Journal of the Indian Institute of Science
A Multidisciplinary Reviews Journal
ISSN: 0970-4140 Coden-JIISAD

REVIEWS

Cytoskeletal Remodeling in the Establishment of the Neuronal Circuitry

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Abstract | The stereotyped circuitry of the adult nervous system is a result of instructive guidance of the neuronal processes to their respective synaptic targets. Signal-induced, choreographed remodeling of the cytoskeleton in the neuronal growth cones is at the core of this remarkable feat of establishing precise connectivity. Actin and microtubule cytoskeltons undergo dynamic reorganization in response to guidance cues and enable the growth cone to navigate accurately through the complex environment of the developing embryo.

In this review we discuss the key principles of cytoskeleton regulation in the process of growth cone-driven motility and pathfinding, primarily focusing on the actin and microtubule polymer systems.

1 Introduction

A key process in embryonic development is the establishment of precise neuronal connectivity. This stereotyped neuronal circuitry forms the basis for neural function and results from a highly regulated process of pathfinding by the neuronal extensions (axons and dendrites) towards their synaptic targets. The critical component of the axon's journey to its destination is the specialized motile structure at its tip, the 'growth cone'. Numerous substrate-bound and diffusible chemotropic guidance cues in the environment guide the growth cone to its target tissue. The receptors on the growth cone membrane interact with the extracellular cues providing spatio-temporally resolved information about the environment. This information is integrated and interpreted by the intracellular signaling machinery and transmitted to the effector system to generate an appropriate response. This can range from an alteration of growth rate to directional movement towards (attraction) or away (repulsion) from the guidance cue.

The downstream effector system regulating growth cone-mediated motility involves directed remodeling of the growth cone cytoskeleton in response to external guidance cues. Regulated cytoskeleton dynamics forms the basis for several key processes in growth cone–driven motility, including directional polarization, the generation of protrusive extensions and their stabilization, and the development of mechanical forces and traction necessary for translocation.

The cytoskeleton of the neuronal growth cone is dominated by the actin and microtubule (MT) polymer systems. The actin component is primarily involved in generating protrusive and contractile forces in the cell with the help of myosin motors. MTs, being less flexible, form a rigid, polarized network inside the growth cone and are critical for establishing polarity and sustained directional migration. The intermediate filaments are less abundant in the growth cone and their function remains less well understood. The lack of polarity and the relatively slower dynamics of intermediate filaments have suggested a relatively passive, structural role. While recent reports suggest a more active role for intermediate filaments,[1] these will not be discussed in this review. The actin and MT systems are coordinately regulated in order to bring about successful and efficient cell migration. While Rho-GTPase signaling is implicated in coordinated regulation of actin and MT cytoskeletons, there is a paucity of information regarding other biochemical players and mechanisms mediating actin-MT crosstalk. Growth cones, like other motile cells, form adhesive contacts with the substrate. These sites of focal contact/adhesion

Growth cone: Highly dynamic and specialized structure at the tip of the growing axons and dendrites. This forms the sensory and motile module that guides neurites to their destinations.

Cytoskeleton: Composed of microfilaments (actin), microtubules and intermediate filaments (e.g., neurofilament, lamin, etc.). They form the structural framework of the cell by providing rigid supports and tracks for motor-based movement and force generation. Cytoskeleton polymers can be highly dynamic and undergo tightly regulated cycles of assembly and disassembly.

Actin: Polarized, filamentous polymer (F-actin) made of globular actin monomers (G-actin) attached end-to-end. Involved in generating protrusive cellular structures, contractile forces and myosin motor-driven transport.

Microtubules: Polymer made up of tubulin dimers with a fast growing plus end and a slow growing or depolymerizing minus end. Involved in cellular polarization and movement, transport and structural rigidity.

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mediate mechanical coupling between intracellular cytoskeleton and extracellular matrix (ECM), allow transduction of intracellular forces to the substrate resulting in traction. Efficient directional motility necessitates coordinated spatio-temporal regulation of the focal adhesions across the growth cone surface. However, the mechanisms mediating these processes remain poorly understood.

Focal adhesions/contacts: Multi-molecular assemblies at the sites of attachment of the growth cone to the substrate coupling the cytoskeleton of the growth cone to the extracellular matrix via integrin and other associated molecules.

The cellular and molecular principles underlying the establishment of the neuronal circuitry have intrigued and challenged generations of developmental neurobiologists. It is only in the last two decades, owing to the integration of molecular cell biology, genetics, imaging technology and biophysical techniques that we have begun to develop an integrated framework for the mechanisms governing the process of axon guidance, and the formation and refinement of the neuronal connections. This review will focus on the dynamic remodeling of the actin and MT cytoskeletons and its role in growth cone-mediated neuronal pathfinding.

2 Structure and Dynamics of Growth Cone Cytoskeleton

The growth cone is the specialized, motile tip of growing neuronal processes that explores the external space for guidance cues and brings about directional motility. The growth cone consists of two types of protrusive structures with distinct morphologies—flat, sheet-like extensions called lamellipodia and long, finger-like filopodia. These structures not only differ in their morphologies but also in their underlying sub-cellular actin architecture. The lamellipodia are produced by the protrusive activities of a dense meshwork of cross-linked actin while long bundles of filamentous actin (F-actin) generate filopodial structures.[2] Unlike other motile cells, filopodial extensions dominate growth cone motility. The filopodia are highly dynamic and undergo bouts of extension and retraction, making them ideally suited for interrogating the environment. It has been demonstrated that the filopodia form the key sensory modality in growth cones and bear the relevant guidance receptors.[2,3]

Guidance cue and Guidance receptor: Diffusible or substrate bound molecules that provide spatio-temporal information to the growth cone about its surrounding. Each guidance cue has a cognate receptor on the growth cone and is interpreted as an attractive or repulsive chemotropic signal by the cellular signal transduction machinery.

Lamellipodia: Flat, sheet-like cellular protrusions generated by underlying branched meshwork of short actin filaments.

Filopodia: Finger like cellular protrusions generated by underlying long, unbranched actin filaments.

Based on the underlying cytoskeletal organization, the growth cone is structurally classified into three regions (Figure 1). The region immediately after the axon spreads out into the growth cone is referred to as the C-domain (central domain). The MTs (with the exception of dynamic, exploratory MTs) remain largely restricted to the C-domain along with a large number of vesicles and other cellular organelles. The peripheral region (P-domain) of the growth cone comprises the protruding filopodia and lamellipodia with a highly active actin cytoskeleton. The T-zone forms a narrow transition region between the C- and P-domains. As the growth cone advances, a new P-domain is formed ahead of the previous one and a new C-domain takes up the position of the original P-domain. This cycle continues as long as the growth cone advances.

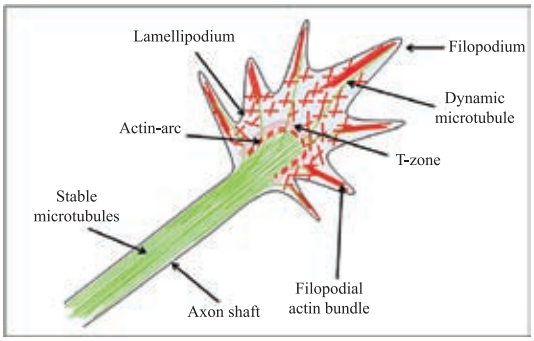

Figure 1: Cytoskeletal organization of the growth cone. Actin (red) forms multiple structures in the growth cone. At the leading edge (P-domain) long bundled F-actin gives rise to filopodia while short branched actin mesh generates lamellipodia. At the T-zone (pink) it forms contractile actin arcs that limit the MTs (green) to the central C-domain. Few dynamic MTs explore the cortex including the filopodial extensions.

The process of growth cone advancement is described in three stages. *Advancement/protrusion* consists of rapid filopodial and lamellipodial extensions due to the actin polymerization at the leading edge. Actin filaments assembled in the P-domain are carried backwards to the T-zone by the activity of myosin motors, where they are disassembled. This retrograde physical movement (retrograde flow of F-actin) balances the actin mediated protrusive activities. Modulation of retrograde flow of actin thereby modulates growth cone advance.[4] Upon substrate adhesion, the ECM is mechanically coupled to the F-actin flow (see substrate-cytoskeleton coupling model described later) leading to a local reduction in retrograde flow. Actin polymerization continues in the region ahead of the attachment, thus pushing the membrane forward. The T-zone is rich in actin arcs formed by end-to-end annealing of actomyosin bundles that form a mechano-chemical barrier for MTs attempting to invade the P-domain. During the *engorgement phase,* these actin arcs depolymerize, thereby clearing the region for MTs to advance into the P-domain. The final step is *consolidation,* where the MTs that have extended to the erstwhile P-domain are bundled together resulting in the addition of a new segment to the axon shaft and an overall forward displacement.

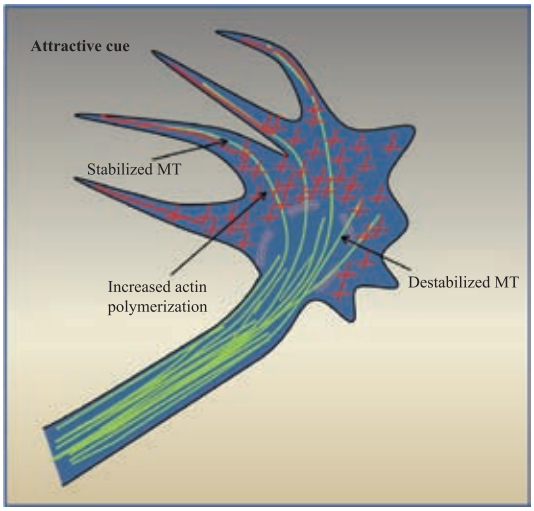

Figure 2: Model for growth cone turning. Turning induced by an attractive cue (in a gradient from top left to bottom right) results in increased filopodial activity at the side engaging the attractive cue. These filopodia are stabilized following the cortical capture of the invading exploratory MTs. Actin polymerization and MT capture activity is reduced at the opposite side of the growth cone. Finally, the C-domain MT core invades the region with stabilized filopodia resulting in a forward displacement towards the attractive cue.

The actomyosin arcs are re-established and MTs are pushed back and limited to the new C-domain. The iterative cycle of advancement, engorgement and consolidation results in axon elongation.[5,6] First articulated using differential interference contrast time-lapse microscopy by Goldberg and Burmeister in 1986, this now forms the basis of growth cone dependent axon outgrowth models. When a growth cone encounters an attractive guidance cue on one side, the filopodial activity on that side increases due to increased actin polymerization. The number of exploratory MTs and the frequency of their capture at the cortex is also enhanced. Concomitantly, there is increased MT destabilization and actin depolymerization on the other side. This results in a net polarization in the direction of the attractive cue (Figure 2). Repetitive cycles of advancement, engorgement and consolidation, persistently biased towards the polarized direction, leads to directional outgrowth.

3 Actin Cytoskeleton

The actin cytoskeleton underlying the leading edge is primarily responsible for the protrusion and retraction of the growth cone P-domain. Growth cones treated with Cytochalasin, which prevents actin polymerization, show slow translocation rates and altered morphology.[7,8] Low magnitude forces exerted by the growing MTs are the most likely source of this compromised residual motility. The growth of actin filaments form the mechanical basis of actin driven protrusions (filopodia and lamellipodia).[9] The dynamic balance between barbed end polymerization on one hand and depolymerization and retrograde flow on the other, determine extension or retraction of filopodia and lamellipodia.[10] The length of the actin filaments is controlled by the activities of elongation factors and capping proteins. Capping proteins provide a check on the length of the filament while elongation factors like the Ena/VASP family counteract capping and promote elongation.[11] The ability to generate long filaments is of particular importance to filopodia-dominated structures like growth cones. In accordance with this, members of this family are found to localize to filopodial tips and regulate filopodia formation and guidance.

Localized actin polymerization/depolymerization at selective regions in the growth cone is a key mechanism regulating the turning of the growth cone. Focal loss of actin bundles on one side of the growth cone using myosin light chain kinase inhibitor, ML-7, results in turning of the growth cone towards the other side.[12] This potentially could be the mechanism for repulsive axon guidance. Receptor engagement on one side of the growth cone leads to local loss of actin bundles, thus destabilizing the force homeostasis. The side with active tension due to myosin II activity prevails and turns the growth cone towards that side. In the case of attractive guidance the reverse is likely to occur. Zhu et al., 2007 provide a comprehensive analysis of the turning model. In this study, direct involvement of Myosin X was found in the repulsive guidance dictated by a diffusible guidance cue Netrin-1 and its receptor DCC. Myosin X interacts with DCC and a related molecule neogenin. Depletion of MyoX from neurons results in abnormal projections of the neurons *in vivo*.[13]

The formation of new actin filaments by nucleation of globular actin (G-actin) monomers is not spontaneous and requires other proteins to facilitate the reaction. These proteins, referred to as actin nucleators, along with accessory nucleation promoting factors (NPFs) regulate key aspects of actin dynamics. In non-neuronal cells, Arp2/3 along with an accessory factor N-WASP is the major actin nucleators, which generates the branched meshwork of actin filaments at the leading edge of the cell. The minimal region of Arp2/3 required for actin nucleation has a WASP homology domain (WH2) which binds to the actin monomers.[14] Role of Arp2/3 and N-WASP in neuronal

cells is debatable since its removal does not have a drastic effect on the growth cone morphology or motility.[15–17] A large GTPase Dynamin-2 (Dyn2) along with an actin binding protein cortactin has been implicated in the formation of branched actin filaments at the leading edge of epithelial cells. Cortactin binds to Arp2/3 directly apart from its binding to Dyn2 and thus is a promising candidate for the formation of a physical link between the membrane and the underlying actin mesh.[18] Unlike the expendable role of Arp2/3 and N-WASP in neuronal growth cones, both cortactin and Dyn2 are found to colocalize at the T-zone in hippocampal neurons, and when overexpressed lead to changes in growth cone morphology.[18]

Formins, another class of actin nucleators, use a very different mechanism of F-actin formation. Several formins have an inactive autoinhibited conformation due to the intramolecular interactions which can be relieved on Rho-GTPase binding. Upon activation, formins nucleate actin filaments using their formin homology (FH1 and FH2) domains. Working as dimers, these proteins processively add actin monomers to the barbed end of the filament.[19] Thus they form straight, unbranched actin bundles similar to those found in the filopodia. Experiments in *Dictyostelium* have implicated a formin, dDia2 in filopodia formation.[20] Another formin family member dDAAM is shown to be important for filopodia formation in *Drosophila* embryonic neurons.[21] Apart from their nucleation activity, formins also act as elongation factors.[22] Being associated with the barbed end of the filament, formins could potentially counteract the activity of capping proteins. Cordon-bleu (Cobl) and Spire are recently discovered classes of actin nucleators. Cobl utilizes its three WH2 domains to nucleate new actin filaments, which unlike the ones generated by Arp2/3, are unbranched. Cobl expression is enriched in the developing nervous system and its function is critical for both induction and branching of neuritis.[23] Spire has four WH2 domains and like Cobl generates unbranched actin filaments. Spire is also found to be neuronally enriched[24] though its role in the nervous system is unclear. In non-neuronal systems, Spire is known to work in combination with formins to regulate processes like cytoplasmic streaming in *Drosophila* oocytes.[25] This is suggestive of cooperative function of actin nucleators.

4 Microtubule Cytoskeleton

MTs are polarized filaments with structural and functional differences at both the ends. GTP bound tubulin is added at the plus end while hydrolysis of GTP leading to loss of tubulin occurs at the minus end. Majority of the MTs in the axon and growth cone are oriented with their plus ends facing the direction of growth. MTs exhibit exploratory behaviour and frequently probe the periphery of the growth cone including the filopodia. Failure of cortical capture leads to rapid retraction of MTs.[26,27] This behaviour can be attributed to the dynamic instability of MTs. An attractive guidance cue allows selective capture and stabilization of MTs and results in polarization of the growth cone. These then provide the structural support and tracks for cargo, eventually leading to movement of the entire growth cone in this direction.

Studies show that MTs in the central domain of the growth cone are bundled together like a zipper as the growth cone moves ahead. This is brought about by MT Associated Proteins (MAPs). Actin arcs are essential for the transport of uncaptured MTs to the C-domain from the periphery of the growth cone.[5] Myosin II activity is crucial for the bundling of the MTs at the growth cone neck and inhibition of myosin activity leads to spreading out of the MTs in the C-domain.[28] Reduced myosin activity results in a loss of tension in the actin arcs compromising their ability to bundle MTs. Another hypothesis regarding this behaviour of MTs is that active suppression of the exploratory behaviour of MTs keeps them in close association with actin arcs which may allow for the MAPs to cross link actin and MTs.[29] As the growth cone advances these MTs are stabilized and the consolidation stage of the growth cone follows. At the growth cone neck MT bundling is also mediated by myosin II. Inhibition of myosin activity by the Rho-ROCK pathway results in splaying of the MTs at this region.[30]

MT plus end-tracking proteins (+TIPs) have generated considerable interest in the field of growth cone motility and axon guidance. As the name suggests they bind to the fast growing +end of MTs. The strategic location of these proteins makes them ideal candidates for actin-MT crosstalk especially at the sites of MT capture. +TIP proteins have been shown to be important for almost every step of MT formation including polymerization, stability and dynamics.[31] APC is a component of the +TIP complex which promotes formation and elongation of neurites by MT stabilization.[32] Others like CLASP1/2 have also been shown to be important in axon elongation and regeneration downstream of GSK3 signaling.[33] The FH2 domains of formins mDia1 and mDia2 also bind MTs and +TIP members like EB1, CLIP170 and APC.[32] This may represent a potential mechanism for actin-MT coordination.

Another important observation is that MTs frequently target the focal adhesions.[34] This targeting

and subsequent stabilization of MTs at these adhesion sites, initially demonstrated in fish fibroblasts by Kaverina and colleagues, could be an efficient way of controlling focal adhesion assembly and disassembly. The activity of +TIPs and MAPs are thought to bring about this interaction. A small number of splayed actin filaments from the adhesion site form a tether with the dynamic MTs (or +TIPs) via hitherto uncharacterized proteins. Early focal contacts are not fully committed to stabilization, and MT probing these regions results in MT shrinkage. In contrast, mature focal adhesions seem to stabilize the invading MTs.[34] Signals from the adhesion site may travel inside the cell via retrograde flow to initiate MT invasion. These early MT invasion events could then trigger the strengthening of the adhesions via interactions between actin and cell adhesion molecules.[35]

MT catastrophe occurs at adhesion sites with high Paxillin phosphorylation.[36] How the phosphorylation status controls MT catastrophe remains unclear. MT shrinkage is thought to be promoted by MT destabilizing proteins like Stathmin or by the depletion of MT stabilizing proteins.[37] The bidirectional regulation of MT stability and paxillin phosphorylation could potentially form a key feedback loop regulating focal adhesion turnover.

5 Signaling and Regulation

For the correct interpretation of the complex molecular diversity presented by the attractive and repulsive cues the growth cone must be equipped with a very sensitive and reliable system of signaling reception, integration and interpretation. There are several studies investigating the role of a particular signaling pathway in an event like protrusion, turning or retraction.[3,38–40] However, the mechanisms for signal amplification and noice filtration necessary to maintain the fidelity of the response in a complex and dynamic environment remain elusive.

Over the years it has become clear that Rho GTPases are critical to growth cone motility.[41] Rho GTPases cycle between active and inactive forms, and this switch is mediated by the activities of GTP exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs activate GTPases by promoting the release of GDP and uptake of GTP while GAPs turn off the GTPase activity by stimulating GTP hydrolysis. Specific combinations of GEFs and GAPs have been shown to be associated with specific receptor-ligand combinations, such as those specific for slit-robo mediated guidance signalling.[42] Although these examples suggest that the regulation of GTPases is very tightly regulated, the literature has plenty of evidence for the existence of crosstalk between various GAPs and GEFs.[43] High throughput studies which looked at the distribution of different GEFs and GAPs in neuronal cells show that there is segregation of various components between the cell body and the axon shaft or growth cone.[44]

The growth cone motility is also regulated by tightly controlled local translation events. β-actin mRNA is localized at the growth cone and undergoes local translation upon encountering Netrin-1, a typically attractive guidance cue.[45] This gives rise to increased polymerization on the side exposed to Netrin-1 resulting in growth cone turning. Similarly, Netrin-1 activation results in a gradient of phosphorylation of the translation initiation factor, 4EBP, leading to a translation bias on one side of the growth cone.[45] Local translation of RhoA and cofilin has been demonstrated in the context of repulsive guidance and implicated in mediating a spatial bias in actin disassembly.[46]

6 The Role of Forces

For advancement, the growth cone has to make stable contacts known as focal adhesions/focal contacts with the substrate. Actomyosin activity at these anchorage sites generate traction forces which propel the growth cone forward. A substrate-cytoskeletal coupling model (molecular clutch hypothesis) has been proposed to mediate the physical link between the ECM and the intracellular F-actin flow. This mechanical coupling causes reduced myosin-driven actin flow and shifts the balance of forces in favour of actin-based protrusions. This leads to the development of traction and forward translocation. Focal contacts are multimolecular assemblages that include structural proteins as well as signaling modules. The strength of these adhesions is an important regulator of the translocation rate of the growth cone. A biphasic model has been proposed where the translocation rate drops at very high and very low adhesion strengths with fast movement in the intermediate regime.[47] Experiments have recently modified this model to incorporate the dynamic organizational states of actomyosin and focal adhesions. Adhesion strength and FA distribution, combined with local myosin II activity, dictate specific spatio-temporal organization states of F-actin. Myosin II activity at the FA affects its spatial distribution and turnover, creating a feedback between the actomyosin contractile module and FAs which results in the adhesion-strength-dependent effects on cell migration.[48]

Traction forces: Forces exerted by the growth cone on the substrate via the focal adhesions.

A critical signaling element at the focal adhesion is the focal adhesion kinase (FAK).[49] FAK is strategically placed upstream of Rho-GTPases and

Src family kinases and is activated in response to integrin engagement and netrin encounter.[50] Surprisingly, the same molecule, FAK, in neurons is necessary to both stabilize and also disrupt adhesions. This suggests that the dynamics of FAK activity rather than its absolute amount is at the core of this regulation.[51]

Traction forces develop in the growth cone as a function of substrate attachment (eg. attachment to immobilized Netrin-1).[52] These mechanical stimuli are known to elicit intracellular signaling cascades involving tyrosine kinase activity. Shibasaki et al. in 2010, demonstrated the stretch activation of thermosensitive TRP channels leading to axon elongation.[53] On the other hand, activation of mechanosensitive calcium channels is known to inhibit axon outgrowth due to Ca^{2+} influx.[54] These studies suggest that growth cones posses a sophisticated discriminatory mechanism based on the nature of the force experienced.

7 Conclusions

It is clear that the cytoskeletal regulation of growth cone motility and ultimately neuronal connectivity remains partially characterized. The interpretation of the complex environmental stimuli and the mechanisms of achieving specificity remain poorly understood. At the level of cytoskeletal dynamics, the processes that regulate the remodeling of individual components is reasonably well characterized but those coordinating the activities of different polymer systems remain elusive. The mechanochemical outputs of cytoskeletal reorganization are only beginning to be revealed. New techniques involving high-resolution imaging, microfluidics and force measurements are likely to lead the next wave of investigation into growth cone–driven motility. Breakthroughs in areas involving ligand presentation and noise filtration, interpretive computations during signal transduction, dynamic modulation of the effector cytoskeletons, and coordinated generation of mechanical forces and its feedback mechanisms will be critical in developing a comprehensive understanding of the processes leading to the establishment of accurate neuronal connectivity.

Acknowledgements

The authors thank Ms. SampadaMutalik for critical evaluation of the manuscript. AS is funded by a CSIR senior research fellowship. AG and AS's research is supported by grants from the Department of Biotechnology, Government of India and intramural funding from IISER, Pune.

Received 20 November 2012.

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