Touch and go: guidance cues signal to the growth cone cytoskeleton
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Growth cones, the highly motile tips of growing axons, guide axons to their targets by responding to molecular cues. Growth cone behaviors such as advancing, retracting, turning and branching are driven by the dynamics and reorganization of the actin and microtubule cytoskeleton through signaling pathways linked to guidance cue receptors. Actin filaments play a major part in growth cone motility, and because of their peripheral locations were thought to be the primary target of molecular cues. However, recent studies have shown that dynamic microtubules can penetrate the growth cone periphery where guidance molecules can influence them directly. Moreover, guidance cues can regulate growth cone steering by modulating dynamic actin–microtubule interactions.

Introduction
Growth cones, the highly motile structures at the tips of growing neurites, guide axons towards their targets by responding to molecular cues in the environment [1,2]. Growth cone motility and guidance behaviors that cause the axon to advance, retract, turn and branch are regulated by reorganization and dynamics of the actin and microtubule cytoskeleton [3]. The central region of the growth cone contains bundled microtubules, and the periphery is dominated by actin filaments that form both a meshwork in veil-like lamellipodia and bundles in fingerlike-like filopodia (Figure 1). Filopodia are essential for sensing guidance cues and steering the growth cone. Actin filaments play a central part in cell motility [4] and are well positioned at the leading edge of the growth cone to be the direct targets of guidance cues. However, live cell imaging has revealed that although many stable microtubules remain in the center of the growth cone, a population of dynamic microtubules can actively explore the periphery and penetrate filopodia, where they could interact with signaling pathways linked to the cytoplasmic domains of guidance cue receptors. Moreover, links between actin filaments and microtubules [5] imply that signaling pathways that regulate the dynamics of one cytoskeletal element will also affect the other. Signaling cascades in growth cones that link cell surface receptors to the cytoskeleton have been recently reviewed [6–9]. In this review we focus on recent studies of organization and dynamics of the cytoskeleton in relation to growth cone behaviors, and the modulatory influence of axon guidance cues.

Actin filament dynamics regulate growth cone motility
Actin polymerization drives protrusion of the plasma membrane [4,10], but how this leads to cell motility is still controversial. The architecture of actin filaments in relation to cell protrusion [11] suggests that different cell types have different strategies for moving and changing shape [12]. Consistent with this view, comparison of the organization of actin filaments in fibroblasts and hippocampal growth cones [13*] revealed that a network of highly branched actin filaments formed by the actin binding complex Arp2/3 is required for membrane protrusion in fibroblasts but not in neuronal growth cones.

Actin filaments also draw the growth cone membrane rearward during retrograde actin flow and are involved in growth cone retraction. Steady state myosin-dependent retrograde flow occurs in both filopodia and lamellipodia [14], and involves cycles of net assembly of actin filaments at the leading edge, retrograde movement of actin networks and disassembly of filaments proximally [15]. Retrograde actin flow might regulate the rate of axon outgrowth and prevent microtubules from invading the peripheral domain of the growth cone [16]. However, it was recently shown that LPA (lysophosphatidic acid), which enhances Rho-dependent myosin contractility, induces growth cone retraction independent of retrograde actin flow [17]. Thus, growth cone collapse and retraction in response to inhibitory cues might not involve changes in retrograde flow [18**], as had been previously assumed. Myosin contractility also plays a part in growth cone retraction and retrograde flow, and recent evidence [19] demonstrates an essential role for myosin II in growth cone turning behaviors at laminin borders.

Microtubule dynamics are essential for growth cone steering
Microtubules, once considered to have a role secondary to actin filaments in steering the growth cone towards...
attractive cues and away from repulsive cues, are now regarded as central players [20]. The dynamic properties of microtubules, that is, their ability to grow and shrink in dynamic instability, enables them to explore lamellipodia and filopodia [21–24](Figure 1). In growth cones, the fast growing (plus) ends of microtubules face outward towards the periphery. Inhibition of microtubule dynamics with pharmacological agents abolished turning behaviors, and localized stabilization and de-stabilization of microtubules are sufficient to induce attractive and repulsive turning [25]. These results demonstrate an instructive role for microtubules in growth cone steering.

How are microtubule dynamics regulated? In addition to microtubule associated proteins (MAPs) [26], a family of highly conserved plus end tracking proteins (+TIPS) [27–29] regulates microtubule dynamics by stabilizing them. Tip proteins bind selectively to the plus ends of microtubules and contribute to their stabilization by reducing the frequency of microtubule shortening (catastrophe) or by promoting their growth (rescue). For example, at the leading edge of fibroblasts the tip protein EB1 (end-binding protein 1), through interactions with another tip protein APC (adenomatous polyposis coli), selectively binds to microtubule plus ends and stabilizes them [30**], a process that is required for cell migration. Other tip proteins such as CLASP 1 and 2 bind to EB1 in non-neuronal cells [31] and locally regulate microtubule plus end dynamics by promoting rescue at the leading edge of the cell. Until recently little was known about the function of +TIPs in neurons [32]. However, EB3 binds to the tips of growing microtubules in hippocampal and Purkinje neurons [33]. Moreover, +TIPs in growth cones seem to play an important part in microtubule stabilization. Overexpression of the tip protein CLASP in Xenopus growth cones results in MTs that form loops in the central region of the growth cone, thus slowing growth cone advance [34**]. Taken together, evidence from neuronal and non-neuronal cells points to the importance of regulating microtubule dynamics in directed cell motility.

Actin–microtubule interactions regulate growth cone guidance
It is now clear that neither actin filaments nor microtubules act alone. Regulatory and structural interactions
between the two systems are required for functions such as cell motility and growth cone guidance [5]. For example, during lamellipodial protrusion in epithelial cells [35] activation of Rac1, a member of the Rho family of small GTPases, regulates both dynamic instability of microtubules and actin polymerization through p21-activated kinases (Paks), demonstrating coordinated regulation of the two cytoskeletal elements. Interestingly, Rac and Pak have recently been implicated in netrin signaling by binding of netrin-1 to the cytoplasmic domain of the DCC receptor [36]. Live cell imaging of growth cones has shown that interactions between the two filament systems can involve coordinated polymerization, growth and guidance of microtubules along actin filament bundles and coupling of microtubules to retrograde actin flow [21,23,24]. Although these studies suggest structural links between actin filaments and microtubules in growth cones, identification of such links is at an early stage. Recently, Dpod-1 [37], the Drosophila homolog of Pod-1, first isolated in C. elegans where it functions in axis formation, was found to crosslink actin filaments and microtubules. In developing neurons in the Drosophila embryo Dpod-1 is enriched at the tips of growing axons in regions of actin–microtubule interactions, especially at navigational choice points. Absence of Dpod-1 or its overexpression disrupted growth cone targeting and pathfinding but not axon outgrowth. Interestingly, defects in target innervation sometimes involved defects in axon branching, which is known to require dynamic actin–microtubule interactions [21]. At present it is unknown whether Dpod-1 functions to link signaling molecules downstream of guidance receptors to the actin and microtubule network or functions as a structural crosslinker to enable actin filaments and microtubules to change in concert.

Guidance cues modulate actin–microtubule organization and dynamics

Receptors to guidance cues belonging to the netrin, semaphorin, slit and ephrin families signal through complex pathways to the Rho family of small GTPases to direct the assembly and disassembly of actin filaments [7–9]. Activation of Rac and Cdc42 by attractive cues promotes actin polymerization in lamellipodia and filopodia and causes growth cone extension, whereas Rho activity is induced by repulsive cues to decrease actin polymerization and cause growth cone retraction through actin–myosin contraction [38]. Guidance cues can also be bifunctional depending on factors such as the receptors with which they interact [38] and levels of cyclic nucleotides in the growth cone [39,40].

Guidance cues such as netrin-1 that attract axons have been shown to promote actin polymerization (Figure 1). Netrin-1 rapidly increases axon branching and growth cone filopodia in cortical neurons [18**] with a concomitant increase in actin filaments. This, in turn, causes microtubules to splay apart in the axon shaft and the growth cone. These dynamic microtubules can then interact with the newly polymerized actin bundles to initiate outgrowth in new directions. Netrin-1 also promotes filopodial formation on hippocampal neurons [41**], which requires phosphorylation of Ena/VASP proteins through activation of PKA (protein kinase A). Ena/VASP proteins promote elongation at the growing (barbed) end of actin filaments by antagonizing capping proteins that normally terminate actin filament elongation [11]. Inactivation of Ena/VASP activity reduces the length and number of filopodia through a reduction of bundled actin filaments, whereas elevation of Ena/VASP increases filopodia formation by increasing actin filament bundles [41**]. Thus, netrin-1 can influence axon guidance by regulating formation of growth cone filopodia through the actin remodeling effects of Ena/VASP proteins. Neurotrophins such as BDNF (brain derived neurotrophic factor) can also increase filopodial length and number, which was recently shown to occur through the activation of ADF/cofilin (actin depolymerizing factor) [42], an actin associated protein that enhances actin dynamics.

Inhibitory guidance cues such as Sema 3A collapse growth cones by depolymerizing actin filaments [43] (Figure 1). This involves the downregulation of cofilin activity through phosphorylation by LIM kinase [44]. Collapsing effects have made it difficult to image changes in cytoskeletal dynamics induced by Sema 3A. However, growth of cortical neurons on a highly adhesive substrate to prevent growth cone collapse permitted visualization of the rapid dissolution of actin filament bundles caused by Sema 3A [18**]. Because microtubules extend along and interact with actin bundles, loss of actin filament bundles and the maintenance of retrograde flow caused microtubules to stop their dynamic exploration of the lamellipodia and collapse rearward into the growth cone center [15**]. These changes in cytoskeletal dynamics did not affect axon elongation but ultimately caused changes in the direction of growth by inhibiting axon branching.

Other guidance cues such as the slits and ephrins can also cause growth cone repulsion [8]. Although results from migrating cells demonstrate that slit downregulates Cdc42 activity [45], direct evidence for the effects of slits on actin dynamics during growth cone repulsion is absent. Abl (Abelson kinase) and Arg (Abl related kinase) regulate actin polymerization [46]. The possible role of Abl and Arg in ephrin signaling [47] and the functional link between EphA receptors, Rho GTPases and the cytoskeleton [48] suggest that the ephrin signaling pathway influences actin cytoskeletal dynamics in the growth cone [8,38]. One caveat is that growth cone collapse through the mechanism of actin depolymerization might be an oversimplification. The pharmacological agent cytochalasin D is known to induce growth cone collapse by capping actin filaments and inhibiting actin filament
polymerization. However, collapsing factors such as serotonin appear to cause growth cone collapse by a different mechanism involving actin bundle loss and a decrease in actin filament assembly at the leading edge [49], suggesting that depolymerization of actin filaments might not be the primary cause of growth cone collapse induced by inhibitory cues [50]. Further study of the effects of guidance cues on specific mechanisms of actin filament assembly and disassembly will be required to address this issue.

Many of the effects of axon guidance molecules on microtubule dynamics might occur as a consequence of changes in actin filaments. However, several recent studies have demonstrated direct effects of guidance cues on microtubules [34**,51**]. At the CNS midline in *Drosophila*, axon crossing is regulated by the repellent effects of slit through the Robo receptor family [52]. In a genetic screen for modifiers of Abl in *Drosophila*, the MAP Orbit/MAST was found to mediate the action of Slit by acting downstream of Abl [34**] to regulate midline axon crossing. As mentioned above, CLASP, a vertebrate ortholog of Orbit/MAST, binds to the tips of dynamic microtubules in Xenopus spinal motor growth cones where its overexpression causes microtubules to form loops, thereby slowing growth cone advance [34**]. Thus, the repellent effects of slit are mediated through Abl, which regulates actin and microtubule dynamics and might coordinate actin–microtubule interaction during growth cone guidance. The effects of neurotrophin signaling on axon elongation are also mediated by a protein that binds to the plus ends of microtubules [51**]. Nerve growth factor (NGF) promotes elongation of DRG (dorsal root ganglion) axons through a novel signaling pathway (PI3K–GSK3β) that regulates microtubule assembly by increasing microtubule interactions with the plus end binding protein APC. Understanding how guidance cues regulate microtubules through their association with tip proteins awaits further studies in living growth cones.

**Conclusions and future directions**

In neuronal growth cones the actin and microtubule cytoskeleton is the ultimate target of signaling pathways from extracellular guidance cues. As we have discussed, our understanding of how guidance cues modulate the dynamics of actin filaments and microtubules in addition to regulating the interactions between these two filament systems is far from complete. Furthermore, as exemplified by the puzzling differences between neurons and non-neuronal cells, the mechanisms by which changes in cytoskeletal organization and dynamics lead to cell motility are still controversial. Recent evidence has shown that localized changes in levels of second messengers, such as calcium, can locally activate downstream signals such as CaMKII (calcium–calmodulin-dependent protein kinase II) and calcineurin phosphatase to switch growth cone turning behaviors from attraction to repulsion [53*]. It will be important to visualize how localized signaling in the growth cone evokes localized modification of cytoskeletal dynamics leading to growth cone turning in specific directions. Finally, the three dimensional environment of the developing nervous system subjects the growth cone to multiple extracellular cues at once. A major challenge for the future will be to understand how the growth cone integrates this complex information into a guidance decision.

**Update**

Eph receptors and their ephrin ligands typically mediate axon guidance through growth cone collapse. Topographic mapping in the retinotectal system depends on opposing EphA7 and ephrin-A gradients [54]. Interestingly, EphA7 influences map formation by suppression of branching of retinal axons anterior to their normal tectal targets. Growth cone collapse and/or repulsion induced by Eph receptor stimulation involves remodeling of the actin cytoskeleton through regulation of the Rho family GTPases [48]. However, the signaling components linking Eph receptors to Rho GTPases in the growth cone are not well understood. Now, two recent studies [55**,56] have identified guanine nucleotide exchange factors (GEFs) ephexin1 [55**] and Vav2 [56] that regulate Rho GTPases and are required for ephrin-dependent axon repulsion. In response to EphA signaling, ephexin1 is tyrosine phosphorylated, which enhances the exchange activity of ephexin1 toward RhoA without changing the basal activity of Rac1 and Cdc42 [55**]. Thus, by changing the balance of Rho GTPase activity toward RhoA mediated actin cytoskeletal contractility, ephexin1 plays an important role in growth cone collapse. This is consistent with the finding that *in vitro* retinal ganglion cells lacking ephexin1 show reduced growth cone collapse in response to ephrin-A. Interestingly, in the absence of ephrin stimulation, ephexin1 promotes axon outgrowth. Vav2, another Rho family GEF [56], is required for Rac-dependent endocytosis of the ephrin–Eph complex, which converts initial cell–cell adhesion into repulsion. In mice lacking several Vav family GEFs, axon projections from the retina to the thalamus are abnormal and growth cones of retinal axons from these mice fail to collapse in response to ephrin-A. These findings suggest that Vav proteins play a role in axon guidance by activating Rho GTPases to stimulate ephrin–Eph endocytosis, which is required for growth cone collapse and axon repulsion. In the future it will be important to understand exactly how these Eph–ephrin signaling pathways can locally modulate actin cytoskeletal dynamics by regulating Rho GTPases to promote localized growth cone extension or retraction.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
- of special interest
- of outstanding interest

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37. These authors demonstrate that netrin-1 promotes expansion of the growth cones of spinal commissural neurons by recruiting an intracellular signaling complex directing actin organization. Netrin-1 induced growth cone expansion is shown to require Cdc42, Rac1, Pak1 and N-WASP.


   This elegant study provides detailed mechanisms by which netrin-1 promotes filopodial formation on hippocampal neurons. This requires global activation of PAK and phosphorylation of Ena/VASP proteins. Elevation of Ena/VASP activity increases actin filament bundles by antagonizing capping proteins that normally terminate elongation of actin filaments, whereas Ena/VASP inactivation reduces filopodia by reducing actin filament bundles. Netrin-1, thus, affects axon guidance by promoting formation of filopodia through regulation of actin filaments by Ena/VASP proteins.


   An elegant study demonstrating mechanisms by which localized calcium signaling in Xenopus spinal growth cones can switch growth cone turning from attraction to repulsion. By directly manipulating calcium levels in the growth cone the authors show that CaMKII and CaN-PP1 mediate calcium-dependent growth cone attraction and repulsion, respectively. Importantly, this CaMKII-CaNPP1 switching mechanism also operates during calcium induced growth cone turning induced by gradients of netrin-1.


   These authors elegantly demonstrate that the GEF ephexin1 is a required signal transduction intermediate by which Eph receptors regulate Rho family GTPases to induce growth cone collapse in response to ephrin. They show that tyrosine phosphorylation of ephexin1 occurs specifically in response to EphA signaling and enhances the GEF activity of ephexin1 toward RhoA, thereby promoting F-actin contractility underlying growth cone collapse. Importantly, growth cones of cultured retinal ganglion cells lacking ephexin1 exhibit reduced growth cone collapse in response to ephrin-A, and in vivo knockdown of ephexin1 disrupts Eph-mediated guidance of motor neuron axons.