### **RESEARCH ARTICLE SUMMARY**

#### **CELLULAR NEUROSCIENCE**

# The actin-spectrin submembrane scaffold restricts endocytosis along proximal axons

Florian Wernert<sup>†</sup>, Satish Babu Moparthi<sup>†</sup>, Florence Pelletier, Jeanne Lainé, Eline Simons, Gilles Moulay, Fanny Rueda, Nicolas Jullien, Sofia Benkhelifa-Ziyyat, Marie-Jeanne Papandréou, Christophe Leterrier\*<sup>‡</sup>, Stéphane Vassilopoulos\*<sup>‡</sup>

**INTRODUCTION:** Endocytosis plays a vital role in neurons, enabling the retrieval and transport of membrane and extracellular components. This essential process organizes the intricate and compartmentalized structure of neurons and maintains the synaptic vesicle cycle fundamental to neuronal communication.

**RATIONALE:** Most endocytosis in neurons is mediated by the assembly of clathrin-coated pits (CCPs) at the plasma membrane. In neurons, CCPs are prevalent in the cell body and dendrites as well as around the active zone in presynapses, hence the focus on these compartments as endocytotic locations. However, there is limited understanding regarding the nanoscale organization or presence of clathrinmediated endocytosis along the axon shaft and at the axon initial segment (AIS), a specialized compartment located along the proximal part of the axon. It was classically assumed that endocytosis along the axon shaft is minimal, an assumption further supported by the presence of a dense complex of spectrins, ankyrins, and anchored membrane proteins at the AIS. Furthermore, spectrins form a periodic scaffold linking actin rings along the axonal plasma membrane. The recent proposal that endocytosis at the AIS is a mechanism for polarized protein sorting and activity-dependent morphological plasticity made the case for reexamining the nanoscale architecture of CCPs at the AIS and their endocytic behavior.

**RESULTS:** We used a combination of superresolution microscopy and electron microscopy to visualize the structural components of the AIS at nanoscale resolution. In addition to the periodic scaffold, AIS spectrins together with actin form well-defined circular exclusion zones we termed "clearings", which allow CCPs to form at the center. We either genetically manipulate neuronal cultures or, by treating them with drugs targeting actin or the spectrin mesh, we found that the dense spectrin mesh restricts the formation of CCPs inside clearings. These ments to show that CCPs are preferentially stabilized along the AIS, consistent with their restrained endocytic activity. Furthermore, these stalled CCPs could be unlocked and endocytosis triggered at the AIS by a plasticityinducing stimulation with *N*-methyl-*D*-aspartate (NMDA). This endocytosis trigger was mediated by the polymerization of branched actin "nests" around the CCPs in clearings which likely help CCP scission from the plasma membrane.

limited endocytosis at the AIS of resting **the area** rons. We use live-cell super-resolution experi-

**CONCLUSION:** Our nanoscale investigation reveals that endocytosis at the AIS is regulated at two levels: First, the dense spectrin mesh restricts the formation of CCPs inside circular clearings where they are stabilized and long-lived, resulting in low endocytic activity at steady state. Secondly, endocytosis from these preformed pits can be triggered by physiological cues such as plasticity-inducing NMDA stimuli. These two levels of regulation explain how endocytosis can be organized within the dense and stable scaffold of the mature AIS while allowing for its structural rearrangement.

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A nanoscale view of clathrin-coated pits and endocytosis at the AIS. At the AIS (top left), clathrin-coated pits (magenta in the STORM-PREM view, bottom left) form in clearings within periodic spectrin scaffold (orange). Endocytosis of these long-lived CCPs inside clearings is triggered after stimulation by the polymerization of branched actin nests (blue on the PREM and illustrations, right). Scale bars, 1 µm (left, axon view), 100 nm (right, zooms on clearings).



### **RESEARCH ARTICLE**

#### **CELLULAR NEUROSCIENCE**

# The actin-spectrin submembrane scaffold restricts endocytosis along proximal axons

Florian Wernert<sup>1</sup>†, Satish Babu Moparthi<sup>2</sup>†, Florence Pelletier<sup>1</sup>, Jeanne Lainé<sup>3</sup>, Eline Simons<sup>1</sup>, Gilles Moulay<sup>2</sup>, Fanny Rueda<sup>1</sup>, Nicolas Jullien<sup>1</sup>, Sofia Benkhelifa-Ziyyat<sup>2</sup>, Marie-Jeanne Papandréou<sup>1</sup>, Christophe Leterrier<sup>1</sup>\*‡, Stéphane Vassilopoulos<sup>2</sup>\*‡

Clathrin-mediated endocytosis has characteristic features in neuronal dendrites and presynapses, but how membrane proteins are internalized along the axon shaft remains unclear. We focused on clathrincoated structures and endocytosis along the axon initial segment (AIS) and their relationship to the periodic actin-spectrin scaffold that lines the axonal plasma membrane. A combination of superresolution microscopy and platinum-replica electron microscopy on cultured neurons revealed that AIS clathrin-coated pits form within "clearings", circular areas devoid of actin-spectrin mesh. Actin-spectrin scaffold disorganization increased clathrin-coated pit formation. Cargo uptake and live-cell imaging showed that AIS clathrin-coated pits are particularly stable. Neuronal plasticityinducing stimulation triggered internalization of the clathrin-coated pits through polymerization of branched actin around them. Thus, spectrin and actin regulate clathrin-coated pit formation and scission to control endocytosis at the AIS.

embrane exchange is a ubiquitous cellular process that allows cells to interact with their environment by processes such as endocytosis, phagocytosis, and exocytosis, as well as exosome release and uptake. Differentiated cells such as neurons rely extensively on endocytosis to organize their complex and compartmented morphology and sustain the synaptic vesicle cycle at the core of neuronal communication (1). This role explains the historical focus on presynapses as the main site of endocytosis in neurons, with a wealth of studies dissecting its molecular mechanisms and coupling to the converse process of exocytosis (2, 3). Neuronal endocytosis has also been extensively studied in dendrites, where it drives receptor internalization near postsynapses (4, 5) and is the source of complex endocytic traffic (6).

Endocytosis in neurons is mainly achieved through clathrin-mediated endocytosis (7). Clathrin forms triskelia made of trimerized heavy chains and light chains that assemble through adaptor proteins to form a cage-like coat around a membrane invagination called a clathrin-coated pit (CCP), before scission of the CCP neck releases a clathrin-coated vesicle. In neurons, CCPs are found at the plasma membrane of the cell body and dendrites as well as

These authors contributed equally to this work.

in the vicinity of the active zone in presynapses, strengthening the prevalent view that endocytosis mainly occurs in these compartments. By contrast, little is known about the nanoscale organization or even the existence of clathrin-mediated endocytosis along the axon shaft and at the axon initial segment (AIS), the specialized compartment that constitutes the proximal part of the axon next to the neuronal cell body (8, 9). It was commonly assumed that little to no endocytosis could take place along the axon shaft (10), a view reinforced by the presence of a dense submembrane assembly of spectrins, ankyrin G, and anchored membrane proteins at the AIS, as well as the discovery of a dense, periodic scaffold of actin rings connected by spectrin tetramers lining the axonal plasma membrane (11-13).

We previously used super-resolution microscopy combined with platinum-replica electron microscopy (PREM) on unroofed neurons to reveal the ultrastructure of the periodic actinspectrin scaffold and AIS submembrane components (14). Despite the presence of this dense undercoat, PREM views showed numerous CCPs present along the AIS of mature neurons, which was notable as studies at the time only detected endocytosis in the nascent AIS in developing neurons (15, 16) or under acute excitotoxic stress (17). More recently, robust endocytic activity at the AIS was proposed as a mechanism for membrane protein sorting, allowing the retrieval of somatodendritic proteins when they enter the axon (18). This prompted us to focus on the detailed architecture of CCPs at the AIS, their endocytic function, and their relationships with the actin-spectrin submembrane scaffold.

#### Clathrin-coated pits are present in circular clearings of the actin-spectrin scaffold at the AIS

We first visualized the distribution of CCPs with regard to the spectrin mesh at the proximal axon of neurons after 14 days in vitro, labeled for  $\alpha$ 2-spectrin and for the AIS-specific adhesion molecule neurofascin (Fig. 1A). We used structured illumination microscopy (SIM), which resolves the 190-nm periodicity of the actin-spectrin scaffold as well as individual CCPs, and found that CCPs were present in areas devoid of  $\alpha$ 2-spectrin as confirmed by the intensity profiles of averaged images centered on CCPs (Fig. 1B). The presence of CCPs in areas devoid of spectrin were also observed along the more distal axons and were similarly observed when labeling the spectrin mesh for  $\beta$ 2-spectrin (fig. S1, A to C). To isolate the axonal plasma membrane and associated cortical cytoskeleton without additional signal from intracellular compartments, we mechanically unroofed cultured hippocampal neurons using ultrasound (Fig. 1C). The presence of CCPs inside circular areas devoid of a2-spectrin was clarified by the unroofing, with a distinct circular hole of a2-spectrin around CCPs seen on the average image (Fig. 1D). This exclusion pattern along the dense spectrin scaffold of axons is an extreme example of the complementary pattern between submembrane spectrins and CCPs previously observed in fibroblasts (19) and at the lateral membrane of epithelial cells (20).

We then produced platinum replicas of unroofed hippocampal neurons to visualize the ultrastructure of CCPs at the AIS by PREM. To identify the axonal process stemming from unroofed cell bodies, we located the characteristic fascicles of AIS microtubules (Fig. 1E). High-magnification PREM views of the axonal membrane-associated cytoskeleton revealed the presence of characteristic actin rings embedded in a dense spectrin mesh as previously described (14) but also provided views of CCPs at the AIS with their characteristic honeycomb patterns (Fig. 1E). In agreement with superresolution fluorescence microscopy, CCPs formed on a patch of bare plasma membrane, forming a circular "clearing" of the actinspectrin scaffold. These clearings were devoid of proteinaceous material as if the mesh had been pushed out, save one or two actin filaments that were often seen reaching the central CCP (Fig. 1F). All AIS CCPs were found at the center of spectrin clearings that measured  $330 \pm 7.0$  nm in diameter whereas the CCPs measured  $83 \pm 1.5$  nm (Fig. 1G; all further exact mean  $\pm$  SEM values together with statistics can be found in data S1). Consistent with the role of cortical actin in clathrin-mediated endocytosis (21), sparse filaments were found to interact with CCPs in all neuronal compartments. However, we only observed the circular clearings of the actin-spectrin scaffold in axons as they were

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Fig. 1. Clathrin-coated pits are present in clearings of the periodic spectrin scaffold at the AIS. (A) SIM image of a cultured hippocampal neuron at 14 days in vitro (div) fixed and stained for clathrin (magenta; CCPs appear as clusters),  $\alpha$ 2-spectrin (green, ~190 nm-spaced bands) and neurofascin (blue, indicates the AIS). Scale bars, 10 µm (left image), 2 µm (center column), 0.5 µm (zooms). (B) (Left) average images centered on CCPs for 591 individual pits showing the distribution of clathrin (magenta on overlay) and  $\alpha$ 2-spectrin (green on overlay). (Right) radial intensity profile corresponding to the average images on the left (gray arrow), showing how CCPs reside in an area devoid of  $\alpha$ 2-spectrin. Scale bar, 100 nm. (C) SIM image of a neuron unroofed, fixed, and stained for clathrin (magenta; CCPs appear as clusters) and  $\alpha$ 2-spectrin (green). The central column shows zoomins corresponding to the area highlighted in the left image while square images on the right correspond to zoom-ins on the areas highlighted in the central column. Scale bars, 10 μm (left image), 2 μm (center column), 0.5 μm (zoom-in). (**D**) (Left) average images centered on CCPs for 562 individual pits showing the distribution of clathrin (magenta on overlay) and  $\alpha$ 2-spectrin (green on overlay). (Right) radial intensity

profile corresponding to the average images on the left (gray arrow), showing how CCPs reside in an area devoid of α2-spectrin. Scale bar, 100 nm. (E) PREM view of an unroofed proximal axon showing the presence of microtubules (blue), ~190 nmspaced actin rings (yellow), the dense mesh of spectrins between actin rings, and CCPs that reside in "clearings" of the spectrin mesh. Right images show zoom-ins of square areas highlighted on the left image corresponding to an actin rings-spectrin mesh area, and an area with two CCPs. Scale bars, 200 nm (left image), 100 nm (zoom-ins). (F) Gallery of PREM views showing CCPs found in circular areas of bare plasma membrane devoid of spectrin mesh ("clearings") along the proximal axon. Arrowheads point to isolated actin filaments contacting CCPs. Scale bars, 100 nm. (G) Quantification of the diameter for spectrin mesh clearings and for CCPs from PREM views. For all graphs unless stated otherwise, plots are individual points, bars are mean ± SEM, and significance are against the control condition (see statistics section of the material and methods for details). All means, SEM, and standard deviations together with statistics (number of points n, number of independent experiments N, significance of differences) are summarized in the data S1.

absent from cell bodies and dendrites that lacked such a dense undercoat (fig. SIC).

### Molecular architecture of CCPs and actin-spectrin clearings at the AIS

We next focused on the detailed architecture of the circular clearings in the actin-spectrin mesh. Along the AIS we found examples of empty clearings as well as clearings presenting nascent CCPs at various budding stages, including empty clearings or those with nascent, mature single, or multiple CCPs (Fig. 2A). Clearings containing single mature pits were the majority population and no change in clearing diameter was measured between maturation stages (Fig. 2B). This suggests that CCPs form within existing clearings rather than induce their formation. We detailed the components and organization of the AIS CCPs and actin-spectrin clearings using single molecule localization microscopy (SMLM), alone and in correlation with PREM. Three-dimensional (3D) stochastic optical reconstruction microscopy (STORM) of clathrin in intact neurons revealed the presence of CCPs distributed around the circumference of the AIS (fig. S2, A to C), although the grazing illumination used in SMLM resolves the CCPs along the ventral plasma membrane more clearlythe only CCPs remaining after unroofing (fig. S2, D to F). Point-accumulation in nanoscale topography (PAINT) allowed us to image CCPs and a2-spectrin simultaneously in 3D at ~20-nm resolution (Fig. 2, C to E). This confirmed the presence of CCPs in areas devoid of the spectrin mesh, both on in-plane views and transverse sections of the AIS (Fig. 2E). In addition, we used 3D-PAINT to show that the adaptor protein complex AP2 colocalizes to CCPs along the AIS (fig. S2, G to I), as classically described (22, 23).

To further dissect the organization of the submembrane scaffold components around CCP-containing clearings, we performed correlative STORM-PREM. After unroofing and fixation of neurons, we labeled and imaged a given AIS component by STORM, then produced platinum replicas of the same unroofed neurons and observed them by PREM (14). We validated our correlative approach by using an anticlathrin antibody to label CCPs at the AIS. The resulting overlays show how we can precisely correlate the 100-nm CCPs on both STORM and PREM images, although the anti-clathrin immunolabeling obscures the characteristic honeycomb pattern of the CCPs (fig. S2J). We then stained the AIS-specific  $\beta$ 4-spectrin, localizing fluorescence clusters corresponding to the center of the spectrin tetramers within the dense mesh that surrounds CCPcontaining clearings (Fig. 2F). We confirmed this localization using immunogold staining of β4-spectrin, which similarly showed gold beads localized within the dense mesh (Fig. 2G). Turning to correlative STORM-PREM of actin, we observed fluorescent phalloidin delineating the 190 nm-spaced actin rings throughout the mesh and actin filaments along the circular border of the mesh for some clearings, as well as individual filaments contacting the CCPs (Fig. 2, H and I). Indirect immunogold staining of actin filaments confirmed the presence of actin along the border of the clearings (fig. S2K). We also examined the nanoscale distribution of the AIS master organizer ankyrin G (ankG) that interacts with the center of the spectrin tetramers (8). Correlative STORM-PREM and immunogold labeling showed that ankG is present within the spectrin mesh, including around the CCP-containing clearings (fig. S2, L and M). Thus, in addition to forming a periodic actin-spectrin lattice, the AIS membrane-associated scaffold accommodates the presence of CCPs on the plasma membrane by forming circular clearings, with spectrins pushed away from the clearings and actin filaments enriched at their border (fig. S7).

## The submembrane actin-spectrin mesh restricts CCP formation at the AIS

Given the formation of CCPs at the center of the circular clearings, we investigated whether the periodic actin-spectrin mesh could control the formation of CCPs at the AIS. To test this hypothesis we knocked down  $\alpha$ 2- and/or β4-spectrin in neuronal cultures using short hairpin RNA (shRNA) adeno-associated viruses (AAVs). These AAVs could knock down spectrins efficiently, as shown by immuno blots (fig. S3A) and immunolabeling (fig. S4, A to D). In addition, AAV-expressed shRNAs against  $\alpha$ 2-spectrin,  $\beta$ 4-spectrin, or a combination of both disorganized the periodic scaffold, as shown by the loss of periodicity for actin rings along the AIS and proximal axon (fig. S4, E to J). PREM views of unroofed neurons depleted for  $\alpha 2$ - and/or  $\beta 4$ -spectrin showed a lack of submembrane spectrin mesh, with only actin filaments visible and the presence of numerous CCPs (Fig. 3A). Quantification confirmed that depletion of spectrins increased the density of CCPs two to three times in comparison with controls (Fig. 3C; refer to data S1 for exact values), while measurement of membrane occupation in three categories (organized mesh, disorganized mesh, bare membrane) revealed increased areas of disorganized mesh and bare membrane area (Fig. 3D). Thus, the dense spectrin mesh indeed restricts the formation of CCPs at the AIS plasma membrane.

We next used drugs to target CCPs, actin filaments, or the spectrin mesh, and assessed their effect on CCP density. We first evaluated the density of CCPs at the AIS of treated neurons using SIM of intact and unroofed neurons labeled for clathrin and  $\alpha$ 2-spectrin (Fig. 3, D to G). Pitstop 2 is a drug that inhibits receptor-mediated endocytosis by blocking the budding of CCPs from the plasma membrane (24). Pitstop 2 treatment resulted in an increase of the CCP density at the AIS compared with controls, both in intact and unroofed neurons. Treatment with swinholide A, which inhibits new actin filament assembly and disassembles existing ones (14, 25), did not result in a significant increase in CCP density. Diamide is a drug that causes oxidation of spectrins and disruption of their submembrane arrangement (26) Acute diamide treatment resulted in a significant increase in CCP density in both intact and unroofed neurons, similar to the effect of Pitstop 2 (Fig. 3, D to G).

PREM views of unroofed neurons allowed us to characterize the effect of drugs at the ultrastructural level (Fig. 3H): More clearings containing multiple CCPs were observed after Pitstop 2 treatment, swinholide A treatment caused the disappearance of actin rings and maintenance of the spectrin mesh after, and diamide treatment caused precipitation of spectrins into reticulated aggregates, leaving numerous patches of bare accessible membrane. Quantification from PREM views confirmed the SIM data for the effect of drugs on CCP density (Fig. 3I) whereas measurement of membrane occupation revealed the subtle effect of swinholide contrasting with the larger effect of diamide on spectrin organization (Fig. 3J). Overall, perturbation experiments showed that CCP formation at the AIS is not substantially affected by actin disassembly, likely because it leaves the AIS spectrin organization relatively intact (14). By contrast, disorganization of the spectrin mesh increases the density of CCPs at the AIS, suggesting that the disorganization of the spectrin scaffold and its defined clearings makes the plasma membrane more accessible, resulting in the assembly of more CCPs.

To assess whether the regulation of CCP formation by the dense actin/spectrin mesh along the proximal axon is specific to neurons, we performed similar perturbation experiments in Rat2 fibroblasts. In these cells most of the plasma membrane is readily accessible, with the presence of loose disordered  $\alpha 2$ - and  $\beta 2$ spectrin networks as well as areas exhibiting denser spectrin arrays (19, 27). PREM views of unroofed fibroblasts showed abundant clathrincoated structures (CCSs) over their inner surface, with more diverse clathrin structures than in neurons (fig. S5A), ranging from flat to fully curved endocytic pits (28, 29). Using immunogold labeling of  $\alpha$ 2- and  $\beta$ 2-spectrin as well as correlative fluorescence microscopy and PREM, we found that spectrins localized on the cortical mesh in the vicinity of CCSs, directly associating with actin filaments into polygonal networks (fig. S5, B and C). Short interfering RNAs (siRNAs) could efficiently deplete α2and B2-spectrin without affecting clathrinheavy chain expression levels (fig. S3, B to D)



**Fig. 2. Molecular architecture of CCPs and actin-spectrin clearings at the AIS.** (**A**) Gallery of PREM views showing actin-spectrin mesh clearings at different stages of CCP formation, from left to right: Empty clearing, clearing containing a nascent, single mature, or multiple mature CCPs. Scale bars, 100 nm. (**B**) Quantification of clearing diameters for empty clearing or clearings containing nascent, single, or multiple CCPs. The percentage of clearings identified for each category is indicated above each bar. (**C**) Widefield image of a neuron fixed and stained for neurofascin (blue). The contour of the whole neuron (gray) is delineated. Scale bar, 10 µm. (**D**) Two-color 3D-PAINT images of the AIS corresponding to the image in (B) with staining for clathrin (magenta) and  $\alpha$ 2-spectrin (green). Scale bar, 2 µm. (**E**) Zoom-ins showing individual CCPs (magenta) in clearing of the periodic  $\alpha$ 2-spectrin mesh (green) on XY images [top row, corresponding to areas highlighted in (C)] and corresponding XZ transverse sections (bottom row, taken between the lines highlighted on the XY images). Scale bars, 0.5  $\mu$ m. (F) Correlative STORM-PREM image of an unroofed AIS labeled for  $\beta$ 4-spectrin (orange). (Right) zoomed-in images showing CCPs in spectrin mesh clearings, with spectrin encircling the bare membrane area. (G) PREM view of an unroofed AIS immunogold-labeled for  $\beta$ 4-spectrin (yellow). Right, zoomed images showing CCPs in spectrin mesh clearings, with 15-nm gold beads bound to the spectrin mesh around the bare membrane area. (H) Correlative STORM-PREM image of an unroofed AIS labeled for actin (orange). (Right) zoomed-in images showing CCPs in spectrin mesh clearings. Scale bars for (F to H): 5  $\mu$ m (left image), 100 nm (zoom-ins). (I) Images of individual CCP-containing clearings from unroofed neurons stained for actin (orange) and imaged by correlative STORM-PREM. Scale bars, 100 nm.

and resulted in a significant increase in the density of CCSs at the ventral plasma membrane, as visualized by PREM after unroofing (fig. S5, D and F). Disorganization of submembrane spectrins with diamide also increased the density of CCSs in fibroblasts (fig. S5, E and G), confirming that the loose spectrin mesh negatively regulates the formation of CCSs. Actin filament disruption with swinholide A had the largest effect on CCS density (fig. S5F) and was able to completely disorganize the submembrane mesh compared with the partial



#### Fig. 3. The submembrane actin-spectrin mesh restricts CCP formation at the AIS. (A) PREM views of the unroofed proximal axon of neurons infected with AAVs expressing control shRNAs (left) or targeted against $\alpha$ 2-spectrin

with AAVs expressing control shRNAs (left) or targeted against  $\alpha$ 2-spectrin (middle left),  $\beta$ 4-spectrin (middle right) or  $\alpha$ 2-spectrin and  $\beta$ 4-spectrin (right), with CCPs colored in magenta on the main image. Insets, zoom-ins of the area highlighted on the main images. Scale bars, 1 µm (main images), 200 nm (insets). (**B**) Effect of spectrin knockdown on the CCP density as quantified from PREM views of unroofed proximal axons. (**C**) Quantification of the submembrane organization (organized mesh, brown; disorganized mesh, gray; bare plasma membrane, purple) from PREM views of neurons in the control or shRNA against α2-spectrin conditions. (**D**) Effect of the treatment with vehicle (0.1% DMSO, 3 hours), Pitstop 2 (30 μM, 45 min), swinholide A (100 nM, 3 hours), or diamide (5 mM, 45 min) on the density of CCPs along the AIS as quantified from SIM images of intact neurons. (**E**) Effect of drug treatments on the density of CCPs along the AIS as quantified from SIM images of the proximal axon of neurons treated with drugs, fixed, and stained for clathrin (magenta on overlay) and α2-spectrin (green on overlay). Scale bars, 5 μm. (**G**) SIM images of the proximal axon of neurons treated with drugs, then unroofed and fixed before being stained for clathrin (magenta on overlay). Scale bars, 5 μm. (**H**) PREM views of the

proximal axon of unroofed neurons after treatment with vehicle (DMSO 0.1%), Pitstop 2 (30  $\mu$ M, 15 min), swinholide A (100 nM, 3 hours), or diamide (5 mM, 15 min), with CCPs colored in magenta on the main image. Zoomed insets correspond to the area highlighted on the main image. Scale bars, 1  $\mu$ m (low-magnification image for diamide), 500 nm (main images), 200 nm (insets). (I) Effect of drugs on the density of CCPs along the AIS as quantified from PREM views of unroofed neurons. (J) Quantification of the submembrane organization (organized mesh, brown; disorganized mesh, gray; bare plasma membrane, purple) from PREM views of neurons in the control or drug-treated conditions.

disorganization induced by  $\alpha$ 2-spectrin knockdown (fig. S5G). This suggests that in the absence of the dense spectrin mesh found along the axons of neurons, inhibition of endocytosis by actin disassembly can induce a notable accumulation of endocytic structures all over the bare plasma membrane of these non-neuronal cells.

### Endocytic cargo concentrates in stalled CCPs at the surface of the AIS

Owing to possible variations in scission efficiency, CCP density at the plasma membrane does not directly translate into amount of endocytosis occurring in a cell; for example, Pitstop 2 treatment leads to more stalled CCPs at the membrane and completely inhibits endocytosis by blocking CCP scission (24). Thus, we sought to probe the extent of clathrin-mediated endocytosis directly after perturbation of the actin-spectrin submembrane scaffold. We first assessed this in Rat2 fibroblasts using a classic fluorescent transferrin uptake experiment. Knockdown of  $\alpha$ 2- and/or  $\beta$ 2-spectrin did not perturb the expression level of transferrin receptors (fig. S3E) and significantly increased the uptake of transferrin (fig. S5, I and J). By contrast, treatment of fibroblasts with Pitstop 2 and swinholide A, which increased the density of CCPs (see above), efficiently inhibited the endocytosis of fluorescent transferrin (fig. S5K).

We then aimed to assess endocytosis at the AIS of neurons by visualizing the uptake of fluorescent cargo. Incubation of neuronal cultures with fluorescent transferrin for up to 1 hour resulted in its accumulation within the cell body and dendrites but no endocytosed transferrin could be detected along the proximal axon (Fig. 4A), consistent with the reported absence of transferrin receptors from the axon (10, 30, 31). To detect endocytic processes independently of any specific receptor, we used fluorescent 10-kDa dextran as a fluid phase marker (32). After 5 to 30 min of incubation, fluorescent dextran clusters were readily detected along the dendrites and the proximal axon (Fig. 4B). Close inspection of SIM images revealed that a fraction of these dextran clusters along the proximal axon of intact neurons were colocalized with CCPs, an observation confirmed by averaged images centered on CCPs, which showed the selective enrichment in dextran at CCPs (Fig. 4C). To verify that a significant proportion of dextran clusters were not found in endosomes but rather were trapped at the cell surface in CCPs, we unroofed neurons after dextran feeding and before fixation to remove the intracellular compartments. SIM images showed that most of the dextran clusters now colocalized with CCPs after unroofing (Fig. 4D). We confirmed that a major population of dextran clusters were still present at CCPs after 30 min of feeding by obtaining correlative SIM-PREM images of unroofed neurons, which showed colocalization of fluorescent dextran clusters at CCPs on PREM views (Fig. 4E). Thus, the numerous CCPs found in spectrin mesh clearings along the AIS are stalled at the plasma membrane and not efficiently engaged in endocytosis.

We then wanted to test whether perturbation of the actin-spectrin mesh, which increases the density of CCPs by increasing membrane accessibility (see Fig. 3), induces more endocytosis or just more stalled CCPs at the AIS surface. We treated neurons with Pitstop 2, swinholide A, or diamide, combining treatments with a dextran uptake assay. Neurons were kept intact before fixation, labeling, and imaging by SIM (Fig. 4, F and G) or unroofed before fixation (Fig. 4, H and I). In intact neurons, inhibition of CCP scission with Pitstop 2 resulted in a decreased density of dextran clusters whereas actin disassembly by swinholide A did not significantly change this density (Fig. 4G). In unroofed neurons that reveal the surface dextran within stalled CCPs, both Pitstop 2 and swinholide A did not affect dextran cluster density, suggesting that the decrease in intact neurons for Pitstop 2 was due to inhibited endocytosis (Fig. 4I). Diamide treatment resulted in an increased density of dextran clusters both in intact and unroofed neurons, suggesting that the enhanced formation of CCPs after disorganization of the spectrin mesh is not followed by a substantial increase in endocytosis (Fig. 4, G and I). Disorganization of the actin-spectrin mesh thus allows for the formation of more CCPs at the AIS membrane, but these CCPs appear to remain as stalled structures not actively engaged in endocytosis.

### Live-cell super-resolution microscopy reveals static CCPs along the AIS and proximal axon

If CCPs are present at the AIS but rarely result in scission and conclusive endocytosis, they should be long-lived and seen as stable structures by live-cell imaging. To visualize the dynamics of CCPs together with the periodic actin-spectrin scaffold along the proximal axon, we used live-cell TIRF-SIM (*33*) of neurons transfected with clathrin light chain (CLC)mCherry and EGFP- $\beta$ 2-spectrin (Fig. 5A and movie S1). The ~110-nm lateral resolution of TIRF-SIM could clearly resolve individual CCPs and the 190-nm periodicity of B2-spectrin along the AIS and proximal axon, along with low intensity illumination coupled to deep learning-based denoising allowed to image every 20 s for 20 min. The resulting movies (movie S1), visualization of the dynamics through kymographs (static CCPs are vertical lines. Fig. 5B) or time-color coded projections (static CCPs are white dots, Fig. 5C) demonstrate how CCPs are more stable at the AIS compared to the dendrites and cell body of the same neuron, with most AIS CCPs being present during the full 20 min of imaging. Temporal autocorrelation analysis of CLC-mCherry intensity profiles from several movies confirmed this visual impression, with a higher temporal stability of CLC-mCherry at the AIS compared with that of the cell body and dendrites (Fig. 5D).

To confirm this specific stability of AIS CCPs and avoid potential stabilizing effects of overexpressed clathrin or spectrin, we next used a modified AAV-mediated HiUGE strategy (34, 35) to endogenously tag clathrin light chain with EGFP or mStayGold (36) in cultured hippocampal neurons, and imaged clathrin dynamics using super-resolution spinning disk microscopy (Fig. 5E and movie S2). The enhanced temporal resolution (one frame every 5 s for 10 min) allowed us to discern scission events for both static pits at the AIS (appearing as interruptions in the vertical traces on kymographs) and transient pits in dendrites (appearing as J-shaped traces in kymographs; Fig. 5F). Kymographs and time color-coded projections (Fig. 5G) showed that endogenously tagged CCPs are more stable at the AIS compared with the dendrites and cell body, an observation confirmed by temporal autocorrelation analysis of clathrin dynamics from several neurons (Fig. 5H). Thus, CCPs are specifically stabilized at the AIS of neurons, consistent with the restrained endocytic activity observed using cargo uptake.

### Endocytosis at the AIS can be triggered by plasticity-inducing stimulation

So far, we have demonstrated that the actinspectrin scaffold regulates the formation of CCPs at the AIS but that these CCPs are longlived, stalled structures. We reasoned that this could allow AIS endocytosis to be "readyto-go" but be dependent on a physiological trigger. It was recently shown that a brief application of *N*-methyl-D-aspartate (NMDA) induces increased neuronal activity and long-term



#### Fig. 4. Endocytic cargo concentrates in stalled CCPs at the surface of the

**AIS.** (**A**) SIM image of a neuron fed for 1 hour with transferrin-Alexa Fluor 647 (100  $\mu$ g/mL, orange), fixed and stained for map2 (dendrites, blue) and β4-spectrin (AIS, green). Images on the right are straightened zooms along the dendrites and axon highlighted on the main image. (**B**) SIM image of a neuron fed for 30 min with dextran-Alexa Fluor 555 (50  $\mu$ g/mL, orange), fixed, and stained for map2 (dendrites, blue) and β4-spectrin (AIS, green). Images on the right are straightened zooms along the dendrites and axon highlighted on the main image. In (A) and (B), dashed lines highlight the position of the AIS in the dextran uptake and clathrin channels. Scale bars in (A) and (B), 10  $\mu$ m (main

image), 2 µm (zooms). (**C**) (Left) SIM image of the AIS of a neuron fed with dextran-AF555 (30 min, yellow), fixed and stained for clathrin (magenta) and neurofascin (blue). Some dextran clusters colocalized with CCPs (arrowheads). (Right) average images of 591 pits centered on CCPs showing the distribution of dextran (orange), clathrin (magenta) and  $\alpha$ 2-spectrin (green). The graph shows the radial intensity profile corresponding to the average images, with the dextran clusters colocalizing with CCPs in an area devoid of  $\alpha$ 2-spectrin. (**D**) (Left) SIM image of the AIS of a neuron fed with dextran-AF555 (yellow), unroofed, fixed and stained for clathrin (magenta) and neurofascin (blue). Most dextran clusters colocalized with CCPs (arrowheads). (Right) average images

of 562 pits centered on CCPs showing the distribution of dextran (orange), clathrin (magenta), and  $\alpha$ 2-spectrin (green). The graph shows the radial intensity profile corresponding to the average images (gray arrow), with dextran clusters colocalizing with CCPs in an area devoid of  $\alpha$ 2-spectrin. Scale bars for (C) and (D): 2  $\mu$ m (AIS images), 1  $\mu$ m (zooms), 100 nm (average images). (**E**) Correlative SIM-PREM image of the AIS of a neuron fed with dextran-AF555 (orange), unroofed, fixed and stained for  $\beta$ 4-spectrin (green) and actin (blue). Zoomed overlays on the right show dextran clusters found at CCPs in areas devoid of spectrin labeling (arrowheads). Scale bars, 5  $\mu$ m (isolated SIM channels), 500 nm (main overlay), 100 nm (zooms). (**F**) SIM images of the AIS of neurons treated with vehicle (0.1% DMSO, 3 hours), Pitstop 2 (30  $\mu$ M,

depression (LTD), in turn causing structural AIS plasticity driven by Nav1.2 voltage-gated sodium channel endocytosis along the distal AIS (37). We thus wanted to test whether this up-regulated AIS endocytosis results from a rearrangement of the actin-spectrin mesh and engagement of CCPs into conclusive endocytosis. Neurons were stimulated with NMDA (50 µM, 4 min) before resting and dextran uptake steps (fig. S6A). Quantification of CCP and dextran cluster densities from SIM images of intact and unroofed neurons (Fig. 6, A to D) showed that NMDA stimulation modestly increased the density of CCPs at the AIS compared with controls. By contrast, NMDA stimulation induced a significant increase in dextran cluster density in intact neurons (Fig. 6C) but it did not change the detected density of surface dextran clusters (Fig. 6D). This indicates that the extra dextran clusters in the intact condition are endocytosed and suggests that NMDA stimulation can trigger endocytosis from existing CCPs at the AIS membrane. We confirmed by performing an uptake experiment using MemGlow/MemBright, a fluorescent probe to stain the plasma membrane and endocytic compartments during the uptake assay (38, 39). After NMDA stimulation and rest, numerous small, round endosomes were detected within the AIS, compared to the fainter, tubule-vesicular profiles seen in control neurons (Fig. 6E).

We next visualized the submembrane ultrastructure of NMDA-treated neurons with PREM. High-magnification images of unroofed proximal axons revealed partial disorganization of the spectrin mesh compared to the control and the presence of CCPs with normal morphology (Fig. 6F). We observed branched actin filaments surrounding CCPs (see fig. S6B for another example), which we quantified by measuring the area of clearings occupied by branched actin (Fig. 6G). Coapplication of latrunculin A (LatA, fig. S6A) during the whole experiment abolished the formation of these branched actin nests around CCPs by inhibiting the polymerization of new actin filaments (Fig. 6, F and G). We used SIM of unroofed neurons to confirm the appearance of clusters of actin around clathrin-coated pits in holes of the β4-spectrin labeling after NMDA stimulation, clusters that are eliminated by LatA (Fig. 6H). In addition to the formation of actin nests, close examination of PREM views of NMDA-treated neurons showed that the moderate increase in CCP density (Fig. 6I), which is consistent with the effect seen on SIM images (see Fig. 6C), corresponded to elevation of the average number of CCPs per clearing (Fig. 6J) and significantly increased diameter of the clearings (fig. S6C). This could be because of increased dynamics of CCP formation and scission from existing clearings thereby resulting in the observed elevated endocytosis. Quantification of the membrane organization showed no significant change after NMDA treatment, with most of the membrane consisting of organized mesh whereas co-application of LatA induced a slight increase of bare membrane areas (fig. S6D). To refine this assessment, we used STORM to image NMDA-treated, intact neurons stained for  $\beta$ 4-spectrin (fig. S6, E and F). This revealed that the periodicity of the spectrin mesh was attenuated but still detected after NMDA treatment (fig. S6, G and H).

Thus, NMDA treatment leads to polymerization of branched actin nests around CCPs in clearings, which is correlated with an increase in endocytosis along the AIS. To demonstrate that these actin nests are driving the engagement of CCPs toward conclusive endocytosis, we used co-application of LatA during NMDA stimulation and rest followed by dextran feeding (fig. S6A). LatA slightly reduced the density of CCPs below that of the control as measured by SIM on intact neurons (Fig. 6L) but the density of CCPs measured on PREM images remained constant (Fig. 6I), indicating that actin polymerization could also act at the level of CCP formation but primarily acts on CCP fission. In addition, LatA was able to reverse the effect of NMDA on dextran cluster densities, bringing their values down to that of the control (Fig. 6, K and M). Thus, the newly polymerized actin plays a role in the triggering of endocytosis by the plasticity-inducing NMDA treatment.

#### Discussion

The AIS and proximal axon are neuronal compartments in which endocytosis has long been overlooked. We discovered that in the AIS, CCPs form on bare areas of the plasma membrane that are precisely delimited by a circular

45 min), swinholide A (100 nM, 3 hours), or diamide (5 mM, 45 min), fed with dextran-AF555 (30 min, yellow), fixed, and stained for clathrin (magenta) and neurofascin (blue). Dashed lines highlight the position of the AIS in the dextran uptake and clathrin channels. Scale bars, 5  $\mu$ m. (**G**) Effect of drugs on the dextran cluster density along the AIS as quantified from SIM images of intact neurons. (**H**) SIM images of the AIS of neurons treated with vehicle, Pitstop 2, swinholide A, and diamide, fed with dextran-AF555 (yellow), unroofed, fixed and stained for clathrin (magenta) and neurofascin (blue). Dashed lines highlight the position of the AIS in the dextran uptake and clathrin channels. Scale bars, 5  $\mu$ m. (**I**) Effect of drugs on the density of dextran clusters along the AIS as quantified from SIM images of unroofed neurons.

clearing of the actin-spectrin undercoat. These ~300-nm clearings are scattered across the inner surface of the AIS and proximal axon. inserted within the previously described periodic scaffold of actin rings connected by spectrin tetramers (14). They exhibit a specific organization wherein actin filaments are present at the border of the clearings, crosslinked by spectrins and ankyrin that form an outer polygonal arrangement. In addition, a single actin filament is often seen to enter the clearing and contact the central CCP (fig. S7). We propose that clearings are structures that allow the formation of CCPs by providing access to the AIS plasma membrane. The formation of CCPs in membrane areas devoid of spectrins was previously shown by diffraction-limited fluorescent microscopy in spectrin-dense regions of fibroblasts (19), and along the lateral membrane of epithelial cells (20), which makes sense along axons where the spectrin mesh is particularly dense.

What are the respective roles of the submembrane actin and spectrin in defining the patterns of CCP formation? An inhibitory role of spectrins on CCP formation and subsequent endocytosis has been proposed for fibroblasts (19), and endocytosis is negatively controlled by the ankyrin/spectrin domains at the lateral membrane of epithelial cells (20). We demonstrate that spectrin knockdown or disorganization by diamide directly up-regulates CCP formation in fibroblasts and along the AIS in neurons, establishing the spectrin mesh as an insulating layer restricting CCP formation. Indeed, the existence of clearings containing various stages of nascent CCPs and the increase in CCP density after spectrin knockdown or disruption suggests that the spectrin mesh regulates the formation of CCPs by defining the location and density of these clearings. Actin, by contrast, has been shown to be important for the late stages of clathrin-mediated endocytosis (21, 40), as confirmed by the presence of numerous stalled CCPs in fibroblasts after swinholide A treatment. In our experiments, swinholide A is not able to alter the density of CCPs significantly along the AIS, suggesting that the remaining spectrin mesh is the primary factor for the formation of clearings and CCPs. Beyond the AIS, the periodic



Fig. 5. Live-cell super-resolution microscopy reveals static CCPs along the AIS and proximal axon. (A) Individual frame from movie S1 showing a neuron transfected with  $\beta$ 2spectrin-GFP (blue) and CLC-mCh (orange) and imaged by TIRF-SIM every 20 s for 20 min. Scale bar, 5  $\mu$ m. (B) Zooms on areas highlighted in (A) with corresponding kymographs (highlighted white lines) along the AIS and proximal axon (top), along a dendrite (bottom left), inside the cell body (bottom center) and a neighboring glial cell (bottom right). Spectrin bands appear as vertical lines spaced by 190 nanometers (blue) and static pits appear as vertical lines and dynamic pits as dots (orange). Scale bars, 2  $\mu$ m (images), 1  $\mu$ m (kymograph horizontal), 5 min (kymograph vertical). (C) Time color-coded projection of the CLC-mCh channel for the neuronal compartments shown in (B): static pits are white and dynamic pits are colored due to their transient appearance. Scale bars, 2  $\mu$ m. (D) Temporal autocorrelation of the clathrin dynamics from tracings within the different compartments of neurons: AIS (orange), dendrites (blue), and cell body (pink). Significances correspond to tests at 60, 120,

180, and 240 s (see data S1). (**E**) Individual frame from movie S2 showing a neuron with CLC endogenously tagged with GFP (orange), and extracellular staining for neurofascin highlighting the AIS (blue), imaged by SoRa spinning disk every 5 s for 10 min. Scale bar, 10  $\mu$ m. (**F**) Zooms on areas highlighted in (E) with corresponding kymographs (highlighted white lines) along the AIS (top left), along two dendrites (top and bottom right), and inside the cell body (bottom left). Static CCPs appear as vertical lines, with putative endocytic events appearing as discontinuities of characteristic J-shaped traces. Scale bars, 2  $\mu$ m (image), 1  $\mu$ m (kymograph horizontal), 5 min (kymograph vertical). (**G**) Time color-coded projection of CLC-GFP for the neuronal compartments shown in (B): static pits are white and dynamic pits are colored due to their transient appearance. Scale bars, 2  $\mu$ m. (**H**) Temporal autocorrelation of the clathrin dynamics from tracings within the different compartments of neurons, showing the relative stability of CLC in the axon (orange) compared to the dendrites (blue,) and 240 s (see data S1).

actin-spectrin scaffold is likely to regulate endocytosis along the whole axon, as shown by the enhanced endocytosis of cannabinoid receptors after activation in neurons depleted for  $\beta$ 2-spectrin, a part of the distal axon periodic scaffold (41).

In addition to the insulating effect of the spectrin mesh on CCP formation, our results

suggest that a second level of regulation is present to control endocytosis at the AIS. We found that CCPs that do form in mesh clearings along the AIS are unusually long-lived



#### Fig. 6. Endocytosis at the AIS can be triggered by plasticity-inducing

**stimulation.** (A) SIM images of the AIS of neurons treated with vehicle (0.1% water), or NMDA (50  $\mu$ M, 4 min followed by 30 min rest), fed with dextran-AF555 (30 min, yellow), fixed and stained for clathrin (magenta) and neurofascin (blue). (B) SIM images of the AIS of neurons treated with vehicle (0.1% DMSO), or NMDA, fed with dextran-AF555 (yellow), unroofed, fixed, and stained for clathrin (magenta) and neurofascin (blue). In (A) and (B), dashed lines highlight the position of the AIS in the dextran uptake and clathrin channels. Scale bars for (A) and (B) 5  $\mu$ m. (C) Effect of NMDA stimulation on the CCP density along the AIS as quantified from SIM images of intact neurons (left bars) or unroofed

neurons (right bars). (**D**) Effect of NMDA stimulation on the dextran cluster density along the AIS as quantified from SIM images of intact neurons (left bars) or unroofed neurons (right bars). (**E**) SIM images of neurons treated with vehicle or NMDA, fed 30 min with MemGlow560 (gray) and anti-neurofascin extracellular antibody (blue), fixed and stained. Scale bars, 5  $\mu$ m (main images); 1  $\mu$ m (zooms). (**F**) PREM views of the proximal axon of unroofed neurons after treatment with vehicle, NMDA, or NMDA+LatA (5  $\mu$ M throughout the experiment) with CCPs (magenta) and branched actin surrounding CCPs (yellow) on the main image. Zooms correspond to the area highlighted on the main image. Scale bars, 1  $\mu$ m (main images), 200 nm (zooms). (**G**) Surface

occupied by branched actin inside clearings in the control, NMDA and NMDA +LatA conditions. (H) SIM images of the AIS of neurons treated with vehicle, NMDA or NMDA+LatA, unroofed, fixed and stained for actin (gray), clathrin (orange) and  $\beta$ 4-spectrin (blue). Arrowheads (gray) point to actin clusters next to CCPs. Scale bars, 5  $\mu$ m (main images), 1  $\mu$ m (zooms). (I) CCP density quantified from PREM views of unroofed neurons treated with vehicle or NMDA. (J) average number of CCPs per clearing quantified from PREM views of unroofed neurons treated with vehicle or NMDA. (K) SIM images of the AIS of

neurons treated with vehicle (0.1% water), NMDA (50  $\mu$ m, 4 min followed by 30 min rest), or NMDA+LatA (5  $\mu$ M throughout the experiment), fed with dextran-AF555 (30 min, yellow), fixed and stained for clathrin (magenta) and neurofascin (blue). Dashed lines highlight the position of the AIS in the dextran uptake and clathrin channels. Scale bars, 5  $\mu$ m. (L) CCP density along the AIS of neurons treated with vehicle, NMDA, or NMDA+LatA as quantified from SIM images of intact neurons. (M) Dextran cluster density along the AIS of neurons treated with vehicle, NMDA, or NMDA+LatA as quantified from SIM images of intact neurons.

and do not readily engage in conclusive endocytosis, accumulating cargoes at the AIS surface. A signal is necessary to unlock these CCPs and engage them in conclusive endocytosis: We found that a chemical long-term depression protocol based on NMDA stimulation, previously shown to induce endocytosis at the distal AIS (37), can indeed provide this signal and trigger the scission of stalled CCPs (fig. S7). In this second level of regulation, actin seems to play a prominent role, as shown by the formation of dense branched actin nests around CCPs in clearings that are necessary for increased endocytosis. This role for branched actin in CCP maturation and scission is consistent with the "edge pushing" model recently proposed for cortical actin around CCPs (21), and we speculate that the isolated actin filaments we consistently saw contacting CCPs inside clearings in resting neurons might be the nucleating structure for these actin nests. It will be important for future studies to decipher the upstream pathway that connects synaptic NMDA activation to local actin polymerization at the AIS clearings.

What could be the functional relevance of these two levels of regulation of endocytosis at the AIS by the submembrane scaffold? First, it implies that the steady-state amount of endocytosis at the AIS is low in mature neurons. This is consistent with the stability of AIS membrane proteins (42) and the existence of mechanisms inhibiting their endocytosis (15); it suggests that the recently proposed role of AIS endocytic retrieval and degradation in the polarized sorting of somatodendritic proteins might primarily occur in developing neurons (18). Secondly, the presence of pre-positioned, static endocytic spots within the dense architecture of the AIS can facilitate the regulation of AIS membrane proteins (sodium channels, neurofascin) that are bound to the remarkably stable ankyrin/spectrin scaffold (43). Endocytosis of these proteins would require two simple steps that do not imply profound structural rearrangements: (i) Phosphorylation-dependent detachment from ankyrin G (44, 45) that allows them to diffuse into the nearest CCPs (ii) followed by triggered engagement of stalled CCPs into conclusive endocytosis. This "ready to go" mechanism is an attractive hypothesis to explain how the AIS length or position can be modified over a short time after physiological stimuli (46). Overall, our results support a model in which CCPs form along the AIS in clearings of the actin-spectrin scaffold, where they are stabilized as long-lived structures with low endocytic activity at steady state. Efficient endocytosis can be triggered by physiological signals, allowing rearrangement of the AIS membrane content or internalization of extracellular components (fig. S7). We look forward to further exploring the mechanisms and roles of this regulated process, which is likely to have physiological and pathological implications for neuronal organization and function.

#### Materials and Methods Animals and neuronal cultures

The use of Wistar rats followed the guidelines established by the European Animal Care and Use Committee (86/609/CEE) and was approved by the local ethics committee (agreement D13-055-8). Rat hippocampal neurons were cultured following the Banker method, above a feeder glia layer (47). Rapidly, 12- or 18-mmdiameter round #1.5H coverslips were affixed with paraffin dots as spacers, then treated with poly-L-lysine (Sigma-Aldrich #P2636). Hippocampi from E18 rat pups were dissected and homogenized by trypsin treatment followed by mechanical trituration and seeded on the coverslips at a density of 4000 to 8000 cells/cm<sup>2</sup> for 3 hours in serum-containing plating medium. Coverslips were then transferred, cells down, to petri dishes containing confluent glia cultures conditioned in B27-supplemented (Thermo Fisher Scientific #17504044) neurobasal medium (NB, Thermo Fisher Scientific #21103049), and cultured in these dishes for up to 4 weeks. For this work, neurons were fixed at 13 to 16 days in vitro (div), a stage where they exhibit a periodic actin/spectrin scaffold along virtually all axons (11).

## Fibroblast cultures and siRNA-mediated knockdown of spectrins

Rat2 fibroblasts were seeded on 18-mm glass coverslips to 80% confluency in DMEM containing 10% FCS, and they were incubated overnight at 37°C. Prior to transfection, cells were washed in PBS. Complexes of Lipofectamine 3000 (Thermo Fisher Scientific) and siRNA (corresponding to the shRNA sequences detailed below) were then introduced to the cells following the manufacture protocol (Thermo Fisher Scientific). The cells were left to incubate for 48 hours.

The validation of siRNAs knock-down in fibroblasts was performed by immunoblot. Rat2 cultures were lysed in a buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40) supplemented with a protein inhibitor cocktail 1:100 (Sigma-Aldrich). Samples denatured by 3 min boiling in Laemmli buffer were separated by electrophoresis on 4 to 12% bisacrylamide gel (Thermo Fisher Scientific), then transferred to 0.45 µm nitrocellulose membranes (Thermo Fisher Scientific) and labeled with a primary antibody then a secondary antibody coupled to horseradish peroxidase (Trueblot immunoglobulin G HRP, Rockland). Proteins in samples were detected using Immobilon Western Chemiluminescent HRP Substrate (Sigma-Aldrich) and image acquisition was performed on a G-Box (Ozyme). The following primary antibodies were used to validate siRNA knock-down in fibroblasts: anti- $\alpha$ 2-spectrin (mouse IgG2b clone D8B7, Biolegend #803201, 1:1000), anti-\2-spectrin (BD Biosciences #612563, mouse IgG1 against residues 2101 to 2189 of human  $\beta$ 2-spectrin, 1:100), anti-clathrin (rabbit polyclonal anti-CHC, Abcam #ab21679, 1:1000), anti-TfR (mouse IgG1, Invitrogen #13-6800, 1:1000); anti-GADPH (mouse IgG1, Santa Cruz #sc-47724, 1:1000) was used as a loading control.

#### Plasmid constructs and neuronal culture transfection

Transfections were performed using the following constructs: clathrin-GFP and clathrinmCherry plasmids were a gift from Subhojit Roy (48). The EGFP- $\beta$ 2-spectrin plasmid was a gift from Damaris Lorenzo (49). The spectrinsilencing shRNA plasmids were constructed in the pRFP-C-RS backbone (Origene). These plasmids allow cloning and expression of shRNA under a U6 promoter and co-expression of the TurboRFP red fluorescent protein under the control of a CMV promoter. Insertion of the shRNA sequences was performed using the SLIC technique using overlapping single strand oligonucleotides (50).

The sequences for each shRNA were:  $\alpha$ 2-spectrin:

**g**ATCAGTTTGTGGAAGAAACTT<u>tcaagag</u> AAGTTTCTTCCACAAACTGAT

β2-spectrin:

**g**CAGAAGAGATCTCCAACTACAtcaagag

TGTAGTTGGAGATCTCTTCTG β4-spectrin:

**g**CACTGGATAGCCGAGAAGG<u>tcaagag</u> CCTTCTCGGCTATCCAGTG Downloaded from https://www.science.org at INSERM on August 23

2024

Luc (negative control):

#### **g**CGCTGAGTACTTCGAAATGTC<u>tcaagag</u> GACATTTCGAAGTACTCAGCG

(First g in bold-lowercase: additional g for efficient U6 transcription, underscore-lowercase: shRNA loop. A shRNA directed against the luciferase coding sequence was used as negative control).

Plasmid constructs (0.25 to  $0.5 \,\mu$ g) per 18-mm coverslip were incubated with 1  $\mu$ L of Lipofectamine 3000 (Thermo Fisher Scientific #L3000001) in 50  $\mu$ L NB for 20 min at room temperature for liposomes to form, before being added to 10 div cultured hippocampal neurons, coverslips flipped cells up in FB12 dishes containing preheated NB. Neurons were incubated for 30 min at 37°C with 5% CO<sub>2</sub>. After incubation, the neurons were returned to their origin culture dishes and left incubating for 2.5 to 3 days before imaging.

#### AAV production and titration

AAV viral vectors were prepared by triple transfection in HEK-293 cells using polyethyleneimine transfection agent: pSMD2 AAV vector plasmid containing the shRNA sequences detailed above, pXX6 plasmid coding for the viral sequences essential for AAV production, and p0009 plasmid coding for serotype 9 capsid. Vector particles were purified on an iodixanol gradient and concentrated on Amicon Ultra-15 100K columns (Merck-Millipore). The AAV vectors were titrated as vg per milliliter by quantitative real-time PCR using ITR2 (inverted terminal repeats) specific primers at a 60°C annealing temperature (forward 5'-CTCCATCAC-TAGGGGTTCCTTG-3' and reverse 5'-GTAGATA-AGTAGCATGGC-3') and the MGB Taqman probe 5'-TAGTTAATGATTAACCC-3.

### AAV infection for shRNA-mediated knockdown in neuronal cultures

Spectrin-silencing shRNAs in adeno-associated virus vectors (AAV) were produced and titrated by the AAV production service of Institut de Myologie (Paris). Vectors identifiers AAV9-U6SCR (p05.1 Origen scrambled sequence GAG-AGCCAGATTCAATCTGACGACTATGG), AAV9-U6beta4 / p10.1 (42), AAV9-U6alpha2 / p09.1 (51), AAV9-U6-shRNA-α2+β4-spectrin / p11.1 (both combined) targeted respectively no protein (scrambled control),  $\beta$ 4-spectrin,  $\alpha$ 2-spectrin,  $\alpha$ 2- and  $\beta$ 4-spectrin. The validation of shRNAs knock-down in neurons was performed by immunoblot. Cells infected with an AAV-shRNAs were resuspended in a loading buffer with DTT, diluted to 50% in ultrapure water. The samples were then heated to 90°C for 3 min. After extensive washes, samples were resolved by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoprobed with one of anti- $\alpha$ 2-spectrin (Biolegend #803201, mouse IgG2b clone D8B7, 1:1000), anti-β2spectrin (BD Biosciences #612563, mouse IgG1 against residues 2101 to 2189 of human  $\beta_2$ spectrin, 1:100), anti- $\beta_4$ -spectrin Cter (from Matthew Rasband, Baylor College of Medicine Austin TX, rabbit against residues 2237 to 2236, 1:100), an anti- $\alpha$  tubulin (Sigma-Aldrich #T5168, mouse IgG1 clone B-5-1-2, 1:1500) was used as control. Hippocampal neurons were infected at 3 div with 1.5 to  $3.10^{10}$  AAV particles for 4 coverslips in a 60-mm dish and left in the presence of the AAV until fixation. The shRNAs effect on the periodicity of the axonal actin-spectrin scaffold was evaluated by fluorescence intensity quantification and autocorrelation of intensity profiles along axonal segments (https://github.com/cleterrier/Process\_Profiles).

#### Endogenous clathrin tagging (knock-in)

We used a rat optimized version of the HiUGE method (*34*, *52*), with an adapted Donor Recognition Sequence (DRS) GCGATCGTAATCA-CCCGAGT-GGG. We used the homologous sequence of the protospacer used by Gao *et al.* to cut the clathrin light chain A subunit gene (*Clta*) as close as possible to the stop codon (5 nucleotides before):

Mouse, Gao *et al.*: ...-GGCTCTTCAGTGCACCA-GGG

Rat, this study: GGT-GGCTCTTCAaTGCACCA-GGG

In italics: Additional nucleotides in 5' to have a 20-nucleotid length protospacer sequence. In bold the PAM sequence on the *Clta* gene. In lowercase the nucleotide that differs from the mouse sequence. This was used for aminoterminal insertion of a sequence coding for EGFP or mStayGold with a c4 linker (*36*).

AAVs were produced by the standard triple transfection in HEK293T in the AAV9/PHP.S serotype. Small scale AAV preparations were obtained by mild lysis of HEK cells 3 days post transfection in a citrate buffer (38.1 mM citric acid, 74.8 mM NaHCO3, 75 mM NaCl, 100 mM MgCl, pH 5) and neutralized subsequently with Tris-HCl pH 9.5. AAV particles were titrated by qPCR with an improved version of the Addgene SYBR green method. In brief, we used a linearized AAV plasmid as standard instead of a supercoiled plasmid and we incorporated a proteinase K step after the DNAse treatment. We usually obtained titers between 10<sup>11</sup> and 10<sup>12</sup> part/mL. Hippocampal neurons were triply infected after plating at 0 div and at a density of 12,000 cells/cm<sup>2</sup> with 10<sup>9</sup> AAV particles per coverslip for each 3 different AAVs: Mecp2-Cas9 AAV, clathrin-Cter sgRNA AAV and DRSR2-EGFP-ORF1 Cter HiUGE payload and left in the presence of the AAVs for 48 hours.

#### Pharmacological treatments

Treatments were applied on neurons and fibroblasts in their original culture medium at  $37^{\circ}$ C, 5% CO<sub>2</sub>. Dimethyl sulfoxide (DMSO) 0.1% (from pure DMSO, Sigma-Aldrich #D2650) was used as a control vehicle, Pitstop 2 30  $\mu$ M (from

30 mM stock in DMSO, Abcam #120687) was applied for 15 min (45 min in neuron feeding experiments), swinholide A 100 nM (from 100 µM stock in DMSO, Sigma-Aldrich #S9810) was applied for 3 hours, diamide 5 mM (from 1M stock in ultrapure water, Sigma-Aldrich #D3648) was applied for 15 min (45 min in neuron feeding experiments). All stock solutions were stored at -20°C. For NMDA stimulation, 14 div neurons were first incubated 5 to 15 min in 500 µL of uncomplemented neurobasal medium (NB) in a 12 wells plate at 37°C 5% CO<sub>2</sub>, then NMDA (from 50 mM stock in ultrapure water, Sigma-Aldrich #M3262), diluted to 100 µM in preheated NB, was added to the wells for a final concentration of 50 uM. For control experiments, NB without NMDA was used. After 4 min, neurons were gently washed 3 times with 500 µL NB, then returned to their original complemented medium and incubated for 30 min. Neurons were then either fixed and stained as described in the immunocytochemistry section or incubated with an endocytosis probe as described in the uptake assays section. LatA coapplication at 5 µM (from 10 mM stock in water, Merck #5163) was performed during all steps of the NMDA stimulation experiments (pretreatment, NMDA treatment, rest, uptake).

### Unroofing and fixation of fibroblasts and neuronal cultures

Unroofing was performed by sonication as previously described (53). Coverslips were quickly rinsed three times in Ringer+Ca (155 mM NaCl, 3 mM KCl, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 4.5 mM HEPES, 10 mM glucose, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.2), then immersed 10 s in Ringer-Ca (155 mM NaCl, 3 mM KCl, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 4.5 mM HEPES, 10 mM glucose, 3 mM EGTA, 5 mM MgCl<sub>2</sub>, pH 7.2) containing 0.5 mg/mL poly-L-lysine, then quickly rinsed in Ringer-Ca then unroofed by scanning the coverslip with rapid (2 to 5 s) sonicator pulses at the lowest deliverable power in KHMgE buffer (70 mM KCl, 30 mM HEPES, 5 mM MgCl<sub>2</sub>, 3 mM EGTA, pH 7.2). Unroofed cells were immediately fixed in 4% PFA in KHMgE for 10 min for epifluorescence microscopy, SIM and SMLM, for 45 min for PREM of immunogold-labeled samples, and for 15 min for PREM, correlative SIM-PREM and SMLM-PREM. After fixation and rinses, all neuronal samples for PREM were placed in individual sealed transport wells containing 5 mL of KHMgE with 2% glutaraldehyde and shipped immediately the following day. Samples for correlative light microscopy-PREM were immunolabeled and imaged immediately and kept in KHMgE with 2% sodium azide (Sigma-Aldrich #08591) at 4°C for a maximum of 1.5 days before being shipped in the same manner.

#### Fluorescence immunocytochemistry

Fluorescence immunocytochemistry of neuronal cultures for widefield microscopy, SIM and

SMLM was performed as in published protocols (54). Cells were fixed using 4% PFA in PEM buffer (80 mM PIPES pH 6.8, 5 mM EGTA, 2 mM MgCl<sub>2</sub>) for 10 min at room temperature (RT). After rinses in 0.1 M phosphate buffer (PB), neurons were blocked for 2 to 3 hours at RT in immunocytochemistry buffer (ICC: 0.22% gelatin, 0.1% Triton X-100 in PB), and incubated with primary antibodies diluted in ICC overnight at 4°C. After rinses in ICC, neurons were incubated with secondary antibodies diluted in ICC for 1 hour at RT and rinsed. Actin staining was then performed by incubating in fluorescent phalloidin at  $0.5 \,\mu M$  for either 1 hour at RT or overnight at 4°C. Stained coverslips were kept in PB + 0.02% sodium azide at 4°C until imaging by SMLM. For widefield microscopy and SIM, coverslips were mounted in ProLong Glass (Thermo Fisher Scientific #P36980).

Immunofluorescence of unroofed neurons for correlative SIM-PREM was performed using a fast protocol, beginning with 3 rinses in KHMgE buffer, then 30 min blocking in detergent-free buffer (DFB: KHMgE, 1% BSA), 30 min primary antibodies incubation at RT, 3 DFB rinses with 5 min incubation between them, 30 min secondary antibodies incubation at RT, 3 DFB rinses as before, then a KHMgE rinse, keeping the unused coverslips in KHMgE at 4°C until acquired in SIM.

Immunogold labeling of unroofed samples was performed in the same detergent-free solution: samples were blocked for 30 min, incubated for 90 min with the primary antibodies diluted to 1:20, rinsed, incubated two times 20 min with the gold-coupled secondary antibodies, then rinsed.

For immunogold labeling of actin, unroofed neurons were fixed with 2% PFA in KHMgE, then quenched for 10 min in KHMgE, 100 mM glycine, and 100 mM NH4Cl. After blocking in KHMgE, 1% BSA, they were incubated with phalloidin-Alexa Fluor 488 (0.5  $\mu$ M, Thermo Fisher Scientific #A12379) for 45 min, then immunolabeled using a polyclonal rabbit anti-Alexa Fluor 488 primary antibody (1:20, Thermo Fischer Scientific #A11094) and a gold coupled goat anti-rabbit secondary antibody as described above for immunogold labeling.

#### Antibodies labeling, probes, and other reagents

Rabbit polyclonal anti  $\beta$ 4-spectrin antibody (against residues 2237 to 2256 of human  $\beta$ 4spectrin, 1:500 dilution for immunofluorescence IF, 1:20 for immunogold IG) was a gift from Matthew Rasband (Baylor College of Medicine, Austin, TX). Mouse monoclonal anti  $\beta$ 2spectrin (clone 42, against residues 2101 to 2189 of human  $\beta$ 2-spectrin, 1:100 for IF) was from BD Biosciences (#612563). Mouse monoclonal anti  $\alpha$ 2s-spectrin (clone D8B7, 1:100 for IF) was from Biolegend (#803201). Rabbit polyclonal anti 480-kDa ankyrin G (residues 2735

to 2935 of rat 480-kDa ankyrin G, 1:300 for IF, 1:100 for IG) was a gift from Vann Bennett (Duke University, Durham, NC). Chicken polyclonal anti-map2 (against residues 2 to 314 of human map2, 1:1000 for IF) was from Synaptic Systems (#188006). Chicken polyclonal anti pan-neurofascin (against residues 25 to 1031 of rat neurofascin, 1:400 for IF) was from R&D System (#AF3235). Mouse monoclonal antineurofascin (clone A12/18, against extracellular residues 25 to 110 common to rat neurofascin 155 and 186, 1:250 for live staining) was from NeuroMab (#75-172). Rabbit polyclonal anticlathrin heavy chain (against residues 1650 to C terminus of human clathrin heavy chain, IF 1:150) was from Abcam (#ab21679). Mouse monoclonal anti-clathrin light chain (clone X22, 1:100 for IF) was from Thermo Fisher Scientific (#MA1-065); we also used clone CON.1 (1:1000 for IF) from Sigma-Aldrich (#c1985) and guinea pig polyclonal anti-clathrin light chain (1:500 for IF) from Synaptic Systems (#113004). Mouse monoclonal anti-AP2 (clone AP6, 1:100 for IF) was from Abcam (#ab2730).

#### Secondary antibodies and reagents

Donkey and goat anti-rabbit, anti-mouse, and anti-chicken secondary antibodies conjugated to Alexa Fluor 488, 555, and 647 were from Life Technologies or Jackson ImmunoResearch (1:200-1:400 for IF). Goat anti-rabbit and antimouse secondary antibodies conjugated to DNA-PAINT handles were obtained from Massive Photonics (MASSIVE-sdAB 2-PLEX). Goat antirabbit and anti-mouse secondary antibodies conjugated to gold nanobeads were from Aurion (15 nm gold, #815011 and #815022, respectively, 1:20 for IG) or Jackson ImmunoResearch (18 nm gold, #111-215-144 and #115-215-146, respectively, 1:15 for IG). For light microscopy, we used Alexa Fluor 488 and Alexa Fluor 647 Plus-conjugated phalloidin from Thermo Fisher Scientific (#A12379 and #A2287, respectively) and Atto488or Atto643-conjugated phalloidin (#AD48881 and #AD643-8, respectively) from Atto-Tec. Paraformaldehyde (PFA, #15714, stock 32% in water) was from Electron Microscopy Sciences, and glutaraldehyde (#G5882, stock 25% in water) was from Sigma-Aldrich.

#### Transferrin, dextran and MemGlow uptake assays

For pulse-chase uptake assay of transferrin internalization in rat fibroblasts, cells were cultured on 12-mm glass coverslips in DMEM and 10% FCS to 80% confluency. Transferrin from human serum conjugated to Alexa Fluor 488 (Thermo Fisher Scientific #T3342) was used at 20  $\mu$ g/mL for 10 min at 37°C. Cells were acid-stripped (0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M citric acid), rinsed three times in PBS, and then fixed in 4% paraformaldehyde (PFA) for 20 min. Glass slides were mounted with coverslips using Vectashield containing DAPI (Vector Laboratories #H-1200). Regions of interest were de-

fined based on the cell shape. Individual cells were manually outlined and corrected total cell fluorescence = integrated density – (area of selected cell × mean fluorescence of background readings) was calculated using Fiji imaging software.

For transferrin uptake experiments in neurons, cells that were incubated for 1 hour in Neurobasal medium, then Alexa-Fluor-647conjugated transferrin was used (Thermo Fisher Scientific, #T23366, stock 5 mg/mL in water) diluted to  $100 \,\mu\text{g/mL}$  in the same medium and incubated for 1 hour at 37°C and 5% CO2. After incubation, the cells were quickly rinsed 3 times with pre-heated medium and fixed with PFA 4% in PEM buffer. For dextran uptake experiments, the same procedure was used, with Alexa Fluor 555-conjugated dextran 10 kDa (Thermo Fisher Scientific #D34679) diluted to 50 µg/mL in Neurobasal medium and incubated for 30 min at 37°C and 5% CO<sub>2</sub> before rinses and fixation. For MemGlow (commercial name of the MemBright probes) uptake experiments, MemGlow560 (Cytoskeleton Inc #MG02-02) was diluted to 200 nM (from a 100 µM stock in DMSO) in Neurobasal medium and incubated for 30 min at 37°C and 5% CO<sub>2</sub> together with 1:250 mouse anti-neurofascin antibody directed against an extracellular epitope, before rinses and fixation.

#### Live-cell imaging probes and AIS staining

All live-cell experiments were performed in livecell imaging medium (Hibernate E low fluorescence, Brainbits/Thermo Fisher Scientific #NC0285514) supplemented with 3% glucose, 2% B27, and 0.5 mM L-glutamine). Live-cell AIS staining was performed using a mouse anti-neurofascin antibody targeting an extracellular epitope conjugated with CF647 (Mix-N-Stain CF647 antibody labeling kit, Sigma-Aldrich #MX647S50) following the manufacturer protocol. The conjugate was diluted to 1:50 and incubated for 10 min at 37°C and 5% CO<sub>2</sub> before proceeding to SIM or SoRA spinning disk microscopy.

#### Structured illumination microscopy

SIM of neurons was performed on an N-SIM-S microscope (Nikon Instruments). The N-SIM system uses an inverted Nikon Eclipse Ti2-E microscope with a perfect focus system 4, an integrated Nikon laser launch with 405, 488, 561, and 640 nm solid-state excitation lasers, a 100X NA 1.49 oil objective (SR HP Apo TIRF), a Mad City Labs Nanodrive piezo stage and a Hamamatsu Fusion BT CMOS camera. On fixed cells, after locating a neuron of interest using low-intensity illumination, an image was acquired in 2D-SIM/TIRF-SIM (9 images per image) or 3D-SIM (15 images per z plane) mode. Reconstruction was performed using the N-SIM module in the NIS-Elements software (AR 5.30.05), resulting in a ~120-nm lateral (all modalities) and ~250-nm axial resolution (for 3D-SIM).

For live-cell SIM, live-cell imaging medium was used, and the stage was kept at 37°C by a Tokay Hit STX heater. TIRF-SIM movies were captured at a frequency of 0.05 Hz for 15 to 20 min, alternating the 488- and 561-nm illumination to image **β2-spectrin-GFP** and CLCmCherry, respectively. Movies were processed using a deep-learning based denoising module (Enhance.ai in NIS-Elements software, Nikon) using a model trained from 20 pairs of fixed samples imaged at low (10 to 20%) and high (100%) laser intensities. Neurons used for model training were transfected with CLCmCherry and EGFP-82-spectrin, then fixed and mounted in live-cell imaging medium. Movies were stabilized using the Image Stabilizer plugin (https://imagej.net/plugins/image-stabilizer), corrected for bleaching by histogram matching (https://imagej.net/plugins/bleach-correction) and presented using the "Noice complementary" LUTs from Kevin Terretaz (CRBM, Montpellier).

For super-resolved spinning disk microscopy of Rat2 fibroblasts, images were acquired using a Nikon Ti2 microscope, driven by Metamorph (Molecular Devices), equipped with a motorized stage and a Yokogawa CSU-W1 spinning disk head coupled with a Prime 95 sCMOS camera (Photometrics) equipped with a 100X oil-immersion objective lens. Super-resolution images were obtained using the LiveSR module (Gataca Systems). DAPI, Alexa Fluor 488, Alexa Fluor 568 and Alexa Fluor 647 were sequentially excited. Z-series from the top to the bottom of fibers were sequentially collected for each channel with a step of 0.1-0.3  $\mu$ m between each plane.

Live-cell super-resolved spinning-disk imaging of endogenously tagged clathrin-EGFP in neurons was performed on a Yokogawa CSU-W1 spinning disk microscope equipped with 405, 488, 561, and 640 nm solid-state excitation lasers, a SoRa pixel reassignment module, a Ti2-E stand (Nikon), a 60× NA 1.49 oil objective, and a Fusion BT sCMOS camera (Hamamatsu). Live-cell imaging medium was used, and the stage was kept at 37°C by a stage enclosure (Okolab). After capturing a single image of the neurofascin live-cell staining using 647-nm laser excitation, super-resolved images of clathrin-GFP were obtained using 488-nm laser excitation, with the SoRa module 4× magnification lens and a camera binning of 2×2 at a frequency of 0.2 Hz. Movies were processed using a pre-trained deep-learning based denoising module embedded in the NIS-Elements software (Denoise.ai, Nikon).

#### STORM and PAINT

Both STORM and DNA-PAINT acquisitions were performed on an N-STORM microscope (Nikon Instruments). The N-STORM system uses an Agilent MLC-400B laser launch with

405 nm (50 mW maximum fiber output power), 488 nm (80 mW), 561 mW (80 mW), and 647 nm (125 mW) solid-state lasers, a 100× NA 1.49 objective, and an Ixon DU-897 camera (Andor). After locating an axon using lowintensity epifluorescence illumination, followed by either a STORM or DNA-PAINT acquisition using laser illumination in HiLo (grazing angle) configuration. An astigmatic lens was added to the light path to achieve 3D imaging. For STORM, stained coverslips were mounted in a silicone chamber filled with STORM buffer (Smart Buffer Kit; Abbelight), and 30,000 to 60,000 images (256×256, 15ms exposure time) were acquired at 100% 647-nm laser power. Reactivation of fluorophores was performed during acquisition by increasing illumination with the 405-nm laser. For DNA-PAINT, stained coverslips were mounted in a Ludin chamber filled with imaging buffer (Massive Photonics). Imaging strands conjugated to Atto565 and Atto655 (Massive Photonics) were added from a starting concentration of 0.1 nM each, then adjusted to optimize blinking density. 30,000 to 45,000 images (256×256, 40 ms exposure time) were acquired at 25 to 50% laser power for 561 nm, 60 to 100% laser power for 647 nm by alternating frames with 561-nm and 647-nm laser excitation.

For STORM and DNA-PAINT images, acquired stacks were processed using DECODE (55). Briefly, PSFs were modeled using spline fitting in SMAP (Li et al., 2018) and used to simulate sequences of blinking events using characteristics (photon number range and lifetime distribution) inferred from real acquisition data from the N-STORM microscope. A PyTorch model was trained to infer the 3D coordinates and uncertainty of the simulated blinking events and then applied to the experimental acquired sequence (55). The resulting localizations (fitted blinking events) were filtered based on uncertainty, and drift during acquisition was corrected in 3D using a redundant cross-correlation algorithm (56) implemented as an independent module of SMAP (57). After translation of the coordinate files, image reconstructions were performed using the ThunderSTORM ImageJ plugin (58) in Fiji software. Custom scripts and macros were used to translate coordinate files, as well as automate image reconstruction for whole images at 16 nm/pixel for visualization and at 8 nm/pixel for cluster analysis (https://github. com/cleterrier/ChriSTORM).

#### Electron microscopy of platinum replicas

Adherent plasma membranes from rat hippocampal neurons and rat fibroblasts grown on glass coverslips were disrupted by sonication (53). Unroofed cells were fixed and processed as described previously (14). Cells were sequentially treated with 0.5%  $OsO_4$ , 1% tannic acid, and 1% uranyl acetate prior to graded ethanol dehydration and hexamethyldisilane substitution (Sigma-Aldrich). Dried samples were rotary-shadowed with 2 nm of platinum and 6 nm of carbon using a high vacuum sputter coater (Leica Microsystems). The resultant platinum replica was floated off the glass with hydrofluoric acid (5%), washed several times with distilled water, and picked up on 200 mesh formvar/carbon-coated EM grids. Replicas on EM grids were mounted in a eucentric sideentry goniometer stage of either a transmission electron microscope operated at 80 kV (model CM120; Philips) equipped with a Morada digital camera (Olympus), or a transmission electron microscope running at 120 kV (Jeol) equipped with a Xarosa digital camera (EMSYS GmbH). Images were adjusted for brightness and contrast in Adobe Photoshop (Adobe) and presented in inverted contrast.

#### Correlative SIM-PREM/SMLM-PREM microscopy

Unroofed and stained samples were imaged by SIM and an objective-style diamond scriber (Leica) was used to engrave a 1-mm circle around the imaged area. Low-magnification,  $10 \times$  and  $40 \times$  phase and fluorescence images were taken as a reference for relocation. After the acquisition session, samples were stored, shipped and platinum replicated as described in the PREM section. After replica generation and prior to its release, the coated surface of each coverslip was scratched with a needle to make an EM grid sized region of interest containing the engraved circle. Grids were imaged with low-magnification EM to relocate the region that was previously imaged by SIM or SMLM, and high magnification EM views were taken from the corresponding axonal region. The SIM or SMLM reconstructions were mapped and aligned by affine transformation to the corresponding high-magnification EM view using the eC-CLEM plugin in ICY software (59).

#### Data quantification and analysis

Clathrin pits and dextran clusters segmentation (SIM data)

Clathrin and dextran clusters were segmented using a custom script in Fiji (60). After manually delineating ROIs corresponding to the proximal axon on a stack of SIM images, fluorescence within each axon ROI was thresholded (threshold levels were determined by the Moments method for clathrin and dextran intact. Otsu method for clathrin and dextran unroofed, eventually fine-tuned manually). Thresholded areas were then segmented using the StarDist plugin in Fiji (61), with a baseline threshold of 0.5, eventually fine-tuned manually (other StarDist parameters were: StarDist2D Versatile model, normalize bottom 0.1 top 99.8, overlap threshold 0.33). Only the clusters inside the axon ROI or overlapping it by more than half their area were kept, and segmented aggregates of several clusters were removed. The remaining clusters were quantified for area within Fiji and the quantifications exported to a CSV file for each image. The particles CSV files were analyzed in a dedicated JupyterLab notebook, pooling the experiments per condition, and performing particle area, count, density, and pixel-occupation averages.

### Average image of clathrin pits and dextran clusters

The ROI sets for clusters obtained after the segmentation of SIM images were used to generate average intensity plots of clathrin pits and dextran clusters.  $15 \times 15$  pixels centered areas were extracted from each ROI and averaged over all ROIs for each channel. The radial average fluorescence profile for each channel was obtained from the averaged image using the Radial Profile Extended ImageJ plugin.

### Live-cell temporal autocorrelation and kymographs

Live-cell imaging movies were corrected for drift using the Image Stabilization plugin and for bleaching by histogram matching, both in Fiji. Line ROIs were traced on the axon, dendrites, and cell body using NeuronJ (62) on the maximum projection of the movie frames. These line ROIs (360 to 390 nm line thickness) were used to generate kymographs using the KymoResliceWide plugin (https://github.com/ UU-cellbiology/KymoResliceWide). In addition, the intensity profile along thick line ROIs was used to calculate a temporal correlation profile with the ImageCorrelationJ plugin, comparing the image similarity between all pairs of frames distant by 1, 2, ..., (n-1) frames within an n-frames movie (https://www.gcsca.net/LJ/ ImageCorrelationJ.html). Temporal correlation profiles were averaged for ROIs of the same type (axon, dendrite, cell body), resulting in an average temporal correlation profile for this compartment.

### Morphological quantification on PREM views

Clathrin structures and branched actin filaments within clearings were manually delineated on unroofed PM pictures using ImageJ. Each object was classified and the object area was measured. For each image, the total membrane area was also measured to calculate the clathrin structure density corresponding to the object count normalized by membrane area.

#### Membrane occupation on PREM views

Membrane areas were manually delineated on unroofed PREM images using ImageJ and subdivided into either organized mesh, disorganized mesh, and bare membranes (for neurons) or mesh and bare membrane (for fibroblasts). For neurons, areas that showed a distinguishable cytoskeletal network were considered as organized mesh, areas that showed undistinguishable cytoskeletal components were considered as disorganized mesh, and areas that were devoid of any cytoskeletal network were considered as bare membrane. On each image, the total membrane area was also measured to calculate the percentages of each classified membrane area.

#### Statistics

Individual measurement points (n) from independent experiments (N) were pooled. Intensity profiles, graphs, and statistical analyses were generated using Prism. On bar graphs, dots (if present) are averages of each independent experiment, bars or horizontal lines represent the mean, and vertical lines are the SEM unless otherwise specified. Significances were tested using one-way, non-parametric ANOVA with Kruskal-Wallis post-hoc significance testing between selected conditions. In Figures, the results of the post-hoc significance are indicated as follows: ns or ns, non-significant; \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001. All means, SEM and standard deviations together with statistics (number of points n, number of independent experiments N, significance of differences) are summarized in data S1.

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#### SUPPLEMENTARY MATERIALS

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