REVIEW ARTICLE

Structure and function of subcortical periodic cytoskeleton throughout the nervous system

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ABSTRACT

The cytoskeleton plays an essential role in various functions of different cell types and is involved in the pathogenesis of several neural diseases. With the development of super-resolution fluorescence imaging technologies, which combine the molecular specificity and simple sample preparation of fluorescence microscopy with a spatial resolution comparable to that of electron microscopy, numerous new features have been revealed in the organization of the subcortical cytoskeleton. A novel periodic lattice cytoskeleton is prevalent in different cell types throughout the nervous system. Here, we review the current studies of the molecular distribution, developmental mechanisms, and functional properties of this periodic cytoskeleton structure.

Keywords: Cytoskeleton · Super-resolution microscopy · Nervous system · Spectrin · Actin

Introduction

The formation of complex nervous systems depends on cytoskeleton-based structural organizations and their dynamic remodeling, which plays a crucial role in the development, migration, and differentiation of neurons. Typical neurons possess axons and dendrites, two types of neurites (processes extending from one neuron to another) with different structures and functions. Axons are typically single long and thin neurites that transmit signals to other neurons. Dendrites are relatively short and thick, and are composed of multiple processes and spines that receive electrical and chemical signals from other neurons' axons. The formation and maturation of these distinct cellular compartments are crucial for the morphology and function of the nervous system. In neurons, tight regulation of cytoskeleton organization and remodeling has emerged as a key element in polarization, axon growth, maturation and degeneration (1).

Recently, using super-resolution imaging techniques, a novel periodic distribution of cytoskeletal elements has been observed in neurons. Actin and associated proteins form a lattice structure with a periodicity of ~180 to 190 nm in axons (2-5). This lattice structure has been observed throughout the nervous system and in many types of cells, including both neurons and oligodendrocytes (4-7). The periodic cytoskeletal structure is disrupted at presynaptic sites, and interestingly, the periodic pattern is observed in some dendrite spine necks (7, 8). These new findings provide us a better understanding of the structure and function of cytoskeleton in neurons and other types of cells. In this review, we aim to provide an overall insight into the current understanding on the structure and function of the subcortical periodic cytoskeleton organization.

I. Periodic cytoskeleton structures of neurons

The cortical cytoskeleton was first described in red blood cells using electron microscopy (EM) technique. A lattice of repeating hexagons and pentagons, arranged in a two-dimensional plane, has been observed in the cytoskeleton underneath the cell membranes (9-11). This cytoskeleton structure is composed of spectrin dimers that are formed by α- and β-spectrin subunits. Spectrin heterodimers are associated head-to-head to form tetramers, and are linked by short actin filaments of 40 nm to generate a hexagonal shaped cytoskeleton (12, 13). Several proteins, including ankyrins, adducin and tropomyosin, participate in the assembly and maintenance of the actin-spectrin cytoskeleton in red blood cells (14-16).

Like red blood cells, neurons maintain their basic structure with the help of an internal cytoskeleton. Since EM can obtain structural information with nanometer resolution, it is a key tool in discovering cytoskeleton

organization, especially the structure of polymorphic actin in neurons. Recently, using platinum replica electron microscopy, more details of delicately organized actin structures at dendrite spines and axon initial segment (AIS) have been revealed (17, 18). However, harsh conditions like detergent extraction during the labor-intensive sample preparation procedures may disrupt the fragile actin network.

With the development of super-resolution fluorescent microscopy, many novel features of subcortical cytoskeleton structures have been revealed. Xu and his colleagues recently used stochastic optical reconstruction microscopy (STORM) and discovered that actin, and its related molecules, form a periodic structure in axons (3). In addition, a similar structure with a similar period was also observed in some dendrites (2). Along neuronal axon shafts, short actin filaments, spectrin, and adducin form a highly regular lattice with a periodicity of 180 nm to 190 nm (**Figure 1A-B**). Furthermore, using stimulated emission depletion (STED) nanoscopy, these periodic cytoskeleton structures have been confirmed in the axons and dendrites of living neurons (4, 5, 8). The periodic spectrin distribution in *Drosophila* and *C. elegans* neurons was also found by another super-resolution fluorescence imaging technology, termed structured-illumination microscopy (SIM) (7). Indeed, the very first study of periodic subcortical cytoskeleton structure in the nervous system was suggested by the observation of ankyrin2 in presynaptic nerve terminal at the neuromuscular junction of *Drosophila*, observed with SIM (19). These periodic structures in the subcortical cytoskeleton, observed by various super-resolution imaging techniques, indicate that they truly exist and are unlikely to be artifacts of one particular method.

Initial studies show that the periodic pattern of cytoskeleton predominantly exists in hippocampal or

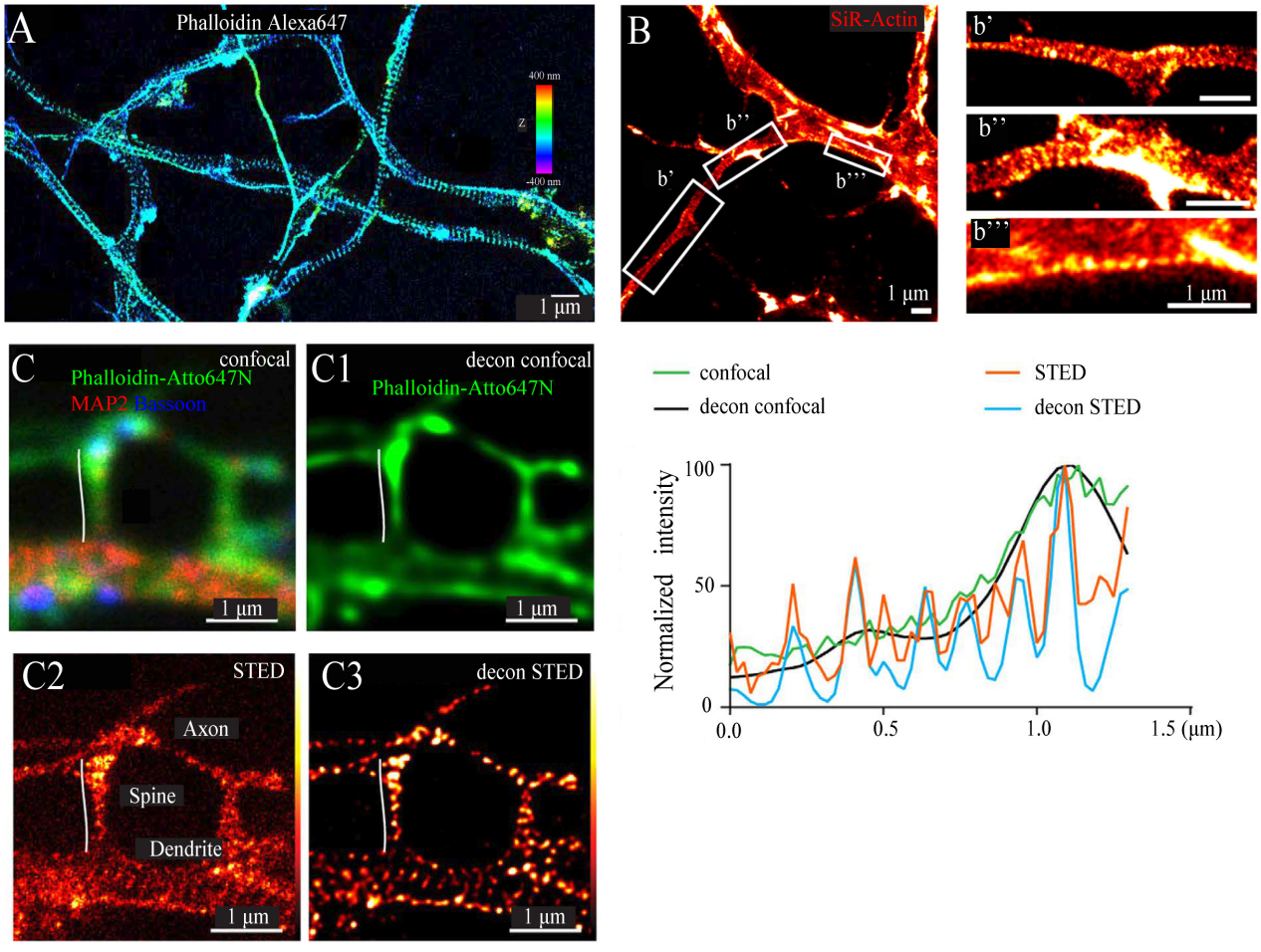

Figure 1. Cytoskeleton structures in the nervous system. (A) 3D STORM image showing periodic organization of actin filaments stained with Phalloidin-Alexa 647 in neurons. A is adapted from Zhong et al., 2014 (2). (B) STED image shows actin pattern in a living oligodendrocyte stained with SiR-Actin. c', c''and c''' are the enlarged figures indicated in C showing the periodic actin structures in dendritic-like processes. B is adapted from D'Este et al., 2016 (6). (C and C1) Conventional images show a dendrite with spines, stained with Phalloidin-Atto647N (green), dendritic marker MAP2 (red), and pre-synaptic marker Bassoon (blue). (C2) STED and (C3) deconvolution STED image shows periodic distribution of actin filaments in dendrite shafts as well as in dendrite spines. C is adapted from Bär et al., 2016 (56). In right panel, normalized line profiles of phalloidin-Atto647N intensity along the spine necks indicated in (C-C3) for raw and deconvolved confocal and STED images.

tcortical neurons (2-5). For a comprehensive understanding of this periodic cytoskeleton, it is important to address whether this periodic structure is a common feature in the nervous system. Here, four related questions should be asked carefully: 1) Do the inhibitory neurons form such a similar periodic lattice structure? 2) Do neurons in the peripheral nervous system develop a periodic actin-spectrin structure? 3) Do structurally and functionally specialized compartments in neurons form the periodic cytoskeleton structure? 4) Do glial cells have the ability to develop the periodic cytoskeleton structure? Multiple lines of evidences show that this periodic lattice cytoskeleton structure is formed in different types of cells throughout the central nervous system and peripheral nervous system, including excitatory and inhibitory neurons from various brain regions, as well as striatal neurons, granule cells, dopaminergic neurons, olfactory neurons, bipolar cells of the retina, peripheral motor neurons and dorsal root ganglia neurons (6, 7). In addition, the periodic cytoskeleton pattern is formed in different locations of neuronal axons, like the actin ring at the internodes of sciatic nerve fiber underneath of the myelin sheath, and the periodicity of ankyrin-G at AIS or the nodes of Ranvier (3, 5, 6). **Table 1** shows a summary of periodic cytoskeleton structures in different neuron cell types at different developmental stages (Table 1).

The expression of spectrin in glial cells raises the question whether the periodic lattice cytoskeleton structure is present in these cells. Studies show that the periodic structure is rarely observed in the majority of glia cells, including astrocytes and microglia (6, 7). In differentiating oligodendrocytes, the ring-like structure of actin, and, intriguingly, actin filaments, develop a periodic feature with a length of approximately 190 nm. Further, the βII spectrin is periodic and alternates with the actin ring structure. This observation was similar to the findings

Table 1: the summary of periodic cytoskeleton structure in the different neuron cell types.

Species	Neuron cell type	Region	Cytoskeleton proteins	Ref.
mouse or rat	hippocampal neuron	axon		Xu et al., 2013, Zhong et al., 2014
		dendrites		
		AIS		
	cortical neurons		actin	D'Este et al., 2016b, He et al., 2016
	HPN dendrites		actin	
	HPN axons Phall		actin	
	HPN axons		actin	
	striatal neurons		actin	
	granule cells		actin, betaII spectrin	
	bipolar cells of retina	axon	actin, betaII spectrin	
		dendrites	actin, betaII spectrin	
	DRG neurons		actin (DIV2), betaII spectrin (DIV6)	
	sciatic nerves	axon	actin	
	sciatic nerve	node of Ranvier	actin, ankyrinG	
	oligodendrocytes		actin (DIV5), betaII spectrin (DIV6)	
	dopaminergic neurons		betaII spectrin	
	olfactory neurons		betaII spectrin	
	peripheral motor neurons			
	parvalbumin neuron	cortex	betaII spectrin	
		midbrain		
		hippocampus		
	golgi cell		betaII spectrin	
	purkinje cell		betaII spectrin	
	mES motor neuron	axon	betaII spectrin	
chicken	neuron		betaII spectrin	
C. elegans	neuron		beta spectrin	
Drosophila	neuron		beta spectrin	

in neurons, though the periodicity in neurons is much less regular (6). Thus, the periodic lattice cytoskeleton structure is likely prevalent in the nervous system (**Figure 1**). How a periodic structure formed in development is an interesting question worthy of further study.

II. Developmental mechanism of the periodic cytoskeleton structure in neurons

The 180 - 190 nm spaced actin ring structures expand along the neurites. The existence of this periodic structure raises two interesting questions. First, why are actin filaments spaced so regularly, with a periodicity of approximately 190 nm? One straightforward possibility is that these short actin filaments are physically connected by a ladder-like protein, which should be stably formed and have a length around 190 nm. Second, why are the actin filaments so short, and why do they wrap around neurites? To study this, we need to find molecules which can cap the actin filaments and regulate their length, and also find the mechanism responsible for these actin filaments wrapping around axons.

Spectrin molecules are the most likely candidate to connect the actin filaments in neurons, because βII spectrin molecules are periodic with the same length as actin filaments in neurons, and also alternate with the actin rings. After using the shRNA strategy to knock down spectrin expression, the periodic pattern of actin is disrupted, implying the periodicity of actin filaments relies on the normal expression of spectrin (3). Adducin might be the candidate to control the length of actin filaments since it forms periodic, ladder-like structures in axons with a similar periodic pattern compared to the actin and spectrin structures (2), and knocking out adducin in mice can lead to enlarged neuronal actin rings and axon diameters (20).

How does the periodic cytoskeleton structure develop in neurons? At earlier developmental stages, the periodic pattern of βII spectrin could first be detected in the proximal region of axon near the cell body in neurons at 2 days *in vitro* (DIV), when normally one neurite fast outgrows other processes and becomes an axon (2). As neurons continue to mature, the periodic distributed βII spectrin extends to more distal regions of axons and eventually fills the entire axon (2). With STED imaging and SiR-actin labeling, the actin filaments are also found as early as DIV2 and throughout the whole developmental stages, forming the same periodic structure as spectrin (5). Interestingly, the periodic adducin structure begins to appear in axons at around DIV6 (2). If the adducin forms a periodic structure at a relatively late stage, such a lack of adducin in capping actin filaments during early development might explain why actin filaments exist in a less stable form in the earlier developmental stages of neurons (2). These findings indicate that the periodic structure starts to form early during axon development and originates in the starting region of axons near the cell body. After the initiation of the lattice cytoskeleton structure's formation, it continues to mature with the actin filaments becoming more stable, capped by adducin. Once matured, the supercomplex of the periodic cytoskeleton structure possesses a relatively fixed molecular organization, becomes stable and rarely moves (2). While most signaling proteins in axon differentiation and development are highly expressed at the growing tip of axons (21), the subcortical periodic cytoskeleton structures instead originates at the proximal axon region. This indicates that the formation of this actin-spectrin-adducin lattice structure may be independent of the mechanisms for establishing or maintaining the polarity of neurons. Earlier studies show that the periodic cytoskeleton structure is predominantly observed in axons (3), only small, isolated patches of dendritic shafts exhibit periodic pattern of βII spectrin, and the periodicity of βII spectrin in dendrites appears less regular (2, 7). Ankyrin-B highly expresses in axons, and specifically targets βII spectrin (11). In wild type neurons, βII spectrin concentration in axons is about 2 times higher than in dendrites (2, 22, 23). Coincidentally, the spectrin mainly forms an obvious periodic structure in axons of wild type neurons (2, 7). A concentration-driven hypothesis can be formed based on these studies by inferring that higher concentrations of spectrin lead to a capability to form the periodic structure in neurons. Studies indicate that ankyrin-B knockout can cause substantial redistribution of βII spectrin in neurons (2, 24). Interestingly, the expression level of βII spectrin increases in dendrites and becomes indistinguishable from axons in ankyrin-B knockout neurons (2). Overexpression of βII spectrin substantially increases the chance of dendrites with the periodic distribution of βII spectrin (2). However, several studies report that the periodic structure of actin ring can be observed in a small fraction (~10-30%) of dendrites in living neurons labeled by SiR-actin using STED nanoscopy (5). Recently, Sidensteinet et al. found that βII spectrin showed a sharp periodic organization along all the dendrites decorated with spines, especially in the spine necks (8). These studies showed that, in dendrites, the periodic structure appeared more frequently than reported by earlier reports (2, 3).

The contradicting results, in terms of presence or absence of the periodic structure, could be due to many possible reasons. First, different actin populations may exist in cells, and different sub-populations of actin protein may have unequal affinity to labeling tools (25, 26). It is possible that phalloidin has a higher binding affinity to long actin filaments in cytosol versus subcortical actin rings, while SiR-actin, which labels endogenous actin in living cells with high specificity to subcortical actin, prefers binding to the actin rings. Since there are usually much higher concentrations of long actin filaments in dendrite shafts, this may explain the reason why with phalloidin labeling, researchers always visualize long F-actin signals in dendrites and only occasionally observe periodic actin ring structures, differing from results gained by SiR-actin labeling. Also, in dendrites, a dense layer of long filament mesh net structure of F-actin exists (3). If the periodic actin network is evident in dendrites, it would be very challenging to separate this periodic form

of cytoskeleton from the dense long F-actin filaments. In addition, the contradicting presence or absence of observed periodic structures in dendrites may also be caused by observations at different developmental stages and/or at different sizes of dendritic processes. Currently, it is generally believed that the periodic structure of cytoskeleton exists in dendrites, though with less regularity.

How this periodic structure develops in dendrites remains unknown. From the perspective of axons we know that βII spectrin may regulate the assembly of the periodic actin cytoskeleton (2, 4). But in dendrites additional investigations are needed to reveal the molecular mechanism. βIII spectrin, which is enriched in dendrites but not in axons (27-29), has been reported periodically distributed in dendrites (3), and may contribute to the assembly of periodic structures in dendrites.

The regulation of the periodic membrane skeleton seems to be more complex than previous thought, because other molecular factors may participate in this regulation. For example, intact microtubules are required for the formation of the periodic structure. A microtubule depolymerizing drug disrupts the periodic structure in axons, whereas stabilizing the microtubule by using drugs like taxtol or SB216763 promotes the formation of the periodic structure (2). The periodic structure is destroyed and not observed in STORM (2) or EM (18) images after membrane detergent extraction. Other unknown factors might also regulate such a precise periodic cytoskeleton structure. To systematically study these components related to the periodic structure, it is necessary for us to understand the regulatory mechanism. Proteomics techniques, like mass spectrometry, can help identify these unknown components and better understand the mechanism of the formation of the periodic cytoskeleton in neurons.

III. Function of the periodic cytoskeleton structures

What is the function of the periodic cytoskeleton in neurons and in other types of cells in the nervous system? Actin filaments form ring-like structures and are evenly spaced by spectrin tetramers in neurons. The first function of such a periodic actin-spectrin might be to physically support the cell membrane in neurons. Neurons possess multiple thin processes which are often needed to survey the surrounding environments and respond to multiple stimuli. In some cases, these thin processes may have to squeeze around the brain tissue and might need a relaxed and robust cytoskeleton to support such an action. Indeed, one spectrin subunit is comprised of many elastic repeats, permitting the flexible feature of submembranous cytoskeleton (23, 30), and thus the spectrin-based cytoskeleton might provide a robust and flexible mechanical support to the thin processes in neurons (2, 3). Disruption of the periodic structure by spectrin depletion in *C. elegans* not only led to axon collapse and breakage when animal was moving (31), but also impaired its sensitivity to external touch (32), supporting that the periodic skeleton might play a critical role in maintaining mechanical stability of axons.

Besides mechanical support, this highly periodic submembrane skeleton could organize important membrane molecules, such as voltage-gated sodium channels and cell adhesion molecules, into a periodic distribution along the axon (3). Anchoring proteins with varying biochemical and mechanical properties on the axonal plasma membrane might not only affect the generation and propagation of action potentials, but also influence other signaling pathways (33). The periodic subcortical actin-spectrin-adducin cytoskeleton may tightly connect with a variety of membrane molecules in dendrites. It is interesting to speculate whether the specialized organization of certain membrane molecules in dendrites may play essential roles in controlling the dendrite branches, regulating dendrite size, or instructing the morphogenesis of dendrite spines. How the periodic subcortical cytoskeleton establishes and carries out all of these fundamental biological functions remains to be answered and deserves further investigation. Investigating the generation of extended polymeric filaments *in vitro* by self-assembling may provide a possible strategy to illustrate the detailed mechanisms for the organization, interaction and remodeling of cytoskeletal elements. A recent study of ankyrin-G in the somatodendritic plasma membranes of hippocampal neurons showed its function in promoting GABAergic synapse stability through the inhibition of endocytosis (34). Moreover, ankyrin/spectrin networks have also been reported moving along the membrane and preventing lateral membrane endocytosis (35). It is likely that the subcortical periodic structures may be involved in the regulation of endocytosis.

The molecular components of the periodic skeletal structure are not evenly distributed. Specific isoforms of ankyrin and spectrin molecules, as well as other proteins, are found at discrete sites along the axon (3, 24, 33). With the different components and organization at discrete locations, do the heterogeneous structures have specific functions accordingly?

AIS, an essential subcellular compartment, assembles at the proximal axonal region during early developmental stages while neurons further mature (33, 36, 37). AIS is characterized by high expression of ankyrin-G and βIV spectrin, which replaces the βII spectrin isoform during development (23). Notably, both isoforms of spectrin have the same periodic pattern. βII spectrin's periodic pattern appears first while βIV spectrin's periodic pattern comes later during development. It is speculated that the periodic pattern forms first during an early developmental stage when a structural foundation is laid out. Later, other molecules add or replace existing molecules within this foundation. Other proteins, such as sodium channels and neurofascin (NF), are integrated in the lattice (3, 18, 33, 38, 39). These periodic molecules in AIS are not added randomly, instead they are added in a precisely controlled manner (2, 3, 33). AIS has been recognized as an essential functional compartment in neurons. It is critical for maintaining neuronal polarization, is generally considered to be the site of action potential generation, and is an important site for protein transportation. Whether the specific periodic cytoskeleton has any relation to the

above functions of AIS is unknown and deserves further investigation. Interestingly, the AIS structure is highly resistant to drugs that cause depolymerization of actins or microtubules, and is otherwise remarkably stable (2, 33), further supporting its critical function as the specific excitable domain of an axon.

Thus far, we have discussed the possible function of the periodic cytoskeleton structure in neurons without myelination. Most vertebrate axons in the central and peripheral nervous systems are myelinated, forming nodes of Ranvier which are electrically active domains of axon (40). Ankyrin-G is an essential scaffolding protein which could regulate sodium ion channel clustering at nodes of Ranvier (41). During early development neuron-glia interactions first cluster the cell adhesion molecule NF186, resulting in the recruitment of ankyrin-G (39). Sodium channels are subsequently recruited by ankyrin-G to the developing nodes (42). Then βIV spectrin can further stabilize the NF186-AnkG-sodium channel complex by interacting with ankyrin-G, resulting in a mature node of the cytoskeleton (43). The high density of sodium channels at nodes of Ranvier is a crucial feature in myelinated axons, and confers several important advantages, including decreased energy and space requirements, for the rapid propagation of action potentials (44). It is relevant to consider how the cytoskeleton is organized underneath the myelin coat, as well as at nodes of Ranvier. The most recent studies show that similar periodic cytoskeleton patterns exists under the myelin coat as compared to unmyelinated axons (6). The nodal cytoskeleton consists of ankyrin-G and βIV spectrin, with clear periodic organization just like the AIS (6). However, the actin concentration is very high at the nodes, and the fine subcortical actin structure requires future study. More glial and axonal proteins have been recently reported to exist at the nodes of Ranvier in sciatic nerve fibers with a periodic spatial arrangement (45). Nevertheless, the latest studies indicate that ankyrin-R/βI spectrin can compensate for loss of ankyrin-G/βIV spectrin, resulting in a secondary reserve method of sodium channel clustering in nodes of Ranvier (46). Whether the periodic cytoskeleton exists after this type of functional compensation is an interesting question to be studied. These multiple mechanisms to node of Ranvier formation ensure the stable molecular composition of the nodes and highlight their importance in efficient nervous system function.

Besides AIS and nodes of Ranvier, another highly specialized compartment of axons and dendrites is the synaptic site, including presynaptic and postsynaptic ends, which are essential for neuronal signal transmission. Actin is highly concentrated at synapses and involved in their assembly and development (18, 47). Spectrin is also found in synapses and plays crucial roles in the stabilization of synapses and formation of dendritic spines (48, 49). Does the actin-spectrin cytoskeleton exist at synapses? Interestingly, the axonal periodic cytoskeleton structure is disrupted at most presynaptic sites, but is observed in some dendrite spine necks from the dendritic shaft regions (7). Another study indicated that the periodic actin-spectrin lattice pattern was absent at presynaptic and postsynaptic sites (8). As previously proposed, the periodic cytoskeleton structures provide robust mechanical support in processes. Synapses are adaptable structures, which are built, pruned, and modified throughout the organism's whole life (50). These structural re-arrangements are believed to require the disassembly of the subcortical lattice structure. Furthermore, the presence of a tight periodic lattice might disrupt the fusion of synaptic vesicles and thus influence synaptic transmission (7, 8, 51). Thus, the absence of periodic cytoskeleton structure at synaptic sites might be due to the need for plasticity and rapid reorganizations which occur there (7).

Besides its functions in neurons, the periodic structure is sparsely observed in the processes of glial cells (6, 7), with functions which are still unclear and need further investigation. One possible reason for the sparse presence of periodic structures in glial cells may be due to the dynamic and transient nature of these cells. Furthermore, the degree of periodicity for spectrin distributions is positively correlated with the expression level of βII spectrin. βII spectrin levels in glial cells are similar to those in neuronal dendrites, but much lower than those in axons (52, 53). The high actin density in the cytosol of glial cells also hinders the ability to observe actin periodicity which may already be rarely present. Since *in vitro* glia cultures are quite different from their physiological conditions, further *in vivo* studies are important for illuminating the possible functions of periodic lattice in the development of glial cells.

Conclusions and perspectives

The ubiquity of the cytoskeleton periodicity throughout the nervous system underscores its fundamental importance to the development of various types of neurons (and likely some glial cells as well). Besides mechanically supporting and maintaining the membrane structure, the precisely organized periodic lattice may also affect many other cellular structures and functions, such as protein transport, cellular polarity, and excitability. Recently, Zhou et al. used super-resolution imaging to visualize colocalization of the cannabinoid type 1 receptor (CB1) and other related signaling proteins on the membrane-associated periodic skeleton (54). CB1 is one of the most abundant G protein-coupled receptors (GPCR) in the central nervous system and a therapeutic target for regulating appetite, pain, motor, mood and memory, as well as for treating neurodegenerative diseases (55). These new findings indicate that the periodic cytoskeleton structure play a role in GPCR's intracellular signaling and may be important for specific behavior and disease mediated by GPCRs.

However, more questions have been raised and many aspects need further investigation. What is the function of the periodic structures in dendrites and what factors determine its location in dendrites? Furthermore, why are the periodic structures disrupted in most presynapticboutons, yet formed in a significant fraction of dendritic spine necks? What is the relationship of periodic structures

and neuronal synapses? Beyond neurons, even more is unclear about how the periodic structure is formed or functions in glial cells. Thus, further studies on glial cells are essential for a more comprehensive understanding of the periodic cytoskeleton.

To study the formation, regulation and function of periodic cytoskeleton structure remain challenging. To meet these challenges, powerful labeling tools and novel imaging strategies to improve the spatio-temporal resolution, as well as enhancing the imaging sensitivity in thick samples, will be necessary. As novel methods are being developed today at a rapid pace, it will be exciting to see the elucidation of mechanisms of how cytoskeletal proteins control and regulate cellular development, function, and plasticity. Future studies could provide critical insights into future therapeutic interventions for human diseases of the nervous system related to the dysfunction of the cytoskeleton.

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Conflict of interest

The authors declare that they have no conflict of interest.

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