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The trip of the tip: understanding the growth cone machinery

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Abstract

Preface—The central player in the road trip of axon guidance is the growth cone, a dynamic structure located at the tip of the growing axon. During its journey, the growth cone comprises both `vehicle' and `navigator'. Whereas the `vehicle' maintains growth cone movement and provides the cytoskeletal structural elements of its framework, a motor to move forward, and a mechanism to provide traction on the road, the `navigator' aspect guides this system in a spatially-biased way to translate environmental signals into directional movement. Understanding the functions and regulation of the vehicle and navigator provides new insights into the cell biology of growth cone guidance.

Introduction

During nervous system development, each neuron extends an axon to find its ultimate destination amidst a complex and changing environment. At the tip of each axon is the growth cone (BOX 1), and its highly dynamic behaviour and responsiveness to multiple sources of spatial information allows it to find its target with an impressive level of accuracy. The growth cone vehicle cannot move forward without a road upon which to travel, made up of adhesive molecules presented on a neighbouring cell surface (such as transmembrane cell adhesion molecules (CAMs)[1]) or assembled into a dense extracellular matrix (ECM) (including Laminin and Fibronectin[2]) (FIG. 1). These molecules provide defined `roadway' surfaces to which growth cone receptors can adhere, but they also activate intracellular signalling pathways utilized by the growth cone guidance machinery. Additionally, anti-adhesive surface-bound molecules (such as Slits and Ephrins[3,4]) can prohibit growth cone advance and thus provide `guardrails' that determine roadway boundaries. Finally, diffusible chemotropic cues represent the `road signs' that present further steering instructions to the travelling growth cone (FIG. 1). These include a whole spectrum of molecules, including classic factors that were identified explicitly in axon guidance assays[3,4], as well as morphogens[5], secreted transcription factors[6,7], neurotrophic factors[8,9] and neurotransmitters[10]. Whereas it was originally thought that some cues always function as attractive `go' signals (for example, Netrins) and others as repulsive `stop' signals (for example, Ephrins), it is now clear that the response of attraction versus repulsion is not due to the intrinsic property of the cue, but rather to the specific growth cone receptors engaged and the internal signalling milieu of the growth cone. In particular, the `navigator' function of the growth cone comprises the intracellular signalling elements that determine how environmental directions lead to a given guidance response[4].

Despite significant advances following decades of research, our current understanding of how the growth cone achieves its impressive road trip is far from complete. In this Review, we examine the basic cell biological features of growth cone guidance, focusing on cytoskeletal mechanisms that the growth cone uses as its vehicle to move forward, as well as elements of the navigation system that converts spatial bias into steering by translating environmental

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guidance cues into localized cytoskeletal remodelling. Whereas changes in membrane dynamics, including regulation of endocytosis and exocytosis, also have crucial roles in growth cone migration and are likely targets of guidance cue signalling[11,12], this topic is beyond the scope of this Review. We conclude by highlighting some of the key unsolved questions in growth cone dynamics, and propose how recent technological advances will allow future investigations to further the knowledge in these areas.

The growth cone vehicle: under the hood

The growth cone engages its cytoskeleton to drive forward and turn, continuously progressing through three stages of advance that are influenced by environmental factors: protrusion, engorgement and consolidation[13,14] (BOX 2). In order for the growth cone to navigate based on spatial landmarks, the motility machinery that drives forward movement must have the capacity to be spatially biased to achieve accurate steering, and in fact, the steering and drivetrain are intimately connected at a physical level. Therefore, it is essential to understand the underlying cytoskeletal mechanisms that propel the vehicle forward and that have the potential to be affected asymmetrically if we are to fully grasp how `guidance' occurs.

Turning on the engine: F-actin retrograde flow

Growth cone motility and protrusion of the leading edge membrane depend on the dynamic properties of actin (BOX 3). Whereas actin might not be the sole engine that powers axon elongation per se (axons without actin polymerization can still move forward, albeit with abnormal growth cone morphology and substratum selectivity)[15], actin is a central part of the mechanism that controls growth cone exploration. A combination of F-actin treadmilling and F-actin retrograde flow (the continuous movement of F-actin from the leading edge towards the centre of the growth cone) provide the `motor' that keeps the growth cone engine idling (FIG. 2a) and available to drive movement in response to directional cues[16]. Following the increased technological advances in live cell imaging, the past few years have seen significant strides in our molecular understanding of F-actin retrograde flow and how it relates to growth cone motility and protrusion.

It has been convincingly demonstrated that F-actin retrograde flow is driven both by contractility of the motor protein myosin II, which seems to be tethered through protein-protein interactions in the T-zone (the transition (T) region between the peripheral and central domains of the growth cone), and the `push' from F-actin polymerization in the P-domain (the peripheral (P) region of the growth cone that includes the filopodia and lamellipodia-like veils)[17]. Myosin II-driven compression across the T-zone circumference causes buckling of the F-actin bundles (FIG. 2a), which might be enhanced by pushing from leading edge actin polymerization[17]. This leads to bundle severing near the proximal ends[17], likely involving actin filament severing proteins of the actin-depolymerizing factor (ADF)/cofilin family[18]. A recent paper suggests that myosin II might also actively depolymerize actin filaments[19]. After severing, the actin fragments are recycled into individual actin subunits and are available for transport to the periphery for further actin polymerization at the leading edge[20] (FIG. 2a).

Engaging the clutch and forming traction to push ahead

How does the growth cone utilize the actin engine to move forward? Mitchison and Kirschner first proposed the `clutch' hypothesis[21], also called the `substrate-cytoskeletal coupling' model[22], which links growth cone protrusion to actin dynamics[16,23]. They suggested that growth cone receptor binding to an adhesive substrate leads to the formation of a complex that acts like a molecular `clutch', mechanically coupling the receptors and F-actin flow, thus anchoring F-actin to prevent retrograde flow and driving actin-based forward growth cone protrusion on the adhesive substrate (FIG. 2a). Indeed, growth cone-substrate adhesions have

long been shown to be important for growth cone migration[24], and in fact the generation of traction also requires myosin II[25].

Filopodia, in particular, are considered to be guidance sensors located at the `front line' of the growth cone and might have a major role in establishing growth cone-substrate adhesive contacts during environmental exploration[26]. Studies show that filopodia function as points of attachment to the substrate and produce tension utilized for growth cone progression[27,28]. Whereas earlier studies blocking filopodia formation using general F-actin inhibitors led to abnormal growth cone steering[29,30], a recent study specifically targeting filopodial F-actin suggested that filopodia are dispensible for accurate growth cone guidance but are indeed required for normal growth cone motility[31], supporting its role in forming adhesive contacts.

Accumulating evidence in recent years supports the `clutch' model, in particular from *in vitro* live growth cone imaging experiments utilizing APCAM, an NCAM orthologue in *Aplysia californica*[32], a model system with large growth cones that allow high resolution imaging of their cytoskeletal dynamics[33]. Following APCAM-mediated growth cone-substrate adhesion, dramatic local reorganization of actin occurs. Increased levels of localized actin assemble at the site of adhesion, followed by regional slowing of retrograde flow and growth cone protrusion[16,34,35]. Subsequently, F-actin bundles disappear between the adhesion site and C-domain (the central (C) region of the growth cone where the microtubules from the axon shaft enter the growth cone), and F-actin arcs reorient from the C-domain towards the adhesion site, creating a corridor between the two regions. Actomyosin-driven tension builds-up between the actin assembly at the adhesion site and the actin arcs with its associated microtubules (MTs), and the growth cone undergoes engorgement as the C-domain moves forward[35] (BOX 2).

Whereas many studies that investigated the clutch hypothesis focused on the cell adhesion molecule APCAM in *A. californica*, recent studies have begun to provide molecular bases for similar clutch linkages between actin and other CAMs and ECM receptors in growth cones. In non-neuronal systems, focal adhesion proteins Talin and Vinculin provide the prototypic molecular clutch mediated by the Integrin ECM receptors[36]. More recently discovered examples of growth cone-specific clutch machinery include Catenins, shown to mechanically couple N-cadherin receptors and F-actin flow in rat neurons[37], and the novel protein Shootin1, which mediates linkage between L1 cell adhesion molecule (L1CAM) receptors and F-actin flow[38]. A complete molecular understanding of the clutch mechanism will allow the transition from *in vitro* findings to embryonic systems and will provide a framework for understanding the overall logic that governs forward progression of the growth cone *in vivo*.

MTs help steer the vehicle

While actin structures are rapidly remodelled in response to guidance cues, actin is not the only component of the vehicle, as growth cones cannot move forward without MT function[39]. Whereas the mechanisms that control MTs have generally received less attention than those of actin, recent studies have confirmed early seminal experiments[40] by demonstrating that MTs have a significant role in the process of growth cone steering, in two complementary ways: individual P-domain MTs act as `guidance sensors', whereas bulk C-domain MTs steer growth cone advance[41].

First, prior to growth cone protrusion (BOX 2), a population of individual MTs actively explore the P-domain[42] (FIG. 2b), utilizing their property of dynamic instability (BOX 3). Because introduction of a localized adhesive cue leads to an increase in the number of exploratory MTs interacting with the adhesion site[35], it has been proposed that these MTs might be acting as guidance sensors (FIG. 2b). This might occur either by carrying signals involved in steering to and/or from the cortical membrane, or by acting as a scaffold for the localized recruitment of key signalling components needed for navigation[43]. For example, dynamic MTs are required

for the localized accumulation of active Src kinase signalling at sites of adhesion, which is necessary for the growth cone turning response to an adhesive substrate[43], and it is likely that the MTs might be bringing other signalling molecules as well (for example, Rho-family GTPase regulators).

The second major role MTs have in steering is during engorgement (BOX 2), after initial actin remodelling in response to cues. At this point, stable bundled C-domain MTs move into the area of new growth, as consolidation of a new region of axon shaft occurs behind them, thereby fixing the axonal direction[16,44]. Further supporting the instructive role of MTs in growth cone steering, the inhibition of MT dynamics prevents growth cone turning in response to guidance cues, whereas localized MT stabilization induces turning[44].

MT interactions with actin

The role of MTs during growth cone steering clearly requires the participation of, and interaction with, actin[45,46]. Recent live imaging studies show that the function of actin dynamics might be to provide spatiotemporal guidance to MTs in order to steer the growth cone in the right direction. In particular, actin has a pivotal role in determining MT localization within the growth cone, acting as both a barrier to premature MT invasion and as a guide to MTs during their advance[45,47]. Furthermore, local perturbation of actin structures leads to redistribution of MTs and a change in direction of growth[48]. Here, we discuss two significant interactions between MTs and actin: P-domain MTs and F-actin bundles, and C-domain MTs and actin arcs (FIG. 2c).

As dynamic MTs preferentially explore the growth cone periphery, they usually follow the trajectories of F-actin bundles[42], thought to guide MT advance into the P-domain[41,45]. A new study, however, showed that these F-actin bundles, specifically, are not required for MT advance and that they actually inhibit MT penetration into the P-domain when the MTs are coupled to F-actin bundle-specific retrograde flow[47] (by MT-actin crosslinking proteins). As MT coupling to F-actin retrograde flow directly affects the ability of MTs to explore the P-domain, it seems likely that regulation of MT-actin coupling and uncoupling (defined as release of MTs from F-actin retrograde flow) would have an effect on MT dynamics, and this prediction was demonstrated in a more recent study examining P-domain MTs[35]. Not only does increased MT-actin uncoupling permit dynamic MTs to explore growth cone sides more frequently than central regions under uniform conditions, which might account for increased sensitivity to guidance cues not on the current axon outgrowth path, it also allows an increased number of MTs to explore sites of APCAM-mediated adhesion[35]. It is possible that repellent guidance cues induce an opposite response in the actin-MT coupling state, thus leading to an opposite effect on the cytoskeletal machinery.

Interestingly, absence of F-actin bundles does not lead to the inappropriate advance of C-domain MTs[47], suggesting that while F-actin bundles regulate exploratory P-domain MTs during protrusion, stable C-domain MT movement into the growth cone during engorgement might be regulated by another mechanism, namely the actin network and actin arcs. Indeed, another study showed this to be the case. Disruption of actin arcs results in failure of MT consolidation during axon outgrowth, leading to an abnormally broad C-domain[49]. During engorgement, as the C-domain advances towards an adhesion site, actin arcs on the sides of the C-domain become more prominent (BOX 2), and mechanical connectivity between extracellular adhesion, actin arcs and the C-domain is apparent[49]. Thus, actin arcs normally form a barrier around the C-domain that regulates MT advance by capturing MTs on the sides of the growth cone and transporting them into the C-domain. In a separate study, it was demonstrated that myosin II, which mediates contraction of anti-parallel actin filaments found within actin arcs, has a significant role in actively transporting MTs from the sides into the C-domain, compressing them into bundles and perhaps holding them in place[50] until they are

stably crosslinked by MT-associated proteins (MAPs) in the growth cone neck[51]. Myosin II in the growth cone neck has also been shown to suppress F-actin protrusion to allow axon shaft consolidation[52], and this function might contribute to its function during growth cone turning[53]. Therefore, distinct classes of actin structures seem to regulate different populations of MTs. Whereas F-actin bundles can inhibit the protrusive activities of P-domain MTs when they are coupled together, F-actin arcs regulate the engorgement and consolidation activities of C-domain MTs (FIG. 2c). An obvious question, then, is how MT-actin interactions are regulated in a spatiotemporal manner in response to guidance cues, and this topic is addressed below.

The growth cone as a `navigator'

Thus far, we have described how changes in the cytoskeletal machinery drive forward progression of the growth cone vehicle. However, growth cone pathfinding obviously does not consist solely of moving forward; it is a dynamic process in which the growth cone progresses, pauses, turns and retracts, as it navigates its way through the embryonic landscape and encounters various directions for its trip. Spatial bias in a given direction can occur through either positive cues that increase protrusion (towards the side of new growth) or negative cues that decrease protrusion (occurring on the side away from new growth). In order for spatial discontinuities in the environment to drive growth cone steering and, in particular, to accurately interpret numerous cues simultaneously, the growth cone requires a `navigation' system that translates multiple environmental directions and integrates separate signalling pathways to locally modulate the dynamics of the cytoskeletal machinery. The overall logic that governs this process is still emerging. There is a vast literature describing specific aspects of the growth cone navigation system, but many studies focus on individual pathways engaged by particular cues or receptors. While there are numerous signal transduction molecules that convey guidance information including kinases[54,55], phosphatases[56] and calcium ions[57], we have the most comprehensive understanding of Rho-family GTPases, a class of molecules that control cytoskeletal dynamics downstream of virtually all guidance signalling receptors[58,59] (FIG. 3).

Although increasing numbers of studies have been analyzing the functions of Rho-family GTPases and their cytoskeletal effectors within the growth cone, much of our understanding of these molecules still comes from non-neuronal systems (for example, fibroblasts, neutrophils, and *Dictyostelium discoideum*[60,61]). As significant differences in molecular content between cell types dictates that we must be careful to not assume that the systems act identically, numerous studies do suggest some parallels in cytoskeletal signalling between neuronal and non-neuronal cells[60,61].

Additionally, common cell biological mechanisms underlie growth cone guidance and regulation of other aspects of axon biology, such as axon initiation and modelling of secondary axonal branches[62]. Thus, studies on cytoskeletal signalling in other systems might provide insights into the mechanisms of growth cone guidance (and vice versa).

Rho-family GTPases act behind the wheel

Rho-family GTPases, which include RhoA, Rac1 and Cdc42, act as signalling nodes to couple upstream directional cues and downstream cytoskeletal rearrangements to either enhance actin polymerization for protrusion or promote disassembly and actomyosin contraction for retraction[58,59] (FIG. 3). If regulation of Rho-family GTPase activity is to convey guidance information, then its upstream regulators must be activated in a spatially-specific manner, internally reflecting the extracellular environment. Upstream regulators include proteins that activate Rho-family GTPases, guanine nucleotide exchange factors (GEFs), and those that inactivate them, GTPase activating proteins (GAPs)[58,63] (FIG. 3; another newly implicated method of Rho-family GTPase regulation is local translation downstream of guidance

signalling[64], which is briefly discussed in Box 4). A great number of Rho-family GTPase regulators have been studied in non-neuronal systems, but our understanding of their specific functions in the growth cone is not quite as advanced[63]. Growth cone guidance receptors can contain their own GTPase regulatory domains, such as the Plexin receptor for Semaphorin[65], but many other receptors transduce their activation state through separate regulators recruited by cytoplasmic domains. For example, the guidance cue EphrinB3/ EphA4 receptor combination, activates the regulatory Rac GAP α-Chimerin to inhibit growth cone extension[66], the guidance cue receptor EphA can trigger activation of Rho GEF Ephexin to activate RhoA but inhibit Cdc42 and Rac1 to induce growth cone collapse[67], and guidance cues Slit and Netrin can both signal through the Rac and Rho-GEF Trio to regulate growth cone dynamics[68,69].

A potentially confusing feature of Rho-family GTPase signalling is that possible GEF/GAP/GTPase signalling network combinations are numerous and complex. Multiple GTPases (with antagonistic functions) can be activated in response to the same guidance cue. For example, EphrinA4 can lead to RhoA activation, Rac inactivation or Rac activation, depending on which receptors and GEFs or GAPs are engaged[58], and there are over 70 GEFs and 80 GAPs described in mammals[70]. Many of them regulate several different Rho-family GTPases, and a particular GTPase might be regulated by numerous GEFs and GAPs that are all residing within the same cell. How can this complex network of interactions be functionally explained within the growth cone? A recent proteome profiling study in neuroblastoma cells suggests that Rho-family GTPase spatial localization and activation might be the answer, a popular idea supported by previous studies and speculations[71]. This particular study found that specific GEFs and GAPs are differentially localized between the cell body and the axonal process[72], with 11 out of 14 GEFs and 6 out of 7 GAPs expressed in neuroblastoma cells being enriched in axonal processes compared to the cell body. The authors propose that spatial compartmentalization of Rho-family GTPase regulators might allow the same GTPase to be regulated by distinct GEFs or GAPs in different locations throughout the growth cone. Moreover, time-lapse microscopy after individual knockdown of these GEFs/GAPs showed distinguishable axonal phenotypes. Whereas depletion of several regulators (including Rac1 and Cdc42-GAP ArhGAP30 and the Rac1 GEF Dock4 led to an increase in axon extension on an ECM substrate but normal cytoskeletal structure, silencing of others led to changes in axon extension along with distinct and obvious pertubations in the actin cytoskeleton[72]. For example, knockdown of Cdc42-GAP SrGAP2 resulted in increased filopodia and cell spreading, knockdown of Rac1-GAP BCR led to increased filopodia without cell spreading, and loss of RhoA and Rac1-GEF Trio led to long but unstable filopodia. Thus, even though Rac1 GTPase could be targeted by seven different GEFs and three different GAPs, and Cdc42 could be targeted by four GEFs and five GAPs, all located within the same cell, each upstream regulator is likely to be required for distinct cellular functions. These data suggest that the same GTPase might control various aspects of growth cone cytoskeletal dynamics, such as F-actin assembly, disassembly and retrograde flow, by receiving different upstream inputs of GEFs and GAPs in time and space.

Upon activation, how do distinct Rho-family GTPases mediate downstream growth cone responses to affect growth cone steering? Intriguingly, activation of the same GTPase can lead to opposite responses of the growth cone; for example, whereas RhoA activation leads to growth cone retraction (by promoting myosin II contractile activity)[73], it can also be required for axon outgrowth[74] (by inhibiting F-actin severing protein ADF/cofilin)[75]. Again, as with the upstream regulators, an explanation for this discrepancy is that Rho-family GTPases perform different functions within the growth cone depending on their localization, and specifically, depending on which downstream effector molecules are activated. Numerous Rho-family GTPase effectors have been identified[76,77], but only a few (such as Rho kinase (ROCK)) have been well-studied in the growth cone (see[59] for review). Following activation by Rho-family GTPases, these effectors either directly or indirectly regulate numerous

downstream targets to modify the cytoskeleton, in order to direct the growth cone vehicle in a spatially-biased manner.

Control of actin dynamics at the leading edge

Rho-family GTPase cytoskeletal effectors are known to regulate all aspects of the actin cycle which affect growth cone steering, including F-actin assembly at the periphery, F-actin retrograde flow towards the C-domain, and disassembly and recycling of actin at the T-zone.

In order for the engine to run, actin polymerization at the leading edge must occur. This process is controlled by multiple regulators, which include the actin nucleators Actin-Related Protein (Arp)2/3 complex and Formins, and the F-actin polymerization factors Enabled/vasolidator-stimulated phosphoprotein (Ena/VASP). The Arp2/3 complex is a major effector of Rac1 and Cdc42 and is thought to control nucleation of F-actin polymerization and F-actin branching by binding to existing F-actin[78]. Several studies have shown that Arp2/3 is required for guidance[79,80], but there has been some question as to whether this complex functions similarly in neuronal and non-neuronal cells[79]. However, it was recently shown that Arp2/3 is present in the branched F-actin networks of growth cones and does affect their protrusion dynamics[81,82]. Inhibition of Arp2/3 in neurons blocked protrusion of both lamellipodial-like veils and filopodia and also increased RhoA activity[81], but future studies will be needed to determine its full role in growth cone motility. Downstream of Rho-family GTPase signalling, Formins nucleate and then remain continuously associated with elongating F-actin barbed ends[83]. Formin is required for growth cone filopodia formation, and the *Drosophila melanogaster* Formin DAAM was recently shown to act together with Rac GTPases and Enabled during axonal growth regulation[84]. The novel actin nucleator, Cordon-Bleu, is also highly enriched in rat brain and might have a role during growth cone guidance[85].

Ena/VASP proteins are a family of proteins that enhance F-actin elongation by several methods, including binding to F-actin barbed ends at the leading edge to antagonize capping proteins (that inhibit F-actin elongation), like Formins, and also by recruiting actin subunit complexes to the P-domain for further polymerization[86]. While Ena/VASP proteins are mainly thought to be regulated by protein kinases downstream of guidance cue signalling (such as the cyclic-nucleotide activated kinases protein kinase A (PKA)[86]), there are genetic interactions between Ena and the Rac and Rho GEF Trio in *D. melanogaster*[87], and Ena regulator Ableson tyrosine kinase functions through Rac and Rho GTPases in both neuronal and non-neuronal cells[88, 89], suggesting that there is cross-talk between Rho-family GTPase signalling and Ena/VASP proteins.

Additionally, cytoskeletal effectors downstream of RhoA GTPase have crucial roles regulating F-actin retrograde flow and disassembly of actin in the T-zone. ROCK, one of the most widely studied downstream effectors of RhoA, has multiple phosphorylation targets implicated in actin dynamics in the growth cone, including myosin light chain kinase (MLCK), LIM domain kinase (LIMK) and Ezrin-Radixin-Moesin (ERM) proteins. Phosphorylation of MLCK induces myosin II activity and promotes its association to F-actin, leading to actomyosin contraction and driving F-actin retrograde flow[59,90]. Active LIMK inactivates the actin severing protein ADF/Cofilin by phosphorylation, thereby stabilizing actin filaments and promoting vehicle forward progression[75]. Finally, ERM proteins are another group of actin-binding proteins that have an important yet unclear role in growth cone actin dynamics. Not only are they directly downstream of ROCK[91], they can interact with specific growth cone receptors (such as L1CAM)[92], suggesting they might be having a role in cross-talk between different signalling pathways. Activated ERM proteins are asymmetrically localized in response to guidance cues in the growth cone and promote growth cone motility in response to the guidance cue Semaphorin 3A[93], but how they function to remodel actin dynamics has not yet been clarified.

These examples are just a few of the many effectors of actin dynamics downstream of Rho-family GTPase signalling. Additionally, there are other important actin effectors which are not clearly linked to Rho-family GTPases. The complete picture of how guidance cues affect all aspects of actin dynamics on a global growth cone scale is still in the process of emerging.

Steering and MT-F-actin interactions

As with actin, there are many controllable aspects of MT dynamics, including nucleation, polymerization, stabilization, and translocation along F-actin, all of which have roles in steering the growth cone vehicle and which are coordinated by cytoskeletal effectors downstream of guidance signalling. However, while numerous studies have elucidated the functions of a multitude of actin-binding proteins, fewer studies have analyzed the detailed functions of MAPs. Whereas Rho-family GTPase signalling has a central role in regulating actin dynamics, this is not as evident for MTs, although there are several examples of MT-specific Rho GEFs in non-neuronal cells, such as RhoGEF2 in *D. melanogaster* 94 and Rho-GEF XLFC in *Xenopus laevis*[95] and Rac1 can regulate MT dynamics in non-neuronal cells[96]. However, this connection has not yet been clearly demonstrated in growth cones. Based on recent studies, it is clear that the ability of MTs to explore the growth cone periphery and enter into filopodia (an important early step necessary for proper steering in response to environmental cues) is highly dependent on how MTs interact with the F-actin bundles and network, and thus Rho-signalling at least indirectly affects MT dynamics.

A central focus of studies on MT dynamics in the growth cone revolves around the control of MT-actin interactions, specifically, the coupling and uncoupling of MTs and F-actin retrograde flow. For example, positive guidance cues such as the adhesive molecule APCAM increase the frequency of MT-actin uncoupling, leading to increased MT exploration at sites of cue binding[35]. In contrast, repellent cues might increase coupling between MTs and actin, thus reducing MT exploration and increasing MT `looping', as the growing plus-ends of MTs are transported back towards the C-domain by their linkage to F-actin retrograde flow, thereby forming MT `loops' within the growth cone. Thus, one function of the growth cone navigation system is to locally control MT-actin interactions in response to asymmetric guidance cues. The detailed signalling pathways by which this occurs are still unclear, but it is obvious that components of the navigation system can control MT dynamics by directly or indirectly modulating the activity of MAPs, and in particular, those that function as MT-actin crosslinking proteins.

Revealing the roles of MAPs in growth cone dynamics

One group of MAPs that has recently been shown to play a crucial role in growth cone dynamics is the family of `plus-end tracking proteins' (`+TIPs', such as End-binding proteins (EBs) and Adenomatosis Polyposis Coli (APC)), which bind specifically to plus-ends of MTs[97,98]. These proteins were originally implicated in affecting MT plus-end stabilization, but they also mediate MT cross-linking to actin, either in a positive or negative way. Interestingly, +TIP EB1 is required for RhoGEF2 association with MT plus-ends in non-neuronal cells[94], suggesting the intriguing notion that navigator signalling molecules such as Rho-family GTPase regulators might actually harness MT dynamics to move themselves into areas of guidance cue signalling[99]. This could be one explanation for how exploratory MTs are required early for steering.

Evidence suggests that at least two +TIP members, Lissencephaly1 (LIS1) and APC, promote MT-actin uncoupling in the growth cone. In particular, LIS1 cooperates with the MT motor protein Dynein to allow uncoupling of dynamic MTs from actin retrograde flow in chick and rat neurons plated on a laminin substrate[100,101]. Inhibition of Dynein and LIS1 prevents MT advance into the periphery and reduces the ability of MTs to resist F-actin retrograde

flow[101]. Consequently, the growth cone is unable to accurately steer along a Laminin border[100]. This is further confirmed by blockage of retrograde flow using the Myosin II inhibitor blebbistatin, leading to recovery of MT extension into the P-domain in Dynein-depleted growth cones and demonstrating that actin retrograde flow can prevent MT exploration[100]. Whereas the pathways by which guidance cues lead to LIS1 and Dynein activity in the growth cone have not been elucidated, LIS1 can specifically interact with Rho-family GTPase Rac1 and Cdc42 regulator, IQGAP1, in migrating mouse neurons[102], and LIS1 deficiency is associated with deregulation of Rho-family GTPases Cdc42, Rac1 and RhoA[103]. Additionally, connections exist between Dynein and Rho-family GTPase members in other systems (for example, the Dynein regulator Nudel binds to and inhibits the Rho-family GTPase regulator Cdc42GAP in migrating mouse cells[104]), suggesting that Rho-family GTPases control LIS1 and Dynein-dependent uncoupling downstream of guidance cues. Interestingly, a recent study showed that the motor protein Kinesin-5, working antagonistically with Dynein, has a key role in controlling MT extension into the P-domain during growth cone turning and is specifically phosphorylated on the side opposite the invasion of MTs prior to turning[105], further demonstrating that motor proteins are likely targets of guidance cue signalling.

Similar to LIS1, APC is another +TIP that promotes MT-actin uncoupling in the growth cone and also directly interacts with IQGAP1[106]. APC binds to only a subset of MTs in the growth cone, and its binding location indicates the future growth direction of the axon. Locally enhancing APC association with MTs leads to growth cone steering towards that axis[107], possibly by preventing MT binding to F-actin. This speculation is supported by a recent study showing that APC loss from MT plus-ends leads to increased MT looping and prevention of growth cone progression[108]. APC loss occurs in response to the morphogen Wnt3a, which is acting through the intracellular Wnt signalling pathway member Dishevelled-1 and which can induce axonal remodelling in mouse dorsal root ganglia neurons. In this case, the Wnt signalling pathway might be regulating APC-MT association by modifying the APC phosphorylation state. When APC is lost from the MT plus-ends, a possible hypothesis is that this might allow other MT-actin crosslinkers to bind to the plus-ends and couple MTs to F-actin retrograde flow, leading to MT looping. For example, the +TIP member, Cytoplasmic Linker Protein (CLIP)-Associated Protein (CLASP), promotes MT looping in the growth cone when over-expressed[109] and it also can link MT ends to actin in non-neuronal cells[110], although its actin-binding activity in the growth cone has not yet been examined. If this function holds true in growth cones, then perhaps CLASP is a +TIP member that drives MT-actin coupling and MT looping behaviour downstream of environmental `stop' signals. Interestingly, Glycogen Synthase Kinase 3β (GSK3β), which is downstream of a number of guidance cues including Wnt, Netrin and Semaphorin signalling[111], can regulate whether CLASP binds to the MT plus-ends or along the entire MT, and this change in localization might regulate its function[112].

MT-associated protein 1B (MAP1B), considered to be a scaffold protein which stabilizes MTs but can also bind actin, also regulates growth cone steering and motility of mouse neurons by controlling MT-actin dynamics[113]. In particular, the role of MAP1B in growth cones might be to couple MTs and F-actin in response to guidance cues, as MAP1B function is required for MT looping which occurs in response to treatment with Lysophosphatidic acid (LPA) (a phospholipid derivative that triggers axonal process retraction and growth cone collapse)[113]. As MAP1B function can be modulated by phosphorylation[111], this suggests a mechanism by which the ability of MAP1B to couple MT and actin depends on upstream guidance cue signalling. For example, MAP1B is a phosphorylation substrate of GSK3β[114], like CLASP.

While coupling MTs to F-actin retrograde flow prevents MT extension to the periphery in the above cases, interactions between MTs and F-actin can also promote MT extension. For

example, a recent study showed that the F-actin-associated protein drebrin binds directly to +TIP protein EB3 in embryonic rat growth cones, and this interaction occurred specifically in the proximal region of filopodia when EB3-bound MTs enter and align alongside drebrin-bound F-actin bundles[115]. Strikingly, loss of drebrin function prevents MT extension into filopodia, suggesting that the drebrin-EB3 interaction is important for allowing MT exploration of filopodia[115].

These recent studies examining the growth cone functions of MT-actin regulatory proteins are beginning to uncover the complete and detailed mechanisms by which environmental cues are translated to changes in the cytoskeleton, and, especially, they seek to answer the intriguing question of how MTs interact functionally with the actin network during growth cone steering. However, there is still much to understand as to the role of MTs and MT-actin interactions in the growth cone, and this is a promising area of growth cone guidance research for the future.

Conclusion and future perspectives

Like an experienced driver, the growth cone navigation system can integrate multiple environmental variables, including road conditions, stop lights and street signs, to direct the vehicle and reliably reach its destination. Navigation system signalling through Rho-family GTPases and downstream cytoskeletal effectors is superimposed upon the cytoskeletal mechanisms of the vehicle, including F-actin retrograde flow and F-actin guidance of MTs, in order to introduce spatial bias for steering the growth cone in the right direction. Understanding how multiple cytoskeletal effectors work in concert to achieve this, in combination with elucidating additional signalling pathways mediating growth cone navigation, will give an integrated picture for the logic of growth cone dynamics over time.

Whereas much work has investigated how individual cues are interpreted, the next horizon will be to understand how the growth cone interprets multiple overlapping gradients of cues. Previously, it has been difficult to examine complex effects on the growth cone cytoskeletal machinery, in part due to technical limitations of experimental assays. Recent work utilizing microfluidic devices has allowed the production of stable, precisely controlled gradients in various combinations of both diffusible and substrate-bound factors[116,117]. These types of combined cues can more faithfully recapitulate the complexity of the *in vivo* environment, and thus, future experiments utilizing this method, in combination with high-resolution cytoskeletal imaging, hold considerable potential for refining our understanding of the growth cone guidance mechanism.

In the past decade, there has been a great advance in high-resolution imaging methods that can be used for analyzing growth cone cytoskeletal dynamics, including fluorescent speckle microscopy (FSM)[118] and total internal reflection fluorescence microscopy (TIRF)[119]. Combinations of these microscopy methods with new cross correlation speckle tracking algorithms for quantitatively measuring the details of cytoskeletal dynamics have provided further insights into the roles that actin and MTs have during normal vehicular function of the growth cone[36,120], including the finding that exploratory MTs might have a role in early signalling from guidance cues, whereas actin dynamics guide and control more stable MTs to fix the direction of new growth. The same types of quantitative methodology will need to be applied to understand protein-protein interactions and catalytic activations of signalling molecules, through advanced fluorescent sensors and tags[121-123]. This is an exciting time for studying growth cone dynamics, with new techniques and microscopy tools that are now providing even more extensive opportunities to explore the outstanding questions of the growth cone vehicle and its navigation.

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Appendix

Online summary

- During axon guidance, the growth cone, which comprises both the `vehicle' and the `navigator', progresses through stages of protrusion, engorgement and consolidation to move forward in a spatially-directed manner.
- Growth cone guidance is an integrated process that requires both substrate-bound cues (such as cell adhesion molecules (CAMs), laminin and fibronectin to provide the roadway for traction, and chemotropic cues (such as Netrins and semaphorins) that present road signs for steering directions.
- F-actin retrograde flow, driven by myosin II contractility in the transition (T)-zone and F-actin bundle treadmilling, keeps the growth cone engine idling and responsive to directional cues. Growth cone receptor binding to an adhesive substrate leads to the formation of a complex that acts like a molecular `clutch' mechanically coupling receptors and F-actin to stop retrograde flow and driving actin-based forward growth cone protrusion.
- Microtubules (MTs) have a role in steering the growth cone vehicle; individual peripheral (P)-domain MTs might act as guidance sensors and carry signals to and from receptor binding sites, and bulk central (C)-domain MTs steer growth cone advance.
- Live imaging studies suggest that the function of actin dynamics is to guide and control MTs in order to steer the growth cone in the right direction, and interactions between actin and MTs are tightly regulated. F-actin bundles regulate the activities of exploratory MTs, whereas F-actin arcs constrain C-domain MTs.
- In order for spatial discontinuities in the environment to drive growth cone steering and, in particular, in order to accurately interpret numerous cues simultaneously, the growth cone `navigation' system integrates and translates the multiple environmental directions to locally modulate the dynamics of the cytoskeletal machinery. Rho-family GTPases control cytoskeletal dynamics downstream of virtually all guidance signalling pathways, and they are spatially regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Localized control of actin dynamics at the leading edge through actin-binding proteins, and coordination of MT-actin cross-linking, are two key outputs of the navigation system required for growth cone steering.

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Glossary term suggestions

Filopodia, Thin, transient actin protrusions that extend out from the cell surface and are formed by the elongation of bundled actin filaments in its core.
Lamellipodia-like veil, Thin, sheet-like extensions of cytoplasm between the filopodia and are formed by branched actin networks.
F-actin arcs, actomyosin contractile structures that lie perpendicular to F-actin bundles, forming a hemicircumferential ring within the T-zone.
F-actin treadmilling, The process in which there is continual addition of actin subunits at the barbed-end of an F-actin polymer and disassembly at the pointed-end, so that the polymer stays of constant length, but individual subunits move along.
F-actin bundles, long actin filaments that are cross-linked together in parallel, forming the core of filopodia.
F-actin network, actin filaments that are cross-linked in branched pattern, forming the structure of the lamellipodia-like veils.
Chemotropic cue, external chemical cue, often found in a gradient, that leads to directional growth in response.
Neuroblastoma, tumour derived from primitive ganglion cells that can partially differentiate into cells having the appearance of immature neurons.
Dynamic instability, The state that has been used to describe microtubule polymer dynamics, in which the microtubule polymers cycle through periods of growth, shrinkage and occasional pausing.
Protrusion, The stage of growth cone progression in which there is extension of filopodia and lamellipodia-like veils.
Engorgement, The stage of growth cone progression in which microtubules invade further into the growth cone, fixing the new axonal growth direction.
Consolidation, The stage of growth cone progression in which F-actin at the neck of the growth cone depolymerises and the membrane shrinks to form a cylindrical axon shaft around the bundle of microtubules.

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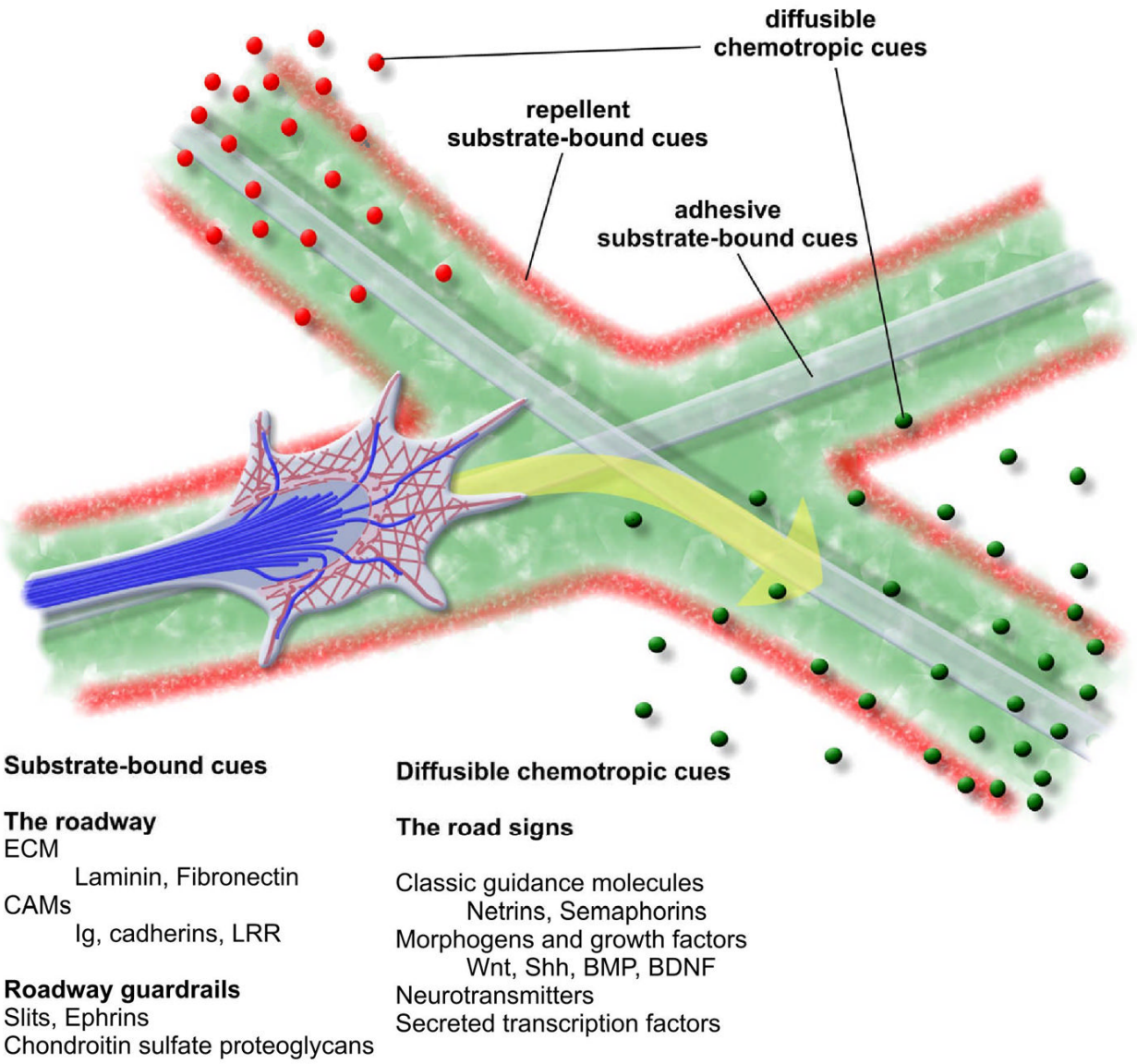


Figure 1. Directions for the trip

The growth cone encounters different types of cues in the environmental terrain. It travels upon a roadway, made up of adhesive molecules presented directly on a neighbouring cell surface (such as transmembrane cell adhesion molecules (CAMs)[1]) or assembled into a dense and complex extracellular matrix (ECM) (including laminin and fibronectin[2]). Additionally, anti-adhesive surface-bound molecules (such as Slits, Ephrins, and Chondroitin sulphate proteoglycans) can prohibit growth cone advance and thus provide the roadway `guardrails' that determine roadway boundaries. Finally, diffusible chemotropic cues represent the `road signs' that present further steering instructions to the growth cone, and include various diffusible chemotropic molecules (including Netrins and Semaphorins[3,4]), as well as morphogens (Wnt, Shh, BMP)[5] and growth/neurotrophic factors like BDNF[8,9], secreted transcription factors[6,7] and neurotransmitters[10]. Whereas it was originally thought that some cues function as attractive `go' signals (for example, Netrins) and others as repulsive `stop' signals (for example, Ephrins), it is now clear that the response of attraction versus repulsion is not due to the intrinsic property of the particular cue, but rather to the specific growth cone

receptors engaged and the internal signalling of the growth cone. Green circles are attractive cues and red circles are repulsive cues.

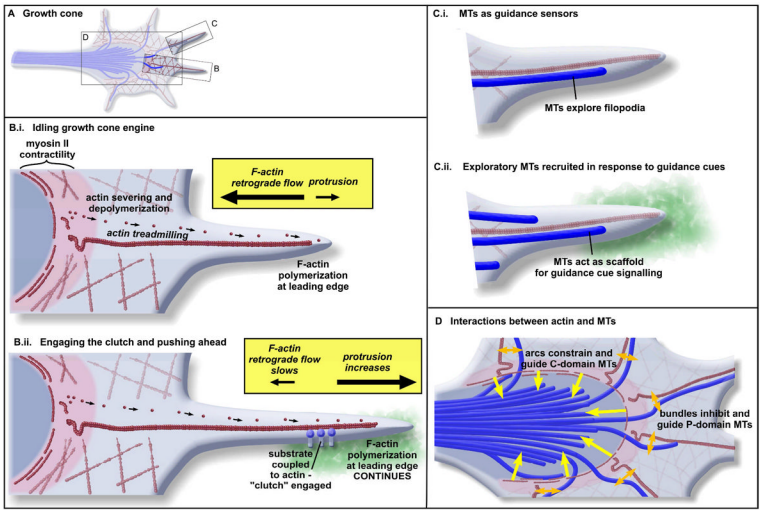


Figure 2. The growth cone `vehicle'

A. Boxed regions of the growth cone are shown in subsequent panels. B | Together, F-actin treadmilling (consisting of F-actin polymerization at leading edge, F-actin severing at transition (T)-zone, and recycling of these subunits back to leading edge) and F-actin retrograde flow (F-actin moving backwards towards T-zone) keep the growth cone engine idling. When the retrograde flow and polymerization forces are balanced, no protrusion occurs. When filopodia encounters an adhesive substrate, growth cone receptors bind to substrate and are coupled to F-actin through `clutch' proteins. This engages the clutch, anchoring F-actin with respect to the substrate and attenuating F-actin retrograde flow. Further F-actin polymerization pushes the membrane forward. This results in growth cone protrusion. C | Peripheral (P)-domain microtubules (MTs) explore filopodia along F-actin bundles and might be acting as guidance sensors. As a filopodium encounters a guidance cue, exploratory MTs might be acting as scaffolding for further signalling, and additional MTs are recruited to the region. D | Actin has a role in determining MT localization within the growth cone. Actin arcs constrain and guide C-domain MTs (simple arrows), and F-actin bundles inhibit and guide P-domain MTs (double arrows).

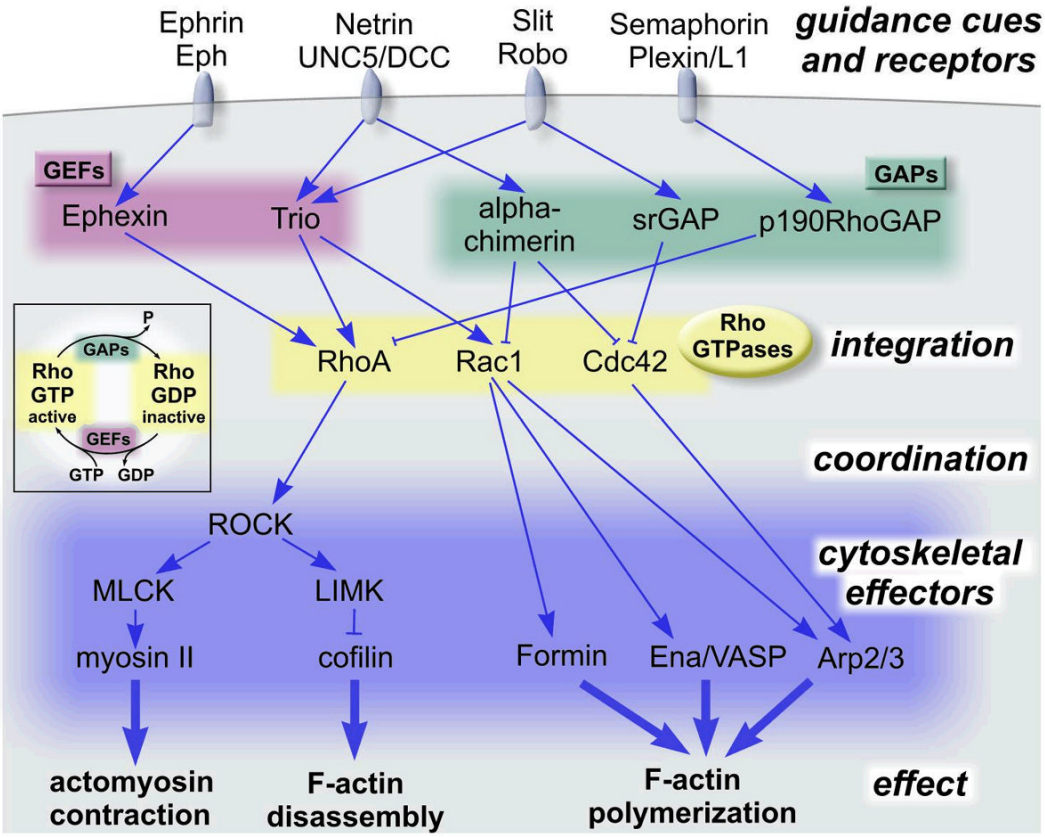
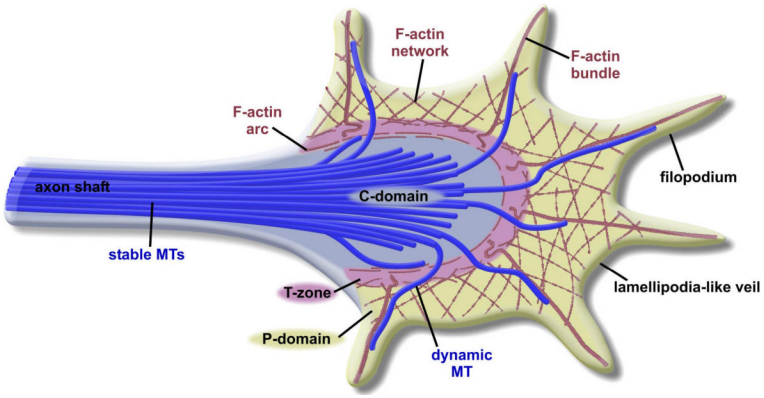


Figure 3. The growth cone as a `navigator'

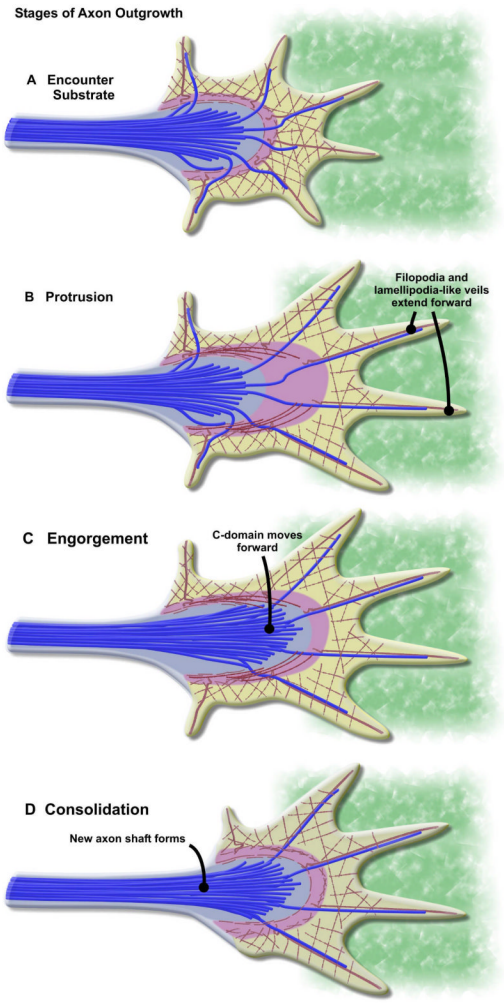
Rho-family GTPases act as key navigation signalling nodes to integrate upstream directional cues and coordinate downstream cytoskeletal rearrangements. Activation of receptors by guidance cues leads to activation of Rho-GTPase regulators. These include proteins that activate Rho-family GTPases, guanine nucleotide exchange factors (GEFs), and those that inactivate them, GTPase activating proteins (GAPs). Rho-GTPases integrate the responses of upstream pathways and coordinate downstream effects by modifying the function of cytoskeletal effectors. Activation or inactivation of cytoskeletal effectors leads to responses such as actomyosin contraction, F-actin disassembly or F-actin polymerization. The resulting growth cone turning response depends upon the localization of the guidance signalling within the growth cone.

Only some examples of guidance cues and receptors, GEFs and GAPs, and cytoskeletal effectors downstream Rho-family GTPases are shown in this figure. Arrows do not necessarily denote direct interaction. Boxed inset shows the Rho-GTPase activation/inactivation cycle, in which GAPs lead to the hydrolysis of GTP to GDP, whereas GEFs catalyze the exchange of GDP for GTP. Actin-Related Protein(Arp)2/3, Enabled/vasolidator-stimulated phosphoprotein (Ena/VASP), LIM domain kinase (LIMK), myosin light chain kinase (MLCK) and Rho kinase (ROCK).



Box 1. The structure of the growth cone

The structure of the growth cone is fundamental to its function. The leading edge consists of dynamic, finger-like filopodia that explore the road ahead, separated by lamellipodia-like veils, sheets of membrane between the filopodia (see the figure). The cytoskeletal elements within the growth cone underlie its shape, and the growth cone can be separated into three domains based on cytoskeletal distribution[14]. The peripheral (P)-domain contains long bundled actin filaments (F-actin bundles), which form the filopodia, as well as mesh-like branched F-actin networks, which give structure to lamellipodia-like veils. Additionally, individual dynamic `pioneer' microtubules (MTs) explore this region, usually along F-actin bundles. The central (C)-domain encloses stable, bundled MTs that enter the growth cone from the axon shaft, in addition to numerous organelles, vesicles and central actin bundles. Finally, the transition (T)-zone (also called T-domain) sits at the interface between the P- and C-domains, where actomyosin contractile structures termed actin arcs lie perpendicular to F-actin bundles, forming a hemicircumferential ring within the T-zone[33]. The dynamics of these cytoskeletal players determine growth cone shape and movement during its journey.

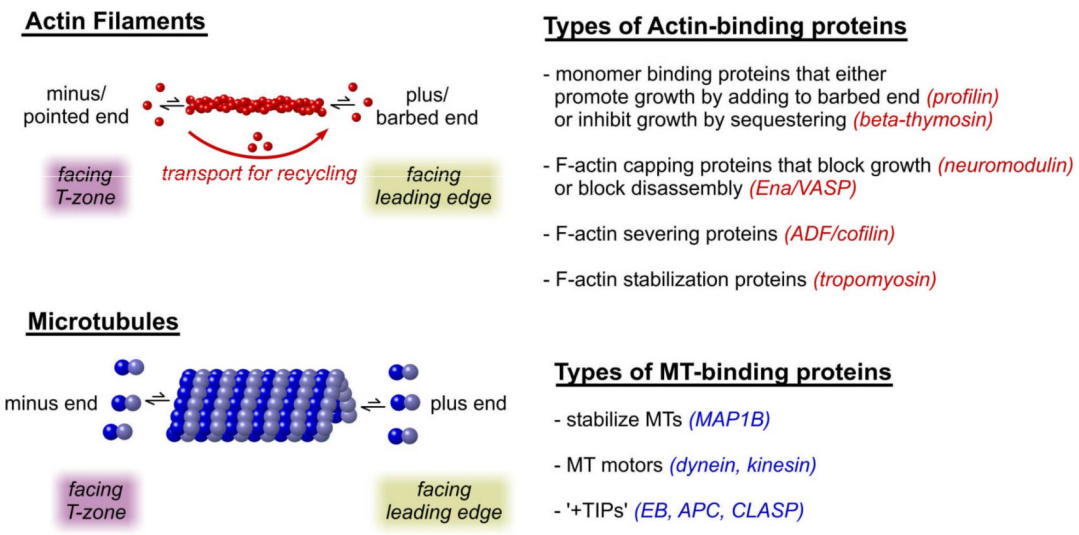


Box 2. Stages of axon outgrowth

One traditional description of the axon outgrowth process separates it into three stages[13,14]: Protrusion, Engorgement and Consolidation, which occur upon encountering attractive, adhesive substrates. This sequence during growth cone progression provides a framework for understanding detailed molecular mechanisms, and we assume that some of the same mechanistic events are utilized in response to diffusible chemotropic cues.

The distal end of the growth cone contacts adhesive substrate (see the figure, panel **a**). Binding of growth cone receptors activates intracellular signalling cascades and begins formation of a molecular `clutch' that links the substrate with the actin cytoskeleton. During protrusion, the `clutch' strengthens, resulting in regional attenuation of F-actin retrograde flow (see the figure, panel **b**). This anchors the actin with respect to the substrate, so that as F-actin polymerization continues in front of the clutch site, the filopodia and lamellipodia-like veils of the peripheral (P)-domain move forward to extend the leading edge (see[124] for discussion of molecular ratchet model for membrane protrusion). Engorgement occurs after actin clears from the corridor between the adhesion and the central (C)-domain, perhaps as F-actin behind the clutch is severed and removed (see the figure, panel **c**). F-actin arcs reorient from the C-domain towards the site of new growth[16,34,35], followed by C-domain microtubules (MTs) invading this region, guided by T-zone actin arcs and C-domain actin bundles. Finally, consolidation of the recently advanced C-domain occurs as the proximal part of growth cone compacts at the growth cone neck to form a new segment of axon shaft (see the figure, panel **d**). The myosin II-containing

actin arcs function to compress the MTs into the newly localized C-domain (followed by MT-associated protein stabilization). Retraction of filopodia away from the area of new growth occurs as F-actin protrusive activity is suppressed in these regions (also promoted by myosin II activity[52]), further promoting axon shaft consolidation. These three continuous and overlapping stages occur during formation of nascent axons, and also when new growth cones form from an axon shaft during axon branching[14,125].



Box 3. Cytoskeletal dynamics

Actin filaments are polarized polymers composed of actin monomers and their formation, stability and destruction are carefully regulated at every stage in the growth cone. Actin monomers can be added to either end, but changes in equilibria of polymerization dynamics depend on whether ATP or ADP is associated with actin (see the figure). In the growth cone, ATP-actin is usually added to the `plus' (or barbed) end pointing towards the cell membrane, ATP hydrolyzes to form ADP-actin, and ADP-actin disassembled at the `minus' (or pointed) end facing the transition (T) zone. Monomer-binding proteins then transport the actin back to the leading edge to support further growth. Other actin-binding proteins include nucleation factors that create new actin `plus-ends' for new growth, capping proteins that block growth or disassembly, antagonists of capping proteins, filament severing proteins and filament stabilization proteins such as those that assemble F-actin into higher-order structures such as bundles and networks, and those that anchor F-actin to specific regions of the membrane (reviewed in[126]).

Microtubules (MTs) are polarized structures composed of tubulin α/β dimers assembled into linear arrays. A linear array of alternating α- and β-tubulin subunits form a protofilament, and between 11-15 protofilaments form the wall of the MT. GTP-tubulin dimers are added to plus end, GTP hydrolysis can occur, and GDP-tubulin dimers dissociate from the minus end (see the figure). In growth cones, MT `plus' ends, which face outward towards the periphery, exhibit `dynamic instability', where they cycle through periods of growth, shrinkage and occasional pausing[127]. Numerous proteins bind to MTs; some stabilize MTs (such as microtubule associated protein 1B (MAP1B)[111]), some act as MT motors (for example, dynein and kinesin[128]) and others are part of a family called `plus-end tracking proteins' (`+TIPs'), which have been implicated in plus-end MT dynamic control and linking MTs with actin- or membrane-associated structures (for example, End-binding proteins (EBs), Adenomatosis Polyposis Coli (APC) and Cytoplasmic Linker Protein (CLIP)-Associated Protein (CLASP)[97,98]).

Box 4. Regulation of localized protein translation and degradation in the growth cone

Localized translation in response to guidance cues has emerged as an important mechanism mediating cytoskeletal dynamics, including RhoA signalling, during growth cone steering events[129-131]. In particular, attractive cues, such as Netrin and brain-derived neurotrophic factor (BDNF), induce the asymmetrical mRNA localization and translation of cytoskeletal components like β-actin on the side of the growth cone where new F-actin polymerization (and thus growth cone extension) occurs[132,133]. Furthermore, repulsive cues, such as Slit, induce asymmetric translation of proteins that break down the cytoskeleton, such as actin-depolymerizing factor (ADF)/cofilin (a family of actin severing proteins that disassemble F-actin filaments)[134] as well as β-thymosin (an actin monomer-sequestering protein that inhibits actin polymerization)[135]. The microtubule (MT)-F-actin cross-linking protein, Short stop, which is required for growth cone steering, also binds directly to a newly-discovered translation inhibitor, Krasavietz[136]. Further, the `plus-end tracking protein' (`+TIP') family member Adenomatosis Polyposis Coli (APC), which also binds to MT `plus-ends' and has a role in steering, has recently been shown to be required for RNA localization to cell protrusions in non-neuronal migrating cells[137], and thus, it might be functioning similarly in growth cones. In addition to cytoskeletal elements, local translation of the signalling molecule RhoA GTPase is required for the collapsing response of *Xenopus laevis* growth cones to the repulsive cue Semaphorin 3A (Sema3A)[64], suggesting that localized translation is a common theme for many molecules involved in growth cone steering.

Finally, localized protein degradation might also have a role in regulation of growth cone dynamics. This has been shown to be true for RhoA at the leading edge in migrating fibroblasts[138], and seems to be true in neuronal cells as well, during their outgrowth[139,140].