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Review

Axonal cytomechanics in neuronal development

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For more than a century, mechanical forces have been predicted to govern many biological processes during development, both at the cellular level and in tissue homeostasis. The cytomechanics of the thin and highly extended neuronal axons have intrigued generations of biologists and biophysicists. However, our knowledge of the biophysics of neurite growth and development is far from complete. Due to its motile behavior and its importance in axonal pathfinding, the growth cone has received significant attention. A considerable amount of information is now available on the spatiotemporal regulation of biochemical signaling and remodeling of the growth cone cytoskeleton. However, the cytoskeletal organization and dynamics in the axonal shaft were poorly explored until recently. Driven by advances in microscopy, there has been a surge of interest in the axonal cytoskeleton in the last few years. A major emerging area of investigation is the relationship between the axonal cytoskeleton and the diverse mechanobiological responses of neurons. This review attempts to summarize our current understanding of the axonal cytoskeleton and its critical role in governing axonal mechanics in the context of neuronal development.

Keywords. Axon; cytoskeleton; mechanical forces; neuronal development; tension

1. Introduction

How mechanical forces shape neuronal development is a fundamental question, and accumulating evidence suggests that neurons are exquisitely sensitive to mechanical cues and respond with precision and specificity. Neuronal cells encounter various mechanical cues during development, which influence mechanical properties, dynamics and biological functions of neurons. As examples, substrate stiffness affects axonal branching (Flanagan *et al.* 2002; Leach *et al.* 2007), geometrical constraints influence axonal polarization (Roth *et al.* 2012) and osmolarity induces shape instabilities in neurons (Bober *et al.* 2015; Edmonds and Koenig 1990; Pullarkat *et al.* 2006).

Axons are micron-thin, long tubular processes and have to overcome several mechanical challenges in order to be maintained throughout the lifetime of the animal. Apart from the above-mentioned cues, mechanical tension in axons is a critical mechanical property that has been extensively studied and implicated in a number of physiological functions (figure 1). Axons experience stretch throughout the lifetime of an organism and show stretch-dependent growth response (Harrison 1935; Suter and Miller 2011; Weiss 1941). Mechanical tension in neurites has been demonstrated to influence various processes, including neurite growth (Bray 1984; Dennerll *et al.* 1989; Lamoureux *et al.* 2010; Pfister *et al.* 2004), axonal transport (Ahmed *et al.* 2012; Ahmed and Saif 2014), synaptogenesis (Siechen *et al.* 2009) and neuronal excitability (Fan *et al.* 2015). Further, as neuronal processes like axons are subjected to sudden mechanical stretch during normal movements, it is necessary to ensure mechanical resilience in these thin elongated structures to avoid damage. The mechanical resilience may come from both the cytomechanical properties of individual axons (Dubey *et al.* 2019; Krieg *et al.* 2017b) and also from the arrangement of axons in nerve tracts. One extreme example of the latter is the waviness and

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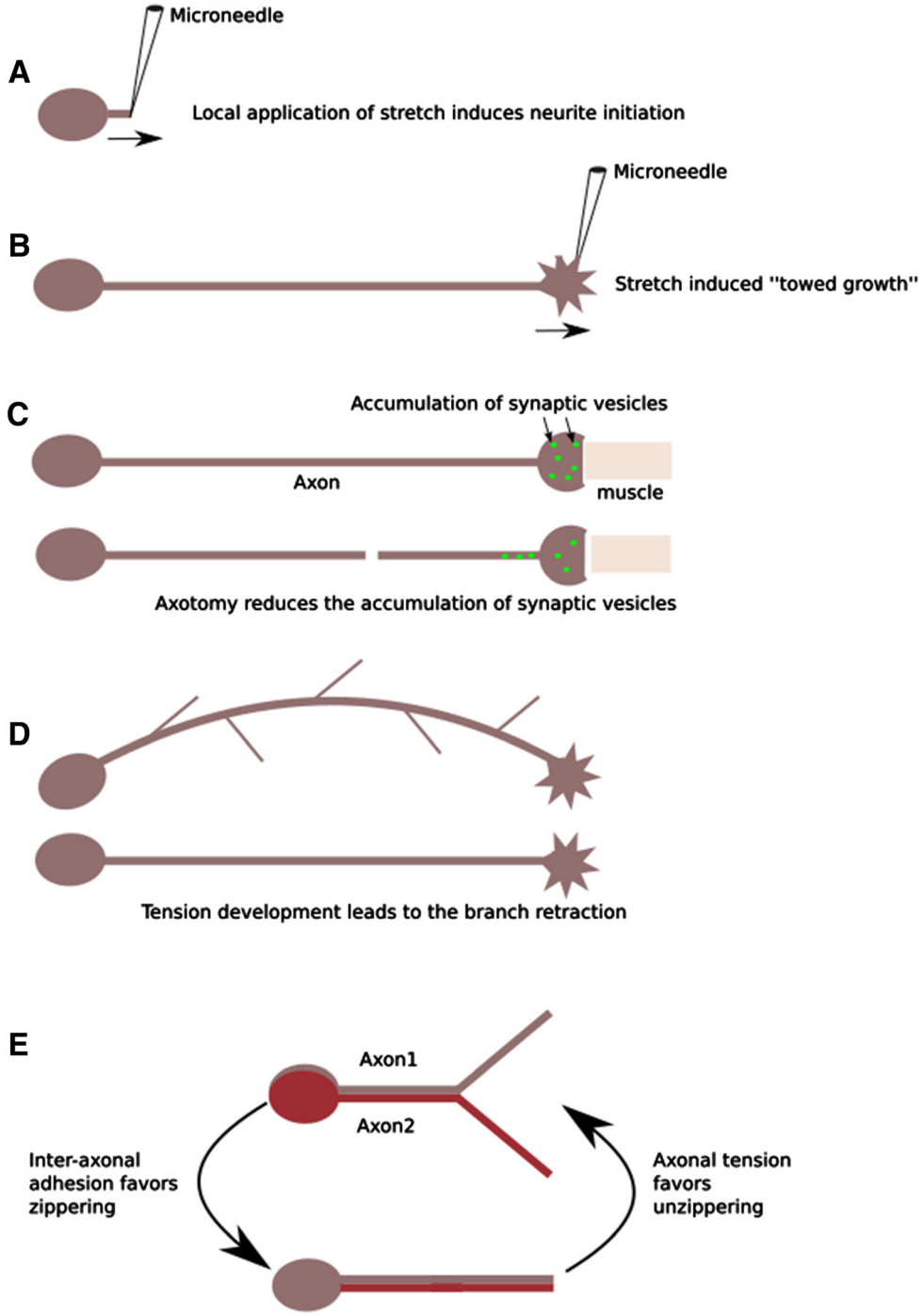

Figure 1. Functions of mechanical tension in neuronal development. (**A**) Neurite initiation (Bray 1984; Fass and Odde 2003; Zheng *et al.* 1991). Focal application of mechanical tension can initiate the formation of neurites. Arrow indicates stretch induced by mechanical pulling using a micro-needle or magnetic beads. (**B**) Elongation (Dennerll *et al.* 1989; Zheng *et al.* 1991). Development of tension in the axon triggers elongation. Arrow indicates stretch induced by growth cone translocation or by applying external stretch using a micro-needle (growth cone towing or lateral distension). (**C**) Synaptogenesis (Siechen *et al.* 2009). Accumulation of synaptic vesicles is regulated by mechanical tension. At the fly neuromuscular junction, development of tension on the motor neurons facilitates the accumulation of synaptic vesicles (top). Release of tension by laser-based axotomy prevents the accumulation of synaptic vesicles at presynaptic termini (bottom). (**D**) Branch dynamics (Anava *et al.* 2009). Tension development in neurites during the straightening of curved axons leads to collateral branch retraction. (**E**) Fasciculation-defasciculation of two axons resulting from competition between two forces: tension in the individual axons and axon-axon adhesion. Tension favours unzippering while inter-axonal adhesion (shown with arrows) promotes zippering (Šmít *et al.* 2017).

coiling of peripheral nerves observed in the floor of the mouth of rorqual whales. During lunge feeding when the floor of the mouth is highly stretched, the nerve uncoils to accommodate the stretch and avoids stretch-induced injury (Lillie *et al.* 2017).

The axonal shaft is filled with a crosslinked network of microtubules and neurofilaments along with thin circumferential cortical F-actin (Kevenaar and Hoogenraad 2015; Leterrier *et al.* 2017, Papandreou and Leterrier 2018) (the organization of the axonal cytoskeleton is described in detail later). It is likely that the axonal cytoskeleton and cytoskeleton-associated motor proteins are intimately involved in both regulating stretch-mediated functions and also conferring mechanical resilience. Here, we review the influence of mechanical tension on neuronal development and function from the perspective of recent advances in our understanding of the cytoskeletal organization in axons.

2. Stretch-induced axon initiation and elongation

Axons undergo continuous, slow stretch as growth cones translocate to their targets and develop synaptic contacts. The bulk of neuronal connectivity is established in early development, following which the animal grows in size. During this later growth phase, the axons again experience sustained mechanical stretch as the surrounding tissue grows (Harrison 1935; Suter and Miller 2011; Weiss 1941).

How neurons respond to continuous stretch is a fascinating question that continues to be actively investigated. The field emerged several decades back with experimentation involving microneedle-based perturbations of neurites, which demonstrated stretch-induced growth. The focus has now shifted towards understanding how neurite elongation and responses to stretch involve the bulk and local properties of the axonal cytoskeleton (Heidemann and Bray 2015; O'Toole *et al.* 2008). Progression of the field is discussed in the following subsection.

Using *in vitro* cultures of chick sensory neurons, the branching geometry of neurites was mapped and it was inferred that neurites are likely to be under tension (Bray 1979). Interestingly, the direction of the growth cone movement was found to be dictated by tension in the neurite shaft thus implicating mechanical tension in neurite growth (Bray 1979). A series of elegant experiments using microneedle-based axon pulling, significantly advanced the understanding of stretch-induced responses (Bray 1979; Bray 1984; Lamoureux *et al.* 1989; Lamoureux *et al.* 2010; Zheng *et al.* 1991). Observations on chick sensory neurons showed that growth cone translocation resulted in a concomitant increase in axonal tension (measured by a microneedle attached to the cell body) and implicated growth cone motility in generating tension in neurites (Heidemann and Buxbaum 1990). Experiments in PC12 neurites suggested that neurons grew above an applied force, while below another (lower) force threshold they retracted. In between these two set points, the axon behaved like a passive viscoelastic solid (Dennerll *et al.* 1989). Threshold-dependent responses were also reported in chick sensory neurons using an improved protocol of step-wise increase in tension (Zheng *et al.* 1991).

Further, it was demonstrated that the tensional set-points are insensitive to substrate composition suggesting minimal influence of extrinsic chemical cues on the intrinsic sensitivity to applied force (Lamoureux *et al.* 1992). However, these results need to be confirmed with a larger range of substrates (in terms of ECM composition) and also varying the substrate stiffness (as substrate rigidity is known to influence other processes like growth and branching (Flanagan *et al.* 2002; Georges *et al.* 2006)).

Complementary studies using neurons grown on stretchable substrates have also been employed to study stretch-dependent growth of neurons. Axons of rat DRG neurons could be stretch-grown to achieve elongation rates of up to 8 mm/day and lengths reaching $\sim$10 cm. Increase in axon caliber (up to 35%) was also observed in response to the substrate stretch (Pfister *et al.* 2004). Follow up studies demonstrated that stretch-grown neurons retain their ability to fire action potentials with normal amplitudes and durations (Pfister *et al.* 2006). Further, the density of Na^+ and K^+ channels increased in the stretch growth paradigm suggesting that stretch growth is a well-regulated physiological response (Loverde and Pfister 2015; Pfister *et al.* 2006).

Leg lengthening is commonly used to fix leg length discrepancies and offers an interesting paradigm to evaluate stretch-dependent neuronal growth in intact animals. Such experiments on the rat sciatic nerve demonstrated stretch growth *in vivo* with the internodal lengths nearly doubling (Abe *et al.* 2004). Another study using the same strategy on rabbit hindlegs found up to 33% increase in internodal lengths of tibial nerve axons with no change in diameter, myelin distribution and conduction velocities (Simpson *et al.* 2013).

Collectively, the studies discussed above demonstrate that stretch-induced axonal elongation is a

common and pervasive phenomenon and underscores the importance of identifying the underlying mechanism. It was proposed that external stretch during body growth or stretch generated by the advancement of the growth cone initiate a cascade of growth-promoting events, like cytoskeletal assembly and rearrangement (Heidemann and Buxbaum 1990). This suggestion was consistent with early experiments showing microtubule stabilization prevented neurite retraction (Joshi *et al.* 1985). Conversely, taxol (microtubule stabilizing agent)-treated PC-12 neurites also failed to show a growth response when subjected to stretching over and above the growth threshold force (Dennerll *et al.* 1989).

Initially, axonal elongation was thought to be via microtubule assembly at the growth cone (Bamburg *et al.* 1986) with the axonal microtubule array considered to be static relative to the growth substrate. However, later experiments indicated that the axon itself is subjected to viscoelastic stretch (Miller and Sheetz 2006), capable of force generation (Bernal *et al.* 2007) and intrinsically contractile (Mutalik *et al.* 2018; O'Toole *et al.* 2015; Tofangchi *et al.* 2016). Axonal stretch resulting in bulk flow microtubules has been modelled to drive neurite elongation (Athamneh *et al.* 2017; de Rooij *et al.* 2018; Miller and Sheetz 2006; Miller and Suter 2018; O'Toole *et al.* 2008, 2015). Thus a major shift in modelling the axon, over long time scales relevant to elongation, have been from considering it to be a solid to an active fluid (de Rooij *et al.* 2018; Miller and Suter 2018; O'Toole *et al.* 2015).

Where mass addition occurs in axons during stretch growth and the underlying mechanisms has been an active area of investigation. A model has been proposed for stretch-dependent axonal elongation (O'Toole *et al.* 2008). It suggests that when the forces produced at the growth cone are weak but the adhesion along the axon is high or the viscosity (or thickness) of the axon is increased, then growth is restricted at the growing tip. In this scenario, the forces get dissipated along axonal length due to strong adhesion and viscosity (O'Toole *et al.* 2008). Thus, mass addition is either intercalated (increase in axonal caliber) or restricted to the tip depending on how strongly the growth cone pulls, and on the distribution of the stretch along the axon. This suggests that localized force generation is central to axonal growth. While recent studies involving trypsin-induced detachment followed by retraction (O'Toole *et al.* 2015) or axonal straightening (Mutalik *et al.* 2018) have probed local force generation, more detailed investigations are warranted.

Experimentally, intercalated growth in response to the stretch-induced elongation has been demonstrated using axonal caliber recovery as a readout (Lamoureux *et al.* 2010; Suter and Miller 2011). There are two possibilities for how this stretch or strain accommodating growth may occur. A pre-synthesized pool of material can be transported from the cell body to the site of expansion and deposited there (Reinsch *et al.* 1991; Suter and Miller 2011). There is evidence to suggest that axonal transport is sensitive to axonal tension (Ahmed and Saif 2014) though if this process directly affects mobilization and the addition of new material is not known. Another possibility is that new material is locally synthesized and incorporated in response to the applied strain. Recently, using mTOR upregulation, local protein synthesis in response to the applied strain has been demonstrated in *vivo* in a rat sciatic nerve model (Love *et al.* 2017). Both the mechanisms mentioned above may not be mutually exclusive and need to be systematically tested. For example, it is possible that prevailing mechanisms depend on strain rates, spatial distribution of growth-promoting proteins/local translation hotspots along axons and local signaling. It may be possible to resolve these questions using devices like compartmentalized microfluidic chambers, however highly sensitive probes for the detection for mass addition will be required.

Membrane dynamics during neuronal growth (Pfenninger 2009; Quiroga *et al.* 2018) and especially during stretch-induced growth has been relatively poorly investigated (Miller and Suter 2018). Early *in vitro* studies in cultured neurons showed that membrane addition occurs near the growth cone (Bray 1970; Feldman *et al.* 1981). Another study suggests that excessive membrane is generated near the growth cone and then flows towards the cell body (Dai and Sheetz 1995). Later studies using polyetheleneimine-coated microspheres attached to the growing neurites, demonstrated that membrane addition takes place interstitially along the entire length of the axon but and with a higher frequency at the distal parts (Zheng *et al.* 1991). However, it is debatable if the bead dynamics reflect membrane flow or the dynamics of the cortical cytoskeleton (Lamoureux *et al.* 2010). Hence how membrane dynamics and recycling is regulated in stretch-dependent growth remains a critical though elusive problem and needs further experimentation and microscopic analysis.

Formation of neurites, *de novo* from cell bodies, has been shown to be influenced by tension (Bray 1984; Chada *et al.* 1997; Fass and Odde 2003; Zheng *et al.* 1991). Application of the tension (using microneedles) on the soma, above a certain threshold, resulted in the

formation of neurites (Zheng *et al.* 1991). These tension-induced neurites showed normal microtubule assembly and also developed growth cones, suggesting normal functionality.

Magnetic bead-based force application has also been used to initiate neurites from chick forebrain neurons with a detailed analysis of the force kinetics (Fass and Odde 2003). This study documents time-resolved events in stretch-induced initiation; elongation is often interrupted by sudden retractions, suggesting dynamic, alternating phases of growth and retraction during initiation.

How cytoskeletal and membrane remodeling occurs during stretch-induced initiation and elongation of neurites remains poorly understood. The original pioneers of the field have recently proposed an attractive model which is worth testing (Heidemann and Bray 2015). They suggest that stretch-induced thinning of the axon leads to the longitudinal drawing away of microtubules and neurofilaments and may result in breaks in the filaments. The associated constriction is also likely to compact the filaments leading to reduced transport and accumulation of material at the site of constriction. Over time, the accumulated material will reorganize and add to the cytoskeletal network resulting in the recovery of the axon thickness. It is thus important to conduct sensitive and dynamic measurements of the axonal diameter during stretch growth.

3. Role of mechanical tension in synaptogenesis and synaptic function

Accumulation of synaptic vesicles at the presynaptic terminal is a crucial step during synaptogenesis. Axotomy of fly motor neurons revealed that vesicle accumulation at the presynaptic terminal is dependent on axonal tension. Presynaptic clustering could be restored by mechanical pulling of the severed axon, confirming tension dependency. This study speculates that the stretch-dependent actin polymerization may lead to the formation of an actin scaffold that facilitates the accumulation of vesicles (Siechen *et al.* 2009). Formation of adhesive contacts with an island of carbon nanotubes and subsequent development of tension was sufficient to cause the accumulation of presynaptic markers in the absence of any postsynaptic structures in locust neurons (Anava *et al.* 2009). Collectively, these studies support the contention that tension is necessary to initiate synaptogenesis.

Applied stretch is also implicated in synaptic functions. Neurotransmitter release, both spontaneous and evoked, has been shown to be stretch-dependent in frog neurons (Chen and Grinnell 1997). Recent studies have shown that synaptic excitability can be regulated by an applied stretch. Using stretch regimes within physiological ranges, modulation of neuronal activity was documented in mouse brain slices (Fan *et al.* 2015).

The nature of the stretch-induced signaling that drives synaptogenesis is currently not known. Synaptogenesis depends on a myriad of proteins at the presynaptic side (along with postsynaptic signals). Axonal cytoskeleton may have a structural role and also enable transport and accumulation of key molecules to the nascent pre-synapse. The current challenge is to understand how biochemical events during synaptogenesis are regulated by mechanical signaling. Assessment of the cytoskeleton scaffold and its dynamics upon application of stretch needs to be studied in greater microscopic detail to uncover the possible mechanisms.

4. Influence of axonal tension on branching and neuronal network geometry

Observations of *in vitro* cultures of chick sensory neurons suggest that the geometrical arrangement of the branched neurite network is constrained by balance of tension across all the branches. Perturbations like branch retraction, either spontaneous or induced, led to branching in a sister neurite. Similarly, slackening by ablation or acute release of the growth cone also induced branching to accommodate the change in tension (Bray 1979). Interestingly, neurons grown on compliant substrates, where one expects lower neurite tension (though not formally demonstrated), show increased branching and support the hypothesis that reduced axonal tension favors branch formation (Flanagan *et al.* 2002).

Complimentary evidence comes from locust neurons grown on substrates patterned with highly adhesive islands of carbon nanotubes. This work demonstrated that increase in neurite tension is concomitant with collateral branch retraction (Anava *et al.* 2009). In this study, anchor points were provided by the adhesive islands that allow tension build up in the neurite shaft between two attachment sites. Development of tension subsequently led to the retraction of collateral branches. Using a mechanical model for local geometry at the branch point, it has been suggested that the geometry depends on volume optimization and tension balance between the branches (Shefi *et al.* 2004). The experimental observations and models discussed above

have been developed for two-dimensional neuronal cultures *in vitro*. It is therefore important to extend these studies, both empirical and theoretical, to three dimensional *in vivo* contexts to determine if indeed tension as is an intrinsic regulator of branch pattern geometry.

Tension balance in the individual neurites can decide the positioning of the cell bodies in the neuronal network. However, if this influences neuronal network functions is not clear (Hanein *et al.* 2011). Demonstration of tension being a major player in maintaining conserved arbor geometry has been challenging due to the difficulty in generating controlled perturbations. An *in vivo* study on embryonic CNS neuropil of grasshopper *Schistocerca americana*, suggests that the bifurcated geometry of a specific neuron is maintained by the balance of tension in the two branches (Condron and Zinn 1997).

Tension in the individual axons and dendrites might facilitate minimizing wiring lengths and result in the compaction of brain tissue. Indeed it has been proposed that axonal tension may drive the development of species-specific patterns of cortical folding (Hilgetag and Barbas 2006; Van Essen 1997). However, axonal tracts are typically oriented radially in the gyri (Xu *et al.* 2010) and suggests that axonal tension between gyri are not the primary drivers of cortical folding. Though not extensively tested, the orientation and extent of axonal tension may influence cortical folding indirectly by modulating and amplifying the folding process driven by differential cortical growth (Garcia *et al.* 2018; Llinares-Benadero and Borrell 2019; Richman *et al.* 1975; Xu *et al.* 2010).

Recently, axonal fasciculation has been demonstrated to be dependent on axonal rest tension. The competition between the tension in individual axons and the strength of axon-axon adhesion appears to determine fasciculation patterns (figure 1E) (Šmít *et al.* 2017). Tension-induced network geometry and its functional implications are significantly understudied. Analysis of geometrical patterns of complex neuronal networks and characterization of progressive changes during development will be necessary to understand the tension-based mechanisms in neuronal morphogenesis.

5. Mechanical signaling in axons

As discussed above, axonal initiation, elongation, retraction, branch dynamics and synaptogenesis can be regulated by mechanical tension. However, the signaling mechanisms underlying the tension-regulated processes are largely unknown. Mechanosignaling along axons could be similar to those observed in growth cones. For example, tension-dependent tyrosine phosphorylation downstream of Netrin and apCAM-mediated growth cone steering has been documented (Moore *et al.* 2012; Suter and Forscher 2001). However, whether axons have similar mechanosensing activities acting via adhesion complexes to promote stretch-dependent growth is not known.

Activation of stretch-activated channels could also be a potential mechanism for the stretch-induced axonal elongation and initiation. The membrane stretch-dependent activation of TRPV2 and subsequent Ca^{2+} entry in mice sensory and motor neurons are known to support the neurite outgrowth (Shibasaki *et al.* 2010). A recent study shows that axons prefer soft substrates, suggesting well-regulated mechanosensory mechanisms in axons. Using pharamacological inhibition and genetic knockdown approaches, this study establishes the role of the mechanosensitive ion channel Piezo-1 in substrate rigidity sensing by retinal ganglion cells both *in vivo* and *in vitro*. Localization of Piezo-1 along axons, apart from growth cones underscores mechanosensitivity along the axonal length (Koser *et al.* 2016). More recently, Piezo activity has also been implicated in regulating axon regeneration in flies and rodents (Song *et al.* 2019).

Another example where mechanosensing is likely to be involved is the induced retraction of neurites of NG-108 15 cells. Retraction of the axonal shaft can be triggered by the mechanosensitive growth cone upon encountering stiff substrates. Axonal retraction is dependent on Ca^{2+} influx through stretch-activated channels. This is an example where signaling at the growth cone has a direct influence on the response of the neurite (Franze *et al.* 2009).

These studies collectively suggest that mechanosensing through mechanosensitive ion channels is required for neuronal durataxis (growth towards soft substrate and active avoidance of stiffer substrates). However, there is a dearth of studies focusing specifically on axonal mechanotransduction.

In order to probe local signaling in axons without influencing the growth cone or the cell body, the axonal segments need to be isolated microfluidically to facilitate localized perturbations. However, as the axon responds as a mechanical continuum, it remains technically challenging to study local stretch-mediated signalling in axons.

6. The axonal cytoskeleton and its dynamics

How the axonal cytoskeleton responds and remodels following axonal stretch and contributes to the stretch-induced processes discussed above is not completely clear. Although the axonal cytoskeleton has been a focus of studies on stretch-induced responses for some time, the details are still emerging. The microscopic mechanisms underlying local cytoskeletal responses and their coordination at larger length scales to elicit an axon-wide response remain especially understudied. Some of the studies mentioned earlier used pharmacological inhibitor-based approaches to understand the contribution of the cytoskeleton in driving tension-induced processes. On the other hand, the response of the subcellular cytoskeleton has been primarily mapped using fiduciary markers like docked mitochondria (Athamneh *et al.* 2017; Chetta *et al.* 2010; Miller and Sheetz 2006; Mutalik *et al.* 2018; Wang *et al.* 2001).

The axonal cytoskeleton is crucial for driving various processes during neural development (Kevenaar and Hoogenraad 2015; Leterrier *et al.* 2017, Papandreou and Leterrier 2018). Further, abnormal changes in the cytoskeletal elements have been associated with neurodegeneration (Griffin *et al.* 1995; Kirkcaldie and Collins 2016; Tang-Schomer *et al.* 2010). However, cytoskeletal organization and its dynamics in axons have only been sparingly investigated and are consequently poorly understood. It is imperative to develop an explanatory framework for axonal mechanics based on the recent advances in our understanding of the axonal cytoskeleton.

Axonal shafts are filled with microtubules which have a polarized arrangement with their plus-ends predominantly pointing towards the distal growth cones (figure 2A,B) (Baas *et al.* 1988). Based on Atomic Force Microscopy (AFM) indentation studies on the axons of DRG and sympathetic neurons, it was suggested that microtubules contribute significantly to the mechanical stiffness to axons (Ouyang *et al.* 2013). Although a direct link between local radial stiffness, as established by AFM measurements, and axial stretch along the axon length is not established, these studies highlight the importance of the axonal microtubule cytoskeleton. Other studies have associated mechanical breakage and loss of microtubule filaments with degeneration and axon loss during rapid stretch-induced injury (Tang-Schomer *et al.* 2010). Within the axon, how microtubule bundling and dynamics of individual microtubules regulate axonal stability is not well understood. It has been established that the stability of microtubules along axons differ spatially; distal segments have a smaller proportion of stable microtubules than proximal (Ahmad *et al.* 1993). It is also known that tubulin post-translational modifications (PTMs) regulate the stability of microtubules (Wloga and Gaertig 2010) and the influence microtubule mechanics *in vitro* studies (Portran *et al.* 2017). Cellular studies in muscle cells have also reported elevated detyrosination of microtubules increasing cytoskeletal stiffness (Kerr *et al.* 2015). While these studies prefigure attractive possibilities for microtubule-dependent axonal mechanics, whether spatial differences in tubulin PTMs on the microtubule network affects mechanical properties of axons is yet to be explored.

Electron microscopy studies show that the microtubule network in axons is heavily crosslinked (figure 2B) (Hirokawa 1982). How crosslinking activities of microtubule-associated proteins contribute to mechanical responses is an interesting question. Among the various microtubule-associated proteins (MAPs), tau, has been extensively studied in this context. Strain rate-dependent disruption of microtubule ultrastructure has been modelled based on the stiffness and force-dependent detachment/re-attachment kinetics of the crosslinkers (Ahmadzadeh *et al.* 2015; de Rooij and Kuhl 2018). At slow loading rates, detachment and re-attachment kinetics of tau and intramolecular extension may allow axons to withstand considerably large strains without structural failure. At lower strain rates, tau dynamics can facilitate microtubule sliding and extension of the axon resulting in relaxation of the stress. However, at fast strain rates the microtubules experience considerably large tensile stresses and are disrupted. These models are consistent with experiments that demonstrate stretch-induced mechanical failure of the microtubule bundle in axons (Tang-Schomer *et al.* 2010). Recently, the worm orthologue of tau has been shown to be involved in dissipating the torque within the microtubule bundles. It has been proposed that the axonal axial tension and the bending stiffness of the microtubule bundle balance the microtubule torque and together contribute to the mechanical stability of the axon (Krieg *et al.* 2017a, Krieg *et al.* 2017b). These observations need to be extended to investigate the role of tau and other MAPs in regulating axonal mechanical properties. Microtubule crosslinkers, like tau, may not be uniformly distributed along the axon (Black *et al.* 1996; Kempf *et al.* 1996). This suggests that there could be spatial heterogeneity in mechanical behavior along the axon depending on the extent and nature of MT crosslinking activity. A general model of axon homeostasis,

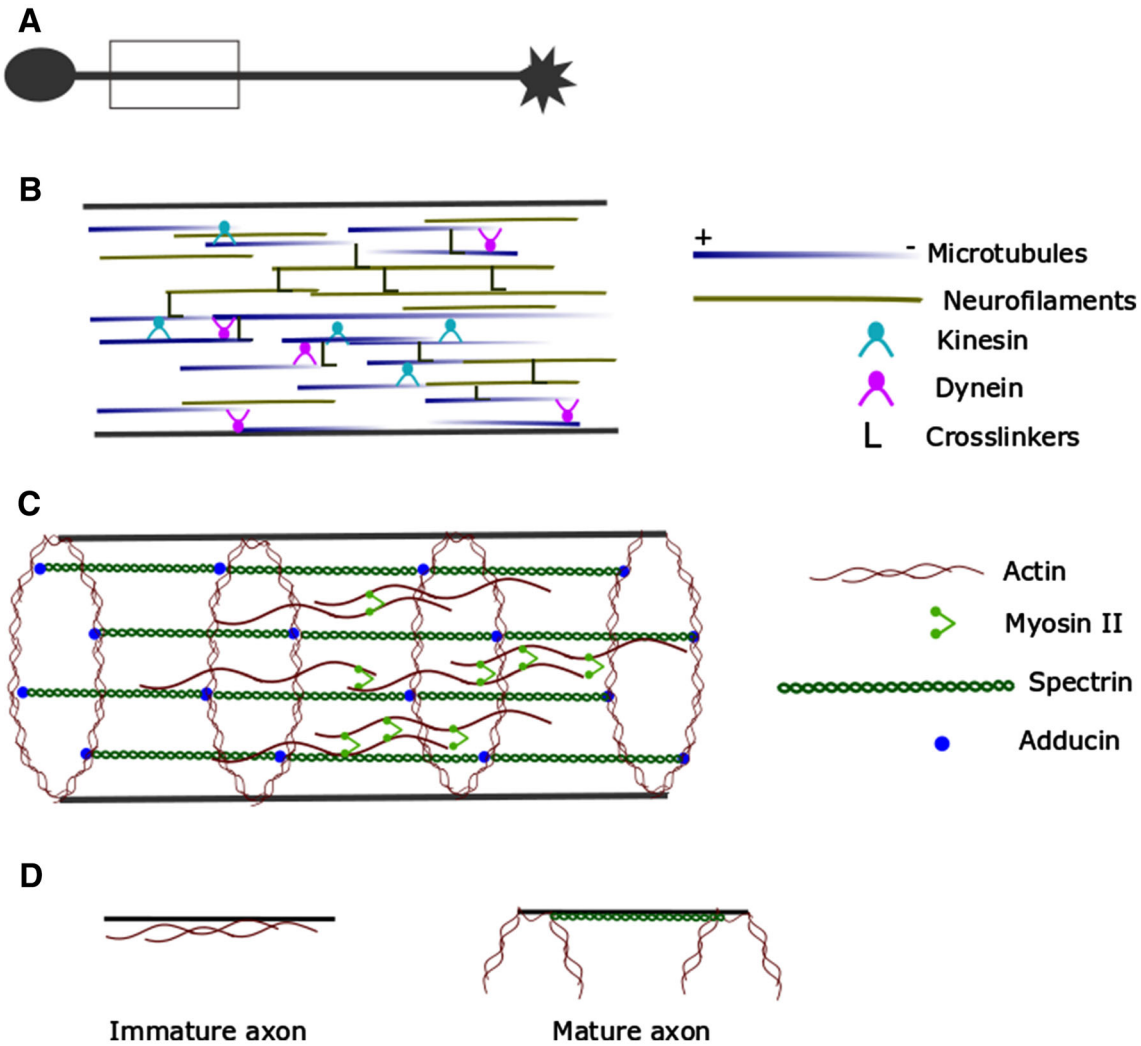

Figure 2. Cytoarchitecture of axons. (A) Schematic representation of a neuron with the cytoarchitecture of the boxed region shown in (**B**) and (**C**). (**B**) Two-dimensional illustration of the organistaion of the microtubule (blue; gradient indicates the polarity) and neurofilament (olive green) cytoskeletons in axons, along with associated proteins. (**C**) Three-dimensional illustration of the organisation of actin and associated proteins. The membrane-associated periodic skeleton (MPS) consists of F-actin rings (maroon) capped by adducin (blue) arranged periodically along the axon with spectrin (green) tetramers bridging consecutive actin rings. Other F-actin structures, like actin patches and trails have also been observed in the axon shaft (**D**) (left) in axons of immature neurons, the periodic organization of cortical F-actin is limited in its prevalence. However, periodic organization of F-actin dominates the entire length of axons (and dendrites) in mature neurons (right).

involving microtubule regulating and bundling activities balancing the propensity of microtubules in the axonal shaft to get disorganized due to rearrangement driven by the dynamics and activities of motor proteins has been proposed (Hahn *et al.* 2019). While such integrative models offer powerful testable predictions, much remains to be done to probe the underlying mechanisms.

Early experiments concluded that while the axonal microtubule network was stationary, there were local dynamics along the length of the axon (Ma *et al.* 2004; Okabe and Hirokawa 1990). However, later studies identified bulk flow of the axonal microtubule lattice (Athamneh *et al.* 2017; Reinsch *et al.* 1991) and these collectively suggest that microtubules exhibit a dynein-driven bulk forward movement that drives axonal elongation (Athamneh *et al.* 2017; Roossien *et al.* 2014).

Neurofilaments are also critical load-bearing polymers involved in maintaining neural morphology (Helfand *et al.* 2003; Laser-Azogui *et al.* 2015). Neurofilaments are implicated in regulating the axonal caliber through electrostatic interactions of their side-arms and subunit composition (Kriz *et al.* 2000; Ohara

et al. 1993; Sakaguchi *et al.* 1993). Like the MAPs mentioned above, the non-covalent cross-bridges between neurofilaments (Mukhopadhyay *et al.* 2004) might be important to (acutely) regulate polymer dynamics and bundling in response to the mechanical cues. Rheological characterization of rat cortical neurons demonstrate that neurofilaments contribute to the fluidization of the axonal shaft through the sliding of cross-bridges (Grevesse *et al.* 2015). AFM-based studies (discussed earlier) also suggests that neurofilaments contribute axonal stiffness, though not to the extent of microtubules (Ouyang *et al.* 2013). The role of neurofilaments in regulating forces along axons have remained poorly investigated due to the lack of specific inhibitors.

F-actin organization and dynamics in axons are critical for various functions like neurite initiation, elongation, branching, transport and maintaining a submembrane scaffold (Letourneau 2009). F-actin remodeling, while very well studied in growth cones (Dent and Gertler 2003; Lowery and Van Vactor 2009), remains poorly explored in axons. However, recent advances in super-resolution imaging techniques and the development of specific fluorescent probes (Lukinavičius *et al.* 2014) have suddenly animated this area.

Early electron microscopy studies in frog and squid neurons identified the cortical localization of F-actin, forming a scaffold immediately below the membrane (Hirokawa 1982; Tsukita *et al.* 1986). Later, patches of F-actin were identified in the axonal shaft and shown to be precursors for axonal filopodia that develop into collateral branches (Spillane *et al.* 2011).

Unlike neurofilaments and microtubules, the contribution of actin to mechanical stiffness of axons (as determined by radial AFM indentation studies) has been assumed to be minimal (Ouyang *et al.* 2013). However, an older study using microneedle-based deformation in PC-12 neurites had reported a reduction of axial axonal tension following actin depolymerization (Dennerll *et al.* 1988). While the contribution of axonal actin is under active investigation, the actin-associated tetrameric protein, spectrin, has been implicated in conferring mechanical stability to axons (Hammarlund *et al.* 2007). This study found that axons of worms with mutations in spectrin were fragile and prone to breakage by the strain induced by body movements. Later investigations also implicated spectrin in regulating pre-stress in worm touch receptors neurons (Krieg *et al.* 2014).

The advent of super-resolution microscopy revealed that spectrin and actin form a periodically organized structure underneath the plasma membrane, commonly named as the membrane-associated periodic skeleton (MPS), in mature neurons (figure 2A,C) (He *et al.* 2016; Xu *et al.* 2013). Short actin filaments, capped by the capping protein adducin, form periodic radial F-actin rings that alternate with spectrin tetramers. This organization was found to be dependent on actin and spectrin, but independent of adducin (Leite *et al.* 2016). A recent study, combining electron microscopy and super-resolution imaging has updated the F-actin organization and suggests that rings comprise of extended, intertwined F-actin filaments as opposed to short filaments (Vassilopoulos *et al.* 2019).

STORM (Stochastic optical reconstruction microscopy) of rat hippocampal neurons suggest that MPS at early developmental stages is limited to the proximal region of the axon and progressively develops along the length of the axon as it matures (Zhong *et al.* 2014). Adducin accumulation is seen at later stages, suggesting that at the early stages the actin rings may be more dynamic (Zhong *et al.* 2014). However in mature neurons, Fluorescence recovery after photobleaching (FRAP) studies suggest that the MPS spectrin is quite stable (Zhong *et al.* 2014). This observation is consistent with a mechanical scaffolding role for the MPS and is consistent with the observation that in worm neurons, spectrin is under tension and mediates axonal pre-stress (Krieg *et al.* 2014).

The molecular mechanisms underlying MPS assembly are a very active area of investigation and is crucial for understanding the physical aspects of the MPS and their contribution to the mechanical responses of axons. In *Drosophila* neurons, MPS assembly is Arp2/3 and formin-dependent. Microtubules also appear to regulate the formation and stability of the MPS (and vice versa) (Qu *et al.* 2017). Stabilization of microtubules by taxol treatment was found to promote the membrane lattice formation in rat hippocampal neurons (Zhong *et al.* 2014). The involvement of microtubules was further confirmed by depolymerizing microtubules with Nocodazole treatment, which resulted in disruption of the MPS. Conversely, disruption of MPS with the actin destabilizing agent cytochalasin D resulted in gaps in the axonal microtubule indicative of microtubule loss (Qu *et al.* 2017). Together, these studies suggest that microtubule bundles in the core of axons and the MPS may have co-regulatory functions; the stiff internal core of microtubules support MPS assembly and once developed, the MPS protects microtubules from mechanical damage. Recently, axons from mice lacking ßII-spectrin have been reported to severe transport deficits, though microtubule dynamics was not significantly affected (Lorenzo *et al.* 2019). It is clear that research in this area is at its infancy and carefully

controlled studies involving actin/microtubule regulatory proteins, motor proteins and precise manipulation of the MPS will be necessary to develop a mechanistic framework for MPS development and maintenance.

MPS is present in a wide range of neurons across different species (He *et al.* 2016). Notably, there are progressive changes in both the prevalence and the spacing of the MPS as neurons mature (figure 2D). This observation is indicative of a developmentally regulated molecular mechanisms underlying MPS assembly (D'Este *et al.* 2015; He *et al.* 2016; Mutalik *et al.* 2018). Studies using a live cell compatible probe for F-actin in hippocampal neurons show that ∼35% of axons from 2 DIV neurons show clear MPS. This increases to 100% by 5 DIV (D'Este *et al.* 2015). Similarly, in 2 DIV chick DRG neurons, the periodic arrangement of spectrin is detectable in ∼20% of the axonal length (Mutalik *et al.* 2018). It is likely that at early developmental stages, the MPS is less abundant and perhaps more flexible and facilitates functions like rapid elongation, branching and branch remodeling. However, at later stages its contribution to the mechanical stability of axons dominates. MPS disassembly associated with axonal degeneration upon trophic factor withdrawal is consistent with our speculation (Unsain *et al.* 2018). Further, spectrin is a major target of calpain-mediated proteolysis. It is possible that such cue-triggered proteolytic mechanisms may facilitate local remodeling of the MPS. Most studies investigating mechanical properties and responses of axons have been undertaken in relatively young neurons. It is now necessary to systematically conduct new investigations taking into account the developmental changes in the organization of the axonal cytoskeleton.

Single molecule force spectroscopy has revealed that spectrin repeats can unfold in response to stretch (Rief *et al.* 1999). In erythrocytes, spectrin repeat unfolding in response to shear stress has been demonstrated ((Johnson *et al.* 2007; Krieger *et al.* 2011; Lee and Discher 2001; Zhu and Asaro 2008). In this cell type, dynamic, short actin filaments also contribute to membrane flexibility (Gokhin *et al.* 2015). Thus, the axonal actin-spectrin MPS may contribute to the load-bearing ability of axons. A recent study combining experiment and theory has suggested that force-dependent domain unfolding-refolding dynamics of axonal spectrin acts as a 'shock absorber' to buffer axonal tension (Dubey *et al.* 2019). Future investigations probing the kinetics of spectrin unfolding in response to stretch and the role of actin will be critical to develop this potentially unifying mechanism of MPS-mediated mechanical resilience.

As mentioned earlier, F-actin dynamics in axons are largely understudied. Early attempts of F-actin dynamics in axons using FRAP suggested slow turnover and indicated lack of bulk polymer movement along axons (Okabe and Hirokawa 1990). More recently, axonal F-actin was found to be dynamic, showing bidirectional movement, though its functional relevance remains speculative (Chetta *et al.* 2015). A tug-of-war like scenario has been proposed, where the mobile F-actin densities may bind to stable F-actin pools via myosins and also to microtubules via actin-microtubule crosslinkers. The balance between the active force generating components and strength of attachment with the stable F-actin/microtubules regulates the mobility of the F-actin densities. Another actin-based structure described in axons *in vitro* is the actin wave. F-actin waves are growth cone-like structures which translocate towards growth cone to replenish F-actin at growing tips (Katsuno *et al.* 2015). Recently, F-actin densities in axons have been reported to be dynamic with regulated F-actin assembly and disassembly. These F-actin 'hotspots' were also found to generate formin-dependent, dynamic actin trails (Ganguly *et al.* 2015). The dynamics of these F-actin trails have been suggested to result in processive transport of actin in axons (Chakrabarty *et al.* 2019). However, it remains to be seen if these dynamic F-actin pools are directly involved in biomechanical responses of neurites.

7. Motor proteins and their role in regulating mechanical forces in axons

Due to their stiffness and long persistence lengths (Blanchoin *et al.* 2014), microtubules are well suited as tracks for motor protein-based transport. Motors, like dyneins and kinesins, drive transport towards the minus- and plus-ends of the microtubules, respectively (Maday *et al.* 2014; Vale 2003) (figure 2B). Actin-based motors, like myosin-II, drive contractility in several different cell types, including in neuronal growth cones and axons (Mutalik *et al.* 2018; Tofangchi *et al.* 2016). Motor proteins have been implicated in maintaining a force balance in neurons (Ahmad *et al.* 2006; Ahmad *et al.* 2000; de Rooij *et al.* 2018; Myers *et al.* 2006). Dynein and myosin-II activities generate counterbalanced forces in neurons, contractile forces generated by myosin-II (discussed later) balanced by the pushing forces of dynein. Other studies have also concluded that dynein drives extensile forces by bulk displacement of the microtubule

lattice while myosin activity generates cortical contractility (Roossien *et al.* 2014). Recent modelling approaches and simulations conducted with different motor protein densities have augmented the force balance model (de Rooij *et al.* 2018). This model remains attractive as it allows the framing and testing of several critical questions, like the nature of motor activity-dependent reorganization of cytoskeletal polymers to achieve such a balancing function.

Functions of kinesin in regulating mechanical forces have not been explored in detail. It has been shown, however, that Kinesin I promotes axonal initiation through microtubule sliding (Lu *et al.* 2015).

With the recent discovery of periodic actin rings, the localization of myosin II activity along axons has acquired great significance. There are limited studies on the organization of myosin II in axons. Recently, phosphorylated myosin light chain (pMLC; indirectly indicating active myosin II) was found to be localized heterogeneously in 2 DIV (days *in vitro*) chick DRG neurons (Mutalik *et al.* 2018). Localization of active myosin in actin rings has been shown in mature hippocampal neurons to be limited to the axon initial segment (AIS) and not extending to the distal axon (Berger *et al.* 2018). Myosin II-driven axial axonal contractility has been demonstrated recently both in invertebrate (Tofangchi *et al.* 2016) and in vertebrate neurons (Mutalik *et al.* 2018). Using cytoskeleton-docked mitochondria as a readout of the cytoskeletal strain, the latter study suggests that myosin-dependent contractile units are distributed heterogeneously along axons. However, the detailed organization of the actomyosin network in axons and its regulation of the inherent contractility of axons (discussed in the next section) needs further systematic investigation.[1]

8. Axonal contractility, regulation of tension and its functional implications

Regulation of rest tension is required to achieve efficient mechanotransduction in elongated processes, like axons. Axonal contraction could be a potential mechanism of regulation of the rest tension. Intrinsic contractility of axons has been demonstrated both *in vitro* (Dennerll *et al.* 1989; Mutalik *et al.* 2018) and *in vivo* studies. (Rajagopalan *et al.* 2010; Tofangchi *et al.* 2016). Axons tend to retract upon detachment or ablation due to inherent contractility; hence, retraction has been used previously as an assay to probe mechanisms of contractility. Axotomy-induced retraction has been used to demonstrate the ATP-dependence of axonal shortening (George *et al.* 1988). Increase in tension in DRG axons, following induction of slackening, also suggested that axonal rest tension is actively regulated (Dennerll *et al.* 1989). In these neurons, axonal retraction is actomyosin-dependent and requires actin turnover (Gallo *et al.* 2002). Recently, in *Drosophila* neurons, actomyosin contractility was demonstrated *in vivo* (Tofangchi *et al.* 2016). Our recent work has also implicated actomyosin-dependent axonal contractility using a trypsin-mediated deadhesion assay (Mutalik *et al.* 2018). The latter study also demonstrated the inherent, spontaneous contraction of axons when the growth cone and cell body are anchored on adhesive islands (Mutalik *et al.* 2018). This intrinsic contractility might be important for the length minimization (Van Essen 1997) and mechanical signaling *in vivo*. A resistive role for microtubules in regulating axonal contraction has been suggested in some studies (Athamneh *et al.* 2017; Tofangchi *et al.* 2016) but have not been obvious in others (Mutalik *et al.* 2018). Careful experimentation is needed to disambiguate microtubule function in axonal contractility.

Circumferential tension has been investigated in *Drosophila* neurons (Fan *et al.* 2017). Disruption of axial tension by inhibiting actomyosin activity resulted in a concomitant reduction in circumferential tension, indicated by an increase in axonal diameter. These observations suggest that circumferential and axial tensions are coupled and are actomyosin-dependent (Fan *et al.* 2017). Although the activity of myosin II is central, the localization of myosin activity along the axon is unclear. In hippocampal neurons, pMLC is distributed along the entire length of axons at early stages, but in mature neurons it is restricted to the AIS and co-localize with the MPS in this region (Berger *et al.* 2018). In 2 DIV chick DRG neurons, pMLC is localized along the length of the axons (Mutalik *et al.* 2018). The interplay between the MPS and myosin II activity in mediating axonal contractility is a fascinating problem but it remains challenging to visualize and probe these processes in live, contractile neurons.

Recently, the actin capping protein, adducin has been implicated in regulating axonal diameter (Leite *et al.* 2016). The depletion of adducin does not disrupt the assembly or periodicity of the MPS but increases the

[1] **Note added in proof:** Two new publications now demonstrate the presence of myosin-II associated with the periodic actin rings in the axons of mature rodent hippocampal neurons (Costa *et al.* 2020; Wang *et al.* 2020). These finding highlight our assertion that mechanical studies of axons need to be undertaken along with a careful analysis of the developmental maturation of the axonal cytoskeletal.

diameter of the axon and the F-actin ring. Consistent with these observations, axons from ßII-spectrin mice show significantly disrupted MPS and increased axonal caliber (Lorenzo *et al.* 2019). These observations suggest that actin filament growth in the rings is a key regulator of axonal caliber though the detailed mechanisms remain unknown. The possibility exists that dysregulation of actin turnover may lead to the loss of axonal contractility and, in turn, influence the axon diameter (see discussion on circumferential tension above).

In this review, we have discussed the structural role of microtubules, neurofilaments in maintaining axonal shape and the function of actomyosin activity in regulating mechanical tension. As discussed earlier, the MPS regulates the organization of axonal microtubules but is itself sensitive to perturbation to microtubules. Collectively, this suggests that axonal structure is maintained by the balance of microtubule and neurofilament bundles in the core and the cortical MPS/actin cortex at the periphery. It appears that actomyosin activity and the MPS may collaborate to actively regulate axonal tension while also contributing to the mechanical resilience.

9. Summary and outlook

In this review, we have discussed the different cytoskeleton components, their mechanical properties, organization and functions, including the recently discovered MPS in the context of axonal mechanics. As discussed here, the field of axonal mechanics has, over the last few decades, enriched our understanding of axonal tension and its functions in neuronal development. However, a definitive model of the underlying axonal cytoskeleton dynamics is in its early phases. A detailed understanding of the organization, dynamics, signaling and regulatory components of the axonal cytoskeleton will be the key to bridge the gap between axonal mechanics and underlying cytoskeletal mechanisms regulating it.

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