



The role of the membrane-associated periodic skeleton in axons

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Abstract

The identification of the membrane periodic skeleton (MPS), composed of a periodic lattice of actin rings interconnected by spectrin tetramers, was enabled by the development of super-resolution microscopy, and brought a new exciting perspective to our view of neuronal biology. This exquisite cytoskeleton arrangement plays an important role on mechanisms regulating neuronal (dys)function. The MPS was initially thought to provide mainly for axonal mechanical stability. Since its discovery, the importance of the MPS in multiple aspects of neuronal biology has, however, emerged. These comprise its capacity to act as a signaling platform, regulate axon diameter—with important consequences on the efficiency of axonal transport and electrophysiological properties— participate in the assembly and function of the axon initial segment, and control axon microtubule stability. Recently, MPS disassembly has also surfaced as an early player in the course of axon degeneration. Here, we will discuss the current knowledge on the role of the MPS in axonal physiology and disease.

Keywords Adducin · Actin ring · Axonal degeneration · Axonal diameter · Axon initial segment · Membrane periodic skeleton (MPS)

Introduction

With the development of super-resolution microscopy, new details on cell ultrastructure were unraveled. So far, the most striking new structure identified by nanoscopy is the axonal membrane periodic skeleton (MPS). The MPS consists of regularly distributed actin rings spaced by approximately 190 nm, interconnected by bipolar spectrin tetramers [1]. In cultured hippocampal neurons, this structure is observed as early as DIV2 in the proximal region of the axon and then extends to the distal end of the axon, occupying the entire axon shaft [2]. Similarly to the erythrocyte membrane cytoskeleton (reviewed in [3]), tetramers of spectrin connect the MPS actin rings [1]. Spectrins are large flexible molecules that exist mainly as heterotetramers of α and β subunits, formed upon the arrangement head to head of antiparallel α/β dimers [4, 5]. In axons, α II/ β IV-spectrin is found proximally to the cell body in the axon initial segment

(AIS) [6], whereas α II/ β II-spectrin is enriched in the remaining axon shaft [6], and α II/ β III-spectrin is mostly observed in dendrites [7]. Although β IV and β II-spectrins present a periodic pattern within the AIS and axon shaft, respectively, the regular lattice is detected only in small patches in dendrites [2, 8, 9]. In cell bodies, the MPS is present as a two-dimensional polygonal scaffold [8]. In the initial MPS model, given the periodic distribution of the actin-capping protein adducin along the axon after DIV6 [2], actin filaments within rings were thought to be short and capped [1]. Binding of adducin was predicted to provide stability to the lattice [1, 2], similarly to the actin-spectrin arrangement present in the erythrocyte membrane. This model was latter supported by the fact that in *Drosophila* axons, MPS abundance is reduced by interfering with actin nucleators (Arp2/3 and formins), but was less sensitive to decreased activity of actin elongators [10]. This argues that actin rings are possibly made of short actin filaments. However, recently, platinum replica electron microscopy combined with super-resolution microscopy, indicated that rat hippocampal axon actin rings are probably organized as two long intertwined filaments. According to this view, adducin was suggested to enhance the lateral binding between actin and β II-spectrin instead of capping actin filaments [11]. The braided actin arrangement was suggested to provide for more stability of the structure,

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as it is expected to be stiffer than a ring made of short actin filaments [11]. Further experiments are now needed to fully understand how actin is nucleated in the MPS.

This unique arrangement of the cytoskeleton is observed in axons across species, from invertebrates (*C. elegans* and *D. melanogaster* [12]) to vertebrates (chicken [12], mouse, rat [1, 2, 12–14], and humans [12]), supporting its importance for neuronal function. The MPS is present in every neuron type inspected so far, including different excitatory neurons, such as cerebellar granule cells and dopaminergic neurons, inhibitory neurons, such as Purkinje cells and in bipolar cells of the retina [12, 13]. In addition to central nervous system neurons, motor neurons [12], and dorsal root ganglion neurons [12, 13, 15] that elongate their axons to the peripheral nervous system, also display a MPS. Similarly to unmyelinated axons in the CNS, myelinated sciatic nerve axons contain the periodic lattice [13]. Within the nervous system, the MPS might not be exclusive of neurons as the processes of different glial cell types including astrocytes, microglia, NG2 glia, Schwann cells, and oligodendrocytes show patches of a similar periodic skeleton [2, 13, 16].

Beyond actin, spectrin, and adducin, an ever increasing number of proteins has been shown to be associated with the MPS, such as ion channels [1], non-muscle myosin II (NMII) [17–19] and tropomyosin 3.1 [20]. Recently, the use of state-of-the-art biochemical approaches [21–23], allowed to confirm the presence and identify additional MPS proteins. Specifically, with co-immunoprecipitation and mass spectrometry, hundreds of candidate MPS-interacting proteins were found including motor proteins, cell adhesion molecules, ion channels, and signaling molecules [22]. Determining the MPS proteome is essential to further comprehend its function(s) in neuronal biology.

Although novel insights regarding MPS structure and function have been untied in the past years, there are still many questions that remain unanswered in the field. Further details are needed to comprehend how the lattice is assembled and what are the molecules involved in its maintenance and dynamics, as well as their localization. The relevance of the MPS to different aspects of the maintenance of neuronal physiology, beyond the initially anticipated mechanical support of the thin long structure of axons, is emerging. In this review, we will discuss the current knowledge on the relevance of the MPS to axonal biology and function, and its importance in neurodegeneration.

The MPS regulates multiple aspects of neuronal physiology

Since its initial discovery, the contribution of the MPS to the integrity and mechanical stability of the axon is widely accepted [24–26]. The data implicating the MPS

on additional aspects of neuronal physiology is currently emerging. This include the formation and function of the AIS [2, 17, 27–29], the regulation of axon diameter [15, 18, 19] in the course of action potential conduction [18] and axonal transport [19], and the modulation of the stability of axonal microtubules [10]. Disruption of the MPS causes widespread neurodegeneration [30, 31] and a variety of neurological impairments are seen when its components are mutated and downregulated [15, 32, 33]. We will dedicate this section to explore the importance of the MPS to neuronal physiology.

The MPS provides mechanical support for the long thin structure of axons

Axons suffer large stretch deformations under a variety of standard and abnormal conditions. Mammalian sciatic nerves, for example, can experience localized strains during regular limb movements [34]. It is also known that the brain, one of the softest tissues in our bodies, undergoes substantial shear deformations (2–5% strain) even under normal activities, such as jumping [35]. When increased longitudinal tension takes place, axons suffer rapid elongation [36], in a process known as “axon stretch growth” (reviewed in [37] and [38]), that alters their intrinsic rest tension and induces axonal thinning [39]. Axonal diameter is restored when tension is released, supporting the need of structural adaptations [40]. The main components of the axonal cytoskeleton, microtubules, neurofilaments, and actin are tension modulators being able to adapt to changes occurring during axon stretch growth. Tension will draw microtubules and neurofilaments apart longitudinally and possibly cause them to break [41]. It is possible that actin rings within the MPS may function as an important element in the process of axon elasticity. The first retrospective evidence suggesting that the MPS provides mechanical support to axons, enabling axon integrity during movement, derives from studies in β -spectrin *C. elegans* mutants [42]. In these mutants, axons spontaneously break as a consequence of the acute strain imposed by movement [42]. Beyond mechanical stability, β -spectrin contributes to touch sensation in *C. elegans* [43]. Antero ventral microtubule (AVM) touch receptor neurons from the worm body are able to sense gentle mechanical stimulus. These neurons can shorten during ventral bending and elongate during dorsal bending, without suffering physical damage. However, in moving worms, mutations in the tetramerization domain of *C. elegans* β -spectrin lead to defects in sensory neuron morphology under compressive stress [43].

To clarify how the MPS protects axons from disrupting, atomic force microscopy (AFM), rheology measurements and computer modelling have been valuable tools [24–26].

When chick DRG neurons are depleted of β II-spectrin, not only they lose the MPS but also they show a significant reduction in the elastic modulus as measured by AFM [24]. Moreover, rheology measurements of neurons treated with latrunculin A, a F-actin destabilizer that disrupts the MPS [1], induces a reduction in the elastic modulus and a decrease in tension relaxation [24], indicating that actin confers mechanic protection to axons. Computational modeling has shown that axons in particular, are characterized by the presence of two distinct elastic moduli, a higher one probably associated with the actin ring structure, and a lower one conferred by spectrins that connect the rings [26]. These observations support that spectrins maintain axonal elasticity [26], which may be ensured by the intrinsic flexibility of spectrin repeats [44]. Together, these data indicate that the MPS can act as a tension buffer allowing axons to undergo significant deformations without being morphologically or functionally compromised [24].

The MPS and the axon initial segment (AIS): from the establishment of a diffusion barrier, to fine-tuning neuronal excitability

The AIS is a specialized axonal structure found in inter- and motoneurons from vertebrates (as detailed in [45]) located proximally to the cell body that is essential for the maintenance of neuronal polarity and for the generation of action potentials (reviewed in [46]). In cultured hippocampal neurons, the AIS is formed as neurons mature and as its master regulator ankyrin-G [47] clearly accumulates from DIV8 on [2]. AnkyrinG recruits the main AIS components including β IV-spectrin [48] (which will replace β II-spectrin within the MPS [2]), voltage-gated sodium and potassium channels [49], and neurofascin [50]. It has been hypothesized that by setting a periodic arrangement of voltage gated channels in the AIS [1, 51], the MPS might interfere in action potential generation and propagation [52]. Of note, in the AIS, the MPS is more resistant to drug-induced perturbations of actin and microtubules, possibly as the incorporation of ankyrin-G and sodium channels further stabilizes the axonal actin-spectrin cytoskeleton [51].

Several studies support that the AIS behaves as a physical barrier, preventing the free exchange of molecules, such as pre- and postsynaptic proteins and lipids [53], between the cell body and the axon, a concept known as the picket fence model [46]. This diffusion barrier is lost upon ankyrin G depletion or actin disruption [53, 54]. The current evidence indicates that the MPS may be involved in the formation of the diffusion barrier at the AIS. Supporting this hypothesis, glycosylphosphatidylinositol-anchored GFP presents reduced lateral movements in the membrane, confined to a repetitive array of small areas spaced by approximately

190 nm [27]. In addition, in a computational model of the axonal plasma membrane, actin rings were found to limit the longitudinal diffusion of the proteins of the inner leaflet of the phospholipid bilayer but not of the proteins of the outer leaflet [26]. This supports that actin and probably other associated proteins, forms a fence capable of restricting the longitudinal diffusion of molecules. The authors suggest that beyond actin, spectrins may also alter the radial diffusion of proteins depending on the strength of their interaction with lipids [26]. More recently, the mobility of small cytoplasmic proteins was also show to be reduced within the AIS and seemed correlated with the MPS periodicity [55]. The future development of technologies allowing to simultaneously track the live movement of molecules and image the MPS, will certainly bring new light to our understanding of the importance of the MPS in the establishment of a barrier at the AIS.

Further than maintaining neuronal polarity and generating action potentials, the AIS is crucial for fine-tuning neuronal excitability as it can relocate proximally or distally from the cell body and suffer changes in length [56]. The mechanisms underlying structural alterations of the AIS are just starting to be unveiled. AIS long-term relocation and rapid shortening are blocked by blebbistatin [57], an NMII inhibitor, supporting that NMII might regulate activity-dependent morphological alterations of the AIS. NMII is a key player in many biological processes given its crucial role in contraction. When NMII bipolar filaments bind antiparallel actin filaments, actomyosin contractility occurs through the hydrolysis of ATP (reviewed in [58]). A later study has shown that phosphorylated myosin light chain (pMLC), the active NMII contraction unit, participates in AIS assembly where it accumulates simultaneously with ankyrin-G, being associated with MPS actin rings [17]. This suggests that the MPS might be a player in fine-tuning neuronal excitability. The role of NMII within the MPS will be further explored in the following section.

The MPS regulates axon diameter: implications to action potential conduction and axonal transport

The axon is not a static structure. Instead, it is a very dynamic compartment, and its diameter can suffer alterations depending on several variables, such as distribution of organelles (particularly relevant in unmyelinated axons [59]), activity-dependent mechanisms [60], thinning upon stretch [36, 39], and deformations triggered by movement or induced by axon degeneration. It is well known that axons are contractile along their longitudinal axis [61, 62] and that they also exert contractility/tension along their circumferential axis, in an actin and NMII-dependent manner [63]. Thus,

the role of axonal actin, specifically of MPS actin rings, has been investigated as a possible regulator of axon diameter.

The actin-binding protein adducin was one of the initial components found to be part of actin rings within the MPS. When adducin is depleted, MPS actin rings have an increased diameter, progressive axon enlargement occurs, followed by axon degeneration and loss [15]. In vitro, hippocampal neuron from WT and α -adducin KO mice reduce their axonal actin ring diameter with time, supporting that actin filaments within the MPS are more dynamic than initially anticipated, and that additional actin-binding proteins beyond adducin probably regulate this process. Since the temporal analysis of F-actin content in actin rings highlighted that axon constriction is related to increased actin intensity per ring, suggesting bundling of actin filaments [15], we and others investigated if additional actin-binding proteins, namely NMII, might participate in actin ring constriction.

NMII is a hexameric protein composed by two regulatory light chains and two essential light chains tightly bound to two heavy chains (reviewed in [58, 64]). Although being enriched in the AIS [17], NMII (including the active phosphorylated light chain) is also found in the axon shaft [18, 19]. Although pMLC is organized in a circular, periodic conformation colocalizing with actin rings, NMII heavy chain is distributed as multiple filaments with approximately 300 nm of length along the longitudinal axonal axis [18]. NMII filaments can crosslink adjacent rings, as previously suggested by platinum-replica electron microscopy [11], but may also be located within individual actin rings [18, 19], with distinct biomechanical roles [18]. Although NMII crosslinkers are expected to provide scaffold properties, intra-ring filaments represent the conformation capable of generating contraction leading to actin filament sliding [18].

When NMII activity is decreased either by using chemical inhibitors or shRNA, axonal diameter is increased [18, 19, 63]. These results indicate that the actomyosin cytoskeleton regulates radial tension throughout the entire length of the axon shaft. This finding has important implications in axonal biology. By controlling radial contractility, NMII within the MPS regulates action potential conduction [18]. Specifically, inhibition of NMII results in a higher propagation velocity [18] as expected considering that in unmyelinated axons, velocity conduction is proportional to the square root of the axonal diameter [65]. In addition to the neuronal electrophysiological properties, the efficiency of transport of large cargoes mainly along thin axons is also dependent on axonal diameter [19]. In hippocampal neurons, when NMII is acutely inhibited by blebbistatin (leading to increased axon diameter), large cargoes move faster, as their movement triggers a transient radial expansion, which is immediately restored [19]. Importantly, the role of the MPS in controlling axon diameter may also be crucial for

the onset of degeneration as the inhibition of NMII for prolonged time periods induces disruption of the MPS, entailing the formation of focal axonal swellings [19].

There are; however, several questions that remain unanswered to fully understand the biomechanical properties of NMII in MPS actin rings. The fact that modulation of NMII activity controls axon diameter [18, 19], supports the presence of antiparallel actin filaments within the MPS. Still, how NMII provides for sliding of short or intertwined actin filaments is yet to determine. These uncertainties are common to actin rings that exist in other biological contexts, as is the case of the actomyosin ring that assembles during cytokinesis [66, 67]. It is also unclear how MPS actin rings (either long intertwined or short filaments) may widen, as this will probably imply a rapid rearrangement and quick de-/polymerization. One should also bear in mind that actin is thought to form relatively brittle filaments that rupture under elongational strains less than 10% of their resting length [68]. The adaptation of MPS actin rings to dynamic fluctuations in axon diameter is an exciting field of research in neuronal cell biology. Variations in axonal caliber also raise important questions on how the meshwork of spectrin tetramers adapts.

The crosstalk between the MPS and axonal microtubules

Microtubules (MTs) are essential for neuronal physiology, not only during the establishment of neuronal polarity [69], but also to sustain intracellular transport by providing the tracks to molecular motors. The possible crosstalk between MTs and the subcortical MPS has emerged shortly after the discovery of this axonal cytoskeleton arrangement. When hippocampal neurons are treated with the microtubule-depolymerizing drug nocodazole, the periodic pattern of β II-spectrin is largely disrupted [2]. In contrast, when treatment is done with the microtubule stabilizer taxol, neurons present multiple axon-like long processes that present a MPS [2]. These observations suggest that MTs may regulate the assembly of the MPS. On the other hand, the assembly of the MPS may modulate MT stability. In *D. melanogaster* neurons, when axons are treated with the actin-destabilizing drug cytochalasin D, a significant reduction in the percentage of MPS abundance occurs [10], as expected. Concurrently, there is MT fragmentation and loss, and diminished MT polymerization [10] supporting that the MPS may confer stability to the MT cytoskeleton. One can speculate that the interdependence of the MPS and MTs can be related to the fact that the MPS acts as a signaling platform [22, 23] (as will be detailed in the next section). Given that multiple signaling molecules are recruited to the MPS in response to extracellular stimuli [23], it is possible that specific pathways

regulating MT organization and dynamics may be engaged. Alternatively or in parallel, mechanobiology can also play an important role as MTs respond to, and generate, mechanical forces (as detailed in [70]). Further studies should address the interplay between these two cytoskeleton components and the identity of the players involved in their interaction.

The MPS as a signaling platform

In the initial description of the MPS, sodium channels were shown to be distributed in axons in a periodic pattern, coordinated with the underlying actin-spectrin-based cytoskeleton [1]. This was the first indication that in neurons, the MPS might function as a platform organizing the interaction of important signaling proteins, enabling signal transduction [1]. Subsequent studies further supported that the MPS can organize transmembrane proteins, such as ion channels and adhesion molecules, into regular distributions along axons [8, 16, 23, 27, 28]. Specifically, STORM analyses of hippocampal neurons have shown that G protein-coupled receptors (GPCRs), cell adhesion molecules (CAMs) and receptor tyrosine kinases (RTKs) are recruited to the MPS as a response to extracellular stimuli, resulting in RTK transactivation by GPCRs and CAMs, and the downstream signal-regulated kinase (ERK) signaling [23]. ERK signaling in turn caused calpain-dependent MPS degradation, providing a negative feedback that modulates signaling strength [23]. Recently, using co-immunoprecipitation and mass spectrometry, several signaling molecules were identified as components of the MPS [22], further supporting its role as a signaling platform. In addition, the signaling platform provided by the MPS is suggested to recruit signaling and adhesion molecules enabling axon–axon and axon–dendrite interactions [22]. This is consistent with previous observations of alignment of the MPS in contacting axons [23]. Future research should be considered to clarify the pathways that might be regulated by MPS assembly, and also the mechanism of recruitment of signaling molecules to the MPS.

The MPS in the course of axon degeneration

Multiple evidence show that the impairment of MPS components is associated with neurologic conditions and neurodegeneration [71]. In the case of adducin, mutations in

this actin-binding protein lead to amyotrophic lateral sclerosis [32] and cerebral palsy [33], and α -adducin knockout mice have axon degeneration and loss [15]. In relation to β II-spectrin, its deficiency in mice causes reduced axon growth, impaired axonal transport, MPS loss, and axon degeneration [72]; in humans β II-spectrin mutations give rise to spinocerebellar ataxia (SPTBN2; Online Mendelian Inheritance in Man [OMIM] ID: 600,224, 615,386). Ankyrin and actin mutations are associated respectively with mental retardation (ANK3; OMIM ID: 615,493), and with Baraitser–Winter syndrome and dystonia with neurodegenerative traits (ACTB7; OMIM ID: 243,310, 607,371).

The participation of the MPS in sensory axon degeneration triggered by trophic deprivation was investigated by two independent groups [30, 31]. After trophic factor withdrawal, the loss of MPS occurred independently of caspase apoptotic pathways and preceded axonal fragmentation [30, 31], the ultimate sign of neurodegeneration. On the other hand, when F-actin was chemically stabilized with cucurbitacin E, the MPS abundance was increased in deprived axons and axonal fragmentation was ameliorated. These data support that the MPS is disrupted before axonal fragmentation and that its loss is required for axon degeneration to take place. This evidence places the MPS as a new target to prevent neurodegeneration. The increased understanding of the ultrastructural organization of the MPS will certainly guide us to better comprehend and interfere with axon loss.

Conclusion

The periodic axonal subcortical cytoskeleton was initially considered to play an important role in the support of the long thin structure of axons. During the last years, this concept has evolved and the MPS has been implicated in multiple aspects of neuronal biology (Fig. 1). We are nevertheless just starting to unravel how this exquisite structure is generated, organized and maintained throughout the lifetime of a neuron. The complete identification of the components involved in MPS biogenesis will ensure a deeper understanding of its biological relevance. This will certainly be powered as new advances on nanoscopy arise, especially in the context of living cells.

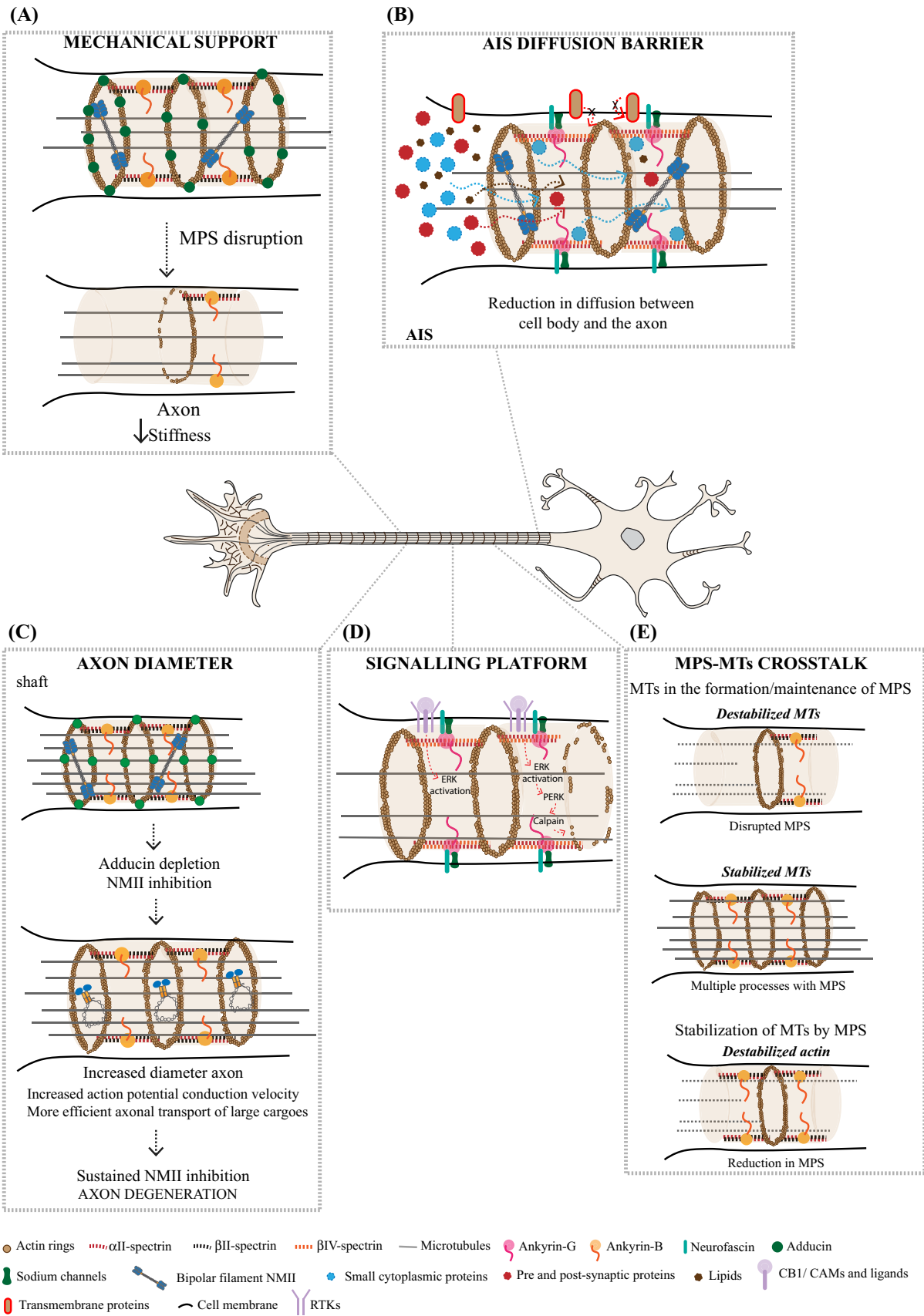


Fig. 1 The impact of the MPS in neuronal biology. The MPS provides mechanical support to neurons (A). Recent findings indicate that the MPS is important for the assembly and function of the AIS (B). In addition, the MPS participates in the regulation of axon diameter, with an important impact on axonal transport and electrophysiological properties (C). The role of the MPS as a signaling platform has recently been investigated (D) and the interplay between the MPS and MTs has been highlighted by drugs that modulate MT and actin stability (E)

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Declarations

Conflicts of interests The authors declare no conflicts of interest.

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