Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance

Review

Erik W. Dent* and Frank B. Gertler
Biology Department
68-270
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Recent studies indicate the actin and microtubule cytoskeletons are a final common target of many signaling cascades that influence the developing neuron. Regulation of polymer dynamics and transport are crucial for the proper growth cone motility. This review addresses how actin filaments, microtubules, and their associated proteins play crucial roles in growth cone motility, axon outgrowth, and guidance. We present a working model for cytoskeletal regulation of directed axon outgrowth. An important goal for the future will be to understand the coordinated response of the cytoskeleton to signaling cascades induced by guidance receptor activation.

Introduction

Ramón y Cajal first described the growth cone in 1890 from sections of embryonic spinal cord stained with silver chromate (Cajal, 1890). Amazingly, Cajal correctly described the growth cone from his fixed specimens as "a concentration of protoplasm of conical form, endowed with amoeboid movements." However, it wasn't until 1907 that Harrison, using newly devised tissue culture techniques in conjunction with long-term time-lapse observation, provided definitive proof that the growth cone was indeed a motile structure. In doing so he demonstrated unambiguously that axons extended from a single neuronal cell body, rather than from a merging of cells into the elongated cylindrical shape of the mature neuron (Harrison, 1907). Importantly, Speidel (1933) confirmed Harrison's observation in a living embryo. In his descriptions of isolated neural tissue from *R. pipiens* embryos, Harrison wrote, "…[these] experiments show that two elementary phenomena are involved in nerve development: (a) the formation of the primitive nerve fiber through extension of the neuroblastic protoplasm into a filament—protoplasmic movement; (b) the formation of the neurofibrillae within the filament—tissue differentiation…" (Harrison, 1910). It is this protoplasmic movement of the growth cone and the underlying dynamic cytoskeletal structures (neurofibrillae) that are the subject of this review.

In this review, we focus in particular on the cytoskeletal dynamics of actin and microtubules in neurons that have been studied in culture. The high-resolution optical methods available to image cultured neurons have permitted detailed study of cytoskeletal dynamics at a subcellular level, which have been technically impossible for the most part in neurons of living animals. Although clearly it will be important to expand these studies into the complex three-dimensional environment of the or-

ganism, by isolating neurons in a relatively simple, easily manipulated, two-dimensional environment, we have learned much about the cytoskeleton and its role in motility and guidance. Our focus in this review will be on the final common target of signaling cascades in the growth cone, the actin, and the microtubule cytoskeleton and the proteins to which they bind directly. There are several aspects of cytoskeletal signaling which, due to the scope of this piece, we will not emphasize. First, there has been much effort to elucidate the signaling cascades downstream of specific axon guidance receptors, and especially prominent in these studies is the importance of Rho GTPases, which are known to play an important role in axon outgrowth and pathfinding in vivo and in culture. This work has been thoroughly covered in a number of recent reviews and so will not be discussed here (Dickson, 2002; Huber et al., 2003; Lee and Van Vactor, 2003; Luo, 2000, 2002; Meyer and Feldman, 2002; Mueller, 1999; Song and Poo, 2001). Second, a number of reviews have focused on the similarities between cytoskeletal reorganization in growth cone guidance and those associated with neuronal migration and polarization of axons and dendrites (see reviews by da Silva and Dotti, 2002; Feng and Walsh, 2001; Lambert de Rouvroit and Goffinet, 2001); these topics will also not be discussed in detail here. By focusing on the cytoskeleton in relation to axon outgrowth and guidance, we hope to provide a framework for those studying guidance cues, their receptors, and downstream signaling cascades in their efforts to connect these pathways to the molecules that ultimately control morphological and mechanical responses of neurons to their environment.

Throughout this review we will refer to several morphological features of the growth cone. Starting at the distal extent of the growth cone, filopodia or microspikes are narrow cylindrical extensions capable of extending tens of microns from the periphery of the growth cone (Figure 1). Lamellipodia are flattened, veil-like extensions at the periphery of the growth cone. We will also refer to different regions of the growth cone following the operational definitions of previous work (Bridgman and Dailey, 1989; Forscher and Smith, 1988; Smith, 1988). These regions include the peripheral (P) domain, composed primarily of lamellipodia and filopodia; the transitional (T) domain, a band of the growth cone at the interface of the P domain and the central (C) domain; and the C domain, composed of thicker regions invested by organelles and vesicles of varying sizes. Generally, growth cones from all species examined to date contain filopodia and lamellipodia, as well as P, T, and C domains, although the shapes and sizes of the domains and number of filopodia and lamellipodia vary dramatically. Before describing what is currently known about growth cone cytoskeletal dynamics and how these dynamic structures play an essential role in motility and guidance, it is necessary to delineate the morphological changes that occur as a growth cone is converted into a stable axon shaft.
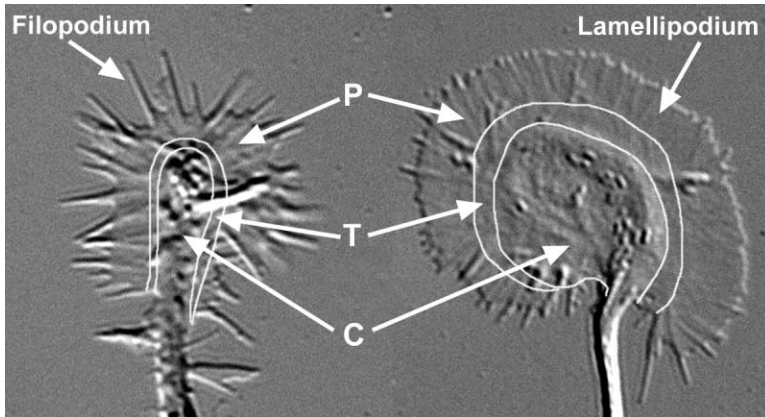
*Correspondence: ewdent@mit.edu

Figure 1. Growth Cones Vary in Shape and Size

Differential interference contrast images of two hippocampal growth cones in culture. Growth cones can exhibit a wide variety of shapes and sizes. An example of a "filopodial" growth cone is shown at left and a "lamellipodial" growth cone is shown at right. Generally, growth cones contain both filopodia and lamellipodia and peripheral (P), transition (T), and central (C) regions, although these vary dramatically in shape and size. Both growth cones are shown at the same magnification.

Stages of Axon Outgrowth

Early phase contrast studies of growth cone dynamics showed that the rate of advance and the shape of the growth cone were linked. Studies in sympathetic neurons demonstrated that the rate of growth cone advance directly correlated with the size and dynamics of lamellipodia and filopodia (Argiro et al., 1984, 1985). Quantitative analysis of filopodia from dorsal root ganglion neurons demonstrated that filopodial movement and growth cone advance were directly correlated as well (Bray and Chapman, 1985). This group showed that growth cones exhibited retrograde flow of material from the peripheral to the central region and into the axon shaft itself. Importantly, they also demonstrated that the growth cone undergoes a systematic maturation that is continuously repeated during elaboration of the axon. This maturation process consists of the following series of events: filopodia and lamellipodia form at the leading edge of the growth cone, followed by flow of the filopodia around the lateral aspects of the growth cone and subsequent retraction of filopodia at the base of the growth cone. These investigators also proposed that filopodial movements were driven by the flow of actin filaments and associated proteins, which make up the filopodia and lamellipodia (see below).

Nevertheless, these phase-contrast images were incapable of discerning fine details of growth cone structure. With the invention of video-enhanced contrast differential interference contrast (VEC-DIC) microscopy (Allen, 1985), it was possible to determine that growth cones from the mollusk *Aplysia californica* progressed through three morphologically distinct stages to form new axon segments (Goldberg and Burmeister, 1986). These stages are termed protrusion, engorgement, and consolidation (Figure 2). Protrusion occurs by the elongation of filopodia and lamellipodia (often between two filopodia), apparently through the polymerization of actin filaments. Engorgement occurs when veils become invested with vesicles and organelles, likely through both Brownian motion and directed microtubule-based transport. Consolidation occurs as the proximal part of the growth cone assumes a cylindrical shape and transport of organelles becomes bidirectional, thus adding a new distal segment of axon. These three stages, iterated many times, give rise to the elongated axon.
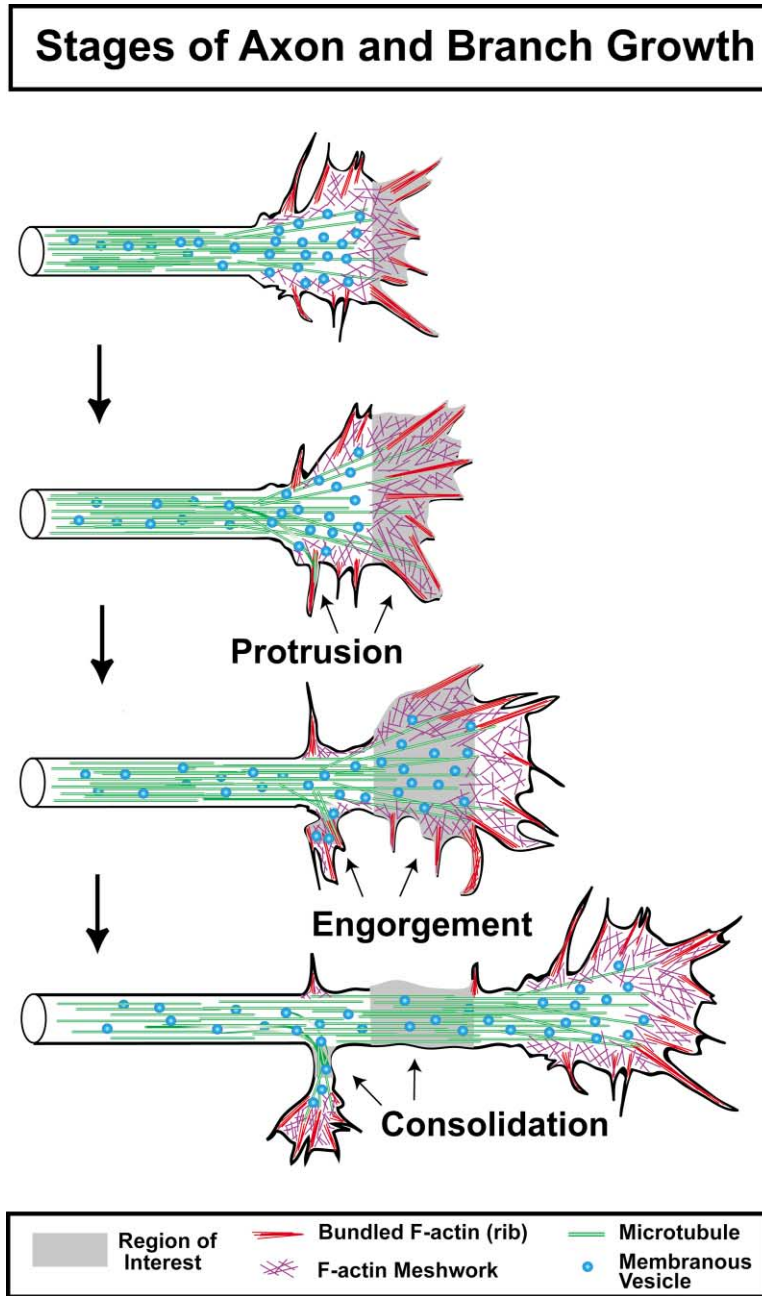
The three stages outlined for *Aplysia* neurons were thus extrapolations of the phases outlined a year earlier for chicken sensory neurons. Subsequent studies demonstrated that PC12 cells, rodent sympathetic (Aletta and Greene, 1988), and cortical neurons (Kalil, 1996) progressed through the same phases of development during formation of new axons. All of the above studies documented growth cone motility and random outgrowth in cell culture; however, growth cone motility and axon outgrowth in the developing embryo also is known to occur through protrusion, engorgement, and consolidation (Godement et al., 1994; Halloran and Kalil, 1994; Harris et al., 1987). However, in vivo outgrowth is not random, but directed to specific postsynaptic targets. We propose that the difference between random or induced outgrowth in culture and directed outgrowth in vivo is likely to be that gradients of guidance cues bias one side of the growth cone to progress through these stages toward (by an attractant) or away from (by a repellent) the guidance cue more rapidly than the other side of the growth cone. Thus, axon guidance can be thought of as directed protrusion, engorgement, and consolidation.

In the mammalian central nervous system (CNS), axons often elongate past their eventual targets and only later branch into these targets through a process of delayed interstitial axon branching (O'Leary et al., 1990; Kalil et al., 2000). This process of forming a growth cone from an axon shaft also occurs through protrusion, engorgement, and consolidation, with the new axon branching off of the parent axon (Figure 2). Therefore, axon guidance, through directed turning or branching, occurs through a conserved mechanism. The rest of the review will elaborate the underlying cytoskeletal mechanisms that regulate protrusion, engorgement, and consolidation, leading to directed outgrowth.

Pioneering Studies in Growth Cone Cytoskeleton

Although microtubules (MTs), microfilaments (filamentous actin or F-actin), and neurofilaments were observed in sections of nervous system tissue under the electron microscope throughout the 1950's and 1960's (Hughes, 1953; Nakai, 1956), it was not until the experimental studies of Wessels' group that MTs and F-actin polymers were shown to be necessary for neurite outgrowth (Yamada et al., 1970, 1971). Yamada and colleagues incubated chick embryo dorsal root ganglia cultures in

Stages of Axon and Branch Growth

Protrusion

Engorgement

Consolidation

Region of Interest	Bundled F-actin (rib)	Microtubule
F-actin Meshwork	Membranous Vesicle	

Figure 2. Stages of Axon and Branch Growth

Three stages of axon outgrowth have been termed protrusion, engorgement, and consolidation (Goldberg and Burmeister, 1986). Protrusion occurs by the rapid extensions of filopodia and thin lamellar protrusions, often between filopodia. These extensions are primarily composed of bundled and mesh-like F-actin networks. Engorgement occurs when microtubules invade protrusions bringing membranous vesicles and organelles (mitochondria, endoplasmic reticulum). Consolidation occurs when the majority of F-actin depolymerizes in the neck of the growth cone, allowing the membrane to shrink around the bundle of microtubules, forming a cylindrical axon shaft. This process also occurs during the formation of collateral branches off the growth cone or axon shaft.

cytochalasin B (CB), which at high concentrations results in F-actin depolymerization, and colchicine, which at high concentrations causes MT depolymerization. Exposure to CB induced retraction of growth cone filopodia, which made the growth cone adopt a club-like appearance and resulted in cessation of axon outgrowth. However, subsequent experiments in dissociated culture (Dent and Kalil, 2001; Lafont et al., 1993; Marsh and Letourneau, 1984), in the grasshopper limb (Bentley and Toroian-Raymond, 1986), in the *Xenopus* retinotectal system (Chien et al., 1993), and in the *Drosophila* peripheral nervous system (Kaufmann et al., 1998) demonstrated that CB-induced F-actin depolymerization actually resulted in continued extension but caused misdirected outgrowth. Depolymerization of

MTs did not immediately affect the filopodia but eventually resulted in retraction of the axons (Yamada et al., 1971). From these studies it can be concluded that F-actin is the primary cytoskeletal element that maintains the growth cone shape and is essential for proper axon guidance, whereas MTs are essential for giving the axon structure and serve an important function in axon elongation.

Subsequent studies suggested that there was actually an interaction between the F-actin cytoskeleton and MTs. Bray and colleagues demonstrated that after addition of colchicine, neurites retracted, as demonstrated by others (Yamada et al., 1970), but that depolymerization of MTs also caused new growth cone-like lamellipodial and filopodial protrusions along the usually
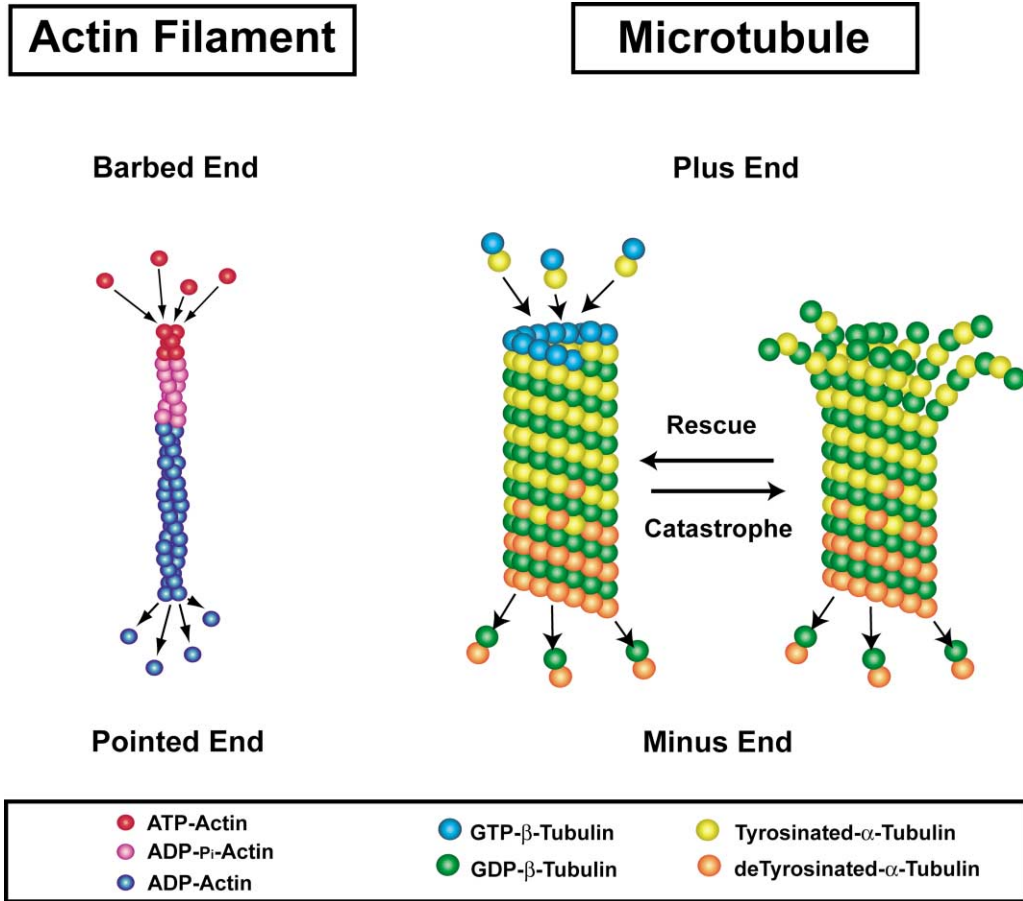
Actin Filament   # Microtubule

Barbed End   **Plus End**

Pointed End   **Minus End**

● ATP-Actin	● GTP-β-Tubulin	● Tyrosinated-α-Tubulin	
● ADP-Pi-Actin	● GDP-β-Tubulin	● deTyrosinated-α-Tubulin	
● ADP-Actin			

Figure 3. Actin Filaments and Microtubules Are Polarized Polymers

Actin filaments in vitro are capable of adding and removing ATP-actin and ADP-actin from both the barbed and pointed ends. However, the equilibrium constant for ATP dissociation is greater at the pointed end. Consequently, at steady-state, actin filaments devoid of actin-associated proteins undergo slow treadmilling through the addition of ATP-actin to the barbed end and release of ADP-actin from the pointed end (Pollard and Borisy, 2003). Actin filaments also exhibit aging, in which ATP-actin is hydrolyzed rapidly to ADP-pi-actin, followed by a slow dissociation of the γ-phosphate, giving ADP-actin. Microtubules are also polarized structures with α/GTP-β-tubulin dimers adding to the plus or growing end and α/GDP-β-tubulin dimers dissociating from the minus end. Microtubules also contain an internal mechanism of GTP hydrolysis that occurs rapidly, giving a "GTP-cap" to the polymers. They also exhibit posttranslational modifications (detyrosination shown here) that correlate with the age of the polymer.

quiescent axon shaft (Bray et al., 1978). The authors concluded that MTs suppress actin polymerization in the axon shaft, which maintains the axon in a cylindrical form. Another group, using EM after stabilization of the cytoskeleton, documented that MTs oftentimes abut or run parallel to F-actin bundles in peripheral regions of the growth cone (Letourneau, 1983). Thus, we have known for more than twenty years that there is likely to be a dynamic interplay between cytoskeletal elements. To understand this interplay, it is necessary to identify what cytoskeletal components are present in growth cones and what higher-order forms they take.

The Growth Cone Cytoskeleton Is Composed of Polarized Polymers

The two principle cytoskeletal components in growth cones are actin filaments and microtubules (Figure 3). However, in developing peripheral nervous system neurons, neurofilaments are also present in the axon and C domain of the growth cone (Bunge, 1973; Shaw et al.,

1981; Tennyson, 1970), but not during the initial axon outgrowth of hippocampal (Shaw et al., 1985) and cortical (E.W.D., unpublished data) neurons in culture. Transgenic mice lacking axonal neurofilaments are viable, with few abnormalities in their neural connections (Eyer and Peterson, 1994). Furthermore, *Drosophila* develop fully functional nervous systems without neurofilaments. Although neurofilaments are clearly an important cytoskeletal component during development of the vertebrate nervous system (Lin and Szaro, 1995), their function in growth cone motility and axon guidance is unknown. Therefore, this review will focus on actin filaments and microtubules.

Actin Filaments

Actin filaments are helical polymers composed of actin monomers, often referred to as globular actin (G-actin) (Figure 3). Neurons contain approximately equal amounts of nonmuscle isotypes of actin, β-actin and γ-actin (Choo and Bray, 1978), although most research has focused on the β-actin isotype. It is not known if these

two isotypes of actin have distinctive functions in neurons and growth cones, although both their mRNA and protein show differential regulation in neurons (Bassell et al., 1998; Gunning et al., 1998). It is also not known whether the composition of individual actin filaments in growth cones is homogeneous, composed of either β-actin or γ-actin, or heterogeneous, containing both β-actin and γ-actin.

Globular actin can exist as ATP-actin, ADP-pi-actin, and ADP-actin (Figure 3). Although ATP- and ADP-actin can associate and dissociate from both barbed and pointed ends in vitro, ADP-actin dissociation from the pointed ends is kinetically favored (Pollard and Borisy, 2003). This results in slow addition of monomers at the barbed end and slow dissociation of monomers at the pointed end. In growth cones, the barbed end of the actin filament generally faces the distal membrane and the pointed end faces the T region. Actin is also modified as it "ages." G-actin polymerizes onto actin filaments as ATP-actin and is hydrolyzed first to ADP-p_i-actin and then finally into ADP-actin upon phosphate release. Interestingly, several actin-associated proteins have been found to bind preferentially to these different forms of actin (Gungabissoon and Bamburg, 2003).

In growth cones, F-actin content is highest in the P and T regions of the growth cone and diminishes to varying levels in the C region of the growth cone (Figure 4). The bulk of F-actin forms two types of arrays. A polarized bundled array of F-actin composes the core of filopodia and often extends proximally into the T region of the growth cone (Figure 4). These bundled F-actin structures are generally termed F-actin ribs or actin ribs if they are present within the lamellipodial P and T regions of the growth cone. F-actin can also adopt a meshwork-like array. It is this meshwork array of F-actin that forms the bulk of the lamellipodia in the P region of the growth cone (Bridgman and Dailey, 1989; Forscher and Smith, 1988; Lewis and Bridgman, 1992; Smith, 1988).

F-actin can also take the form of other dynamic and stable structures in the growth cone. Dynamic comet-like structures that emanate from the T region and extend into the P region are termed intrapodia (Dent and Kalil, 2001; Katoh et al., 1999; Rochlin et al., 1999). The actin structure of intrapodia forms an elongated meshwork array, similar to the tails of bacterial and viral pathogens, about 1–2 μm wide and up to 5–10 μm in length (Rochlin et al., 1999). Thus, intrapodia are a hybrid of the bundled and meshwork arrays that compose the bulk of growth cone F-actin. Actin can also adopt an arc-like structure in the T region of the growth cone that has distinct kinetics from lamellipodial and filopodial actin (Schaefer et al., 2002). Other forms of F-actin include puncta, located within the central region of growth cone and axon shaft, and a thin subplasmalemmal cortical meshwork in the axon shaft (Letourneau, 1983; Schnapp and Reese, 1982). These actin puncta and subplasmalemmal network appear to be quite stable because they are insensitive to long-term incubation with the F-actin capping or G-actin sequestering drugs CB and latrunculin A, respectively (Dent and Kalil, 2001).

Microtubules
Microtubules are polarized structures composed of tubulin dimers assembled into linear arrays. Tubulin dimers are assembled from one α-tubulin subunit and one β-tubulin subunit, resulting in an α/β dimer. In mammals there are six known α-tubulin genes and seven known β-tubulin genes. Of these, three α-tubulins (α1, α2, and α4) and five β-tubulins (I, II, III, IVa, and IVb) are found in brain (reviewed in Luduena, 1998). These αβ-tubulin heterodimers are arranged in a linear array of alternating α- and β-tubulin subunits, which forms a protofilament (Figure 3). Between 11 and 15 protofilaments constitute the wall of the microtubule (usually 13 in mammalian cells), giving rise to a tubular structure approximately 25 nm in diameter (Luduena, 1998). Because these αβ dimers are arranged in a head-to-tail configuration, the MT is inherently polarized, with one end termed the "plus" end and the other the "minus" end. In most cells the plus end of the MT grows and shrinks, while the minus end of the MT is inherently unstable and shrinks unless it is stabilized, presumably by minus end capping proteins. Neuronal MTs can be extremely stable and long lived (Li and Black, 1996). Therefore, nervous system tissue is likely to be an excellent source of MT minus end capping proteins.

Any MT present in a neuron is likely to be a heterogeneous polymer composed of several combinations of α/β dimer isotypes. However, the actual isotypic makeup of individual MTs in neurons is not known. Nevertheless, it appears that different isotypes may confer distinct properties to the MT. For example, in vitro studies have shown that MTs composed exclusively of αβIII-tubulin are more stable than those assembled from αβII-tubulin (Schwarz et al., 1998) and less sensitive to vinblastine and taxol, well-known microtubule destabilizing and stabilizing drugs, respectively (Derry et al., 1997; Khan and Luduena, 2003). Other studies indicate that different α- and β-tubulin isotypes can change microtubule plus end dynamics (Bode et al., 2003; Panda et al., 1994). βIII-tubulin is generally found only in postmitotic neurons. Interestingly, the upregulation of this isotype in gliomas and in lung and prostate cancer correlates with the grade of malignancy and resistance to taxol (Katsetos et al., 2003; Ranganathan et al., 1998; Verdier-Pinard et al., 2003).

A major way in which tubulin and MTs are functionally modified is by several forms of posttranslational modification including tyrosination/detyrosination, acetylation, phosphorylation, polyglutamylation, and polyglycylation (reviewed in Luduena, 1998). Free α/β-tubulin dimers generally contain a C-terminal tyrosine residue on the α subunit. However, after assembly into microtubules, this tyrosine residue can be cleaved by tubulin carboxypeptidase, yielding detyrosinated tubulin (reviewed in Barra et al., 1988; MacRae, 1997). The penultimate amino acid, glutamate, can also be cleaved, giving delta2-tubulin (Lafanechere and Job, 2000). Most of these modifications occur at the highly divergent C terminus of both α- and β-tubulins, which is exposed on the outside of the MT. However, acetylation occurs at lysine-40 of α-tubulin, which faces the MT lumen (Nogales et al., 1999).

It is well known that MTs are heterogeneous polymers in terms of posttranslational modifications. As an example, individual neuronal MTs have been shown to be highly acetylated and sparsely tyrosinated at their minus ends, with a fairly abrupt transition to highly tyrosinated
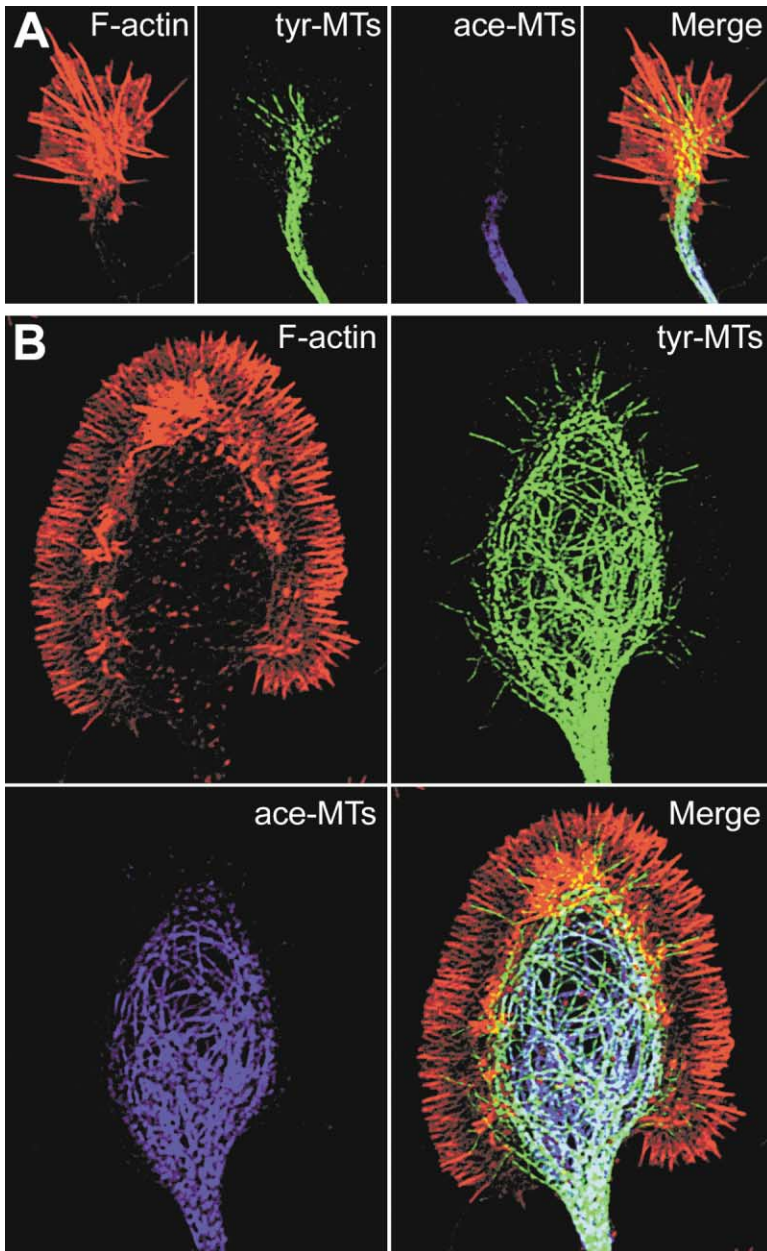
Figure 4. Distribution of Actin Filaments and Microtubules in Growth Cones

(A) A small, rapidly extending hippocampal neuron growth cone was fixed and simultaneously labeled for F-actin (with phalloidin), tyrosinated MTs (tyr-MTs), and acetylated MTs (ace-MTs) with specific antibodies. Note the prominent F-actin bundles and the splaying of tyr-MTs into the actin-rich region, often along the F-actin bundles. Acetylated MTs are much farther back in the C region and axon shaft and do not colocalize with F-actin. (B) A large, paused hippocampal growth cone fixed and labeled as above. Note the halo of F-actin around the prominent looped microtubules in the C region. Tyr-MTs also extend into the actin-rich P region, but ace-MTs are limited to the central region and do not show colocalization with F-actin. Both growth cones are shown at the same magnification.

and sparsely acetylated at their plus ends (Figure 3; Brown et al., 1993). Although degree of acetylation and detyrosination of microtubules correlates directly with the age of the microtubule, these modifications do not confer stability to the microtubule (Khawaja et al., 1988). However, it is likely that such posttranslational changes, like the hydrolysis of ATP-actin to ADP-actin, affect binding of associated proteins and thus interactions with other cytoskeletal components and intracellular signaling pathways (Bonnet et al., 2001; Boucher et al., 1994; Gurland and Gundersen, 1995; Kreitzer et al., 1999; Larcher et al., 1996; Liao and Gundersen, 1998).

MTs form a dense parallel array in the axon shaft. When they enter the growth cone, they generally splay apart (Figure 4A; Dailey and Bridgman, 1991; Forscher and Smith, 1988; Letourneau, 1983; Tanaka and Kirsch-

ner, 1991). This splaying is thought to result from MT dynamics (Rochlin et al., 1996). MTs can also adopt a looped morphology when growth cones are in a paused state (Figure 4B; Dent and Kalil, 2001; Dent et al., 1999; Morfini et al., 1994; Tanaka and Kirschner, 1991; Tsui et al., 1984). Interestingly, this looped morphology was recently discovered to be associated with synapse formation in the *Drosophila* neuromuscular junction (Roos et al., 2000). To sprout new synaptic boutons, the MT loop would transiently break down, allowing MT polymerization and transport, followed by reformation of the loop at both the old and newly formed synaptic bouton.

It was thought that MTs rarely extended into the periphery of the growth cone and were inhibited by the dense F-actin meshwork present in the P region (Forscher and Smith, 1988). However, others had found

that MTs were often present in the P region of fixed and stained growth cones, sometimes extending well into filopodia (Dailey and Bridgman, 1991; Gordon-Weeks, 1991; Letourneau, 1983). Once fluorescently labeled MTs were observed with time-lapse fluorescent microscopy, it became obvious that they could rapidly extend into and retract from the peripheral actin-rich regions of growth cones. In fact, over a period of tens of minutes, MTs can explore almost the entire P region of the growth cone through dynamic polymerization/depolymerization and transport (Dent and Kalil, 2001; Dent et al., 1999; Kabir et al., 2001; Schaefer et al., 2002; Tanaka and Kirschner, 1991, 1995).

Polymer Dynamics

Actin filaments and MTs are in a constant state of flux. An essential feature of both polymers is that they are required by the cell to exist sometimes in a stable state and other times as dynamic structures. For neurons to extend long axons and dendrites and steer these processes to their eventual synaptic partners, they must exert precise control over the dynamics of both actin filaments and MTs. To accomplish these tasks, neurons contain a complex set of actin- and MT-associated proteins in addition to the variety of isoforms and posttranslational modifications mentioned above (Gordon-Weeks, 2000). Furthermore, some cytoskeletal-associated proteins are able to influence both actin filaments and MTs (Rodriguez et al., 2003).

Actin Filament Dynamics

The dynamics of actin filaments are regulated by both their intrinsic polarity and a cornucopia of actin-associated proteins. More than twenty proteins bind directly to F- and/or G-actin and have been localized immunocytochemically to the growth cone (Table 1). We will refer to these proteins throughout the text as actin binding proteins (ABPs). It is beyond the scope of this review to discuss in detail how each of these proteins is thought to function. Furthermore, most of the functional studies of these proteins have been done in nonneuronal cells and may not always translate directly to neurons. Later in the review we will discuss how some of these proteins may function in axon guidance.

Generally, ABPs can be divided into several categories based upon their function. These categories include proteins that (1) bind and/or sequester actin monomers, (2) nucleate actin filaments, (3) cap the barbed or (4) pointed ends of actin filaments, (5) act as barbed end anticapping proteins, (6) sever F-actin, (7) bundle, cross-link, or otherwise stabilize F-actin, and (8) anchor F-actin to membrane adhesions or specific regions of the membrane. However, many of these proteins are probably capable of functioning in several of these categories depending on the internal state of the growth cone, the area in which they are localized, and whether or not they are phosphorylated. Nevertheless, it is interesting to note that the sheer number and complexity of interactions of these ABPs indicates that actin filaments are probably always well decorated and almost certainly never exist in the "naked" state shown in the cartoon in Figure 3.

Although most of the proteins listed in Table 1 have been localized to the growth cone by immunocytochem-

istry, functional studies (i.e., knockout and overexpression phenotypes, GFP-labeling and dynamic localization, immunoelectron localization, and determination of temporal and spatial activation patterns) have yet to be performed on many of these proteins. Furthermore, very few studies have directly placed ABPs downstream of specific guidance receptors. Examples include studies demonstrating that Ena/VASP proteins function downstream of both Netrin/DCC and Slit/Robo pathways (Bashaw et al., 2000; Colavita and Culotti, 1998; Gitai et al., 2003), Cofilin functions in the Semaphorin3A/Neuropilin pathway (Fritsche et al., 1999; Aizawa et al., 2001), Profilin functions in the Dlar pathway (Wills et al., 1999), AbLIM functions in the Netrin/DCC pathway (Gitai et al., 2003), Capulet functions in the Slit/Robo pathway (Wills et al., 2002), and Abl functions in the Slit/Robo and Dlar pathways (reviewed in Moresco and Koleske, 2003). In general, the role of ABPs in nonneuronal cells have been explored more extensively (reviewed in Pollard and Borisy, 2003). Nevertheless, the complement of ABPs differ between neuronal and nonneuronal cells. Therefore, it will be important to determine how the expression and activities of these neural-specific ABPs are orchestrated in growth cones during outgrowth and pathfinding. For example, are they localized to analogous structures in nonneuronal cells and growth cones (i.e., leading edge, filopodia, actin bundles)? What protein complexes exist between actin and ABPs in the growth cone and how are these regulated? One important area of future study will be to determine how these ABPs are involved in signaling cascades from guidance cues, such as Netrins, Semaphorins, Slits, and Ephrins, to the actin cytoskeleton.

Microtubule Dynamics

Like F-actin, MT dynamics are influenced by their associated proteins and by their intrinsic properties. In neurons, microtubules generally assume a plus end distal distribution in the axon and a mixed polarity distribution, with both plus and minus end distal MTs in dendrites (Baas et al., 1989). The plus ends of MTs exhibit a property termed dynamic instability, where they cycle through periods of growth and shrinkage, punctuated by occasional pauses (Mitchison and Kirschner, 1984). The transition from growth to shrinkage is termed catastrophe, and the transition from shrinkage to growth is termed rescue (Figure 3). One consequence of this intrinsic dynamic instability of MTs is that they are capable of efficiently probing the intracellular space (Holy and Leibler, 1994; Mitchison and Kirschner, 1984).

The dynamic instability of microtubules was first directly observed in vivo in fibroblasts (Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Walker et al., 1988) and later in *Xenopus* spinal neurons (Tanaka and Kirschner, 1991; Tanaka et al., 1995). However, it was only recently that microtubule dynamic parameters such as time spent extending, retracting, and pausing as well as catastrophe and rescue frequencies were measured in living neurons (Kabir et al., 2001; Schaefer et al., 2002). In these studies Forscher and colleagues used large paused growth cones from *Aplysia,* which are particularly advantageous for imaging cytoskeletal dynamics due to their large size and very slow growth rates. In the future it will be important to demonstrate directly how MT dynamics change as growth cones turn toward

Table 1. Actin Binding Proteins in the Growth Cone

Protein	Localization in GC	Proposed Function	References[a]
Abl/Arg[b]	unknown	tyrosine kinase, filament organization	Moresco and Koleske, 2003
AbLIM (unc-115)[b]	unknown	crosslinking, membrane anchor	Lundquist et al., 1998
ADF/cofilin	throughout, high in T/C region	severing, recycling	Meberg and Bamburg, 2000; Endo et al., 2003
α-Actinin	throughout	bundling	Sobue and Kanda, 1989; Sobue, 1993
Arp2/3	throughout, high in T and C regions	nucleating, side binding	Goldberg et al., 2000
Calponin	throughout	myosin ATPase inhibitor	Plantier et al., 1999
CAP (capulet)[b]	unknown	G actin buffer, sequestration, turnover	Wills et al., 2002
Ciboulot[b]	unknown	monomer binding, recycling	Boquet et al., 2000
Clipin C (coronin)	throughout	membrane anchoring	Nakamura et al., 1999
Cortactin	throughout	nucleation, membrane anchor	Du et al., 1998; Weaver et al., 2003
Ena/VASP family (Mena, VASP, Evl)	punctate throughout but concentrated at tips of filopodia	barbed end anticapping	Lanier et al., 1999; Bear et al., 2002
Fascin	prominent along actin ribs	bundling, filament stabilization	Cohan et al., 2001
Filamin	throughout	bundling, filament stabilization	Letourneau and Shattuck, 1989
GAP-43/Neuromodulin (GAP-43, CAP-23, MARCKS)	punctate throughout GC	barbed end capping/ anticapping	Dent and Meiri, 1992; He et al., 1997; Frey et al., 2000
Gelsolin	throughout GC but concentrated along actin ribs	severing, capping	Tanaka et al., 1993; Lu et al., 1997
Dpod1 (Pod-1)[c]	throughout	bundling, MT crosslinking	Rothenberg et al., 2003
Profilin (chickadee)	throughout	monomer binding	Faivre-Sarrailh et al., 1993; Wills et al., 1999; Kim et al., 2001
ERM family (Ezrin, Radixin, Moesin)	throughout GC but concentrated along actin ribs	barbed end capping	Paglini et al., 1998; Castelo and Jay, 1999
Myosin family (Ic, IIa, IIb, Va,[c] VI, X)	punctate, variable localization	transport of cargo along actin/MTs, retrograde actin flow, contractility, bundling into actin ribs	Rochlin et al., 1995; Lewis and Bridgman, 1996; Evans et al., 1997; Suter et al., 2000; Zhou and Cohan, 2001; Berg and Cheney, 2002
Spectrin/Fodrin	throughout, prominent in central region	membrane anchoring	Letourneau and Shattuck, 1989; Sobue, 1993
Synapsin I, II, III	C region and actin ribs	nucleating	Fletcher et al., 1991; Benfenati et al., 1992; Ferreira et al., 2000
Talin	punctate throughout, high in C region	membrane anchoring	Letourneau and Shattuck, 1989; Sydor et al., 1996
Thymosin-β4	throughout	sequestration, G-actin buffer, filament turnover	Roth et al., 1999
Tropomodulin	throughout	pointed end capping, filament stabilization	Watakabe et al., 1996; Fowler, 1997
Tropomyosin (5a/b)	throughout	side binding, filament stabilization	Letourneau and Shattuck, 1989; Schevzov et al., 1997
Vinculin	punctate throughout	membrane anchoring	Sydor et al., 1996; Steketee and Tosney, 2002
N-WASP	punctate, high in C region	monomer binding, nucleation	Ho et al., 2001

[a] These are only representative articles; many more have generally been published on each protein.

[b] These proteins have not been specifically localized to the growth cone but bind actin and play an important role in axon guidance. (There are several proteins that have been found in nonneuronal cells that may be found in the growth cone but have yet to be localized there, and a whole host of proteins that are known to bind to these actin-binding proteins but do not bind actin directly. These proteins have not been included in the table.)

[c] Also known to bind microtubules or act as a microtubule-associated protein.

attractive or away from repulsive axon guidance molecules.

There have been a number of indirect studies, plus a few studies in which MTs have been directly observed in the growth cone, that implicate MT dynamics as a key event in axon outgrowth, guidance, and branching. The first direct demonstration of microtubule dynamics in living neurons was performed in both *Xenopus* neural tube cultures and in grasshopper limb in situ (Sabry et al., 1991; Tanaka et al., 1993). These authors showed that MTs were capable of dynamic exploration of the entire growth cone and interconverted between splayed, looped, and bundled arrays on the order of minutes. Furthermore, they demonstrated that orientation of MTs toward the future direction of outgrowth was an early step in pathfinding, both in culture and in situ (Sabry et al., 1991; Tanaka and Kirschner, 1995; Tanaka et al., 1995). Other work, in which neurons were fixed and stained after visualization of growth cone motility and turning, confirmed and extended these observations by showing that the dynamic (tyrosinated) pool of MTs was instrumental in both axon outgrowth toward a cellular target and the turning away of growth cones from inhibitory substrate bound proteins (Challacombe et al., 1997; Lin and Forscher, 1993, 1995; Rochlin et al., 1996; Tanaka and Kirschner, 1995; Williamson et al., 1996). Recent studies demonstrate directly that MTs are transported in the axon and growth cones and how MTs dynamically reorganize when forming axon branches (Dent and Kalil, 2001; Dent et al., 1999; Kabir et al., 2001; Schaefer et al., 2002; Wang and Brown, 2002). Another study has recently shown that MTs can serve an instructive role for growth cone turning toward guidance cues (Buck and Zheng, 2002).

Like actin filaments, MT dynamics are regulated by a number of MT-associated proteins (MAPs) (reviewed in Cassimeris and Spittle, 2001). For this review we will limit ourselves to those microtubule binding proteins (MBPs) that have been shown to bind tubulin/MTs directly and have been localized to the growth cone. There are fewer MBPs than ABPs in growth cones, but the list is growing quickly (Table 2). Many MBPs that were first discovered in neurons were ascribed the role of stabilizers of MTs (MAP1B, MAP2, tau) and termed structural MAPs (Matus, 1991). Other MBPs were grouped as MT motors (dynein and kinesins) (Sheetz et al., 1989). These two distinctions generally still hold, although the diversity of MBPs has increased greatly. Furthermore, there are a number of interesting MBPs that copolymerize with MTs, termed plus end tracking proteins (+TIPs) (Carvalho et al., 2003; Schuyler and Pellman, 2001). These proteins have been implicated in plus end MT dynamics and linking MTs with actin-associated proteins in the cell cortex (Galjart and Perez, 2003). However, few of these proteins have been studied in the growth cone (Morrison et al., 2002; Stepanova et al., 2003). The functions of these proteins in axon outgrowth and guidance will undoubtedly yield many insights into how MTs respond to guidance cues and how they interact with F-actin and other intracellular targets.

Polymer Transport

In addition to polymer dynamics, F-actin and MTs also undergo directed movement through the cytoplasm as polymers. This movement occurs through the action of molecular motors. Molecular motors are well known for transporting vesicles and organelles throughout the cytoplasm on F-actin and MTs, but they are also capable of directed movement of the cytoskeletal polymers themselves. Movement of F-actin and MTs has a number of implications for axon outgrowth and guidance.

Retrograde F-Actin Flow

A well-documented phenomenon in growth cones is termed retrograde actin flow (Forscher and Smith, 1988). This constitutive phenomenon occurs as ATP-actin is assembled into filaments near the membrane in the distal P region of the growth cone and is transported rearward into the T region of the growth cone as polymeric F-actin (Lin and Forscher, 1995; Suter and Forscher, 2000). In the T region the F-actin, now composed of ADP-actin monomers, is likely severed and depolymerized by several proteins including gelsolin and ADF/cofilin (see Table 1). The ADP-actin becomes recycled into ATP-actin and the cycle is repeated (Gungabissoon and Bamburg, 2003).

The rearward transport of F-actin in the P region of the growth cone occurs in both filopodia and lamellipodia (Dent and Kalil, 2001; Lin and Forscher, 1993, 1995; Mallavarapu and Mitchison, 1999; Schaefer et al., 2002; Welnhofer et al., 1997, 1999) and is a myosin motor-driven process (Brown and Bridgman, 2003a; Diefenbach et al., 2002; Lin et al., 1996). This is sometimes referred to as actin treadmilling but actually is motor-driven transport. The difference is that if a polymer treadmills, the monomers within the polymer do not move; rather, they polymerize from one end and depolymerize from the other. This appears as movement because the polymer as a whole changes position, but the monomers within the polymer do not. Transport of F-actin has been demonstrated directly by either following photoactivation/photobleaching of a small segment of the polymer (Mallavarapu and Mitchison, 1999; Okabe and Hirokawa, 1991) or by tracking speckled filaments and following them over time (Ponti et al., 2003). If F-actin treadmilled, a small labeled region would remain stationary within the P domain of the growth cone. This does not appear to be the case. All live cell imaging of the growth cone after labeling actin filaments indicates that marked or speckled filaments move retrogradely at rates of 1–7 μm/min, depending on the neuronal cell type analyzed (Dent and Kalil, 2001; Mallavarapu and Mitchison, 1999; Schaefer et al., 2002). Interestingly, when two growth cones interact, turning toward one another, retrograde F-actin flow decreases along the axis of contact (Lin and Forscher, 1995). This decrease in F-actin flow is thought to be induced by the engagement of a clutch mechanism that bridges the F-actin cytoskeleton with cell adhesion molecules (Suter and Forscher, 2000). The engagement of this "clutch," through an unknown protein complex, attenuates retrograde flow, allowing protrusion to occur in front of the adhesion point and favoring MT invasion behind the adhesion point (Suter et al., 1998).

Currently, the exact nature of the myosin motor(s) that drive retrograde flow remain somewhat controversial. One group, using microchromaphore-assisted laser inactivation (micro-CALI) on chick dorsal root ganglion growth cones, showed that inhibition of myosin Ic

Table 2. Microtubule Binding Proteins in the Growth Cone

Protein	Localization in Growth Cone	Proposed Function	References[a]
CRMP/TUC family (TUC4,4b,CRMP1A,2A,Bs)	high in C region, extends into T/P regions	MT stabilization, orientation, vesicle transport	Byk et al., 1996; Fukata et al., 2002b; Quinn et al., 2003; Yuasa-Kawada et al., 2003
Dishevelled-1	along MTs in C/T/P domains	stabilization of MTs	Krylova et al., 2000
Doublecortin	high in C region, may extend into T/P regions	stabilization of MTs	Francis et al., 1999
Dynein/Dynactin complex[b]	throughout	minus end directed motor, plus end targeting	Abe et al., 1997; Niethammer et al., 2000
EB1,3	MT ends	dynamics/stabilization of MTs	Morrison et al., 2002; Stepanova et al., 2003
Katanin	throughout	severing of MTs	Ahmad et al., 1999
Kinesin family (Eg5, Kinesin, KIF2A)	high in C region, may extend into T/P regions	minus end motor	Ferhat et al., 1998; Morfini et al., 1997, 2001; Homma et al., 2003
Lis1	high in C region, may extend into T/P regions	plus end targeting	Sasaki et al., 2000
MAP1B[b]	high in C region, may extend into T/P regions	dynamics/stabilization of MTs	Gordon-Weeks and Fischer, 2000; Gonzalez-Billault et al., 2001
MAP2B,C[b]	high in C region, can extend into T/P regions	dynamics/stabilization of MTs, a-kinase anchoring protein (AKAP)	Fischer et al., 1986; Davare et al., 1999; Ozer and Halpain, 2000; Sanchez-Martin et al., 2000
MCAF7/short stop/kakapo[b]	throughout	MT-actin linker	Lee and Kolodziej, 2002
mDia[b]	high in C region, can extend into T/P regions	dynamics/stabilization of MTs	Arakawa et al., 2003
Stathmin family (SCG10/Stathmin/RC3/SCLIP)	high in C region, may extend into T/P regions	dynamics/stabilization of MTs	Di Paolo et al., 1997a, 1997b; Mori and Morii, 2002
Tau[b]	high in C region, can extend into T/P regions	dynamics/stabilization of MTs	Black et al., 1996

[a] These are only representative articles; many more have generally been published on each protein. Several other MT-binding proteins, such as APC, BPAG-1, CLIP-170, and CLASPs, found to play important roles in nonneuronal cells are also likely to exist in growth cones but have yet to be localized there.

[b] Also known to bind actin or act as an actin-associated protein.

caused a marked decrease in retrograde flow, whereas inhibition of myosin IIB slightly increased retrograde flow rates (Diefenbach et al., 2002). Another group, working with myosin IIB knockout mice, showed that retrograde flow was increased, similar to the aforementioned study (Brown and Bridgman, 2003b). However, this group did not find any evidence of myosin Ic staining in the T or P regions of the growth cone, implicating a different myosin, possibly myosin IIA, as the primary motor behind retrograde flow. Future experiments using selective inhibition of each myosin family member alone and in combination will likely sort out this enigma.

Interestingly, myosin II has also been implicated as an important factor for F-actin bundling in growth cones. Cohan and colleagues have recently shown that pharmacological inhibition of myosin light chain kinase, which effectively inhibits myosin II, decreases the number of actin ribs in growth cones by merging adjacent actin ribs, resulting in growth cone collapse (Zhou and Cohan, 2001). Thus, myosin II somehow functions to keep bundled F-actin arrays separated in the growth cone, maintaining the growth cone in a spread state. The way in which myosin II accomplishes this feat is currently not known. Nevertheless, both physiological and nonphysiological agents that induce growth cone collapse, some presumably acting through myosin II,

reduce actin ribs without noticeable actin depolymerization. These data are consistent with a study that showed growth cones from myosin IIB knockout mice also had decreased numbers of actin ribs and a smaller lamellar area (Bridgman et al., 2001). Furthermore, localized application of collapsing agents to one side of the growth cone is capable of inducing repulsive turning through actin rib loss (Zhou et al., 2002). Actin rib loss also causes decreases in the number of MTs on the side of the growth cone nearest the source of collapsing factor, implicating signaling between F-actin bundles and MTs. Interestingly, a recent study has shown that myosin II activity, by overexpressing myosin light chain kinase, is important in both attractive and repulsive guidance pathways (Kim et al., 2002). Thus, in addition to actin bundling/stabilizing proteins, such as α-actinin, fascin, filamin, and tropomyosin, myosin II plays an important role in regulating actin rib dynamics, growth cone spreading, and axon guidance.

Microtubule Transport

Microtubule transport has generated much controversy in recent years, mainly centering on the form that MTs take when they are transported. However, recent advances in imaging cytoskeletal dynamics show that a number of dynamic processes are involved in constructing and maintaining the microtubule array in axons

and growth cones during development (reviewed in Baas, 2002; Black, 1994; Brown, 2003). We will concentrate on the studies pertinent to axon outgrowth and pathfinding.

MTs are assembled at the centrosome, which is located in the neuronal cell body. However, they do not remain attached to the centrosome, as occurs in several other cell types, but are severed by katanin and rapidly transported away from the cell body (anterogradely) into axons, with their plus end leading, by dynein-driven transport (Ahmad et al., 1998, 1999; Dent et al., 1999; Gallo and Letourneau, 1999; Slaughter et al., 1997; Wang and Brown, 2002; Yu et al., 1996). During this transport, MTs also polymerize and depolymerize (Dent and Kalil, 2001; Wang and Brown, 2002), consistent with the fact that the axon contains high levels of tyrosinated MTs, a posttranslational modification associated with highly dynamic MTs (Baas and Black, 1990). Surprisingly, MTs are also capable of rapid retrograde movement as well (Dent et al., 1999; Wang and Brown, 2002). Thus, retrograde movement, depolymerization, and severing are all possible mechanisms for redistributing MTs during axon retraction and branch pruning (Ahmad et al., 2000; Dent et al., 1999; Gallo and Letourneau, 1999; Wang and Brown, 2002).

However, the bulk of MTs in axons are stationary at any given time, probably attached to the axonal membrane skeleton, neurofilaments, and other MTs (reviewed in Brown, 2003). If most MTs are stationary, how are new MT arrays constructed in rapidly elongating axons? As random or directed axon outgrowth occurs, a combination of MT polymer transport, tubulin transport, MT dynamics, MT severing/breaking, and possibly local tubulin synthesis contributes to a readily accessible pool of tyrosinated tubulin. This polymerization-competent tubulin exists throughout the axon but at a higher concentration in areas undergoing active growth. These active areas include the growth cone of the parent axon and dynamic areas along the axon shaft, some of which develop into axon branches. A possible scenario for how F-actin/MT dynamics and transport in the growth cone regulates directed protrusion, engorgement, and consolidation follows.

A Model for Cytoskeletal Regulation of Axon Outgrowth and Guidance

As mentioned above, we have considered axon guidance as the process of favored axon outgrowth toward or away from a particular region. Thus, axon guidance can be thought of as a process of biasing the extension/retraction of one side of the growth cone or axon shaft, as in the case of collateral branching, compared to the other side (reviewed in Tanaka and Sabry, 1995). How can this model be reconciled with the convincing evidence that exists in *Drosophila* that axon branching, turning, and outgrowth are distinct processes, depending on the level of Rac activity in the neuron (Ng et al., 2002)? We propose that the key to resolving this apparent paradox is to keep in mind that many studies have shown that both guidance and branching can be selectively inhibited without affecting axon outgrowth per se (Buck and Zheng, 2002; Challacombe et al., 1996, 1997; Dent and Kalil, 2001; Marsh and Letourneau, 1984;

Tanaka et al., 1995; Williamson et al., 1996). All of these studies specifically targeted actin and/or MT dynamics. Therefore, it is likely that outgrowth, guidance, and branching are all linked to the level of activity of F-actin and MTs, which are probably controlled by the activity of upstream components, such as Rac. Interestingly, in fibroblasts, polymerizing MTs are thought to stimulate Rac activity (Waterman-Storer et al., 1999; Wittmann and Waterman-Storer, 2001; Wittmann et al., 2003). Therefore, there is probably bidirectional signaling between cytoskeletal elements and proteins with which they are associated.

On the cytoskeletal level, the process of axon guidance shares many similarities with the process of polarization and chemotaxis in nonneuronal cells (Song and Poo, 2001; Rodriguez et al., 2003). In this sense the growth cone too is a polarized structure. A recent model that has emerged to explain the underlying microtubule reorganization that accompanies polarization and chemotaxis has been termed microtubule capture (Gundersen, 2002). This phenomenon has been most thoroughly studied in budding yeast and polarizing fibroblasts and may take place in the neuronal growth cone as follows. Dynamic microtubules probe the intracellular environment. During this process, the plus ends of microtubules come into contact with F-actin-rich cortical and/or adhesion sites (Fukata et al., 2002a; Krylyshkina et al., 2003). When microtubules contact these cortical sites along the inner membrane, microtubule tip-associated proteins act as "ligands" for "receptors" in these actin-rich regions. Such a ligand/receptor complex has been documented in fibroblasts in which activated Rac1/Cdc42 demarcate active regions along the cell cortex and act as "receptors," along with IQGAP1, to transiently capture the plus end microtubule tip binding protein CLIP-170 (Fukata et al., 2002b). This interaction is believed to convey signals between the cell cortex and the cytoskeleton that allow for continued actin dynamics and membrane insertion needed for polarization and chemotaxis (Gundersen, 2002).

Can this model for fibroblast polarization be applied to the polarization and turning of a growth cone toward or away from guidance cues? Many of the same ABPs and MBPs found to be instrumental in fibroblast polarization exist in neurons as well (Tables 1 and 2). Thus, it is likely that microtubules will be found to interact transiently with cortical structures in the growth cone, which may allow localized insertion of membrane and signaling proteins essential for growth in a preferred direction (Zakharenko and Popov, 1998). Nevertheless, this process of transient MT capture is unlikely to be sufficient for directed growth to continue. If so, there should be some mechanism operating in tandem that allows MT stabilization and recruitment of more MTs in the favored direction of growth. When microtubule dynamics have been recorded in living growth cones, microtubules have never been shown to attach to the actin-rich cortex for more than a few seconds (Dent and Kalil, 2001; Dent et al., 1999; Kabir et al., 2001; Schaefer et al., 2002). Thus, it is unlikely that growth cone turning involves "pioneer" microtubule capture on one side of the growth cone, followed by recruitment of other microtubules along the pioneer MT.

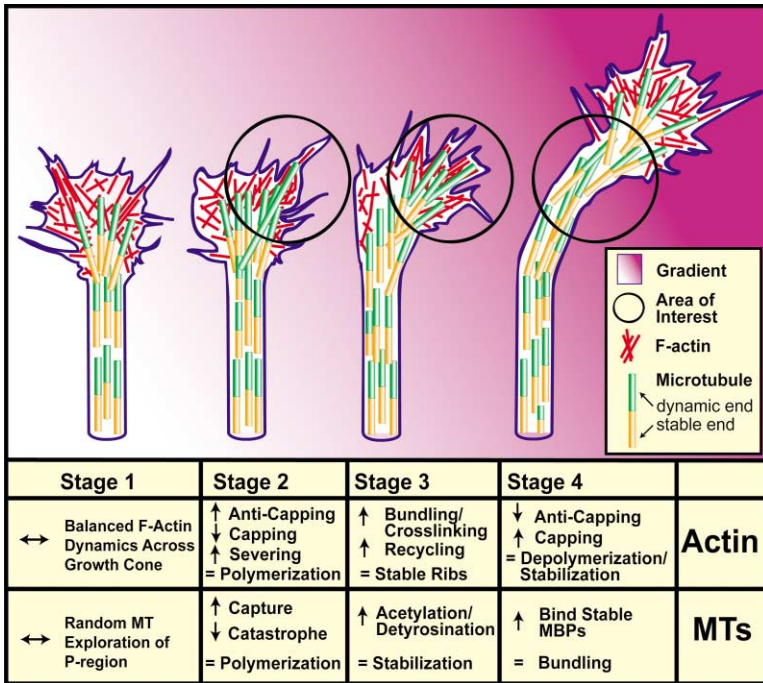If transient interactions are not sufficient, then what

Figure 5. Hypothetical Model for the Cytoskeletal Reorganization Underlying Growth Cone Turning

Stages of growth cone turning in an attractive gradient (pink shading). All stages are in reference to the area of interest (black circle). Stage 1: F-actin takes the form of dynamic bundled filaments (in filopodia) and meshwork array (in lamellipodia). We assume that there are balanced F-actin dynamics across the growth cone. MTs form a thick bundle in the axon shaft and splay apart in the growth cone, stochastically exploring the P region. Stage 2: When a chemoattractant is detected, F-actin selectively polymerizes on the side of the growth cone toward the attractant, forming increased numbers of filopodia and lamellipodia. This bias of actin polymerization to one side of the growth cone causes less polymerization on the opposite side, favoring shrinkage/retraction of lamellipodia and filopodia. Microtubules continue stochastically probing the P region but may be transiently captured at actin-rich regions and undergo fewer catastrophes when they interact with F-actin bundles on the right side of the growth cone. Stage 3: Numbers and size of F-actin bundles (ribs) increase and are stabilized by actin-bundling proteins and linkages to the substrate. Because MTs exploring the actin-rich side of the growth cone are maintained in the polymerized state longer than the MTs on the opposite side, they may be selectively posttranslationally modified. Stage 4: F-actin is depolymerized, capped, and stabilized into puncta and a subplasmalemmal network. MTs are subsequently stabilized by recruitment of stabilizing MBPs, causing them to bundle in the newly oriented axon shaft.

process would be required? It is likely that proximal stabilization of MTs is important (Mack et al., 2000). Indeed, when microtubules have been pharmacologically stabilized on one side of the growth cone by focal uncaging of taxol, growth cones turn toward the side in which MTs have been stabilized (Buck and Zheng, 2002). Conversely, when the microtubule-destabilizing drug nocodazole is locally applied to one side of the growth cone, turning to the opposite side occurs (Buck and Zheng, 2002). If local stabilization/destabilization of the microtubule array is instrumental in directional guidance, then there must be an intrinsic mechanism for this to occur. As noted in Table 2, there are many microtubule stabilizing/destabilizing proteins found in growth cones. Furthermore, neurons are one of the few cell types that contain high levels of posttranslationally modified tubulin (acetylated, detyrosinated, delta2-tubulin) that correlate with microtubule stability (Challacombe et al., 1996; Dent and Kalil, 2001; Paturle-Lafanechere et al., 1994; Williamson et al., 1996).

One possible scenario for the underlying cytoskeletal involvement in directed protrusion, engorgement, and consolidation in response to an attractive cue is as follows (Figure 5). Obviously, similar mechanisms are involved when a growth cone turns in response to a repulsive cue. We assume that there is balanced actin polymerization and depolymerization across the growth cone over time, when the growth cone is involved in random locomotion. This would result in balanced protrusion/retraction so that the growth cone would maintain a straight trajectory. When a chemoattractant is detected on one side of the growth cone, F-actin-driven protrusion is favored on that side of the growth cone

(Bentley and O'Connor, 1994; Lin et al., 1994). This preferential protrusion can take the form of filopodia, lamellipodia/ruffles, and intrapodia (Figure 5, stage 2). Thus, actin anticapping and leaky capping proteins, such as Ena/VASP proteins and GAP-43, respectively, are likely to play important roles in this initial protrusion phase because these proteins are known to enhance actin polymerization (Bear et al., 2002; Dent and Meiri, 1998; He et al., 1997; Lanier et al., 1999). Additionally, actin-capping proteins may be inactivated in these regions of protrusion.

These newly formed protrusions must then be stabilized against retraction. Any number of F-actin bundling/stabilizing proteins are likely to be essential for the continued protrusion of filopodia and lamellipodia (Table 1). Also, gelsolin has been found to destabilize filopodia, possibly through its capping ability, allowing retraction to take place (Lu et al., 1997). Thus, gelsolin and other capping proteins may be inactivated locally. Furthermore, it is not sufficient to simply stabilize these F-actin structures because they are subject to myosin-based retrograde transport, resulting in growth cone collapse (Ahmad et al., 2000; Gallo et al., 2002). Instead, the bundled and crosslinked actin filaments must be stabilized through a mechanism that involves adhesion to the substrate (Suter and Forscher, 2000). In addition, severing/recycling proteins such as cofilin and profilin may be activated to keep the level of free G-actin and barbed ends high for continued polymerization (Endo et al., 2003; Meberg and Bamburg, 2000).

Because MTs stochastically explore the growth cone P region, certain MTs may be transiently captured at actin-rich regions (Figure 5, stage 2) through interactions

of microtubule tip binding proteins with ABPs, or they may be less likely to undergo catastrophe when in association with F-actin bundles (Buck and Zheng, 2002; Dent and Kalil, 2001; Schaefer et al., 2002; Zhou et al., 2002). It follows that by polymerizing and/or being transiently captured on one side of the growth cone, those microtubules are more likely to be acetylated and detyrosinated because the enzymes responsible for these modifications act only on MTs and not free tubulin (Contin and Arce, 2000; MacRae, 1997). As mentioned above, these posttranslational modifications may allow preferential binding of stabilizing MBPs to those specific MTs. When the process of MT polymerization and depolymerization, coupled with transient MT capture at actin-rich regions, local membrane delivery (engorgement), and proximal MT stabilization, is iterated many times over a period of minutes, preferential turning in the direction of chemoattractant occurs (Figure 5, stage 3). If a chemorepellent is encountered instead of a chemoattractant, the preferential interactions and stabilization would occur on the side of the growth cone opposite to the repellent. In either case, the result would be the same—directional guidance.

To continue to turn, the proximal growth cone must consolidate into a cylindrical shaft (Figure 5, stage 4). This process has received relatively little attention to date but would have to include the selective downregulation of dynamic F-actin and MTs. One interesting observation is that acetylated and detyrosinated MTs are rarely found in the P region of the growth cone but become prevalent in the C region concomitant with a marked decrease in F-actin in the same region (Challacombe et al., 1997; Dent and Kalil, 2001). Thus, it will be interesting to see if these posttranslational modifications of tubulin result in recruitment of proteins that suppress F-actin polymerization and/or favor depolymerization. This would cause F-actin dynamics to be suppressed and leave a stable punctate and subplasmalemmal network of F-actin quite resistant to depolymerization. These stable, short actin filaments may be associated with adhesion points along the axon shaft and play a role in maintaining the structure of the axon.

Conclusions and Future Directions

This review has presented a working model for cytoskeletal reorganization during axon elongation and turning. We have proposed that axon guidance results from directed protrusion, engorgement, and consolidation. Thus, although axon outgrowth, guidance, and branching are differentially susceptible to cytoskeletal perturbations, we believe they are likely to be a continuum, rather than truly distinct processes. Nevertheless, to substantiate this hypothetical model, it will be necessary to image actin filaments and microtubule dynamics in growth cones that are exposed to gradients of chemoattractants and chemorepellents both in culture and in vivo. It will also be important to test the functions of ABPs and MBPs by high-resolution imaging of cytoskeletal dynamics after knocking out or overexpressing specific proteins.

We are in a very exciting time in the cell biology of the growth cone. Due to the advances in genetics, molecular manipulations, protein labeling, and fluorescent time-

lapse microscopy, we are beginning to tease out the details of F-actin and MT dynamics and transport as they function in directed outgrowth. There are many actin binding and tubulin binding proteins that have been found in the growth cone but whose functions in axon guidance are just beginning to be discerned. Obviously, high-resolution imaging in cell culture will provide important clues into the function of actin and MTs. However, it is essential to relate these findings back to the living organism. The many elegant genetic studies in *C. elegans*, *Drosophila*, zebrafish, and mice and the molecular studies in chick, grasshopper, and *Xenopus* have provided crucial knowledge about how growth cones are guided to their targets in the living organism. For the most part, these in vivo studies have supported the findings in cell culture, validating this methodology for studying growth cone motility and directed outgrowth. These studies from nonmammalian organisms have also substantiated the importance of proteins whose functions have been conserved over millions of years of evolution.

Nevertheless, there has been a paucity of studies of cytoskeletal dynamics in in vivo or in situ preparations from vertebrates and none that we know of from intact mammalian preparations. We now have the means to genetically and pharmacologically manipulate neurons while imaging them in complex environments, at high resolution and over extended periods of time (Danuser and Waterman-Storer, 2003; Yuste et al., 2000). It will be revealing to probe these complex environments to determine how cytoskeletal dynamics contribute to axon outgrowth, guidance, and branching in the intact developing nervous system. The coming years promise to yield many advances in our understanding of the coordinated dynamics of the actin and MT cytoskeletons and the proteins that link them in neurons as well as other systems (Rodriguez et al., 2003). The most exciting findings are likely to bridge the present gap in knowledge of how guidance factors transduce their signals to the cytoskeleton and the subsequent response of the cytoskeleton to yield directed axon outgrowth.

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