-dimensional (2D) distributions of single-molecule positions belonging to long trajectories ($>10$ frames) were measured by bi-dimensional Gaussian fitting and the resolution determined to be $2.35 \pm 0.2$ pixels, where 1 pixel corresponds to 10 nm. For trajectory analysis, synapses were identified by wavelet-based image segmentation of the Homer1c-GFP postsynaptic marker. The corresponding binary masks were used to sort single-particle data analyses to specific synaptic regions. For NMDA experiments, each mSA and nanobody sequence was analysed separately as described, and data were pooled over 10-min intervals, to obtain representative trajectories of protein populations. The percentage of synapses containing Nlg1 was defined as the ratio between synapses containing at least 10 detections of AP-Nlg1 over the total number of synapses on super-resolved images obtained in the same conditions for mSA and nanobody. Synaptic coverage was determined from super-resolved detections at the ratio between threshold areas containing detections over a whole synaptic region determined from the low-resolution Homer1c signal. The percentage of synaptic detection is defined as the number of detections within synapses defined by Homer1c divided by the total number of detections. For dual-colour imaging of Nlg1 andNr1B, areas with high signal density were identified as domains by wavelet segmentation. Their number and size were estimated from 2D isotropic Gaussian fitting, and their length was determined as the full-width at half-maximum. In the supplemental figures, the number of objects per frame was analysed using the ‘Analyse Particles’ plugin in ImageJ and was determined as the number of trajectories per dendritic segment of 10

**STED imaging and data analysis.** CA1 neurons co-expressing EGFP, BirAER and AP-Nlg1 in organotypic slices from wild-type mice were surface-labelled with high concentration of mSA-Atto594 (100 nM) for 10 min, and then rinsed, mounted in Tyrode solution and observed under the same set-up used for uPAINT. The laser bench (comprising 488, 561 and 642 nm lasers, 100 mW each, Roper Scientific) has a second optical fibre output connected to an illumination device containing galvanometric scanning mirrors (ILAS, Roper Instrument) steered by MetaMorph. It allows precise temporal and spatial control of the focused laser beam at any user-selected region of interest within the sample for targeted photobleaching. Switching between the two fibres for alternating between imaging and bleaching is performed in the millisecond range using a mirror. Oblique illumination acquisition was performed using the 561-nm laser at low power (300 mW at the objective) to avoid photobleaching of the sample. A photobleaching signal was scanned at 50 Hz. In-depth acquisitions were performed by scanning hippocampal slices with an increment of 1 mm in z over 60 mm. When indicated, raw data were deconvolved using the measured point spread function of the system and the Richardson–Lucy algorithm with Huygens Professional (Huygens Software). 3D deconvoluted movies were made using Imaris (Bitplane).

**FRAP experiments and analysis.** Neurons electroporated with Homer1c-GFP, BirAER and AP-Nlg1 or AP-LRRTM2 and cultured for 2 weeks were labelled with high concentration of mSA-Atto594 (100 nM) for 10 min, and then rinsed, mounted in Tyrode solution and observed under the same set-up used for uPAINT. The laser bench (comprising 488, 561 and 642 nm lasers, 100 mW each, Roper Scientific) has a second optical fibre output connected to an illumination device containing galvanometric scanning mirrors (ILAS, Roper Instrument) steered by MetaMorph. It allows precise temporal and spatial control of the focused laser beam at any user-selected region of interest within the sample for targeted photobleaching. Switching between the two fibres for alternating between imaging and bleaching is performed in the millisecond range using a mirror. Oblique illumination acquisition was performed using the 561-nm laser at low power (300 mW at the objective) to avoid photobleaching of the sample. A photobleaching signal was scanned at 50 Hz. In-depth acquisitions were performed by scanning hippocampal slices with an increment of 1 mm in z over 60 mm. When indicated, raw data were deconvolved using the measured point spread function of the system and the Richardson–Lucy algorithm with Huygens Professional (Huygens Software). 3D deconvoluted movies were made using Imaris (Bitplane).

**Confocal microscopy.** Single cell-electroporated neurons from organotypic slice culture co-expressing Gfp, BirAER and AP-Nlg1 were surface-labelled with mSA-Atto647N, biont antibody-Alexa647 or streptavidin-Alexa647 in artificial cerebrospinal fluid (ACSF) (in mM, 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 25 NaHCO$_3$, 1.25 glucose, 105 glutamate, 1 Hz, rinsed and fixed with PFA 0.2% glutaraldehyde plus 0.1% BSA for 2 h at 4°C. Images were acquired on a commercial Leica TCS SP5 confocal microscope using x 63, 1.4 NA oil objective and a pinhole opened to one time the Airy disk. Images of 512 x 512 pixels were acquired at a scanning frequency of 400 Hz.

**Statistics.** Statistical values are given as mean ± s.e.m., unless otherwise stated. Statistical significance was calculated using GraphPad Prism. All data sets containing more than two conditions were compared by one-way analysis of variance test, followed by a post hoc Dunn’s test. Sample size was based on two or three different cultures per condition, 2–10 cells per experiment. Randomization of samples was performed for all experiments. When critical comparison of different labelling conditions was involved, experiments and image analysis were performed blindly.

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Author contributions

I.C., M.S. and O.T. designed research and wrote the article. O.T. did initial proof-of-concept experiments and I.C. performed all subsequent imaging experiments. M.L. provided electroporated organotypic brain slices. C.B. and I.C. performed dSTORM experiments and analysis. B.T. and I.G. generated DNA constructs. B.T. performed biochemistry experiments. K.H.L. and S.P. provided the initial constructs of mSA. I.G., I.C. and M.S. produced and labelled protein probes. J.-B.S. provided the STORM set-up and software for single-molecule analysis. M.S. and O.T. coordinated and oversaw the research project. All authors discussed the results and commented on the manuscript.

Additional information

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