Structural organization of the actin-spectrin–based membrane skeleton in dendrites and soma of neurons

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It was recently discovered that actin, spectrin, and associated molecules form a membrane-associated periodic skeleton (MPS) in neurons. In the MPS, short actin filaments, capped by actin-capping proteins, form ring-like structures that wrap around the circumference of neurites, and these rings are periodically spaced along the neurite by spectrin tetramers, forming a quasi-1D lattice structure. This 1D MPS structure was initially observed in axons and exists extensively in axons, spanning nearly the entire axonal shaft of mature neurons. Such 1D MPS was also observed in dendrites, but the extent to which it exists and how it develops in dendrites remain unclear. It is also unclear whether other structural forms of the membrane skeleton are present in neurons. Here, we investigated the spatial organizations of spectrin, actin, and adducin, an actin-capping protein, in the dendrites and soma of cultured hippocampal neurons at different developmental stages, and compared results with those obtained in axons, using superresolution imaging. We observed that the 1D MPS exists in a substantial fraction of dendritic regions in relatively mature neurons, but this structure develops slower and forms with a lower propensity in dendrites than in axons. In addition, we observed that spectrin, actin, and adducin also form a 2D polygonal lattice structure, resembling the expanded erythrocyte membrane skeleton structure, in the somatodendritic compartment. This 2D lattice structure also develops substantially more slowly in the soma and dendrites than the development of the 1D MPS in axons. These results suggest membrane skeleton structures are differentially regulated across different subcompartments of neurons.

Significance

Actin, spectrin, and associated molecules form a quasi-1D periodic membrane skeleton in neurons, which organizes membrane proteins in periodic distributions and provides mechanical stability for axons. Here, we provide detailed quantifications of this periodic structure in neurons and show that it develops substantially more slowly in dendrites than in axons. Moreover, we observed a 2D, polygonal lattice structure of these molecules in the somatodendritic compartment. The diverse structural organizations and different developmental courses of the membrane skeleton in different neuronal compartments suggest the membrane skeleton is differentially regulated across these neuronal compartments. The observation of the polygonal lattice structure in cells in addition to erythrocytes suggests a potentially general presence of this structure across diverse cell types.

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spanning nearly the entire axonal shaft by DIV 7 (9). How the MPS develops in dendrites is unclear. It also remains elusive whether the molecular components of the MPS can organize into other structural forms in neurons. Here, we addressed these questions by investigating the distributions of βII-spectrin, βIII-spectrin, adducin, and actin in the dendrites and axons at various developmental stages of cultured hippocampal neurons and compared the results with those obtained in axons.

Results

Spectrin, Actin, and Adducin Adopt 1D Periodic Distributions in a Substantial Fraction of Dendritic Regions in Relatively Mature Cultured Neurons. We used 3D STORM imaging (23, 24) to examine the distributions of βII-spectrin, βIII-spectrin, actin, and adducin in dendrites and axons of cultured mouse hippocampal neurons. Actin, adducin and βII-spectrin are abundantly expressed in both axons and dendrites (9, 25), whereas βIII-spectrin is a β-spectrin isoform specifically enriched in dendrites with only a low expression level in axons (26, 27). We used immunofluorescence to label βII-spectrin, βIII-spectrin, and adducin, and dye-conjugated phallloidin to label actin. The antibody against βII-spectrin recognizes the C terminus of the protein, and hence the middle region of the spectrin tetramer; the antibody against βIII-spectrin recognizes the N terminus of the protein, and hence the ends of the spectrin tetramer. We distinguished dendrites from axons by the positive MAP2 immunoreactivity of dendrites.

We observed 1D periodic distributions of actin, βII-spectrin, βIII-spectrin, and adducin in a substantial fraction of dendritic regions in relatively mature mouse neurons at DIV 28. To quantify the degree of periodicity, we randomly selected ~300–400 dendritic regions (~3 μm in length) of DIV 28 neurons from three independent biological replicates and performed 1D autocorrelation analyses. For all four molecules, we observed that some of the dendritic regions exhibited 1D periodic distributions with ~190-nm intervals, whereas other regions exhibited little periodicity (Fig. 1 A–D). In comparison, we analyzed ~200–300 randomly selected axonal regions (also ~3 μm in length) from the same DIV 28 neuron cultures and observed that nearly all axonal regions showed the 1D periodic pattern with ~190-nm periodicity (Fig. S1). Quantitatively, the average autocorrelation amplitude derived from the dendritic regions was only ~40% of that observed for the axonal regions, and the distributions of autocorrelation amplitudes for dendritic regions were generally shifted to lower values compared with those for axonal regions (Fig. 1 E and F and Fig. S2).

Next, we estimated the fractions of dendritic regions showing the 1D MPS structures. Compared with the unbiased analysis of autocorrelation amplitudes, this fraction quantification has some ambiguity. Whereas some regions unambiguously exhibited either a periodic or a nonperiodic distribution of these MPS molecules, some regions are ambiguous in part because periodic and nonperiodic distributions coexist in (different portions of) these regions (Fig. S2). Nonetheless, when the same criteria are applied to such fraction quantification, results obtained from different neuronal compartments or under different conditions can be meaningfully compared. We adopted two methods to estimate the fraction of regions showing the 1D MPS structure. In the first method, we visually inspected the STORM images of these randomly selected ~3-μm regions and classified regions as exhibiting the 1D MPS structure if a ~50% or greater portion of the region exhibited a periodic distribution. Using this approach, we found that ~50–55% of the examined dendritic regions in DIV 28 neurons exhibited 1D periodic patterns for all four molecular markers: βII-spectrin, βIII-spectrin, actin, and adducin.

In the second method, we inspected the 1D autocorrelation functions and classified the regions showing more than two consecutive peaks at 190-nm intervals in the autocorrelation function as exhibiting the 1D MPS structure. The fractions determined this way were similar (~50–60% for the four molecular markers). In both methods, some of the regions classified as being periodic contained

![Fig. 1. Spectrin, actin, and adducin adopt 1D periodic distributions in a substantial fraction of dendritic regions in relatively mature neurons. (A, Left) Conventional images of dendrites from DIV 28 mouse neurons stained for βII-spectrin (green) and a dendritic marker MAP2 (magenta). (Middle) 3D STORM images of βII-spectrin for the dendritic regions indicated by the yellow boxes on the Left and displaying 1D periodic (Top) and irregular (Bottom) spectrin distributions (the z-position information is color-coded). (Right) 1D autocorrelation functions of the white dashed boxed regions in the Middle. (B–D) Similar to A, but for DIV 28 neurons stained for βII-spectrin (B), actin (C), and adducin (D) instead of βII-spectrin. We note that long actin filaments running along axons and dendrites could obscure the detection of periodic actin rings in the MPS. These long actin filaments are better preserved when the neurons are fixed by paraformaldehyde (PFA) plus glutaraldehyde (GA), but substantially diminished in neurons fixed by PFA alone, whereas the actin filaments in the MPS are well preserved under both fixation conditions. Thus, we used PFA fixation to minimize the obscuring effect of the longitudinal actin filaments on the detection of actin in the MPS (see SI Materials and Methods for more details). (Scale bars, 1 μm.) (E and F) Average autocorrelation functions calculated from ~300–400 randomly selected dendritic regions in DIV 28 mouse neurons stained for βII-spectrin, βIII-spectrin, actin, and adducin (E), as well as from ~200–300 randomly selected axonal regions in DIV 28 neurons stained for βII-spectrin, actin, and adducin (F), from three independent biological replicates for each condition.

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subportions that were nonperiodic, and some of the regions classified as being nonperiodic contained subportions that were periodic. The fraction of regions exhibiting the 1D MPS structure and the average autocorrelation amplitudes of these molecular markers observed for spiny dendrites were similar to those observed on nonspiny dendrites. In comparison, we also performed the same fraction quantifications for axons and found that nearly all (>95%) of the axonal regions were classified as exhibiting the 1D periodic distribution for all three markers expressed in axons (βII-spectrin, actin, adducin), using both methods described earlier.

The observed lengths of the periodic domains in dendrites varied from ~1 to ~30 μm, although overlap between neurites and the finite imaging field of view (40 × 40 μm) can truncate periodic domains and cause an underestimate of the domain length. It is worth noting that, because a dendrite often contained both periodic and irregular regions, the fraction of dendrites exhibiting at least one periodic region was quite large (>80%), consistent with previous results (15, 16). We also observed heterogeneity among dendrites with some dendrites being covered by the 1D MPS structure substantially more extensively than the others. Taken together, these results suggest nearly all dendrites have some tendency to form the 1D MPS structure, but the propensity of MPS formation and/or the regularity of the MPS structure within dendrites is lower than that within axons.

Developmental Course of the 1D MPS Structure in Dendrites. To investigate the developmental course of the 1D MPS in dendrites, we imaged the distributions of these MPS components in the dendrites of cultured mouse hippocampal neurons at DIV 7, 14, 21, 28, 47, and 50 (Fig. 1; Figs. S3 and S4). As shown by the quantifications of the average 1D autocorrelation amplitudes and the fractions of regions exhibiting the 1D periodic structure, the MPS appeared to develop substantially slower in dendrites than in axons (Fig. 2). We have shown previously that the 1D MPS starts to form in the proximal axon region near the soma at DIV 2 and then propagates to the distal region, spanning essentially the entire axonal shaft by DIV 6–7 (9), as confirmed here (Fig. 2). The observed increase in the autocorrelation amplitude after DIV 7 was primarily due to the increase in regularity of the 1D MPS structure in the periodic regions. In contrast, only a small fraction of the dendritic regions showed the 1D MPS structure at DIV 7 (Fig. 2), and both the average autocorrelation amplitudes and the fractions exhibiting 1D periodic pattern gradually increased during the next several weeks (Fig. 2). In addition to mouse neurons, we also examined cultured rat hippocampal neurons; although different species and culture conditions resulted in some quantitative differences, the slower development and lower formation propensity of the 1D MPS in dendrites, compared with axons, were also observed in the rat neurons (Fig. S5).

Effect of the Dendrite Diameter and Location on the Formation of the 1D MPS Structure. The average diameter of dendrites (1.1 ± 0.5 μm) was greater than that of axons (0.52 ± 0.15 μm) in our cultured neuron system, raising the question whether the lower propensity of 1D MPS formation in dendrites observed here may stem from their relatively larger diameters. To test this, we analyzed dendritic regions with different diameters ranging from 0.4 to 2.6 μm in DIV 28 mouse neurons. The 1D autocorrelation amplitudes showed little dependence on the dendrite diameter (Fig. 3 A–C), suggesting the lower propensity for the 1D MPS formation in dendrites observed here was unlikely a result of their relatively larger diameters. However, it is worth noting that the range of diameters tested here was limited, and it is possible that over a larger range of diameters, the formation of the 1D MPS may show a dependence on the neurite diameter. In particular, the formation of the 1D MPS structure, which likely requires symmetry breaking, may have a smaller tendency in very wide neurites in which the membrane is nearly flat locally, and the membrane skeleton in such wide neurites may adopt a different structural form.

We also examined whether the probability of 1D MPS formation depends on the location of the dendritic region relative to the soma. We found that the 1D autocorrelation amplitudes of these dendritic regions, as well as the fraction of dendritic regions classified as showing the 1D periodic structure, were independent of their relative distances to the soma (Fig. 3 D–F). This was true both for the relatively mature neurons at DIV 28 (Fig. 3 D–F) and for neurons at an earlier developmental stage (DIV 7) when only a small fraction of the dendritic regions exhibited the 1D MPS structure (Fig. S6 A–C).

Fig. 2. Developmental course of the 1D MPS structure in dendrites in comparison with axons. (A) The average autocorrelation amplitudes of βIII-spectrin, βIII-spectrin, and adducin observed for dendrites and the average autocorrelation amplitudes of βIII-spectrin observed for axons of mouse neurons at DIV 7, 14, 21, 28, 47, and 50. The autocorrelation amplitude is defined as the amplitude of the first peak (at ∼190 nm) in the average autocorrelation function curve. (B) The fractions of dendritic and axonal regions classified as exhibiting the 1D MPS structure at DIV 7, 14, 21, 28, 47, and 50 using the first method. In both A and B, we grouped data from DIV 47 and 50 into a single point, DIV 48.5. Approximately 250–400 dendritic regions and 200–300 axonal regions from two to three independent biological replicates were analyzed for each molecular marker at each DIV. Error bars represent SEM. We also imaged actin at DIV 7 and 28, and the fractions of dendritic regions exhibiting 1D periodic actin distributions are similar to those observed for adducin, βIII-spectrin, and βIII-spectrin.
Role of βII-Spectrin in the 1D MPS Formation. Because βIII-spectrin is the isoform of β-spectrin that is specifically enriched in dendrites (26, 27), it is possible that βIII-spectrin is the major β-spectrin component of the dendritic MPS structure, and that βII-spectrin is not essential for the formation of this structure in dendrites. To test this possibility, we knocked down βII-spectrin in DIV 28 mouse neurons, using a shRNA-expressing adenovirus (28). The mRNA and protein expression levels of βII-spectrin in neurons were indeed greatly reduced (Fig. S7 A), confirming the high knockdown efficiency. As expected, the MPS structure was disrupted in axons (Fig. S7B) (9). Surprisingly, the MPS structure was also largely disrupted in dendrites after βII-spectrin knockdown, as evident from the STORM images of βIII-spectrin and adducin in dendrites (Fig. 4). The average autocorrelation amplitudes for these molecules in dendrites were reduced to a level close to that obtained from neurons treated with actin depolymerizing drugs (CytoD) and latrunculin A (LatA) (Fig. 4C). These results suggest βIII-spectrin also plays a role in the MPS formation in dendrite, consistent with the observation of βII-spectrin in the dendritic MPS. We note that although the mRNA expression levels of βIII-spectrin and adducin were not changed by the knocking down of βII-spectrin (Fig. S7C), their protein levels were slightly reduced (Fig. S7D), potentially as the result of a lower stability of these proteins in the absence of βII-spectrin. This reduction in the βIII-spectrin and adducin protein levels could also contribute to the disruption of the MPS in dendrites by βII-spectrin knockdown.

MPS Components Form 2D Polygonal Lattice Structures in the Somatodendritic Compartment of Neurons. Interestingly, in some of the dendritic regions that did not exhibit a 1D periodic structure, the localization patterns of βII-spectrin, adducin, and actin did not appear entirely random; instead, the clusters of localization appeared to exhibit a regular 2D lattice pattern. This reminded

Fig. 3. Effect of the dendrite diameter and location on the 1D MPS formation. (A–C) Scatter plots showing the autocorrelation amplitudes versus the diameters of ∼100 randomly selected dendritic regions stained for βII-spectrin (A), βIII-spectrin (B), and adducin (C) for DIV 28 mouse neurons. The Pearson correlation coefficients (r) are indicated. (D) Scatter plots showing the autocorrelation amplitudes versus the relative distances to the soma for ∼100–200 randomly selected dendritic regions stained for βII-spectrin (black) and βIII-spectrin (red) for DIV 28 mouse neurons. The relative distance is defined as the distance of the region to the soma along the dendrite divided by the full length of the dendrite containing this region. (E) The binned average autocorrelation amplitude as a function of the relative distance to the soma. Error bars represent SD. (F) Fraction of dendritic regions classified as exhibiting the 1D MPS structure as a function of the relative distance to the soma.

Fig. 4. βII-spectrin dependence of the 1D MPS formation in dendrites. (A, Left) Conventional image of dendrites from βII-spectrin shRNA treated mouse neurons (DIV 28) stained for βII-spectrin (green) and MAP2 (magenta). (Middle) 3D STORM image of βII-spectrin for the dendritic region indicated by the yellow box on the Left. (Right) 1D autocorrelation function of the region indicated by the white dashed box in the Middle. (Scale bars, 1 μm.) (B) Similar to A, but for βII-spectrin knockdown neurons that were stained for adducin instead of βII-spectrin. (C) The average autocorrelation amplitudes obtained for dendritic regions from untreated DIV 28 neurons and the DIV 28 neurons treated with control scrambled shRNA, βIII-spectrin shRNA, and actin-depolymerizing drugs (50 μM CytoD and 20 μM LatA for 3 h), respectively. Because the MPS structure is relatively resistant to actin-depolymerizing drugs, a relatively high drug concentration and long treatment time is used to disrupt this structure. ∼100 dendritic regions were analyzed for each condition. Error bars represent SEM. ***P < 0.001 (unpaired Student’s t test).
MPS components form 2D polygonal lattice structures in some somatodendritic regions of neurons. (A) Conventional image of a dendritic region from a DIV 28 mouse neuron stained for βIII-spectrin (green) and a dendritic marker MAP2 (magenta). (Middle Left) 3D STORM image of βIII-spectrin for the yellow boxed region on the Left. (Middle Right) Zoom-in image of the region indicated by the white dashed box in the Middle Left. (Right) 2D autocorrelation function of this boxed region, which shows a 2D periodic lattice pattern. (Scale bars, 1 μm (left two panels) and 200 nm (right two panels)). (B and C) Similar to A, but DIV 28 neurons were stained for adducin (B) and actin (C) instead of βIII-spectrin. (D) 2D autocorrelation functions of some dendritic regions exhibiting irregular distributions of βIII-spectrin (Top), adducin (Middle), and actin (Bottom). (Scale bars, 200 nm.) (F, Top) Conventional image of a DIV 28 mouse neuron stained for βIII-spectrin (green) and MAP2 (magenta). The image was obtained from the bottom (adhering) surface of the neuron to show the overall morphology of the neuron. (Middle and Bottom, F1 and F2) Two STORM images of βIII-spectrin for the orange arrow indicated regions in the top panel (Middle) and corresponding 2D autocorrelation functions of the regions marked by the white dashed boxes in the middle panels (Bottom). F1 contains regions that exhibit a 2D periodic lattice pattern. F2 exhibits an irregular distribution of βIII-spectrin. The STORM images were obtained from the top (dorsal) surface of the neuron. (Scale bars, 5 μm (Top) and 200 nm (Middle and Bottom)). (G) Similar to F, but for a DIV 28 neuron stained for adducin instead of βIII-spectrin. (H) STORM images of soma regions exhibiting the 1D periodic distribution of βIII-spectrin/adducin and the 2D autocorrelation functions of the regions marked by the white dashed boxes. (Scale bars, 200 nm.) (I) Fractions of the dendritic and soma regions containing the 2D lattice structure determined by the 2D autocorrelation analysis for untreated and LatA/CytoD-treated neurons. (J) STORM image of βIII-spectrin of a soma region (Top) and its reconstituted lattice network, using the method described in the main text (Top and Bottom). (K) Fractions of the dendritic and soma regions containing 2D polygonal lattice structures determined by the lattice reconstitution and connectivity method for untreated neurons, LatA/CytoD-treated neurons, and βIII-spectrin knockdown neurons. *A value too close to zero to be visible in the bar graph. The fraction of dendritic regions exhibiting the 2D lattice structure is defined as the fraction among the regions that do not exhibit the 1D periodic structure (i.e., number of dendritic regions exhibiting the 2D polygonal lattice structure/number of dendritic regions not exhibiting the 1D periodic structure). Approximately 500–600 such dendritic regions not exhibiting the 1D periodic structure and ~100–200 soma regions were analyzed for each condition. Error bars represent SEM. **P < 0.05; ***P < 0.001 (unpaired Student’s t test).
us of the 2D polygonal lattice structure observed from the expanded membrane skeleton of erythrocytes (7, 8). If such a lattice structure is present in neurons, our labeling strategies for βIII-spectrin, adducin, and actin should mark the nodes of this polygonal lattice structure. We used 2D autocorrelation analysis to probe the 2D periodicity in the organizations of these MPS components in the dendritic regions of DIV 28 neurons. Indeed, a small fraction (~10%) of the regions that did not exhibit the 1D periodic pattern showed a 2D lattice pattern with two or three axes of ~190-nm periodicity (Fig. 5A–C), which is distinct from the irregular patterns observed for other regions (Fig. 5D). In contrast, the 2D autocorrelation analysis detected only one axis of periodicity for those dendritic regions that show the 1D periodic patterns (Fig. 5E).

Because βIII-spectrin and adducin also showed an enrichment at the plasma membrane of the soma (Fig. S8), we asked whether the 2D polygonal lattice structure could also be observed at the soma membrane. To answer this question, we randomly selected ~100–200 regions (~1 × 1 μm) on the top (dorsal) soma surface of DIV 28 mouse neurons and performed 2D autocorrelation analysis for βIII-spectrin or adducin distributions. A significant fraction (~20%) of these soma regions showed a 2D periodic lattice pattern with two or three axes of ~190-nm periodicity (average ~187 nm) in the autocorrelation function (Fig. 5F and G). Some soma regions also exhibited the 1D periodic structure (Fig. 5H). The ventral surface of the soma generally exhibited less βIII-spectrin and adducin staining, potentially as a result of adherence of the cell membrane to the glass substrate, and were not further examined.

Because of the finite areas of the regions examined, random distributions of molecules could occasionally appear periodic by chance in such finite-sized regions. The following two controls suggest the 2D periodic patterns that we observed in the somatodendritic compartment were not by random chance but reflected the tendency to form a 2D polygonal lattice-like structure: first, simulated, randomly distributed clusters with the cluster density identical to the experimentally observed values showed a substantially smaller probability to adopt such a 2D periodic pattern in the 2D autocorrelation function (Fig. S9), and second, treatment of neurons with actin-depolymerizing drugs (CytoD and LatA) significantly reduced the fraction of regions exhibiting a 2D periodic pattern in the 2D autocorrelation (Fig. Sf).

In the absence of local lattice defects due to missing molecules in the lattice resulting from incomplete labeling could obscure the periodic patterns in the 2D autocorrelation functions, such analyses might lead to an underestimate of the fraction of regions showing the 2D lattice structure. Hence, we introduced an alternative quantification method by reconstituting the 2D lattice network directly from the STORM images (Fig. 5I and S10A). To this end, we identified clusters of localizations in each of the ~1 × 1 μm soma regions, using a Voronoi tessellation based algorithm (29), and connected adjacent clusters with a line if their distance was close to the 190-nm periodicity of the lattice network derived from the autocorrelation analysis (see SI Materials and Methods for details). The reconstructed lattice network typically did not cover the entire area of the regions examined (Fig. 5J), which could be a result of incomplete labeling and/or lattice distortion, but it is also possible that the network was indeed not continuous everywhere on the cell surface. Next, to quantify the network for each region, we defined a connectivity number (N) as the number of nonoverlapping triangles observed in the reconstituted network per unit area of 1 μm². Indeed, the average N value of the soma regions that exhibited a pronounced 2D periodic pattern in the 2D autocorrelation analysis was substantially larger than the N values obtained from simulated, randomly distributed clusters with the same density over the same area (Fig. S10B), and the scatterplot of N as a function of the cluster density for all the randomly selected soma regions showed that a substantial fraction of these regions had N values significantly beyond the values obtained from simulations of random distributions (Fig. S10C). We then determined a threshold value of N from the simulated distributions and classified the experimentally imaged regions with N above this threshold as containing the 2D lattice structure (Fig. S10C).

Using this approach, the fractions of regions classified as containing the 2D lattice structure were calculated to be ~40–45% for the dorsal surface of the soma at DIV 28 from the STORM images of βIII-spectrin and adducin, and these fractions were substantially larger than the fractions derived from various simulated random distributions (Fig. S10D). The fractions observed for dendrites were smaller than those observed for soma (Fig. S5K and Fig. S6D), but still larger than those derived from the simulated random distributions (Fig. S10E). The fractions observed for both soma and dendrites were substantially reduced after LatA/CytoD treatment (Fig. 5K and Fig. S10F). Likewise, treatment with βIII-spectrin shRNA also led to a drastic reduction in the fractions of regions classified as exhibiting the 2D lattice structure (Fig. 5K and Fig. S10F). In addition, we quantified the densities of localization clusters for βIII-spectrin and adducin in the soma regions and found that the cluster densities decreased by ~50–60% upon LatA/CytoD or βIII-spectrin shRNA treatment, which is consistent with the notion that the 2D lattice structure was disrupted by these treatments.

Taken together, these results suggest a membrane skeleton with a 2D polygonal lattice structure is formed in the somatodendritic compartment of neurons. It is possible that the 2D lattice structure covers the somatodendritic surface more extensively than what the fraction numbers indicate here because lattice deformation or incomplete labeling could obscure the detection of the 2D lattice structure.

Next, we investigated the development of 2D polygonal lattice structures in the soma and dendrites. We used the lattice reconstruction and connectivity analysis as described earlier to determine the fractions of regions in the soma and dendrites containing the 2D polygonal lattice structures for DIV 7, 14, 21, and 28 mouse neurons. The fractions of regions classified as containing the 2D lattice structure were small at DIV 7, and then increased gradually as the neuron matures (Fig. 6), and the developmental course was similar to that of the 1D MPS in dendrites, but substantially slower than that of the 1D MPS in axons.

Discussion

We recently discovered that actin, spectrin, and associated molecules form a 1D periodic membrane skeleton structure in the axons of neurons (1). This 1D MPS structure is ubiquitously present in the axons of all of the neuronal types that have been examined so far (12, 13), and is conserved across diverse animal
species ranging from C. elegans to human (13). We and others have also observed the 1D MPS in dendrites (9, 10, 13, 15, 16).

Here, we systematically investigated the distributions of four MPS components, βII-spectrin, βIII-spectrin, actin and adducin, in cultured hippocampal neurons. We observed that >80% of dendrites in relatively mature neurons contain at least some regions exhibiting 1D periodic distributions of these molecules, but regions with periodic and nonperiodic distributions often coexist within the same dendrites. Overall, ~50–60% of the dendrite regions in relatively mature cultured neurons exhibit the 1D MPS structure. In comparison, more than 95% of axonal regions (essentially the entire axonal shaft) are covered by such 1D MPS structures under the same condition. In addition, the MPS components tend to adopt highly ordered periodic distributions in axons, whereas the spatial distributions of these molecules in dendrites often appear less regular, even for many of the dendritic regions that are classified as exhibiting the 1D periodic structure. The average amplitudes of the 1D periodic autocorrelation functions in dendrites are substantially lower than those in axons.

Our results also showed that the 1D MPS develops substantially slower in dendrites than in axons in the cultured hippocampal neuron system. This structure starts to appear in axons as early as DIV 2, and by DIV 7, essentially the entire axonal shaft is covered by the 1D MPS structure (9, 10). In contrast, the 1D MPS structure begins to appear in dendrites substantially later than DIV 2, and the coverage of the structure in dendrites gradually grows over several weeks. Because our study is based on imaging of fixed neurons, our results do not exclude the possibility that a more labile form of the 1D MPS is formed on imaging of fixed neurons, our results do not exclude the possibility that a more labile form of the 1D MPS is formed earlier or more quickly, but the stable structural form of the MPS that can survive the fixation develops over this slow developmental course. This slow course could partially explain the varying extent to which the 1D MPS has been observed in dendrites in previous reports. Indeed, in the studies that examined relatively young wild-type neurons, a small fraction of the dendritic regions was observed to exhibit the 1D MPS structure (9, 10, 13), whereas in the studies that imaged relatively mature neurons, a more extensive presence of the 1D MPS was observed (15, 16).

Taken together, our results suggest that nearly all dendrites have some tendency to form the 1D MPS structure, but the propensity of formation and rate of development of this structure is substantially lower than that in axons. Whether the dendrite–axon differences observed here for the cultured hippocampal neurons extend to other neuronal types remains to be determined. We also note that cultured neurons, even at their late developmental stage with spines and synapses formed, may still not be as mature in some aspects as neurons in adult animals. Therefore, it remains an open question as to what extent the 1D MPS exists and how fast it develops in the dendrites in vivo.

Notably, in addition to the 1D MPS structure, we also observed a 2D polygonal lattice structure formed by the MPS components in the somatodendritic compartment of neurons. This 2D MPS structure in neurons resembles the structural organization observed for the expanded membrane skeleton derived from erythrocytes, but the membrane skeleton observed in intact erythrocytes appears much more rugged (7, 8). In fact, the 190-nm periodicity we observed in neurons is close to the full length of the spectrin tetramer (7, 8), suggesting the possibility that the neuronal surface is under tension. Our observation of the polygonal lattice-like membrane skeleton structure in cells in addition to erythrocytes suggests this structure may be present in a variety of cell types.

Similar to the 1D MPS structure, which has been shown to organize some functional membrane proteins into 1D periodic distributions along the axons (1, 10, 14), the 2D MPS structure observed here may also organize membrane proteins into 2D periodic distributions on the surface of soma and neurites. It is worth noting that some ion channels and adhesion molecules at the nodes of Ranvier have recently been shown to adopt 2D periodic distributions (14); however, these distributions do not seem to arise from an underlying 2D periodic membrane skeleton but, rather, appear to be imposed by the hexagonal organization of the microvilli of glial cells, as the axonal membrane skeleton at the nodes of Ranvier still adopts a 1D periodic structure (14).

Our quantifications show that the 2D polygonal lattice structure exists in a larger fraction of regions in the soma than in dendrites. This is possibly because the 2D polygonal lattice preferentially forms at relatively flat surfaces, whereas the 1D MPS structure preferentially forms at tubular surfaces with relatively large curvatures. Hence, it is conceivable that the 2D lattice structure may have a higher tendency to form in dendrites, and possibly axons as well, that are substantially wider than those studied here. Similar to the 1D MPS structure in dendrites, we observed that the development of the 2D MPS structure in the somatodendritic compartment is also substantially slower than the development of the 1D MPS in axons.

The diverse structural organizations and the different developmental courses of the membrane skeleton structures observed in the different subcompartments of neurons suggest the actin-spectrin–based membrane skeleton is differentially regulated across these neuronal subcompartments. The functional implications of these differences in structures and dynamics await further investigation.

Materials and Methods

Experimental procedures for neuronal culture; immunofluorescence labeling of jill-spectrin, jill-spectrin, and adducin; and phalloidin labeling of actin, drug and shRNA treatments, STORM imaging, and image analyses are described in the SI Materials and Methods.

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Supporting Information

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SI Materials and Methods
All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Harvard University.

Primary Culture of Mouse and Rat Hippocampal Neurons. Primary cultures of hippocampal neurons were prepared as previously described (13). Timed pregnant CFW mice (Strain code 024; Charles River Laboratories) were killed with CO₂. Hippocampi were isolated from mouse embryos (E18) and digested with 0.25% trypsin–EDTA (1x; Sigma, T4549) at 37 °C for 15 min. The digested tissues were washed with Hanks’ Balanced Salt Solution (Thermo Fisher Scientific, 14175079) for three times and then transferred to culture medium consisting of Neurobasal medium (Thermo Fisher Scientific, 21103049) supplemented with 37.5 mM NaCl, 2% (vol/vol) B27 supplement (Thermo Fisher Scientific, 17504044) and 1% (vol/vol) Glutamax (Thermo Fisher Scientific, 35050-061). The tissues were gently triturated in the culture medium until there was no chunks of tissue left. Dissociated cells were then counted and plated onto poly-d-lysine-coated 12-mm or 18-mm coverslips (NeuVitro, GG-12 and GG-18–1.5-pdl). Wistar rats (Strain code 003; Charles River Laboratories) were killed with CO₂ at postnatal day P0 were used for culturing rat hippocampal neurons. The procedures for preparing the rat hippocampal neuronal cultures were the same as for the mouse hippocampal neuronal cultures, except the dissociated cells were plated on coverslips coated with 100 µg/mL polyornithine (Sigma-Aldrich, P3655) and 1 µg/mL laminin (BD Bioscience, 542322). Both mouse and rat neuronal cultures were maintained in the culture medium in a humidified atmosphere of 5% CO₂ at 37 °C. The neurons were fed with one-half medium volume change every 5 d.

Antibodies. The following primary antibodies were used in this study: guinea pig anti-MAP2 antibody (Synaptic Systems, 188004), rabbit anti-MAP2 antibody (Synaptic Systems, 188002), mouse anti-βIII spectrin antibody (BD Biosciences, 612563), goat anti-βIII spectrin antibody (Santa Cruz, sc-9690), rabbit anti-α-adducin antibody (Abcam, ab51130). The following secondary antibodies were used in this study: Alexa-647-conjugated donkey anti-mouse IgG antibody (Thermo Fisher Scientific, A31571), Alexa-647-conjugated donkey anti-rabbit IgG antibody (Thermo Fisher Scientific, A31573), Alexa-647-conjugated donkey anti-goat IgG antibody (Thermo Fisher Scientific, A21447), Alexa-546-conjugated donkey anti-rabbit IgG antibody (Thermo Fisher Scientific, A10040), Alexa-555-conjugated goat anti-guinea pig IgG antibody (Thermo Fisher Scientific, A21435). For STORM imaging, Alexa-647-conjugated secondary antibodies were used.

Fluorescent Labeling for STORM Imaging. Cultured mouse hippocampal neurons were fixed at DIV 7, 14, 21, 28, 47, and 50, using 4% (wt/vol) PFA in PBS for 20–30 min at room temperature (RT), washed three times in PBS, and permeabilized with 0.15% (vol/vol) Triton X-100 in PBS for 5 min. Neurons were then blocked in blocking buffer containing 3% (wt/vol) BSA in PBS for 1 h, and subsequently stained with primary antibodies in the blocking buffer overnight at 4 °C. We typically immunolabeled two targets for each sample: MAP2 as a dendrite marker and βII-spectrin, βIII-spectrin, or adducin as a marker for the periodic membrane skeleton structure. After incubation with primary antibodies, neurons were washed three times with PBS and stained with secondary antibodies in blocking buffer for 1 h at RT. The secondary antibodies used for MAP2 are labeled with Alexa Fluor 546 or Alexa Fluor 555. The secondary antibody used for βIII-spectrin, βIII-spectrin, or adducin are labeled with Alexa Fluor 647. Cultured rat hippocampal neurons were fixed and immunostained at DIV 5, 8, 14, 21, 30, using the same procedures as used for the mouse hippocampal neurons.

For fluorescent labeling of actin, cultured mouse hippocampal neurons were fixed at DIV 7 and 28, with either 4% PFA in PBS or 3% PFA and 0.2% GA in PBS for 20 min. The GA-fixed neurons were treated with freshly prepared 0.1% (wt/vol) sodium borohydride for 7–10 min to reduce background fluorescence caused by GA fixation. The neurons were permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked in blocking buffer containing 3% (wt/vol) BSA in PBS for 1 h at RT, and incubated with rabbit anti-MAP2 antibody in blocking buffer overnight at 4 °C. After three washes with PBS, the neurons were incubated with Alexa-555 conjugated secondary antibody for 1 h at RT. Finally, the neurons were stained with Alexa-647 conjugated phalloidin (500 nM in PBS, Thermo Fisher Scientific, A22287) overnight at 4 °C or 2–3 h at RT. We noticed that for actin imaging, long actin filaments running longitudinal along the long axis of axons and dendrites, in addition to the periodic distributed actin rings, could be clearly seen in neurons fixed with GA, but these long filaments were largely gone in the PFA-fixed neurons. This is likely because the longitudinal actin filaments are more dynamic than the short, capped actin filaments present in the periodic membrane skeleton structures, and GA, known to be a stronger fixative than PFA, might be better at preserving the longitudinal actin filaments. The presence of longitudinal actin filaments in the GA-fixed axons and dendrites could obscure the detection of the periodic membrane skeleton structures, and hence lead to an underestimate of the autocorrelation amplitude of the periodic actin distribution and of the fraction of neurite regions showing the periodic structure. Indeed, we observed that both the autocorrelation amplitude of the periodic actin distribution and the fraction of neurite regions showing the periodic structure were lower in GA-fixed neurons than in PFA-fixed neurons. Therefore, we used PFA fixed samples to quantify the autocorrelation amplitudes and the fractions of neurite regions exhibiting periodic actin distributions to minimize the obscuring effect of the long actin filaments on the detection of the periodic membrane skeleton.

STORM Imaging. The STORM setup was based on a Nikon Eclipse-Ti inverted microscope: 405-nm (CUBE 405–50C; Coherent), 561-nm (Sapphire 561–200 CW CDRH; Coherent), and 647-nm (F-04306–113; MPB Communications) lasers were introduced into the sample through the back port of the microscope. A translation stage allowed the laser beams to be shifted toward the edge of the objective so that the emerging light reached the sample at incidence angles slightly smaller than the critical angle of the glass-water interface, thus illuminating only the fluorophores within a few micrometers of the coverslip surface. ZT405/488/561/647/752RPC (Chroma) was used as the dichroic mirror and ZET405/488/561/647–656/752 penta-band notch filter (Chroma) was used as the emission filter. For 3D STORM imaging, an adaptive optics device (MicAO 3DSR; Imagine Optic) was inserted between the microscope side port and the EMCCD camera (Andor iXon, DU-897E-CSO-#BV; Andor Technology) to add the astigmatism so that the images of single molecules turned elliptical and the z positions...
of the molecules could be determined from the ellipticity of the images (24).

The sample was imaged in PBS buffer containing 100 mM cysteamine (Sigma), 5% glucose (Sigma), 0.8 mg/mL glucose oxidase (Sigma), and 40 μg/mL catalase (Roche Applied Science). To image the dorsal cell surface of the soma, the neurons were sandwiched between two coverslips in the above buffer and the samples were turned upside down when loaded onto the microscope for imaging, such that the dorsal cell surface was closer to the objective. During imaging, continuous illumination of 647-nm laser (∼2 kW/cm²) was used to excite fluorescence from Alexa Fluor 647 molecules (labeling βII-spectrin, βIII-spectrin, adducin, or actin) and switched them into the dark state. Continuous illumination of the 405-nm laser was used to reactivite the fluorophores to the emitting state. The power of the activation lasers (0–1 W/cm²) was adjusted during image acquisition so that at any given instant, only a small, optically resolvable fraction of the fluorophores in the sample was in the emitting state.

A typical STORM image was generated from a sequence of about 25,000–40,000 image frames at a frame rate of 60 Hz. The recorded STORM movie was analyzed according to previously described methods (24). The centroid positions and ellipticity values of the single-molecule images provided lateral (x and y) and axial (z) positions of each activated fluorescent molecule, respectively (24). Superresolution images were reconstructed from the molecular coordinates by depicting each location as a 2D Gaussian peak.

In addition to the STORM images, for each field of view, a conventional two-color fluorescence image was acquired using the 561-nm laser to excite Alexa Fluor 546 or 555 (labeling MAP2) and the 647-nm laser to excite Alexa Fluor 647 (labeling βII-spectrin, βIII-spectrin, adducin, or actin).

For determining the distances of dendritic regions to the soma, we obtained tiles of conventional images that allowed us to reconstitute the dendrites from the soma to the distal ends of the dendrites. Then we performed STORM imaging of randomly selected regions within these dendritic arbors and aligned the STORM images to the conventional image tiles. This allowed us to determine the distance of any selected dendritic region in the STORM image to the soma and calculate the relative distance to the soma, defined as the aforementioned distance divided by the full length of the dendrite containing this region.

Transfection of Hippocampal Neurons by shRNA Adenoviruses. The adenovirus expressing βII-spectrin shRNA was described previously (28). The sense sequences of the βII-spectrin shRNA are 5'-GCGATGTACGATGTACCACA-3' and 5'-GGATGAAT- GAAGGTGCTA-3'. The adenovirus expressing a scramble shRNA sequence was used as a control (Vector BioLabs, 1122). For transduction of neurons by adenoviruses, the adenoviruses were added to neuronal cultures at DIV 3.

Quantitative Real-Time PCR. Adenoviruses expressing either scrambled control shRNA or βII-spectrin shRNA were added to the cultured mouse hippocampal neurons at DIV 3. Total RNA was extracted from these neurons at DIV 6, using the Direct-zol MiniPrep kit (Zymo Research, R2050), according to the manufacturer's instructions. The quantitative PCR was performed on CFX Connect Real-Time PCR Detection System (Bio-Rad), using the One-Step qRT-PCR Kit (Thermo Fisher Scientific, 11736059). The average relative mRNA levels of βII-spectrin, βIII-spectrin, and α-adducin versus the reference gene (18S rRNA) were determined among four biological replicates, each measured in three technical replicate. After the completion of PCR amplification, a melting curve was obtained for each target gene, and these melting curves all generated a single amplicon, verifying a single PCR product. The sequences of PCR primers used were listed here: βII-spectrin: Spbni1Forward: 5'-TGG-GAATACCTTGTGCTGAACTG-3', Spbni1Reverse: 5'-ATTCA-CACCTTTTACACGTC-3'; βIII-spectrin: Spbni2Forward: 5'-TGGGTGTTCAAGGCGATATC-3', Spbni2Reverse: 5'-TCT-CATCGATCCAGAGTCTCAG-3'; α-adducin: Add1Forward: 5'-TGGTGTCCCTAACCCTGTTCG-3', Add1Reverse: 5'-CTGGT-CCTTCCAGAATGCTGAC-3'; 18S rRNA: Forward: 5'-GCAAATTTCCATCCATAGACG-3', and 18S rRNA Reverse: 5'-GGCTTCATAAACCACCATCCA-3'.

Autocorrelation Analyses. 1D or 2D autocorrelation analyses were performed on regions of neurites or soma (∼3 μm long for 1D autocorrelation analysis of dendrites and axons; ∼1 × 1 μm for 2D autocorrelation analysis of dendrites and soma). For 1D autocorrelation analysis, the autocorrelation curve was averaged from many randomly selected dendritic or axonal regions for each condition. The average 1D autocorrelation amplitude is defined as the difference between the first peak (at ∼190 nm) and the average of the two first valleys (at ∼95 and ∼285 nm, respectively) of the average autocorrelation curve.

Polyhedral Lattice Reconstitution and Connectivity Analyses. The following two steps were taken to reconstruct the polyhedral lattice network from the STORM images of the soma and dendritic regions: step 1: localization clusters in each of the ∼1 × 1 μm regions were recognized using ClusterViSu, a Voronoi tessellation based algorithm (29). To minimize the spurious detection of clusters, recognized clusters containing 10 or fewer localizations were discarded. It has been shown previously, using Monte Carlo simulation, that the number of clusters and the cluster sizes determined by ClusterViSu are insensitive to the presence of background noise (i.e., randomly distributed localizations) when the average density of localizations within individual cluster is 0.01 nm⁻², the number of localizations per cluster is six or more, and the density of background level (number of localizations per unit area) is varied between 0% and 40% of the average localization density within the clusters (29). Our STORM images satisfy these conditions: the average density of localizations within individual clusters in our STORM images is ∼0.012 nm⁻², the number of localizations per cluster is substantially larger than six (∼50–60 on average), and the background level is ∼15% for βII-spectrin images and ∼10–20% for adducin images. Step 2: After the clusters were identified using ClusterViSu, two adjacent clusters were connected with a line if their distance is close to the 190-nm periodicity of the lattice structure (within the range of 190 ± 50 nm). Crossing lines that intersect each other not at the clusters were removed. The connectivity number (N) of each region was then calculated as the average number of nonoverlapping triangles detected in the reconstructed lattice network per unit area of 1 μm².

Simulations of Randomly Distributed Monoclusters and Diclusters. For each examined soma or dendritic regions, simulated clusters were generated adopting a random distribution of the cluster centroid positions in the same area, whereas the density of the clusters was kept the same as the experimentally measured cluster density. Because the fluorescent labeling sites of βII-spectrin, adducin, and actin all mark the two ends of a spectrin tetramer, we simulated two scenarios for random distributions: randomly distributed clusters (termed monoclusters) to mimic the scenario in which spectrin tetramers are not formed (i.e., βII-spectrin exists as spectrin monomers or αβ-spectrin heterodimers) or actin/adducin is not bound to the spectrin tetramers, and randomly distributed dimers of clusters with a 190-nm separation between the two monomers within the dimer (termed diclusters) to mimic the scenario in which spectrin tetramers are formed but randomly distributed without forming a lattice network and actin/adducin is bound to the spectrin tetramers. It is worth noting that without forming a lattice network, the dimer representing a...
The spectrin tetramer is unlikely to extend to its 190-nm full length, and hence the dicluster simulation performed here with a fixed monomer–monomer separation of 190 nm within the dimer is likely to overestimate the fraction of regions that would by chance look like a 2D polygonal lattice structure for randomly distributed spectrin tetramers. For each STORM image of the ~1 × 1 μm soma and dendritic regions, we generated three random distributions of monoclusters and three random distributions of diclusters. For monocluster simulations, the density of the clusters was kept the same as the experimentally measured cluster density; for dicluster simulations, the total density of the monomers was chosen to be the same as the experimentally measured cluster density. For each distribution, we performed lattice reconstitution and connectivity analyses as described earlier, based on the centroid positions of the clusters, and the fractions of regions containing the 2D lattice structure were determined from these hundreds of random distributions. Because the clusters in the STORM images have finite sizes, when two clusters are sufficiently close, they would overlap with each other and appear as a single cluster in the experimental image. Thus, in addition to the simulations described earlier (denoted type 1), we performed two additional types of simulations (type 2 and type 3) to account for the cluster size and merging effect. For type 2, we determined the centroid positions and sizes of individual clusters in each STORM image. When we simulated the random distributions of clusters corresponding to this image, the cluster sizes determined from the STORM image were randomly assigned to each simulated cluster. When adjacent simulated clusters overlapped, we merged them into a single cluster and determined the centroid position of the new cluster. The lattice reconstitution and connectivity analyses were then performed based on the cluster positions after cluster merging. Again, for each STORM image, three random simulations of monoclusters and three random simulations of diclusters were performed. For type 3, we generated random distributions of monoclusters based on each STORM image, but only kept those random distributions that did not contain any overlapping clusters, until three such random distributions without any overlapping clusters were generated for each STORM image. Dicluster simulation was performed similarly, except the monomer–monomer separation within each dimer was fixed at 190 nm. The lattice reconstitution and connectivity analyses were then performed based on the cluster (monomer) centroid positions.

Fig. S1. Periodic distributions of βII-spectrin, actin, and adducin in axons of DIV 28 neurons. (A–C, Left) Conventional images of DIV 28 mouse neurons stained for βII-spectrin (A), actin (B), or adducin (C) (green) and a dendritic marker MAP2 (magenta). (Middle) 3D STORM images of βII-spectrin (A), actin (B), or adducin (C) for the axonal regions indicated by the yellow boxes on the Left. (Right) 1D autocorrelation function of the axonal regions indicated by the white dashed boxes in the Middle. (Scale bars, 1 μm.)
Distributions of autocorrelation amplitudes for dendritic and axonal regions and the STORM images and autocorrelation functions of example dendritic regions. (A) Histograms of the autocorrelation amplitudes for βII-spectrin (Left), βIII-spectrin (Middle), and adducin (Right) derived from ~300–400 randomly selected dendritic regions and ~200–300 randomly selected axonal regions in DIV 28 neurons. The histograms derived from the dendritic regions are shown in gray, and those derived from the axonal regions are shown in red. The autocorrelation amplitude was calculated as the value of the autocorrelation function at 190 nm minus the average value at 95 and 285 nm. (B) STORM images of example dendritic regions stained for βII-spectrin and the corresponding 1D autocorrelation functions. (C) STORM images of example dendritic regions stained for βIII-spectrin and the corresponding 1D autocorrelation functions. (Scale bars, 200 nm.) We observed that the distributions of the autocorrelation amplitudes do not necessarily indicate that the regions adopt a 1D periodic structure for the following reasons: first, the autocorrelation amplitude is operationally defined here as the value of the autocorrelation function at 190 nm minus the average value at 95 and 285 nm, and can show a positive value even when the autocorrelation functions do not show a periodic pattern, and second, some regions that adopt a periodic distribution can contain a small subregion showing 1D periodic distribution, which can give rise to a periodic autocorrelation function with a relatively small positive amplitude.
Fig. S3. Example images and autocorrelation analysis of β-spectrin, βII-spectrin, and adducin in dendrites and βI-spectrin in axons for DIV 7, 14, 21, and 50 mouse neurons. (A–C, Left) Conventional images of dendrite or axons of DIV 7 neurons stained for βI-spectrin (A), βII-spectrin (B), or adducin (C) (green) and the dendritic marker MAP2 (magenta). (Middle) 3D STORM images of the dendritic and axonal regions indicated by the yellow boxes on the Left. (Right) 1D autocorrelation function of the regions indicated by the white dashed boxes in the Middle. (D–F) Similar to A–C, but for DIV 14 neurons. (G–I) Similar to A–C, but for DIV 21 neurons. (J–L) Similar to A–C, but for DIV 50 neurons. (Scale bars, 1 μm.)
Fig. S4. Average autocorrelation functions of βII-spectrin, βIII-spectrin, and adducin in dendrites and βII-spectrin in axons for DIV 7, 14, 21, 47, and 50 mouse neurons. The average autocorrelation functions were calculated from ~250–400 randomly selected dendritic regions of neurons stained for βII-spectrin, βIII-spectrin, and adducin, as well as from ~200–300 randomly selected axonal regions of neurons stained for βII-spectrin at DIV 7 (A), DIV 14 (B), DIV 21 (C), and DIV 47 and 50 (D). These regions were from two to three independent biological replicates for each condition. We grouped data from DIV 47 and DIV 50 into a single point, DIV 48.5.

Fig. S5. Developmental course of the 1D MPS structure in dendrites, in comparison with axons, for cultured rat hippocampal neurons. (A) Average autocorrelation amplitudes of βII-spectrin, βIII-spectrin, and adducin observed for dendrites and average autocorrelation amplitudes of βII-spectrin observed for axons of rat neurons at DIV 5, 8, 14, 21, and 30. (B) Fractions of dendritic and axonal regions containing the 1D MPS structure at DIV 5, 8, 14, 21, and 30.
Fig. S6. Effect of the location of the dendritic region on the 1D and 2D MPS formation. (A) Scatter plots showing the autocorrelation amplitudes versus the relative distances to the soma for ~100–200 randomly selected dendritic regions stained for βII-spectrin (black) and βIII-spectrin (red) for DIV 7 mouse neurons. The relative distance is defined as the distance of the region to the soma divided by the full length of the dendrite containing this region. (B) The binned average autocorrelation amplitude as a function of the relative distance to the soma for DIV 7 mouse neurons. Error bars represent SD. (C) The fraction of dendritic regions classified as exhibiting the 1D MPS structure as a function of the relative distance to the soma for DIV 7 mouse neurons. (D) The fraction of dendritic regions showing the 2D polygonal lattice structure as a function of the relative distance to the soma for DIV 28 mouse neurons. The relative distance is as defined in A. The fraction of dendritic regions showing the 2D lattice was quantified using the lattice reconstitution and connectivity method and was defined as the fraction among the regions that do not exhibit the 1D periodic structure. (i.e., number of dendritic regions exhibiting the 2D lattice structure/Number of regions not exhibiting the 1D periodic structure). Approximately 500–600 such dendritic regions not exhibiting the 1D periodic structure were analyzed.
Fig. S7. The effects of βII-spectrin knockdown on the MPS structure. (A, Left) The normalized mRNA expression levels of βII-spectrin determined for neurons treated with scrambled (control) shRNA and neurons treated with βII-spectrin shRNA. (Right) The average fluorescence intensity of βII-spectrin determined from ~20 dendritic and ~20 axonal regions randomly selected from the untreated, control shRNA-treated, and βII-spectrin shRNA-treated DIV 28 mouse neurons. Error bars represent SEM. (B, Left) Conventional images of axons in βII-spectrin shRNA-treated neurons (DIV 28) stained for βII-spectrin or adducin (green) and the dendritic marker MAP2 (magenta). (Middle) 3D STORM images of the axonal regions indicated by the yellow boxes on the Left. (Right) Average autocorrelation amplitudes obtained for axons from untreated, control shRNA-treated, and βII-spectrin shRNA-treated DIV 28 neurons, plotted together with the average autocorrelation amplitudes obtained for axons from neurons treated with actin-depolymerizing drugs (50 μM CytoD and 20 μM LatA for 3 h). Approximately 100 regions of axons were analyzed for each condition. (Scale bars, 1 μm.) (C) Normalized mRNA expression levels of βIII-spectrin (Left) and adducin (Right) determined for neurons treated with control shRNA and neurons treated with βII-spectrin shRNA. (D) Average fluorescence intensities of βIII-spectrin (from ~20 randomly selected dendritic regions) and adducin (from ~20 randomly selected dendritic regions and ~20 randomly selected axonal regions) from the untreated, control shRNA-treated, and βII-spectrin shRNA-treated DIV 28 neurons. Error bars represent SEM. **P < 0.05; ***P < 0.001 (unpaired Student’s t test).

Fig. S8. Immunofluorescence staining of βIII-spectrin and adducin shows enrichment at the plasma membrane. (A) Confocal fluorescence images of a DIV 28 mouse neuron stained for βIII-spectrin. The main panel shows the XY maximum projection image, and two side panels show the XZ (Bottom) and YZ (Right) cross-sections along the two dashed lines. (B) Same as A, but for neurons stained for adducin. (Scale bars, 10 μm.)
**Fig. S9.** 2D autocorrelation analysis of simulated, randomly distributed clusters. Because both adducin and the N terminus of βIII-spectrin labeled by antibodies mark the two ends of the spectrin tetramer, we simulated two scenarios of random distributions: randomly distributed clusters (termed monoclusters) to represent the scenario in which spectrin tetramers are not formed or actin/adducin molecules are not bound to the spectrin tetramers, and randomly distributed dimers of clusters with a 190-nm monomer–monomer separation with in the dimer (termed diclusters) to represent the scenario where spectrin tetramers are formed and actin/adducin molecules are bound to the spectrin tetramers, but the 2D polygonal lattice network is not formed. It is worth noting that without forming a lattice network, the spectrin tetramer is unlikely to extend to its 190-nm full length, and hence the dicluster simulation performed here with a fixed monomer–monomer separation of 190 nm within the dimer is likely to overestimate the fraction of regions that would by chance look like a 2D polygonal lattice structure for randomly distributed spectrin tetramers. (A, Left) Simulated images of randomly distributed monoclusters at three different cluster densities (13, 32, and 115 clusters/μm²). (B, Left) Simulated images of randomly distributed diclusters at three different cluster densities (13, 32, and 115 monomers/μm²). (C) Fractions of the soma regions exhibiting a 2D periodic pattern with ~190 nm periodicity in the 2D autocorrelation, obtained from STORM images of DIV 28 mouse neurons and simulated images with the cluster density identical to the experimentally observed cluster density. Approximately 100 regions of the soma were analyzed. Error bars represent SEM. **P < 0.05 (unpaired Student’s t test).
Fig. S10. Polygonal lattice reconstitution from the STORM images and the connectivity analysis. (A) Top) A soma region of a DIV 28 mouse neuron displaying a 2D lattice distribution of βIII-spectrin and the reconstituted lattice network determined using the lattice-reconstruction method. The reconstituted lattice network is both superimposed on the STORM image (Left) and plotted alone (Right). Monocluster and diclusters are as described in the caption of Fig. 10 and SI Materials and Methods. For monocluster simulations, the density of the clusters was chosen to be the same as the experimentally measured cluster density; for dicluster simulations, the total density of the monomers was chosen to be the same as the experimentally measured cluster density. (Scale bars, 200 nm.) The connectivity value (N) is defined as the number of nonoverlapping triangles in the reconstructed 2D lattice network per unit area (1 μm²) in each analyzed region. (B) Box plot of the connectivity value (N) for 200 randomly selected soma regions of DIV 28 neurons exhibiting a pronounced 2D periodic pattern in their 2D autocorrelation functions (stained for βIII-spectrin) and simulated images of monoclusters and diclusters with the cluster density identical to experimental images. For each experimental image, 2 simulations of random distributions were performed and the connectivity value (N) of each simulation is plotted. The line in the middle of the box indicates the mean value; the upper and lower bounds of the box indicate 1 SD. (C) Scatter plot of the connectivity value (N) versus the cluster density for ~150 randomly selected soma regions of DIV 28 mouse neurons stained for βIII-spectrin (red) and for the simulated images of randomly distributed monoclusters (green) and diclusters (blue). To extend the cluster density range in the simulations, we used 0.5Nc, 1Nc, and 2Nc of the cluster densities obtained from the STORM images. The dashed line represents the threshold of N used to classify whether a STORM image of a region exhibits the 2D lattice structure. The threshold was set such that 95% of the simulated monocluster images are below the threshold. (D) Fractions of regions containing the 2D lattice structure determined from the lattice-reconstruction method using the threshold defined in C, obtained from the experimental STORM images of ~150-200 randomly selected soma regions of DIV 28 neurons and from the simulated images of randomly distributed clusters with the same cluster densities as those obtained from the STORM images. For dicluster simulations, the total density of the monomers was chosen to be the same as the experimentally measured cluster density. For each STORM image, we generated three random distributions of monoclusters and three random distributions of diclusters, as described in Fig. 15 and SI Materials and Methods. The fractions of regions containing the 2D lattice structure were determined from these hundreds of random distributions. Because the clusters in the STORM images have finite sizes, when two clusters overlap with each other, they would appear as a single cluster in the experimental image. Thus, in addition to the simulations described above (denoted as type 1), we performed two additional types of simulations (type 2 and type 3) to account for the cluster size and merging effect, as described in SI Materials and Methods. In these simulations, not only the cluster densities but also the sizes distributions of the clusters were determined on the basis of the STORM images. In the type 2 simulation, simulated clusters that overlap with each other are merged into a single cluster. In the type 3 simulation, the random distributions of clusters that do not lead to cluster overlapping are generated. The results from type 2 and type 3 simulations for monoclusters are shown in the figure, and the fractions obtained from the simulated data were ~0.08 (type 2) and ~0.15 (type 3). Again, we note that without forming a lattice network, the spectrin tetramer is unlikely to extend to its 190-nm full length, and hence the cluster simulation performed here with a fixed monomer-monomer separation of 190 nm within the dimer is likely to overestimate the fraction of regions that would by chance look like a 2D polygonal lattice structure for randomly distributed spectrin tetramers. (E) Fractions of dendritic regions containing the 2D lattice structure determined by the lattice-reconstruction method, obtained from the experimental STORM images of ~150-400 dendritic regions (DIV 28 mouse neuron) and the simulated images of randomly distributed clusters with the same cluster densities and size distributions as those obtained from the STORM images. Simulations are as described in D. The fractions obtained from type 2 and type 3 simulations were ~0.07 and ~0.12, respectively. (F) Fractions of soma and dendritic regions exhibiting the 2D lattice structure determined by the lattice-reconstruction method with an increased threshold of N value such that 95% of the simulated dicluster images (type 1) are below the threshold. The fractions derived from the STORM images of untreated DIV 28 mouse neurons are shown together with those derived from neurons treated with CytoD and LatA or βIII-spectrin shRNA. The fraction of dendritic regions exhibiting the 2D lattice structure is defined as the fraction among the regions that do not exhibit the 1D periodic structure, as described in Fig. 15D. Approximately 100–400 such dendritic regions and ~100–200 soma regions were analyzed for each condition. Error bars represent SEM. **P < 0.01; ***P < 0.001 (unpaired Student’s t test).