seminars in **THE NEUROSCIENCES,** Vol 8, 1996: pp 67–80

Role of the cytoskeleton in growth cone motility and axonal elongation

Jean F. Challacombe, Diane M. Snow and Paul C. Letourneau

During axonal pathfinding, the direction of nerve fiber extension is established by the growth cone, the motile structure at the distal tip of an elongating axon. It is the growth cone that navigates and directs axonal outgrowth by detecting and responding to complex molecular cues in the nervous system environment. Changes in growth cone behavior and morphology that result from contact with these cues depend on the regulated assembly and dynamic reorganization of actin filaments and microtubules. Therefore, an understanding of growth cone guidance requires resolution of the cytoskeletal rearrangements that occur as navigating growth cones respond to stimulatory and inhibitory molecular signals in their milieu. In this review, we discuss the role of the cytoskeleton in growth cone navigation.

Key words: growth cone / cytoskeleton / actin filaments / microtubules / proteoglycan

THE FUNCTIONAL ORGANIZATION of the adult nervous system depends upon the connections formed during development, when axons grow out from neuronal cell bodies and extend along specific pathways to unite with their targets. This directed axonal elongation results from the exploratory behavior of motile structures at the axon tips, the growth cones. Growth cones establish the direction of axonal elongation by detecting and responding to signals, or guidance cues, in nervous system environment. Navigating growth cones are highly dynamic, displaying complex behaviors during contact with specific guidance cues, such as cell surface and extracellular matrix molecules.[1-7] Growth cone behaviors (protrusion, retraction, branching and turning) depend on the dynamic reorganization of the cytoskeleton, which is composed of actin filaments and microtubules. In the first part of this article, we describe the basic properties of growth cones, emphasizing the role of the cytoskeleton in determining them. Then we present our recent investigations of proteoglycan-mediated growth cone inhibition and turning, suggesting (1) that filopodial contact with chondroitin sulfate proteoglycan (CSPG) elicits changes in microtubule bundling and alignment, and (2) that growth cones with deficient filopodia alter their direction of migration in response to the inhibitory effects of CSPG by sidestepping instead of turning, implying that filopodia are necessary for normal microtubule reorientation at fibronectin (FN)/CSPG borders.

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1044-5765/96/020067+14 $18.00/0

Growth cone motility and actin filaments

Growth cones navigate by constantly extending and retracting sensory protrusions in the form of broad flattened lamellipodia and finger-like filopodia, which comprise the leading edge or peripheral domain. As illustrated in Figure 1, the dynamic protrusive behavior of a navigating growth cone is based on actin filament assembly at the leading edge, retrograde flow of assembled actin filaments, and proximal disassembly.[8-13] Actin filaments are organized into two populations at the leading edge: long bundled filaments in filopodia, and a branching network extending throughout lamellipodia.[14] The dynamics of actin filament assembly, disassembly, and organization into bundles and networks are influenced by actin-binding proteins (reviewed in refs 10, 15-17).

Protrusive force is generated at the leading edge by actin filament dynamics and/or by actin–myosin interactions. In the absence of substratum attachments, these forces result in the retrograde flow of actin filaments.[9] Actin filament polymerization near the plasma membrane is coupled to ATP hydrolysis, which can provide the power to move polymers rearward when they are prevented from forward elongation by the membrane.[8,10] Additionally, myosins I and II have been localized at the leading edge of growth cones.[18-22] This suggests that membrane-associated myosin molecules may interact with actin filaments at the leading edge of growth cones to

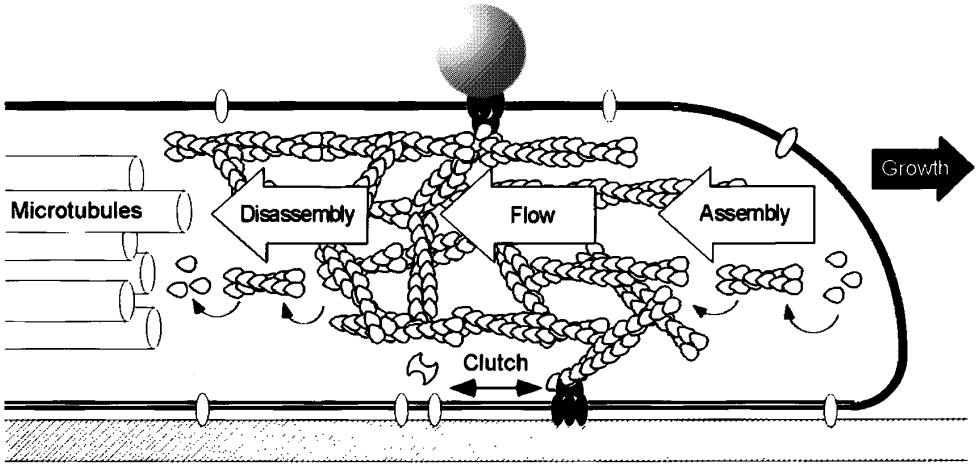

Figure 1. Substratum–cytoskeletal coupling model of growth cone advance. Actin dynamics of a neuronal growth cone lamellipodium are represented by a steady state composed of three superimposed processes (open arrows): actin filament assembly at the leading edge, actin filament disassembly at the back of the P domain, and retrograde flow of actin filaments at a rate matching assembly and disassembly. 'Clutch' protein complexes may regulate the degree of mechanical coupling between the actin filament network in the lamellipodium and adhesive bonds between surface ligands and extrinsic substrata. When the 'clutch' engages the filament network to the cell–substratum contact, the retrograde flow is attenuated, while actin disassembly continues. This removes impedance and accelerates the advance of microtubules by polymerization and/or transport. Reproduced from *Neuron* 14, 763-771 with permission of Dr P. Forscher and Cell Press.

produce the tension that pulls the elongating neurite forward.[10,13,18,21,23,24]

A current model (Figure 1) explains the role of tension and substratum attachments in growth cone advance; this model is based on evidence that the cytoplasmic domains of cell adhesion molecules can be coupled to actin filaments by 'clutch' protein complexes.[9,13,25,26] When the clutch protein complexes are disengaged, actin filaments and cell adhesion molecules are uncoupled, myosin–actin interactions cannot exert tension on the substratum, and actin filaments flow rearward. However, when clutch proteins link actin filaments to cell adhesion molecules, which are bound to the substratum, the tension produced between myosin and actin filaments is transduced into growth cone advance. Different protein complexes link the various cell adhesion molecules to actin filaments.[25] Recent evidence suggests that the involvement of actin filaments in growth cone navigation depends on the nature of the substratum and on how effectively clutch proteins link the different cell adhesion receptors to actin filaments.[26-28]

Axonal elongation and microtubules

Microtubules are the major cytoskeletal components of axons, serving as tracks for the axonal transport of membranous organelles,[29] and contributing to axonal structure by virtue of their rigidity and resistance to compression.[30,31] The organization of microtubules in growth cones suggests that they are also instrumental in axonal formation behind the advancing growth cone.[32-35] The three microtubule activities that contribute to axonal elongation are dynamics, translocation (or advance), and bundling.[36]

Microtubule dynamics

In vitro and in cells, microtubules grow and shrink continuously, a process called dynamic instability.[37] Microtubules can also undergo treadmilling, where net assembly and disassembly occur simultaneously at opposite ends.[38] These dynamic processes can be regulated by the gain and loss of a stabilizing GTP cap from microtubule ends,[37,39] by interactions of various microtubule-associated proteins with microtubules,[40-45] or by the tubulin isotype composition.[46] In addition, microtubule dynamics are linked to post-

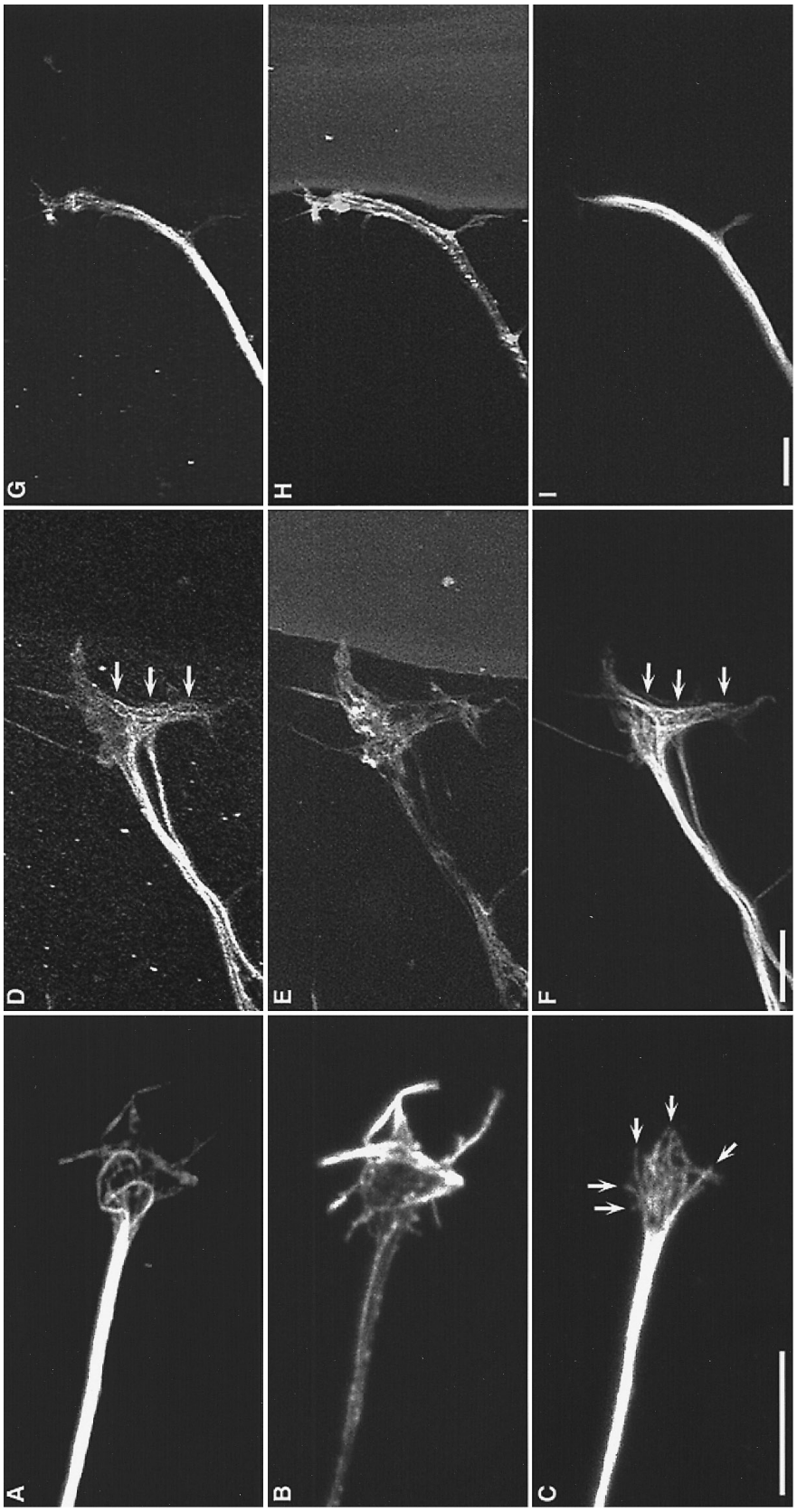

Figure 2. Arrangement of the cytoskeleton in growth cones elongating on FN (A–C), at a FN/CSPG border (D–F), and turning to migrate along the border (G–I). Laser scanning confocal optical sections were projected onto the same plane to show all labeling for each cytoskeletal component. Stable microtubules are labeled with polyclonal antibodies that recognize detyrosinated α-tubulin (A,D,G), actin filaments with rhodamine-phalloidin (B,E,H), and dynamic microtubules with a monoclonal antibody directed against tyrosinated α-tubulin (C,F,I). The rhodamine-labeled CSPG stripe is shown in E and H. Prior to contact with the CSPG stripe, stable microtubules are confined to the central region of the growth cone (A), while dynamic microtubules (C) overlap with actin filaments (B) at the bases of filopodia. The arrows in panel C point to dynamic microtubule ends that overlap with filopodial actin filament bundles. As growth cones begin to turn at the FN/CSPG border, the labeling of stable microtubules overlaps with that of dynamic microtubules (compare D and F). The area of overlap is indicated by arrows in panels D and F. Growth cones that have turned contain tightly bundled stable and dynamic microtubules extending along the border and into actin-dense regions at the growth cone tip (G–I). For each set of images, the scale bar on the bottom image represents 10 μm

translational modifications of tubulin, such that stable microtubules can be distinguished from dynamic ones by specific biochemical markers.[47]

One aspect of neuronal differentiation is that microtubules become resistant, or stable, to disassembly by cold and certain drugs.[42,48-50] While the exact mechanism responsible for microtubule stabilization is unknown, numerous studies implicate microtubule associated proteins.[40-45] Often, neurite microtubules consist of a proximal stable domain and a distal labile domain.[48,51,52] Stable microtubules are abundant in the soma and proximal neurite, while the most distal region of the neurite contains a greater proportion of dynamic microtubules.[51-56] This regional variation in microtubule stability may be important in the dynamic shaping of growing axons.

Microtubule advance

The mechanism of microtubule advance in growing neurites is the focus of an ongoing debate: do microtubules keep up with growth cone migration by polymerization of tubulin onto the ends of existing microtubules, or are microtubule polymers translocated in the direction of axonal elongation?

The view that axons elongate by tubulin polymerization at the growth cone is supported by results showing that local application of microtubule depolymerizing drugs to the growth cone abolishes neurite elongation.[57] However, other evidence indicates that nucleation and initial assembly of axonal microtubules occur in the soma, specifically at the centrosome;[49,58] these newly assembled, short microtubules enter neurites and eventually reach the growth cone by a poorly understood translocation process, elongating and shortening by dynamic instability along the way.[48,49,52,59] Additional studies utilizing low concentrations of vinblastine to arrest microtubule polymerization without net disassembly[60] have shown that axon elongation continues in the absence of tubulin polymerization, presumably by the translocation of short microtubules that were assembled in the soma before drug exposure. In contrast, a recent report states that similar vinblastine concentrations cause growth cone wandering without appreciable forward movement.[36]

Observations of fluorescently labeled microtubules in living growth cones are consistent with the idea that microtubules may advance into growth cones by both mechanisms, namely translocation of stable microtubule polymers, and new assembly of tubulin onto the plus ends of microtubules.[32-34,36,61,62] However, as the above studies used various neuronal types, it has been suggested that neurons from different organisms use different means to advance microtubules;[62,63] this may be related to the growth rates of these neurons. For example, tubulin polymers might be translocated in fast growing frog neurons[62] while in slower growing neurons of mice, grasshoppers[62] and zebrafish,[64] microtubules may advance by addition of distally transported monomers or small oligomers. Another possibility is that the mode of microtubule advance used by a particular neuronal type may reflect the stable or dynamic state of microtubules and/or distinct interactions of microtubules with other cellular elements, such as microtubule-associated proteins, neurofilaments, actin filament bundles, and focal contact components.

Microtubule bundling

The formation of new axon structure involves bundling of microtubules in the growth cone, often preceding or occurring simultaneously with collapse of the growth cone membrane proximally and elongation of the axon.[34] Microtubule bundling often accompanies growth cone advance, and pre-existing bundles sometimes appear to move forward. Bulinski and colleagues suggest that microtubule stabilization facilitates bundle formation by increasing the lifetime of individual microtubules, thus adding to the population of microtubules available for bundle formation.[65] In addition, microtubule bundling, and the subsequent conversion of the dynamic growth cone cytoskeleton to a more stable axonal configuration, may be influenced by compression of the microtubule array by the actin filament network.[9]

Growth cone behavior during pathfinding

Axonal pathfinding arises from the orientation of growth cone filopodia toward favorable cues or away from negative cues, consolidation of the filopodial orientation by microtubule advance, and reorganization of the growth cone cytoskeleton to form the axon (reviewed in refs 9,66). In this section, we will review what is currently known about these events, focusing on the role of microtubules in growth cone navigation, the influence of actin filaments on microtubules, and how substratum contacts may contribute to cytoskeletal reorganization.

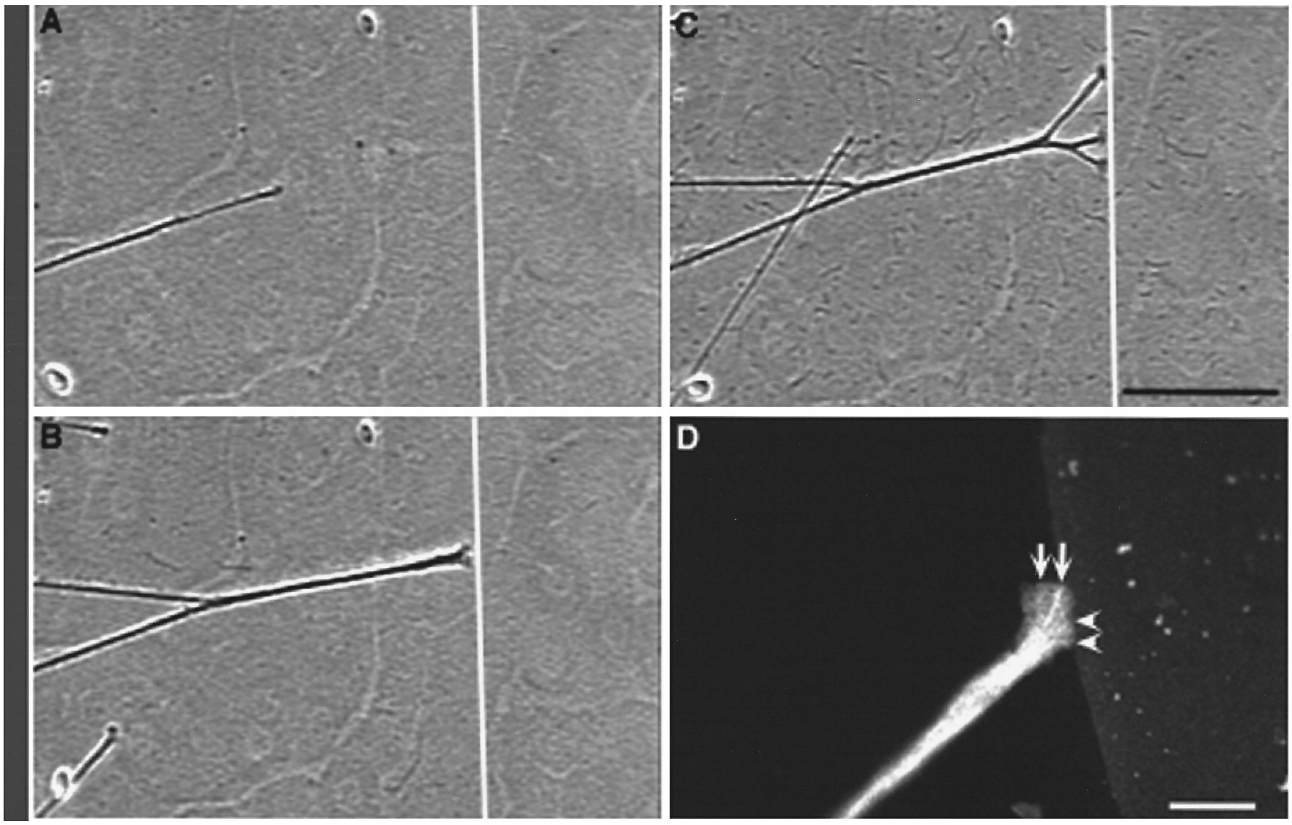

Figure 3. Growth cone sidestepping at a FN/CSPG border in the presence of 0.1 μg/ml cytochalasin B. Phase contrast images of a fascicle containing three growth cones approaching the border (A), stopped at the border (B), and after two of the growth cones had sidestepped along the border by 38 μm (top growth cone) and 17.6 μm (bottom growth cone) (C). The middle growth cone remained at the position shown in (B). The approximate location of the FN/CSPG border is indicated by the white line in panels A-C. Panel D shows a laser scanning confocal image of the top growth cone in (C), following fixation and immunofluorescent labeling for tyrosinated α-tubulin (dynamic microtubules). Note that these microtubules are splayed, and do not extend onto the rhodamine-labeled CSPG stripe (arrowheads). Some microtubule ends (arrows) point toward the direction of movement. The scale bar in (C) represents 100 μm, while that in (D) represents 10 μm.

Microtubule reorganization

Two of the most important navigational behaviors of growth cones, branching and turning, involve the reorganization of microtubules.[33,34,67-70] Evidence indicates that changes in the alignment of microtubules, resulting in microtubule invasion of certain growth cone branches, play a key role in determining the direction of neurite elongation.[33,34] Whether this reorganization is a consequence of redistributing a pre-existing polymer configuration, the result of net assembly, or both is currently unknown.

Influence of actin filaments on microtubules

The advance of microtubules into nascent growth cone branches or into the dominant side of a turning growth cone is influenced by actin filaments.[13,71] Growth cone microtubules often extend into the peripheral domain where they overlap with actin filament bundles at the bases of filopodia.[8,72-74]

Whether actin filaments impede or promote microtubule movement is currently an unresolved issue. Some reports indicate that the force generated by the actin network can inhibit microtubule advance. For instance, depolymerization of actin filaments by treatment with cytochalasins allows microtubules to extend

into the peripheral domain, suggesting that the intact actin network in growth cones can prevent microtubule advance.[8,30,31,75]

In contrast, several lines of evidence derived from observing living growth cones indicate that actin filament bundles can facilitate microtubule advance. For example, when a growth cone contacts a target, mechanical coupling occurs, and tension is transmitted by the growth cone. These events are followed by membrane ruffling, accumulation of actin filaments at the contact site, and microtubule extension toward the target.[56,69-71] These studies support earlier ultrastructural observations that the orientation of microtubules in the growth cone periphery is influenced by interactions with the actin filament bundles of filopodia.[72,73]

Substratum contacts and cytoskeletal reorganization

Growth cone–substratum contacts involve adhesion molecules of the Ig-like, cadherin, and integrin families,[21,76,77] each of which has specific accessory proteins linking actin filaments to adhesive sites (reviewed in ref 25). It has been proposed that adhesive contacts provide anchorage against which actin filaments interacting with myosin can pull and re-orient microtubules.[13,21,71,75] Additional evidence suggests that substratum-bound molecules stimulate microtubule entry into growth cones[78] by regulating the force produced by the actin filament network.[8,30,31,75] Adhesive contacts may also act as sites where second messenger systems are activated, subsequently regulating actin filament and microtubule stability and orientation. It is therefore plausible that the mechanisms responsible for microtubule advance into growth cones are influenced by the signal transduction pathways that are activated when specific growth cone receptors bind to substratum adhesion molecules.

Organization of the cytoskeleton in growth cones turning at inhibitory borders

In this section, we present our recent investigations of growth cone behaviors at boundaries between permissive and inhibitory substratum-bound molecules. We are analysing the organization and functions of dynamic and stable microtubules, and their relationship to actin filaments, using drugs that perturb actin filaments and microtubules with a reliable guidance assay, namely a substratum composed of alternating stripes of fibronectin (FN) and chondroitin sulfate proteoglycan (CSPG). It has been established that CSPGs are present in areas of the developing nervous system that growth cones avoid *in vivo*,[79,80] and that stripes of CSPG elicit chick dorsal root ganglion (DRG) growth cone branching and turning *in vitro*.[7,81] When growth cones on laminin or FN encounter a CSPG stripe, they stop elongating, and sample the border region repeatedly by cyclical filopodial protrusion and retraction. As the growth cone turns at the border, it does not collapse, but remains on the growth-promoting substratum, while filopodia continue to interact with CSPG throughout the directional change.[81,82]

We have observed growth cones of embryonic day 10–11 chick DRG neurons as they (1) elongated on FN, (2) approached CSPG stripes, (3) interacted with the FN/CSPG border, (4) turned, and (5) migrated along the border. At various times during this sequence of events, growth cones were fixed and immunocytochemically stained with rhodamine-phalloidin to label actin filaments, and specific antibodies that recognize detyrosinated (stable) and tyrosinated (dynamic) microtubules,[83,84] followed by FITC- and CY3- or CY5-conjugated secondary antibodies; fluorescent images were obtained with a laser scanning confocal microscope.

Arrangement of actin filaments and microtubules in turning growth cones

Growth cones on FN, and those closely approaching a CSPG border, contain dynamic microtubules that extend through the central region and overlap with actin filaments at the bases of filopodia, while stable microtubules are confined to the central region (Figure 2 (A-C) and Figure 5A). However, in growth cones that have contacted CSPG, the microtubules stop at the border, while filopodia and lamellipodia containing actin filaments constantly probe across the border. As growth cones are starting to turn, the labeling for stable microtubules is often coincident with labeling for dynamic microtubules (Figure 2 (D-I) and Figure 5 (B,C)), suggesting that microtubule rearrangements begin while growth cones are stopped at the border, after filopodia have repeatedly sampled the CSPG. The duration of filopodial sampling is variable, and the length of time necessary for microtubule reorganization is as yet undetermined. Possible microtubule rearrangements include depolymerization and re-assembly of dynamic microtubules,

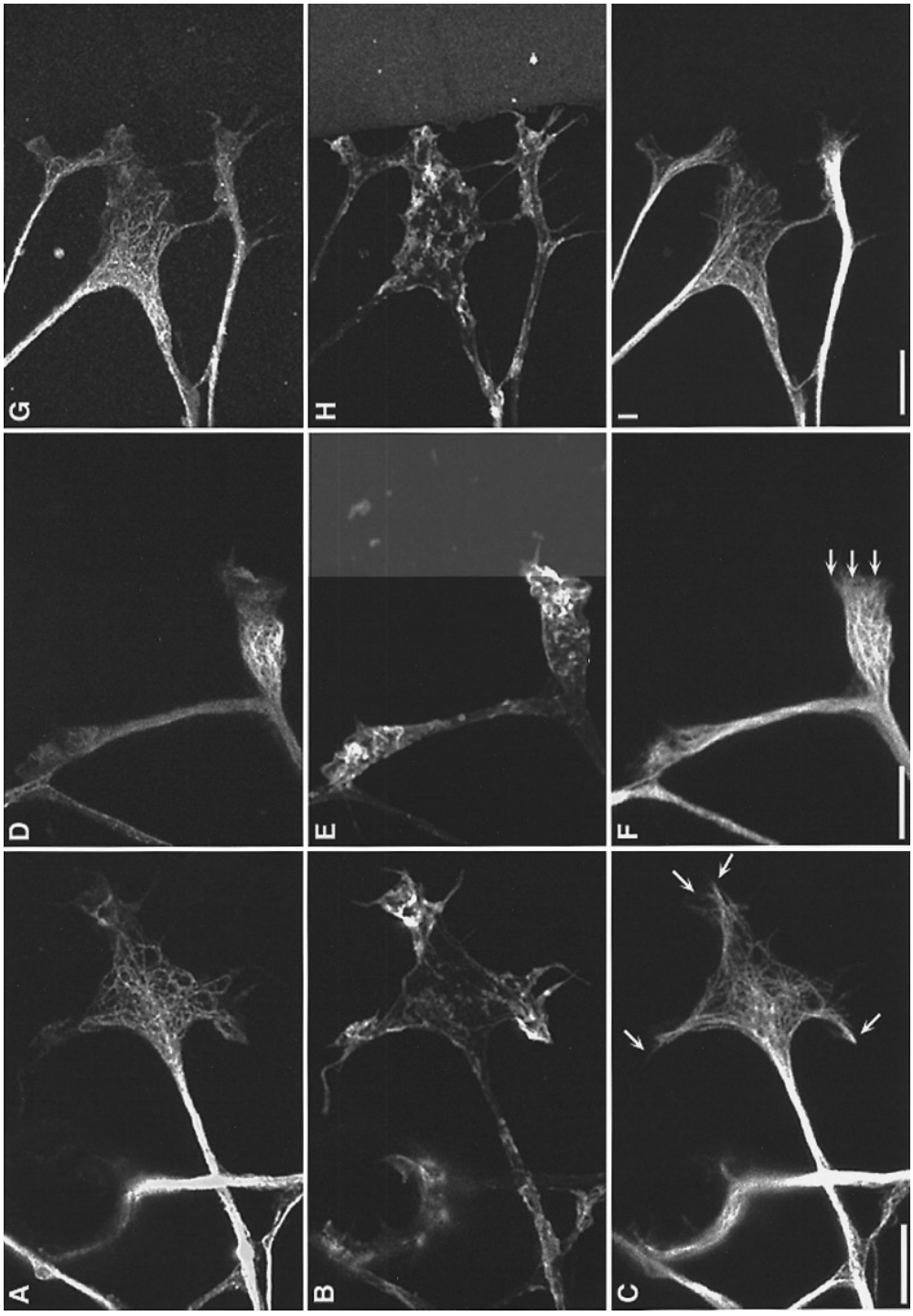

Figure 4. Arrangement of the cytoskeleton in chick sensory neuronal growth cones treated with 0.1 μg/ml cytochalasin B and elongating on FN (A–C), at a FN/CSPG border (D–F) and sidestepping to avoid the border (top growth cone in G–I). Laser scanning confocal optical sections were projected onto the same plane to show all labeling for each cytoskeletal component. Stable microtubules are labeled with polyclonal antibodies that recognize detyrosinated α-tubulin (A,D,G), actin filaments with rhodamine-phalloidin (B,E,H), and dynamic microtubules with a monoclonal antibody directed against tyrosinated α-tubulin (C,F,I). The rhodamine-labeled CSPG stripe is shown in E and H. The stripe in panel E was digitally enhanced to make it more visible. Reduction of the growth cone actin filament network by cytochalasin B results in extension of dynamic microtubules to the leading edge (indicated by arrows in panel C), which contains punctate actin staining (B). Looped stable microtubules (A) overlap with dynamic microtubules to some extent, but are generally excluded from the actin-dense regions. In growth cones that are at a FN/CSPG border (D–F), actin-based protrusions (E) extend onto the CSPG, while dynamic microtubules stop at the border (arrows in panel F). A growth cone that is avoiding the CSPG by sidestepping (top growth cone in G–I) exhibits microtubules that are not as tightly bundled as those in control growth cones (see Figure 2 (G–I)). For each set of images, the scale bar on the bottom image represents 10 μm.

stabilization of dynamic microtubules, and forward translocation of stable microtubules.

Growth cones that have turned at the FN/CSPG border contain tightly bundled stable and dynamic microtubules extending well into the body of the growth cone and often into actin dense regions at the growth cone tip (Figure 2 (G-I) and Figure 5C), indicating that the cytoskeletal rearrangements triggered by CSPG contact include substantial changes in the alignment and bundling of microtubules. It is likely that these changes begin at sites of filopodial contact with CSPG at the FN/CSPG border, as the result of a currently unknown signaling mechanism (see Discussion).

While there are obvious differences in the distribution of stable and dynamic microtubules in growth cones that haven't yet contacted the border (Figure 2 (A-C) and Figure 5A), during the early stages of turning stable microtubules often extend nearly as far forward as dynamic microtubules (Figure 2 (D-I) and Figure 5 (B,C)). This indicates that microtubule stabilization may be a key factor in determining the direction of axonal extension, probably by promoting consolidation of neurites in the direction of growth. We have provided evidence that, in growth cones at FN/CSPG borders, stable microtubules catch up with the leading margin. How might this occur? Stabilization of microtubule ends may serve to establish sites of assembly for further extension of dynamic microtubule polymers,[49] and/or pre-existing stable microtubules may be translocated forward to overlap with dynamic microtubules at the leading margin. Future experiments will address these possibilities.

Low doses of cytochalasin B influence growth cone turning

The drug cytochalasin B binds actin filaments and inhibits addition of actin monomer to filaments.[85] Addition of 10 μg/ml cytochalasin B to established DRG cultures severely reduces protrusion of lamellipodia and filopodia, resulting in neurites with blunt ends that do not exhibit normal motility, but do elongate, presumably by microtubule-associated forward movement of material.[86] Lower concentrations of cytochalasin B (less than 0.1 μg/ml) permit short filopodia-like structures to form.[86-88] While concentrations up to 0.05 μg/ml do not impair navigation,[87] when treated with 0.1 μg/ml cytochalasin B *Xenopus* retinal growth cones *in vivo* exhibit aberrant pathfinding abilities.

For our current studies, we are using low concentrations of cytochalasin B to examine the behavior of microtubules in growth cones with deficient filopodia at CSPG borders. In the presence of 0.05–0.1 μg/ml cytochalasin B, growth cones exhibit diminished protrusive activity and migrate at about one-third the normal rate,[86] confirming that actin filaments are required for normal growth cone motility, and that microtubules can sustain neurite elongation in the absence of normal filopodial and lamellipodial protrusion.

Our initial findings imply (1) that in the presence of cytochalasin B, growth cones avoid CSPG by a mechanism that is different from their normal turning sequence, implicating filopodia as mediators of growth cone turning at borders between growth-promoting and growth-inhibiting molecules, and (2) that the manner in which cytochalasin B-treated growth cones avoid CSPG is concentration dependent. Recall that growth cones typically advance right up to a CSPG stripe, contact the CSPG via filopodial sampling, then turn to migrate along the edge of the border.[81,82] The first part of this sequence of events also occurs in the presence of 0.1 μg/ml cytochalasin B, in that growth cones stop at the FN/CSPG border and do not cross it. However, at this point, cytochalasin B-treated growth cones behave differently. Figure 3 shows a typical example of this altered behavior, where instead of turning at the border to migrate along it, the growth cone sidesteps laterally along the border. At a lower concentration of cytochalasin B (0.05 μg/ml), some growth cones begin to sidestep along the border, then turn like controls (Figure 6). Although growth cones with impaired filopodia can still detect the CSPG cue and react to avoid it by altering their direction of migration, they don't reorient normally, indicating that filopodia play a key role in the growth cone guidance mechanisms that operate at FN/CSPG borders *in vitro*.

Arrangement of microtubules and actin in cytochalasin B-treated growth cones

Our analyses of the cytoskeleton have revealed a difference in phalloidin-stained filamentous actin in growth cones exposed to the two cytochalasin B concentrations mentioned above. As expected, and in agreement with others,[75,87] increasing doses of cytochalasin B reduced the prevalence of filamentous actin at the distal growth cone margin.

In growth cones approaching CSPG stripes in the presence of 0.1 μg/ml cytochalasin B, rhodamine-phalloidin labeling reveals punctate actin condensa-

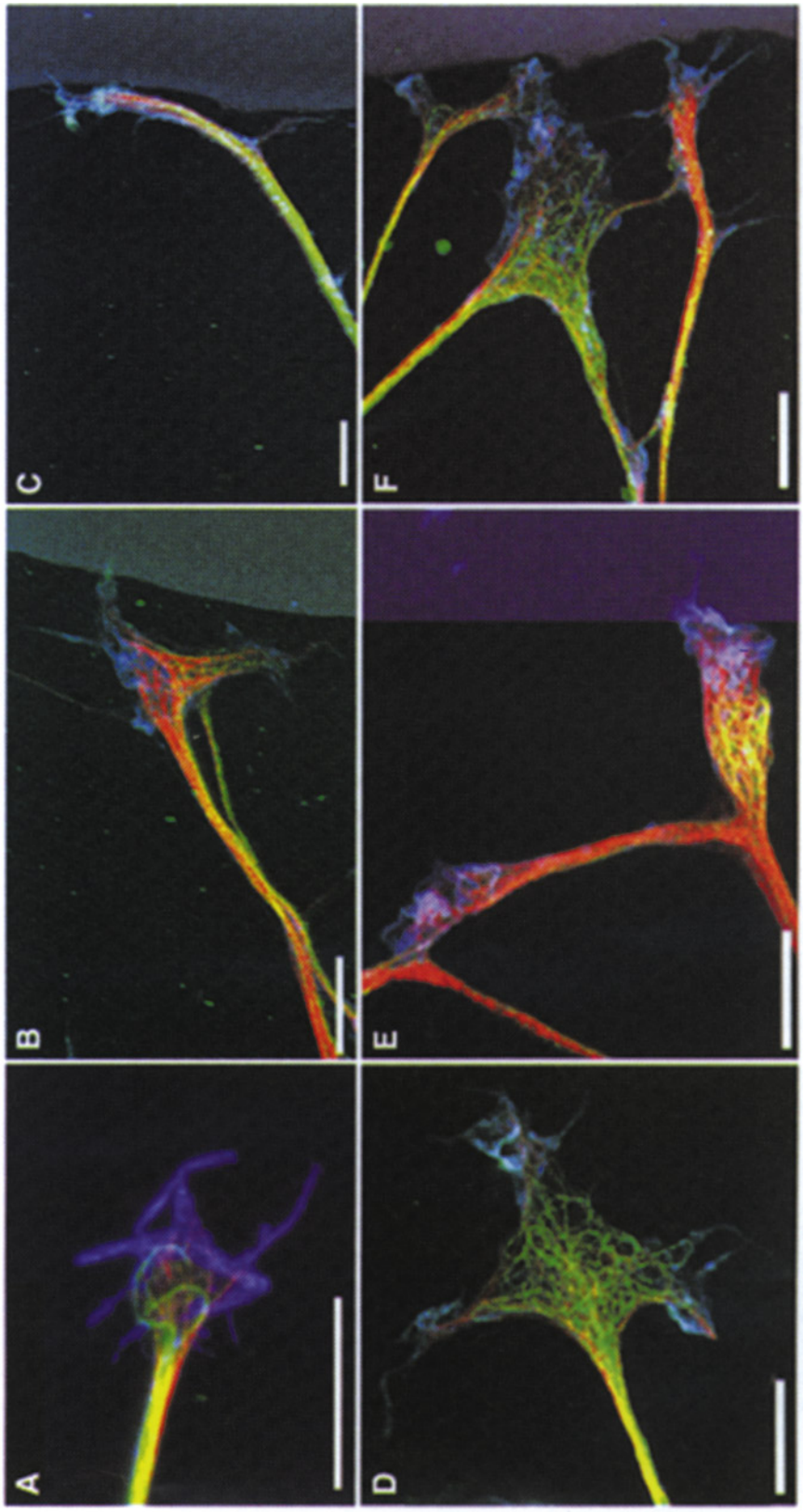

Figure 5. Three color merge of the images shown in Figure 2 and 4 illustrating the arrangement of the cytoskeleton in chick sensory neuronal growth cones as they elongate on FN, interact with a FN/CSPG border, and avoid the border under control conditions (A–C) or in the presence of 0.1 μg/ml cytochalasin B (D–F). Laser scanning confocal optical sections were projected onto the same plane and merged, in three colors, to show labeling of detyrosinated α-tubulin (stable microtubules, green) and tyrosinated α-tubulin (dynamic microtubules, red) relative to rhodamine phalloidin-labeled actin filaments (blue). The CSPG stripe (also fluorescently labeled) is shown in (B,C,E,F). The stripe was digitally enhanced to make it more visible in the images. The control growth cone (C) is turning at the FN/CSPG border, and illustrates the tight bundling and alignment of dynamic and stable microtubules along the border. In contrast, the dynamic microtubules in the cytochalasin B-treated growth cone (F) are splayed with their ends facing the direction of movement, and the stable microtubules are looped. For each set of images, the scale bar on the bottom image represents 10 μm.

tions instead of the well-organized actin filament bundles characteristic of normal filopodia (Figure 4). As in other studies,[8] reduction of the actin filament network resulted in microtubule extension to the leading margin of the growth cone. Our confocal images show that these distally projecting microtubules are dynamic, and that stable microtubules are generally excluded from the actin-containing regions (Figure 4C and Figure 5D). At sites of contact with the FN/CSPG border, actin is present in short protrusions that often extend onto CSPG, while all microtubules are arrested at the border.

In growth cones exposed to 0.1 μg/ml cytochalasin B that were sidestepping along the border prior to fixation, dynamic microtubules are not tightly bundled, but are splayed with their ends facing the direction of movement (Figure 3D; top growth cone in Figure 4 (G-I) and Figure 5F). Like controls, growth cones that had turned at the border in the presence of 0.05 μg/ml cytochalasin B show staining for dynamic microtubule bundles extending toward the new direction of elongation. As shown in Figure 6, labeling of stable microtubules often coincides with these aligned dynamic microtubules.

Discussion

We postulate that chick DRG growth cones normally turn at FN/CSPG borders by filopodia-mediated adhesion and microtubule reorientation. However, it is unclear whether this reorientation process is mediated by adhesion of filopodia to the FN substream, effectively 'pulling' the growth cone away from the CSPG; by reduced adhesion to the CSPG stripes, possibly due to CSPG interference with growth cone integrin binding to FN;[89] or whether the stimulus to turn is generated by transmembrane signals, such as increased intracellular Ca^{2+}[89] or changes in the activity of protein kinases, that result from CSPG contact.

Tanaka and Kirschner (1995) discuss several growth cone turning mechanisms that may operate at substratum borders. They speculate that these mechanisms differ in the extent to which the neurite adheres to the substratum during the turn, such that sidestepping happens when the distal neurite and growth cone are not well-attached, while turning of the growth cone at the border is possible only if the entire neurite and growth cone are adherent. In agreement with Tanaka and Kirschner, our investigations suggest that adhesion plays a role in growth cone turning. Filopodia contain receptors that adhere to the FN substratum,[21] and this adhesion allows tension to be transduced into forward movement that drives the turn. Our results indicate that microtubules become tightly bundled along the border and aligned with the turn, following the direction established by filopodia.

By reducing the length and organization of actin filaments at the leading edge, low doses of cytochalasin B may interfere with microtubule reorientation in several ways. Three of the possibilities include (1) shorter actin filaments resulting in a smaller growth cone with less room to reorient microtubules, (2) less growth cone–substratum adhesion to allow pulling against microtubules, and (3) fewer actin filaments, resulting in less coupling to the substratum and a reduction in the tension that can be used to reorient microtubules. Under conditions of reduced adhesion and/or tension, when actin filaments cannot reorient microtubules, growth cones avoid CSPG by sidestepping laterally along the border. The arrangement of microtubules under these circumstances suggests that microtubule bundling and alignment are usually dependent on the presence of relatively intact actin filaments and associated protein complexes. As mentioned, at very low concentrations of cytochalasin B (0.05 μg/ml), some growth cones exhibit a combination of sidestepping and turning. This may reflect an intermediate level of coupling, as the lower concentration of cytochalasin does not eliminate all filopodia.[87]

Conclusion

Axonal pathfinding depends on the navigational machinery of the growth cone, which includes cell surface receptors that detect and respond to environmental cues, and transmembrane signaling mechanisms that result in reorganization of the cytoskeleton. Our present investigations are focused on elucidating the cytoskeletal changes that occur during growth cone interactions with substratum-bound guidance cues. We have found (1) that filopodial contact with substratum-bound CSPG elicits changes in microtubule bundling and alignment, and (2) that growth cones with deficient filopodia alter their direction of migration in response to the inhibitory effects of CSPG by sidestepping instead of turning, suggesting that filopodia are necessary for normal microtubule reorientation at FN/CSPG borders.

We are further investigating the role of micro-

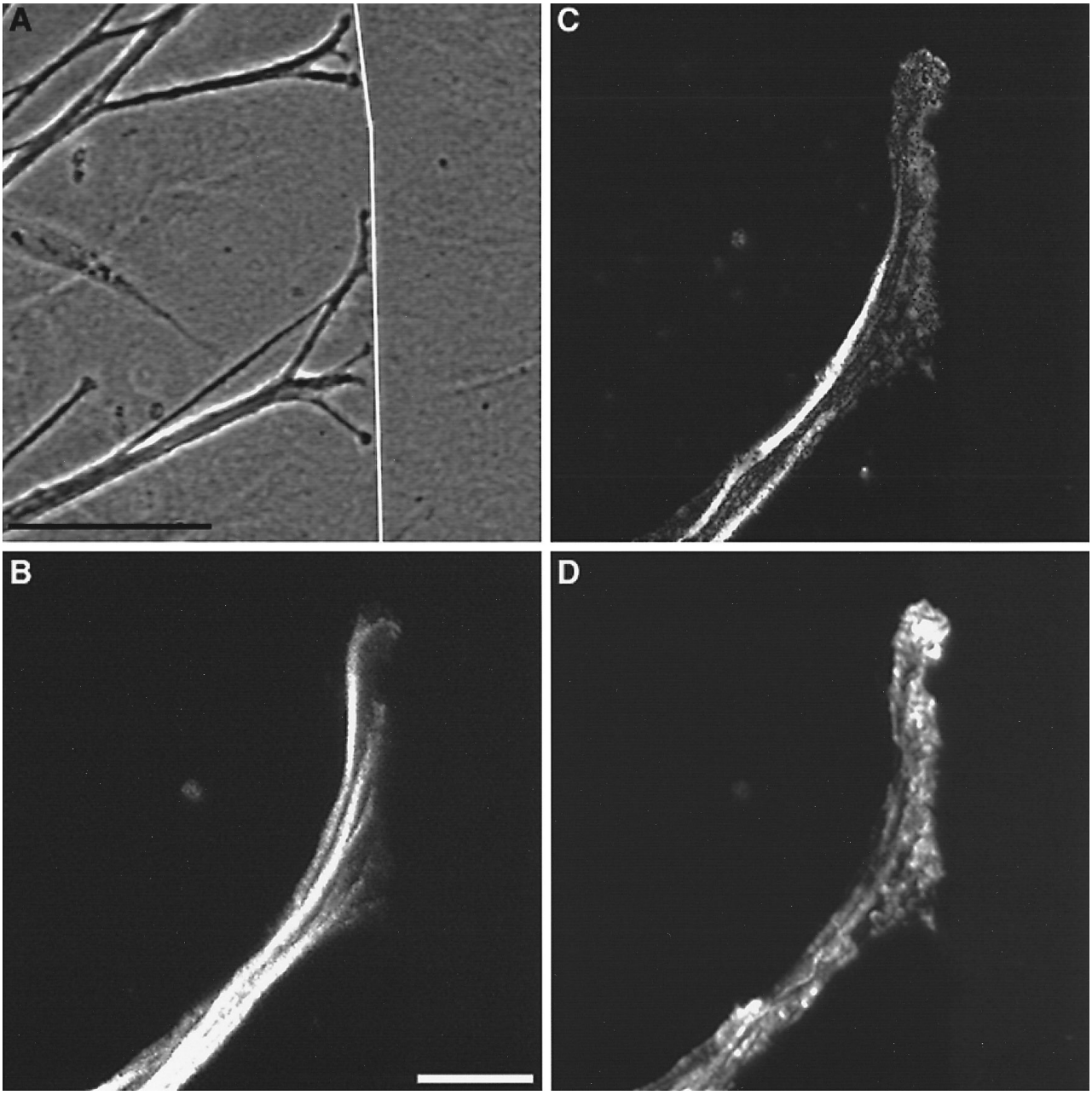

Figure 6. A growth cone exposed to 0.05 μg/ml cytochalasin B that has avoided a CSPG stripe by a combination of sidestepping and turning. Phase contrast image showing several neurites at a FN/CSPG border (A). Laser scanning confocal images (B–D) of the growth cone at the center of the field in (A), following fixation and immunofluorescent labeling for tyrosinated α-tubulin (dynamic microtubules, (B)), detyrosinated α-tubulin (stable microtubules, (C)), and phalloidin-labeled actin filaments (D). Actin labeling is concentrated at the leading edge of the growth cone at the FN/CSPG border, and dynamic microtubules extend right up to the border. Note that the brightest labeling of stable microtubules coincides with that of dynamic microtubules on the inside of the turning growth cone. The scale bar in (A) represents 100 μm, while that in (B) represents 10 μm.

tubules in growth cone turning through the use of various microtubule-disrupting drugs, with a particular interest in determining whether the local control of microtubule stabilization is an important step in growth cone turning at substratum boundaries. To fully understand growth cone guidance, it will be necessary to identify the specific adhesive interactions and second messenger systems that regulate the cytoskeleton.

Acknowledgements

We thank Drs J.C. Bulinski and G.G. Gundersen for providing antiserum against detyrosinated α-tubulin. Preparation of this manuscript and research described from the authors' laboratory were supported by NIH grants HD19950 (P.C.L.), EY10545 (D.M.S.), and F32-NS09971 (J.F.C.), NSF training grant DIR-9113444, and the Minnesota Medical Foundation

References

1. Honig MG, Burden SM (1993) Growth cones respond in diverse ways upon encountering neurites in cultures of chick dorsal root ganglia. Dev Biol 156:454-472
2. Oakley RA, Tosney KW (1993) Contact-mediated mechanisms of motor axon segmentation. J Neurosci 13:3773-3792
3. Myers PZ, Bastiani MJ (1993) Growth cone dynamics during the migration of an identified commissural growth cone. J Neurosci 13:127-143
4. Sretavan DW, Reichardt LF (1993) Time-lapse video analysis of retinal ganglion cell axon pathfinding at the mammalian optic chiasm: growth cone guidance using intrinsic chiasm cues. Neuron 10:761-777
5. Halloran MC, Kalil K (1994) Dynamic behaviors of growth cones extending in the corpus callosum of lung cortical brain slices observed with video microscopy. J Neurosci 14:2161-2177
6. Letourneau PC, Condic ML, Snow DM (1994) Interactions of developing neurons with the extracellular matrix. J Neurosci 14:915-916
7. Snow DM (1994) Neurite outgrowth in response to patterns of chondroitin sulfate proteoglycan: inhibition and adaptation. Neuroprotocols 4:146-157
8. Forscher P, Smith SJ (1988) Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol 107:1505-1516
9. Mitchison T, Kirschner M (1988) Cytoskeletal dynamics and nerve growth. Neuron 1:761-772
10. Smith SJ (1988) Neuronal cytomechanics: the actin-based motility of growth cones. Science 242:708-715
11. Lankford KL, Letourneau PC (1989) Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. J Cell Biol 109:1229-1243
12. Okabe S, Hirokawa N (1991) Actin dynamics in growth cones. J Neurosci 11:1918-1929
13. Lin C-H, Thompson CA, Forscher P (1994) Cytoskeletal reorganization underlying growth cone motility. Curr Opin Neurobiol 4:640-647
14. Lewis AK, Bridgman PC (1992) Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity. J Cell Biol 119:1219-1243
15. Hartwig JH, Kwiatkowski DJ (1991) Actin-binding proteins. Curr Opin Cell Biol 3:87-97
16. Janmey PA (1994) Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu Rev Physiol 56:169-191
17. Letourneau PC (1995) The cytoskeleton in nerve growth cone motility and axonal pathfinding. Perspectives Dev Neurobiol in press
18. Bridgman PC, Dailey ME (1989) The organization of myosin and actin in rapid frozen nerve growth cones. J Cell Biol 108:95-109
19. Cheng TPO, Murakami N, Elzinga M (1992) Localization of myosin IIB at the leading edge of growth cones from rat dorsal root ganglion cells. FEBS Lett 2:91-94
20. Cypher C, Letourneau PC (1991) Identification of cytoskeletal, focal adhesion, and cell adhesion proteins in growth cone particles isolated from developing chick brain. J Neurosci Res 22:259-265
21. Letourneau PC, Shattuck TA (1989) Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones. Development 105:505-519
22. Miller M, Bower E, Levin P, Li D, Chantler PD (1992) Myosin II distribution in neurons is consistent with a role in growth cone motility but not synaptic vesicle mobilization. Neuron 8:25-44
23. Letourneau PC (1981) Immunocytochemical evidence for colocalization in neurite growth cones of actin and myosin and their relationship to cell-substratum adhesions. Dev Biol 85:113-122
24. Sheetz MP, Wayne DB, Pearlman AL (1992) Extension of filopodia by motor-dependent actin assembly. Cell Motil Cytoskel 22:160-169
25. Gumbiner BM (1993) Proteins associated with the cytoplasmic surface of adhesion molecules. Neuron 11:551-564
26. Lin C-H, Forscher P (1995) Growth cone advance is inversely proportional to retrograde F-actin flow. Neuron 14:763-771
27. Abosch A, Lagenaur C (1993) Sensitivity of neurite outgrowth to microfilament disruption varies with adhesion molecule substrate. J Neurobiol 24:344-355
28. Burden-Gulley SM, Payne HR, Lemmon V (1995) Growth cones are actively influenced by substrate-bound adhesion molecules. J Neurosci 15:4370-4381
29. Brady ST, Lasek RJ, Allen RD (1985) Video microscopy of fast axonal transport in extruded axoplasm: A new model for study of molecular mechanisms. Cell Motility 5:81-101
30. Dennerll TJ, Joshi HC, Steel VL, Buxbaum RE, Heidemann SR (1988) Tension and compression in the cytoskeleton of PC-12 neurites II: quantitative measurements. J Cell Biol 107:665-673
31. Lamoureux P, Steel VL, Regal C, Adgate L, Buxbaum RE, Heidemann SR (1990) Extracellular matrix allows neurite elongation in the absence of microtubules. J Cell Biol 110:71-79
32. Reinsch SS, Mitchison TJ, Kirshner MW (1991) Microtubule polymer assembly and transport during axonal elongation. J Cell Biol 115:365-380
33. Sabry JH, O'Connor TP, Evans L, Toroian-Raymond A, Kirschner M, Bentley D (1991) Microtubule behavior during guidance of pioneer neuron growth cones in situ. J Cell Biol 115:381-395
34. Tanaka EM, Kirschner MW (1991) Microtubule behavior in the growth cones of living neurons during axon elongation. J Cell Biol 115:345-363
35. Tanaka E, Kirschner MW (1995) The role of microtubules in growth cone turning at substrate boundaries. J Cell Biol 128:127-137

36. Tanaka E, Ho T, Kirschner MW (1995) The role of microtubule dynamics in growth cone motility and axonal growth. J Cell Biol 128:139-155
37. Mitchison T, Kirschner M (1984) Dynamic instability of microtubule growth. Nature 312:237-242
38. Margolis RL, Wilson L (1978) Opposite end assembly and disassembly of microtubules at steady state *in vitro*. Cell 8:1-13
39. Davis A, Sage CR, Dougherty CA, Farrell KW (1994) Microtubule dynamics modulated by guanosine triphosphate activity of β-tubulin. Science 264:839-842
40. Ferreira A, Busciglio J, Caceres A (1989) Microtubule formation and neurite growth in cerebellar macroneurons which develop *in vitro*: evidence for the involvement of the microtubule-associated proteins, MAP-1a, HMW-MAP2 and tau. Dev Brain Res 49:215-228
41. Gordon-Weeks PR (1993) Organization of microtubules in axonal growth cones: a role for microtubule-associated protein MAP 1B. J Neurocytol 22:717-725
42. Baas PW, Pienkowski TP, Cimbalnik KA, Toyama K, Bakalis S, Ahmad FJ, Kosik KS (1994) tau confers drug stability but not cold stability to microtubules in living cells. J Cell Sci 107:135-143
43. Black MM, Slaughter T, Fischer I (1994) Microtubule-associated protein 1b (MAP1b) is concentrated in the distal region of growing axons. J Neurosci 14:857-870
44. Ezmaeli-Azad B, McCarty JH, Feinsten SC (1994) Sense and antisense transfection analysis of tau function: tau influences net microtubule assembly, neurite outgrowth and neuritic stability. J Cell Sci 107:869-879
45. Hirokawa N (1994) Microtubule organization and dynamics dependent on microtubule-associated proteins. Curr Opin Cell Biol 6:74-81
46. Panda D, Miller HP, Banerjee A, Luduena RF, Wilson L (1994) Microtubule dynamics *in vitro* are regulated by the tubulin isotype composition. Proc Natl Acad Sci USA 91:11358-11362
47. Bulinski JC, Gundersen GG (1991) Stabilization and post-translational modification of microtubules during cellular morphogenesis. BioEssays 13:285-292
48. Baas PW, Black MM (1990) Individual microtubules in the axon consist of domains that differ in both composition and stability. J Cell Biol 1990:495-509
49. Baas PW, Ahmad F (1992) The plus ends of stable microtubules are the exclusive nucleating structures for microtubules in the axon. J Cell Biol 116:1231-1241
50. Baas PW, Ahmad FJ, Pienkowski TP, Brown A, Black MM (1993) Sites of microtubule stabilization for the axon. J Neurosci 13:2177-2185
51. Brown A, Li Y, Slaughter T, Black MM (1993) Composite microtubules of the axon: quantitative analysis of tyrosinated and acetylated tubulin along individual axons. J Cell Sci 104:339-352
52. Ahmad FJ, Pienkowski TP, Baas PW (1993) Regional differences in microtubule dynamics in the axon. J Neurosci 13:856-866
53. Robson SJ, Burgoyne RD (1989) Differential localisation of tyrosinated, detyrosinated, and acetylated α-tubulins in neurites and growth cones of dorsal root ganglion neurons. Cell Motil Cytoskel 12:273-282
54. Arregui C, Busciglio A, Caceres A, Barra HS (1991) Tyrosinated and detyrosinated microtubules in axonal processes of cerebellar macroneurons grown in culture. J Neurosci Res 28:171-181
55. Brown A, Slaughter T, Black MM (1992) Newly assembled microtubules are concentrated in the proximal and distal regions of growing axons. J Cell Biol 119:867-882
56. Smith CL (1994) Cytoskeletal movements and substrate interactions during initiation of neurite outgrowth by sympathetic neurons *in vitro*. J Neurosci 14:384-398
57. Bamburg JR, Bray D, Chapman K (1986) Assembly of microtubules at the tip of growing axons. Nature 321:788-790
58. Ahmad FJ, Joshi HC, Centonze VE, Baas PW (1994) Inhibition of microtubule nucleation at the neuronal centrosome compromises axon growth. Neuron 12:271-280
59. Joshi HC, Baas PW (1993) A new perspective on microtubules and axon growth. J Cell Biol 121:1191-1196
60. Baas PW, Ahmad FJ (1993) The transport properties of axonal microtubules establish their polarity orientation. J Cell Biol 120:1427-1437
61. Okabe S, Hirokawa N (1990) Turnover of fluorescently labelled tubulin and actin in the axon. Nature 343:479-482
62. Sabry J, O'Connor TP, Kirschner MW (1995) Axonal transport of tubulin in Ti1 pioneer neurons in situ. Neuron 14:1247-1256
63. Okabe S, Hirokawa N (1992) Differential behavior of photoactivated microtubules in growing axons of mouse and frog neurons. J Cell Biol 117:105-120
64. Takeda S, Funakoshi T, Hirokawa N (1995) Tubulin dynamics in neuronal axons of living zebrafish embryos. Neuron 14:1257-1264
65. Chapin SJ, Bulinski JC, Gundersen GG (1991) Microtubule bundling in cells. Nature 349:24
66. Goldberg DJ, Burmeister DW (1989) Looking into growth cones. Trends Neurosci 12:503-506
67. Letourneau PC (1982) Analysis of microtubule number and length in cytoskeletons of cultured chick sensory neurons. J Neurosci 2:806-814
68. Letourneau PC, Shattuck TA, Ressler AH (1986) Branching of sensory and sympathetic neurites *in vitro* is inhibited by treatment with taxol. J Neurosci 6:1912-1917
69. Lin C-H, Forscher P (1993) Cytoskeletal remodeling during growth cone-target interactions. J Cell Biol 121:1369-1383
70. O'Connor TP, Bentley D (1993) Accumulation of actin in subsets of pioneer growth cone filopodia in response to neural and epithelial guidance cues in situ. J Cell Biol 123:935-948
71. Bentley D, O'Connor TP (1994) Cytoskeletal events in growth cone steering. Curr Opin Neurobiol 4:43-48
72. Letourneau PC (1979) Cell-substratum adhesion of neurite growth cones, and its role in neurite elongation. Exp Cell Res 124:127-138
73. Letourneau PC, Ressler AH (1983) Differences in the organization of actin in the growth cones compared with the neurites of cultured neurons from chick embryos. J Cell Biol 97:963-973
74. Gordon-Weeks PR (1991) Evidence for microtubule capture by filopodial actin filaments in growth cones. NeuroReport 2:573-576
75. Letourneau PC, Shattuck TA, Ressler AH (1987) 'Pull' and 'push' in neurite elongation: observations on the effects of different concentrations of cytochalasins and taxol. Cell Motil Cytoskel 8:193-209
76. Tomaselli KJ, Reichardt LF (1989) Integrins, cadherins, and cell adhesion molecules of the immunoglobulin superfamily: neuronal receptors that regulate axon growth and guidance, in The Assembly of the Nervous System, pp 81-108. Alan R. Liss, Inc., New York
77. Letourneau PC (1992) Integrins and N-cadherin are adhesive molecules involved in growth cone migration, in The Nerve Growth Cone (Letourneau PC, Kater SB, Macagno ER, eds) pp 181-193. Raven Press, Ltd, New York
78. Burmeister DW, Rivas RJ, Goldberg DJ (1991) Substrate-bound factors stimulate engorgement of growth cone lamellipodia during neurite elongation. Cell Motil Cytoskel 19:255-268
79. Snow DM, Steindler DA, Silver J (1990) Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. Dev Biol 138:359-376
80. Oakley RA, Tosney KW (1991) Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. Dev Biol 147:187-206

81. Snow DM, Lemmon V, Carrino DA, Caplan AI, Silver J (1990) Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. Exp Neurol 109:111-130
82. Snow DM, Watanabe M, Letourneau PC, Silver J (1991) A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Development 113:1473-1485
83. Wehland J, Willingham MC, Sandoval IV (1983) A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. I. Biochemical characterization, effects on microtubule polymerization *in vitro*, and microtubule polymerization and organization *in vivo*. J Cell Biol 97:1467-1475
84. Gunderson GG, Kalnoski MH, Bulinski JC (1984) Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo*. Cell 38:779-789
85. Yamada KM, Spooner BS, Wessells NK (1970 Axon growth: roles of microfilaments and microtubules. Proc Natl Acad Sci USA 66:1206-1212
86. Marsh L, Letourneau PC (1984) Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. J Cell Biol 99:2041-2047
87. Chien C-B, Rosenthal DE, Harris WA, Holt CE (1993) Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. Neuron 11:237-251
88. Letourneau PC, Snow DM, Gomez TM (1994) Growth cone motility: substratum-bound molecules, cytoplasmic [Ca^{++}] and Ca^{++}-regulated proteins, in Progress in Brain Research (van Pelt J, Corner MA, Uylings HBM, Lopes da Silva FH, eds) pp 35-48. Elsevier Science, Amsterdam.
89. Snow DM, Atkinson PB, Hassinger TD, Letourneau PC, Kater SB (1994) Chondroitin sulfate proteoglycan elevates cytoplasmic calcium in DRG neurons. Dev Biol 166:87-100