

# Cytoskeletal Dynamics and Nerve Growth

## Review

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### Introduction

Nerve cells establish their connections by extending neuritic processes away from the rather fixed position of the cell body. In the case of axons, this growth can be extensive and the axon itself can contain more than 99% of the cytoplasmic volume (Heidenhain, 1911). There is abundant evidence that the cytoskeleton is the major internal structure defining the morphology of neurons (Letourneau, 1982; Lockerbie, 1987). We feel that an understanding of how the axonal cytoskeleton is assembled would largely explain the overall intracellular process of neuronal growth. According to this view, the information from both intrinsic and extrinsic sources that governs the direction and morphology of neuronal processes acts either directly or indirectly to influence the spatial organization of the cytoskeleton. While the correct spatial organization of membrane elements is obviously also important, we suggest that this is largely defined by cytoskeletal elements. We will not discuss explicitly membrane dynamics except as they affect the cytoskeleton, nor will we discuss explicitly extrinsic guidance cues. In this review we shall focus on the role of actin microfilaments and microtubules in neuronal growth. We shall try to outline a number of molecular possibilities that could explain cell motility at the growth cone and extension of the axon process. Using knowledge of axonal behavior and the biochemical properties of actin and tubulin, we shall try to narrow down the number of possible models at this time. Much of our information will come from examples from nonneuronal cells, and we will use this information to develop a model for nerve cell growth. We shall not consider the role of neurofilaments. Their slow rate of monomer-polymer exchange seems inconsistent with their playing a major role in the initial elaboration and stabilization of growing axons (Williams and Runge, 1983). Furthermore, from the point of view of axon growth, we note that arthropods apparently manage to establish nervous systems without these polymers (Phillips et al., 1983) and in mammals early neurite outgrowth can occur without their expression (Shaw et al., 1985).

### Nerve Growth Can Be Divided into Three Processes

An early breakthrough in the understanding of neuronal growth came from the realization by Ramon y Cajal that the network of nerve fibers represented highly extended processes of single cells (Ramon y Cajal, 1909). He iden-

tified the growth cone as the actively motile structure at the extremity of these cells during the process of axon outgrowth. As the growth cone advances, the axon is elaborated behind it, and by this simple process, the basic anatomy of the nervous system is generated. We know that the protein building blocks for the construction of new lengths of axon are synthesized in the cell body and transported down the axon (Grafein and Forman, 1980). The question of whether cytoskeletal elements are transported down the axon as monomers or polymers is currently quite controversial, so we cannot presently decide whether actin and tubulin constitute a functional part of the axonal cytoskeleton during transport (Black and Lasek, 1980; Nixon and Logvinenko, 1986; Bamberg, 1988). We shall argue, however, that both actin and tubulin assemble into the stable axonal cytoskeleton at the neck or the central region of the growth cone (a region that can be defined morphologically as the final extremity of the majority of microtubules and membrane-bound vesicles) (Letourneau, 1983) and that it is at this point that the spatial organization of these polymers in the growing axon is defined. Consequently, it is the region around the neck of the growth cone where decisions are made as to which direction the axon will grow.

From these considerations we shall argue that directed neuronal growth results from the combination of three processes: transport of precursors to the growth cone, active motility of the growth cone, and assembly of the axonal cytoskeleton at the neck of the growth cone. We shall organize this review principally on the basis of anatomy, both cellular and molecular. Our aim will be to offer a molecular explanation for the last two of these processes and to analyze how they contribute to directing neural growth toward specific targets.

### The Growing Axon Consists of Two Discrete Cytoplasmic Domains

Already implicit in our discussion of the growth of an axon was the division of its cytoplasm into two domains: the axon tube and the growth cone. We shall refer to the cytoplasm in these two domains as axoplasm and kinetoplasm, respectively (see Figure 1). Axoplasm is specialized for transport, and microtubules dominate its cytoskeleton, although actin is also present in the axon as a cortical shell (Hirokawa, 1982; Schnapp and Reese, 1982). Kinetoplasm is specialized for motility and locomotion and is dominated by actin filaments (Letourneau, 1983). In addition, there is a transition zone between the kinetoplasm and the axoplasm containing less organized vesicles in the central region of neurons (Goldberg and Burmeister, 1986). Despite these differences, the protein content of the kinetoplasm and axoplasm is likely to be quite similar. The major difference lies in the spatial organization and dynamics of their cytoskeletons. In the axoplasm tubulin and actin are

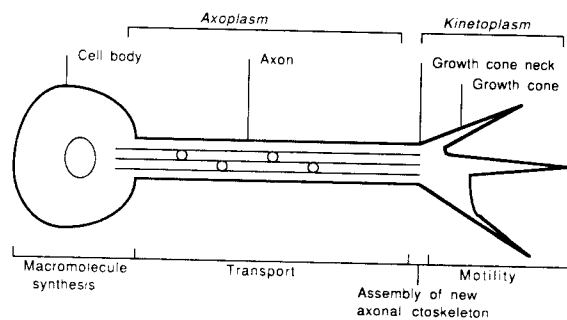


Figure 1. Neuroanatomy for Beginners

present both in their transport states and as part of the stable axonal cytoskeleton, while in the kinetoplasm they are present in highly dynamic polymer and monomer forms (Figure 2). Individual tubulin and actin molecules probably spend a limited time in the kinetoplasm, during the transition between their transport state and their incorporation into the stable axonal cytoskeleton. A molecular picture of nerve growth should explain the compositional and organizational differences between axoplasm and kinetoplasm and, in particular, how changes in the organizational state of actin and tubulin are controlled.

### Actin in the Growth Cone

Actin filaments make up much of the mass of the lamellipodial regions of growth cones. The lamellipodium is an extended, thin veil at the periphery of the growth cone that excludes most organelles. The actin filaments in the lamellipodia constitute much of the density seen by phase or interference contrast microscopy (Yamada et al., 1971). In addition to lamellipodia at the leading edge, filopodia extend out from the periphery. These are long, thin extensions with a dense actin core. The leading edge of the growth cone is homologous both structurally and functionally to the leading edge of other motile eukaryotic cells (Trinkaus, 1984). Both filopodia and lamellipodia in motile cells consist of bundles and meshworks of actin filaments oriented with their barbed or plus ends toward the leading edge. It is not known what generates this polarity of actin relative to the plasma membrane. It is a uniform feature of eukaryotic cells (Small et al., 1982; Tilney, 1983) and is probably intimately related to the mechanism of actin assembly (discussed below).

Actin in the growth cone is extremely dynamic. Both filopodia and lamellipodia are extruded at the front of the growth cone. Most of these structures do not persist and are either retracted or swept backward (Bray and Chapman, 1985; Goldberg and Burmeister, 1986). Since the organization and behavior of filopodia and lamellipodia have important similarities in the structure and dynamics of actin, we will, for simplicity, base all of the discussions in this review on filopodia and assume that they extend to lamellipodia.

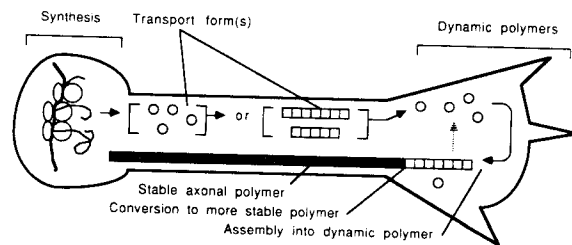


Figure 2. Different Assembly and Transport States of Cytoskeletal Proteins in Growing Neurons

The synthesis of the cytoskeletal proteins (actin and tubulin) is shown in the cell body. Whether they are transported as monomers, in complexes, or as polymers is controversial at this time. They are shown being incorporated into dynamic polymers in the neck of the growth cone.

Direct observation of growth cone motility has failed to produce a clear picture of the mechanisms of growth cone movement. Paradoxically, time-lapse observations of advancing growth cones often give an impression of retrograde movement. In the leading edge of both growing and stationary growth cone inhomogeneities in the cytoplasm, detached filopodia, and small particles on the surface of the cell continuously move backward at similar rates (Bray and Chapman, 1985; Forscher and Smith, 1988). This retrograde flow has been observed in all motile eukaryotic cells that move by protrusive activity of a leading edge (Abercrombie et al., 1970). It was described by Abercrombie for fibroblasts as looking like a fountain in reverse. Retrograde movement probably reflects the basic motor for actin-dependent motility. To understand this motor, we need to explain the filament dynamics that underlie it and the processes that power it. Furthermore, we need to explain how the motor is regulated to both generate and direct motility. We shall discuss a few fundamental questions about actin dynamics, the answers to which would help significantly to define the mechanism for growth cone motility.

### Where Do Actin Filaments Add and Lose Subunits in the Growth Cone?

Actin filaments are polar polymers. The two ends differ in their critical concentration for polymerization and in their absolute rates of polymerization and depolymerization (Korn, 1982; Pollard and Cooper, 1986). The barbed end *in vitro* is the preferred site of actin depolymerization, and despite its association with the plasma membrane, most evidence now suggests that it is the preferred site for monomer addition *in vivo*. It has been shown directly in sea cucumber sperm acrosomes and in permeabilized intestinal brush border microvilli that actin polymerization leads to protrusion of the membrane at the tip of the process, suggesting that subunits are added proximally to the membrane (Tilney and Inoue, 1982; Mooseker et al., 1982). Information about actin dynamics in the leading edge structure of fibroblast cells has come from photobleaching experiments, which demonstrated a net flux of actin filaments toward the

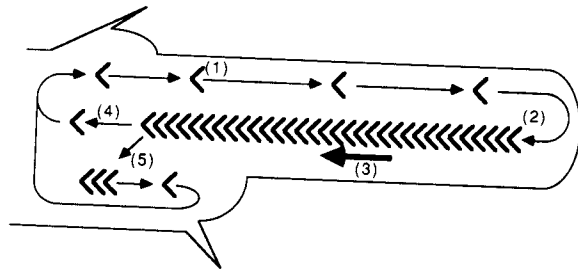


Figure 3. Actin Dynamics in the Growth Cone  
Actin subunits (1) diffuse to the tip of the filopodial process (2) where they add to the barbed end of an actin filament, whose polarity is denoted by the chevrons. The actin filament is being translocated (3) toward the center of the cell where it depolymerizes into monomers from the pointed end (4) or where it is fragmented into pieces, which in turn depolymerize from their barbed ends (5).

center of the cell (Wang, 1985). This flux suggests that the continual addition of subunits at the membrane is coupled to either translocation of the filaments toward the center of the cell or depolymerization at the pointed ends of the actin filaments. Indirect evidence suggests that this flux also occurs in neurons. Video microscopy of *Aplysia* growth cones demonstrated with particular clarity the continuous movement of cytoplasmic density—presumably actin filaments—away from the leading edge (Forscher and Smith, 1988). These data are summarized in Figure 3, which shows the addition of subunits at the membrane and the translocation of the actin filaments toward the interior of the cell. The observable retrograde flow of actin filaments must be balanced by an invisible forward movement of subunits, which then polymerize at the membrane-associated barbed ends. Since only organized polymer, and not individual subunits, gives rise to structures visible by light microscopy, there is a paradoxical impression of retrograde flow in a cell process that can be moving forward.

Is this flux consistent with what we know about actin polymerization *in vitro*? The rate of retrograde flow is about 3  $\mu\text{m}/\text{min}$  (Forscher and Smith, 1988), corresponding to a polymerization rate of 18 subunits per  $s$  (Pollard, 1986). Pure actin filaments *in vitro* elongate at this rate at a monomer concentration of 1.7  $\mu\text{M}$ . Monomeric actin is at a considerably higher concentration than this *in vivo*, but most of the monomer is bound to profilin (Pollard and Cooper, 1986). It is presently difficult to estimate what the expected elongation rate of a free barbed end would be *in vivo*, but the retrograde flow rate is certainly not too fast to be balanced by actin polymerization.

Events at the other end of the polymer present a problem, however. The off rate for ADP actin from the pointed end *in vitro* is only 0.27  $s^{-1}$  (Pollard, 1986), much slower than the flux rate. The kinetic inertness of the pointed end represents an enigma for a system of polymerization-depolymerization in which there is a continuous high flux. Perhaps depolymerization is accelerated by some unknown factor *in vivo*. Alternatively,

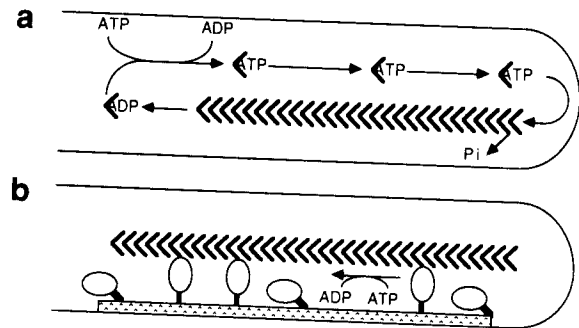


Figure 4. Two Ways of Powering Retrograde Actin Filament Flow

In (a) retrograde actin flow is powered by the insertion of subunits between the membrane and the barbed end of the actin filament and the simultaneous loss of subunits from the pointed end nearer to the center of the cell. This process can occur because it is coupled to ATP hydrolysis. The subunits adding to the tip have bound ATP, which is hydrolyzed soon after polymerization. Hence the bulk of the polymer contains ADP actin. ADP actin disassembles from the pointed end. ATP is exchanged for ADP in the monomer before assembly.

In (b) retrograde actin flow is powered by myosin. This is most likely myosin type I, which is shown as ellipses anchored to an as yet undefined stable and nonmoving submembranous matrix. Force exerted by the myosin accompanying ATP hydrolysis drives the actin filaments to the left of the figure, as dictated by the actin polarity and the properties of myosin.

actin may not depolymerize directly from its pointed ends. We could imagine filament-severing proteins (Stossel et al., 1985; Pollard and Cooper, 1986) cutting the filaments into small pieces at an internal site, thus generating a large number of ADP barbed ends, which could each depolymerize at 7.2  $s^{-1}$  (Pollard, 1986) (Figure 3). Presently we do not know the exact mechanism of actin depolymerization. We do know that the depolymerizing form from the barbed end is ADP actin, while the polymerizing form is ATP actin. Hence assembly is coupled to ATP hydrolysis, and this coupling allows the polymer to escape the rigid thermodynamic constraints that apply to equilibrium polymers (Kirschner and Mitchison, 1986). Were the actin system at equilibrium, it would not be possible to form filaments continuously at one location in the cell, while depolymerizing them elsewhere.

#### How Is the Retrograde Flow of Actin Driven?

Theoretically, force can be generated purely from polymer dynamics when ATP hydrolysis is coupled to polymerization (Figure 4a) (Hill and Kirschner, 1982). The retrograde flux of subunits at the leading edge of the cell could therefore, in principle, be driven by the insertion of subunits between the barbed end and the plasma membrane. The action of cytochalasin on *Aplysia* growth cones renders this explanation for force generation unlikely (Forscher and Smith, 1988). Cytochalasin (which binds to the barbed end of actin filaments and prevents addition to this end while allowing loss at the pointed end) caused the detachment of actin from the leading edge but did not block retrograde flux. This result argues against simple models in which flow is driven complete-

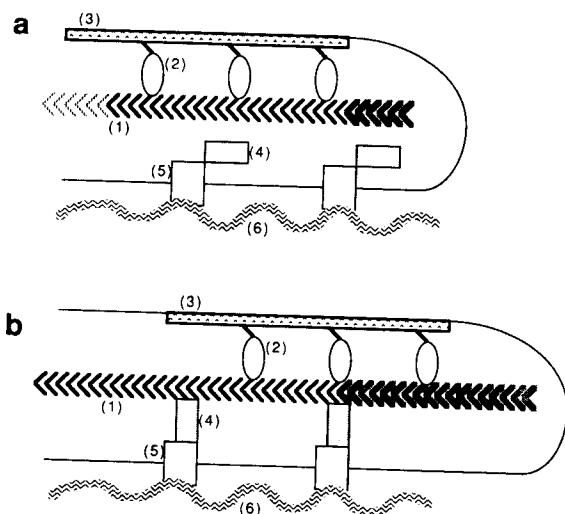


Figure 5. Tension and Forward Movement Is Produced When Actin Retrograde Flow Is Blocked by Binding to Receptors

In (a) a futile cycle of actin assembly (shaded chevrons) and disassembly (faint chevrons) and retrograde flow is produced by the action of myosin on the actin filaments, when there is no mechanical linkage with the extracellular substratum. Actin is shown dissociating from its pointed end (1), while the actin filament is being driven to the left by myosin (2) attached to a rigid submembranous matrix (3). The actin filament is not interacting with the talin (4), which is bound to the integrin receptor (5) through the membrane to the extracellular substratum (6).

In (b) tension and forward movement is produced when there is a mechanical interaction between the talin and the actin filament. Under these circumstances, the actin filament is stationary by virtue of its rigid mechanical interaction with the extracellular substratum (6) via integrin (5) and talin (4), which is now shown attached either directly or indirectly to the actin. The myosin (2) and its stable submembranous element (3) now crawl to the right on the rigid actin filament. New actin subunits add at the barbed end (shown as shaded chevrons), leading to protrusion.

ly by polymer dynamics constrained by a rigid barrier.

Retrograde translocation of actin filaments could be driven by myosin (Figure 4b). Conventional myosin, now called myosin type II, has generally not been found in large amounts near the leading edge (Zigmond et al., 1979; Letourneau, 1981; Yumura et al., 1984). This suggests that its role in motility is at most an indirect or global one, such as extruding cytoplasm by squeezing a distant part of the cell like a toothpaste tube. The recent disruption of the myosin type II gene in slime molds without impairment of motility reinforces the argument that myosin II is not involved in lamellipodial protrusion (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987). Recently, however, type I myosins that may drive the retrograde flow of actin have been described (Korn et al., 1988). These lack a helical tail with which to form filaments, and probably interact with cellular membranes. For myosin to drive retrograde actin flow, as shown in Figure 4b, the myosin must be tethered to some stable submembranous structure that will not tend to move freely with the plasma membrane. In this model force generated by myosin would tend to drive either the

actin filaments backward or the membranous components forward. It has been shown recently that a 110 kd myosin I in the intestinal microvillus links the core actin filaments to the membrane (Mooseker, 1985; Collins and Borysenko, 1984). This myosin has actually been found to exert force in *in vitro* experiments (Mooseker et al., 1988). Interestingly, the maximum velocity that this myosin has been demonstrated to move on actin filaments *in vitro*,  $3 \mu\text{m min}^{-1}$  for myosin membrane plaques, is very similar to the maximum rate of retrograde actin flux in growth cones *in vitro*. We expect that these small myosins may be widely distributed and that ATP hydrolysis by myosin I is the best candidate for providing the motile force on actin filaments (Figure 4b).

### When Does Actin Polymerization Lead to Protrusive Activity?

We have described filopodia in steady state, where the provision of actin subunits at the plasma membrane is balanced by translocation of the filament bundle toward the cell body. Most often filopodia are in fact either advancing or retracting (Bray and Chapman, 1985). Whether the leading edge protrudes or retracts is dependent on the balance between polymerization onto the barbed ends, retrograde movement of the filaments, and whether myosin-containing submembranous structures move freely with the actin or are stationary relative to the actin filaments. Retrograde flux continues in both rapidly moving and stationary growth cones (Bray and Chapman, 1985; Forscher and Smith, 1988), so it may be a relatively constant parameter—perhaps working at the full speed of the type I myosin. Thus whether a filopodium protrudes or retracts is probably decided by the rate of polymerization. This rate is governed by both the local actin monomer concentration and the accessibility of the barbed ends. Accessibility can be controlled by the physical barrier of the membrane or by specific capping proteins. Sudden extension—such as occurs during filopodial or lamellipodial protrusion—could therefore result from any combination of increased monomer concentration, decreased affinity of a capping protein, or decreased membrane resistance (local softening), such as might occur as a result of exocytosis. In the sea cucumber sperm acrosome, protrusion seems to be driven by increased monomer concentration due to dissociation of actin from its complex with the monomer binding protein profilin (Tilney et al., 1978). There is some evidence that the profilin-actin complex can be regulated by second messengers in other cells (Hansson et al., 1988). Recent experiments have shown that receptors can regulate the polymerization of actin off washed membranes (Carson et al., 1986), suggesting that second messenger systems might regulate actin capping factors.

### How Does the Retrograde Flow of Actin Generate Tension?

Advancing growth cones generate tension on the substratum (Bray, 1979). We are interested in the role actin dynamics play in this process. To generate tension, the growth cone must make attachments to the substratum.

Attachment is mediated by receptors such as the integrin family for extracellular matrix (Hynes, 1987) and the cadherin family for proteins on other cell surfaces (Bixby et al., 1987). There is biochemical and morphological evidence that these receptors interact with actin filaments, probably through coupling molecules such as talin (Horwitz et al., 1986). As shown in Figure 5a, when extracellular matrix receptors (5) cannot interact with actin filaments (1) via talin or some other linker (4), the myosin (2) that is semirigidly held to the membrane (3) will generate a continuous translocation of actin filaments toward the pointed end (to the left in Figure 5a). New subunits will add at the barbed end, producing a moving carpet of actin. However, when extracellular receptors latch onto the actin filaments (Figure 5b) and anchor them via linkers (4) to the substrate, tension between the myosin (2) and the substrate (6) can now be exerted. If the membrane-bound myosin can move with no resistance, the entire myosin submembrane structure (2 and 3), possibly including the membrane itself, will be translocated out from the cell (to the right in Figure 5b). If resistance is encountered, tension will be exerted by the myosin, through the actin, the intracellular linkers, and the extracellular matrix receptors onto the substrate. Retrograde flux is continuous, whereas forward movement is variable; therefore, the coupling between actin and receptors must incorporate variable slippage—a molecular clutch. Most likely, the slippage occurs intracellularly, somewhere in the talin interface. The integrin-talin and talin-actin binding affinities are both rather weak, and may be regulated by phosphorylation and proteolysis (Hirst et al., 1986; Beckerle et al., 1987; DeClue and Martin, 1987).

### Actin in the Axon Tube

Actin in the axon tube has properties that are strikingly different from those of actin in the growth cone. Filaments are organized parallel to the membrane and parallel to the axis of the axon tube (Schnapp and Reese, 1982). This organization can be thought of as an extension of the geometry of actin filaments in the growth cone (Figure 3). However, in the growth cone this same organization results in actin filaments extending normally to the plasma membrane of the leading edge. The axoplasm does not possess the protrusive activity of the kinetoplasm, and there is no obvious retrograde movement of actin in the axon tube. There are reasons to think that the axoplasm has a latent capacity for protrusive activity. If the axon process is severed, a new growth cone rapidly arises in both segments at the site of cutting (Shaw and Bray, 1977). Furthermore, colchicine treatment leads to axon collapse, but during this process, extensive protrusive activity occurs all along the axon (Bray et al., 1978).

### How Is Actin Activity Shut Down in the Axoplasm?

The response to colchicine suggests that microtubules play a role in suppressing the actin dynamics. Microtubules have been implicated in suppressing actin-based

motility in fibroblast cells (Vasiliev et al., 1970) and have a poorly understood role in modifying actin polymerization during cleavage (White and Borisy, 1983). Three mechanisms that are not mutually exclusive can be considered for the suppression of actin dynamics in the axon tube:

- Direct protein-protein interactions between microtubule binding proteins and actin filaments. In general, microtubule-associated proteins are bipartite, with one domain interacting with the microtubule lattice and the other free for interaction with other components (Vallee, 1984; Himmier et al., submitted), such as actin filaments (Griffith and Pollard, 1982).
- Modulation by calcium ions. Several actin binding proteins are known to be controlled by calcium over the physiological concentration range (Stossel et al., 1985; Pollard and Cooper, 1986). Microtubules are known to act as tracks for movement of the smooth endoplasmic reticulum (Terasaki et al., 1986), and this organelle is capable of actively modulating calcium ion concentration by sequestration and release (Somlyo, 1984). Axoplasm is densely populated by microtubules and smooth endoplasmic reticulum (Henkart et al., 1978) and for that reason may have a free calcium concentration different from that found in kinetoplasm (Connor, 1986).
- Modulation through tension. Protrusive activity in epithelial cells can be rapidly and reversibly suppressed by exerting tension on the cell; this tension was shown to reorient actin filaments from a radial organization normal to the plasma membrane to a circumferential organization parallel to the plasma membrane, where they could not engage in protrusive activity (Kolega, 1986). Actin filaments in the axon tube are also under tension (Bray and White, 1988; Dennerll et al., 1988), and this may be important in suppressing protrusive activity by imposing an organization parallel to the plasma membrane. The role of microtubules in suppressing actin dynamics could be to act as a compressive element opposing the cortical tension and to allow the force to suppress protrusive activity, while preventing neurite collapse (Dennerll et al., 1988).

### Microtubules in the Growth Cone

Microtubules in the lamellipodial region of advancing growth cones are rather sparse by electron microscopy, but this could in part reflect lability to fixation (Letourneau, 1983). Most growth cone microtubules seem to extend from the axon tube toward the leading edge, and there appears to be some preferred orientation toward, and occasionally into, the base of filopodia (Yamada et al., 1971; Letourneau, 1983). Microtubules in the growth cone, but not in the axon tube, are exceedingly sensitive to depolymerizing drugs (Bamburg et al., 1986). This suggests that they may be very dynamic, since these drugs bind only to monomeric tubulin and act by inhibiting polymerization.

Growth cone kinetoplasm resembles that of motile fibroblasts, so we might expect their microtubules to behave similarly. In fibroblast cells microtubules grow and

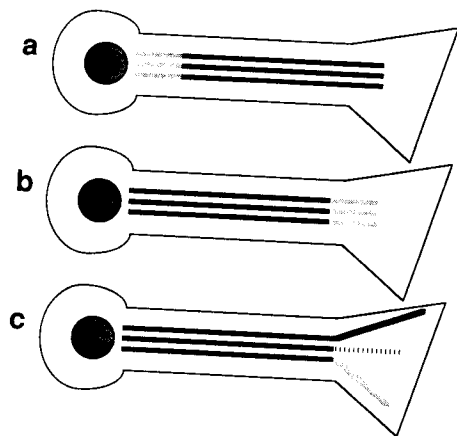


Figure 6. Three Models for Axonal Microtubule Elongation

In (a) subunits are added proximally to minus ends at the cell body and the microtubules are extruded down the axon. The initially unstable polymer shown as light lines is converted to more stable polymers in the axon.

In (b) subunits are added slowly to stable polymers at the neck of the growth cone.

In (c) microtubules grow and shrink intermittently onto the plus ends of stable segments in the growth cone. Initially unstable microtubules (shaded line) either shrink back (dashed line) or are converted into stable segments (dark line).

shrink continuously. Polymerization is mostly random, so that the spatial distribution of microtubules within the cell is constantly changing. The process by which this turnover occurs has been well studied and is called dynamic instability (Mitchison and Kirschner, 1984). Studies of pure microtubules *in vitro* have shown that the polymer lattice is inherently unstable. This is thought to be because tubulin polymerizes with GTP bound to it and then hydrolyzes the GTP shortly after polymerization. The resulting GDP polymer lattice is intrinsically unstable and poised to depolymerize; it will do so unless stabilized in some way. The rapid turnover of microtubules seems intrinsic to the microtubule lattice but cannot in itself generate spatially organized arrays.

In living cells microtubules grow out from an organizing center, usually the centrosome, proceed toward the plasma membrane, and then spontaneously depolymerize. Both polymerization (at a rate of  $5 \mu\text{m}/\text{min}$ ) and depolymerization ( $14 \mu\text{m}/\text{min}$ ) are rapid, and the average microtubule persists for 5 min (Schulze and Kirschner, 1986). Although the majority of microtubules in a fibroblast cell are dynamic and are seemingly polymerized at random, some microtubules are clearly less dynamic. These stable microtubules, which turnover at one-tenth the rate of the majority of the dynamic population are also asymmetrically distributed in the cell and are generally located near the cell center (Schulze and Kirschner, 1987). These microtubules are also often posttranslationally modified, but these modifications are a consequence of their stabilization and not the cause of the stabilization (Schulze et al., 1987). Hence the generation of spatially distinct subsets of stable microtubules out of a randomly polymerized and dynamic population

requires that additional information be provided to the system; this information is generally thought to arise from the extracellular environment.

Recent direct observation of fluorescently labeled microtubules in epithelial and fibroblast cells has revealed new features of these dynamics not observed in *in vitro* experiments with purified tubulin (Sammak and Borisy, 1988; Schulze and Kirschner, 1988). The growth rates of microtubules *in vivo* were found to be extremely variable and to show no consistency within a given microtubule over time. This variability suggests that microtubules are continuously encountering new environments in the cell, and that these environments have a major impact on the rate of polymerization and presumably on the stability of the microtubules. Microtubules that were converted to stable or metastable states were also found. Interestingly, those microtubules that experienced periods of stability depolymerized more slowly than the dynamic population, suggesting that initial stabilization can result in longer term stability. Although most microtubule growth was random in the cell, it was not uncommon for different microtubules to take precisely the same path of polymerization, suggesting that there were barriers or tracks in the cytoplasm that could in some cases direct microtubule polymerization. These new methods of visualization suggest that there are processes in the cell that modulate the intrinsic randomness of microtubule growth. Unfortunately, as yet we cannot connect these processes to external morphogenetic signals.

In nerve cells similar microtubule dynamics may be occurring, particularly at the growth cone. Okabe and Hirokawa (1988) have found continuous incorporation of labeled subunits in differentiated pheochromocytoma cells, suggesting that many microtubules in cell processes are unstable. Recent experiments from our laboratory have extended direct visualization of fluorescently labeled microtubules to living preparations of embryonic frog neurons. Microtubules in the growth cone are also very dynamic and often extend transiently to the tip of the process. The turnover rate of microtubules in growth cones may in fact exceed that in fibroblasts. Ultimately, microtubules in mature neuronal processes are stabilized by microtubule-associated proteins (Horio and Hotani, 1986). However, the choice of which microtubules to stabilize seems to be at the heart of the morphogenetic decisions.

### Microtubules in the Axon Tube

The microtubules in the axon tube are typically closely packed and arranged axially with uniform polarity (Hirokawa, 1982; Schnapp and Reese, 1982; Heidemann et al., 1981; Burton and Paige, 1981). They are rather insensitive to microtubule depolymerizing drugs, and therefore presumably not very dynamic, in sharp contrast to the microtubule behavior in the growth cone. We ask two related questions: How are the microtubules in the axon tube stabilized, and how are these stable microtubules generated as the axon tube elongates?

Microtubules could be stabilized by factors that bind to and cap their ends. Except for the centrosome, which possesses minus end capping activity for the microtubules it nucleates, such factors have not yet been identified, as they have for actin filaments. Their potential importance and relevance in the initial stage of microtubule stabilization are discussed below in the section on pathfinding. Aside from capping, microtubules can be stabilized either by the binding of proteins along the whole length of the polymers or possibly by posttranslational modification of the tubulin subunits. Microtubules in the neuritic processes of PC12 pheochromocytoma and N115 neuroblastoma cells are saturated with the microtubule-associated protein tau, which stabilizes the polymer lattice (Drubin et al., 1985; Drubin and Kirschner, 1986). Microtubules are also subject to specific modifications of the polymer: acetylation, deetyrosination, and phosphorylation (Gard and Kirschner, 1985; Gundersen et al., 1984; Piperno et al., 1987). Posttranslational modification is closely correlated to the stability of microtubules, but it appears that the modifications themselves are a consequence of stabilization, rather than a cause (Schulze et al., 1987). Thus they serve as a marker of microtubule age. The significance of these modifications is presently unknown.

#### How Are New Microtubules Generated in the Growing Axon?

Axoplasmic microtubules could elongate at the growth cone in three ways.

—Sliding of the whole microtubule forward, with concomitant polymerization on its proximal minus end (Figure 6a). According to this model, the dynamic behavior of microtubules in the growth cone is irrelevant to axonal elongation. Experiments following slow axonal transport using isotopic tracers have suggested that the microtubule cytoskeleton slides forward from the cell body (Black and Lasek, 1980). It is difficult to reconcile this model with the sensitivity of the growth cone to colchicine (Bamburg et al., 1986). In addition, the idea that slow axonal transport represents a single cohesive phase has been challenged, as discussed above. In general, we expect the spatial localization of microtubules in cells to be determined by where they polymerize, so we do not favor hypotheses based on the extrusion of the cytoskeleton from the cell body. The experiments on slow transport may be more relevant to the question of the form by which subunits are transported to the end of the axon than to the question of where the microtubules polymerize.

—Addition of subunits slowly and steadily into the neck of the growth cone as the axon elongates (Figure 6b). In this model, microtubules that extend deep into the growth cone will always depolymerize and will not contribute to axonal growth. Although this model may be the simplest, continuous slow growth of microtubules has not generally been observed either *in vitro* or *in vivo*. We expect that free microtubule plus ends will either add subunits at a rather fast rate controlled by the mono-

mer concentration, or shrink back to a nucleating or stabilizing site at an even faster rate. This slow growth mechanism would probably require that the neck of the growth cone be a specialized structure. The only known example in which microtubules seem to elongate slowly and continuously is that of kinetochore microtubules during metaphase of mitosis (Mitchison et al., 1986). These microtubules are attached at their plus ends to a structure that stabilizes them against depolymerization, but allows elongation. Since external force can promote axon outgrowth (Bray, 1984), a kinetochore-like structure could provide a mechanism for coupling microtubule polymerization to force. Although this would provide an elegant coupling of microtubule polymerization to the motility of the growth cone, there is no evidence for such a kinetochore-like structure; furthermore there are other less complicated ways for achieving a coordination between motility and growth.

—Polymerization of microtubules into the growth cone from the ends of stabilized segments in the axon tube and the generation of stable axonal microtubules (Figure 6c). Unlike the other models, this makes use of transient microtubule extensions into the growth cone. A potential disadvantage of this model is that microtubule polymerization when it has been measured directly in fibroblast cells (Schulze and Kirschner, 1988) is often considerably faster ( $4\text{--}10\ \mu\text{m min}^{-1}$ ) than the rate of axonal extension. This implies that the growth is intermittent and that it may be punctuated by episodes of depolymerization. We discuss this model in more detail in the following section.

#### Neuronal Pathfinding and the Mechanism of Directed Axon Growth

*In vivo* neurons can take intricate and convoluted paths to their targets (Fraser, 1985; Goodman et al., 1982). The role of microtubules and actin in this pathfinding process can only be ascertained crudely by drug experiments, and basically the outcome of these drug experiments is what one could expect from the properties of the polymer systems. Microtubules tend to grow in a straight line. When neurons are treated with cytochalasin to depolymerize actin filaments, extension of the axon still occurs but the ability to follow a defined path is lost; the extending neurite follows a linear or gently curving trajectory (Marsh and Letourneau, 1984; Bentley and Toroian, 1986). The actin-based leading edge structures are potentially able to generate protrusive activity all around the cell, and indeed the whole surface of the neuron is capable of generating a process (Bray, 1984). In the absence of polarization, generated by the microtubules, this uniform motility cannot lead to directional movement. Polarization is generated by local suppression of protrusive activity, as discussed above. When neurons are treated with colchicine there is random protrusive activity leading ultimately to the loss of cell asymmetry and the consequent retraction of the neurite (Bray et al., 1978; Solomon and Magendantz, 1981). Somehow during normal nerve growth, these two auto-

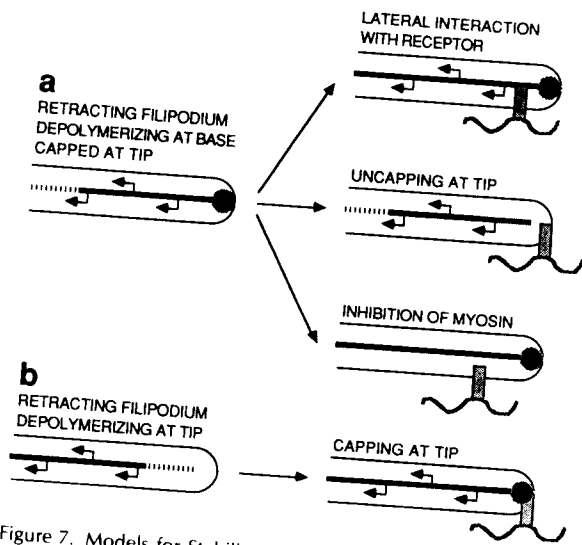


Figure 7. Models for Stabilization of Filopodia

Retracting filopodia are shown on the left, and stabilized filopodia on the right. In (a) retraction is due to depolymerization at the pointed end, failure to polymerize at the barbed end, and retrograde force on the filaments. These filopodia can be stabilized in three ways. First, a guidance cue receptor system (shaded box) makes a rigid mechanical interaction with the filament, converting retrograde translocation into tension. Second, the receptor acts to uncap the barbed end of the filament, allowing polymerization onto the barbed end. The retraction force now acts to generate retrograde flux in the filament, whose length remains constant. Third, the receptor acts to switch off the mechanism generating force for retrograde flux using a second messenger system such as calcium ions, etc.

In (b) retraction is due to depolymerization from the barbed ends of the filaments. This is prevented by the receptor acting to cap the barbed ends against depolymerization.

ous processes must interact to produce directed movement and pathfinding.

In normal axon growth the process of pathfinding can be divided into three subprocesses: First, an orientation phase, in which filopodia interact favorably with specific cues. This phase seems mainly to involve the actin system of the leading edge. Second, a consolidation phase, in which microtubules in the growth cone are stabilized in the future direction of axonal growth (Goldberg and Burmeister, 1986; Aletta and Greene, 1988). Third, a conversion phase in which the oriented microtubules and actin are converted into stable forms and compressed laterally to form the axon tube. This occurs at the neck of the growth cone.

### Orientation

In the grasshopper limb bud system the growth cone seems to explore its environment primarily by extending filopodia that may reach out over 30  $\mu\text{m}$ , subtending an angle of 180° (Caudy and Bentley, 1986). Most of these filopodia retract again, but those that contact a favorable cue, such as a guidepost cell, persist and may even form gap junctions (Goodman et al., 1982). The signal to do this must result from an interaction with receptors near the filopodial tip. It seems likely that filopodial probing

is a random process; there is presently no evidence for preferred outgrowth of filopodia. This would presumably require diffusible signals, whereas most cue molecules are attached to the extracellular matrix or cell surfaces (Letourneau, 1985; Jessell, 1988). Thus the mechanism of orienting growth cones depends on random outgrowth of filopodia and selective stabilization, rather than directed outgrowth.

Given the actin dynamics of filopodia discussed above, how might this selective stabilization work? The answer may lie in the normal mechanism of filopodial retraction and how it is inhibited. Filopodia show continuous actin polymerization at their tips, retrograde polymer flux, and depolymerization at internal sites. When filopodia extend, polymerization exceeds retrograde movement. When filopodia retract, retrograde movement exceeds the net rate of polymerization. Several mechanisms could transduce receptor binding at the filopodial tip into a change in the balance between polymerization and retrograde movement (see Figure 7). These mechanisms, described below, would all stabilize the filopodium, but the consequences for generation of tension and actin dynamics are very different for each. The role of lamellipodia in pathfinding is less clear, but the same mechanisms should also apply.

—Lateral connection of the actin bundle to an extracellular ligand through a receptor. This attachment would prevent the retrograde movement of the filament bundle relative to the position of the ligand. The mechanism that generated movement before would now generate tension. Tension can be communicated to all parts of the growth cone and can redirect the polymerization of actin and influence microtubule dynamics. Tension could also feed back on myosin activity (Coleman and Mooseker, 1985). In many ways tension is an ideal second messenger, quickly distributed to the actin membrane interface. The ensuing response to tension could increase the number and stability of the filopodial actin and microtubules with a direct consequence for the consolidation phase (see below). Three other mechanisms that do not involve tension in the filopodium also can be considered.

—A liganded receptor could act to prevent recapping of the barbed ends. The flux of polymerization at the tip and the movement of the actin filaments away from the tip continue, and the filopodium persists, but not under tension.

—Receptor liganding switches off the force for retrograde flux by an unknown mechanism—blocking calcium entry, for example.

—Capping of dynamically unstable actin filaments. If the normal mechanism of filopodial retraction is in fact depolymerization from the barbed end, rather than the pointed end, as we have been mostly considering, capping of the actin bundle by the receptor could block retraction. Thus binding proteins could regulate growing and shrinking transitions in actin. One feature that would seriously suggest this fourth mechanism would be if the normal rate of retraction was faster than the ongoing rate of retrograde flux. These models could be distin-

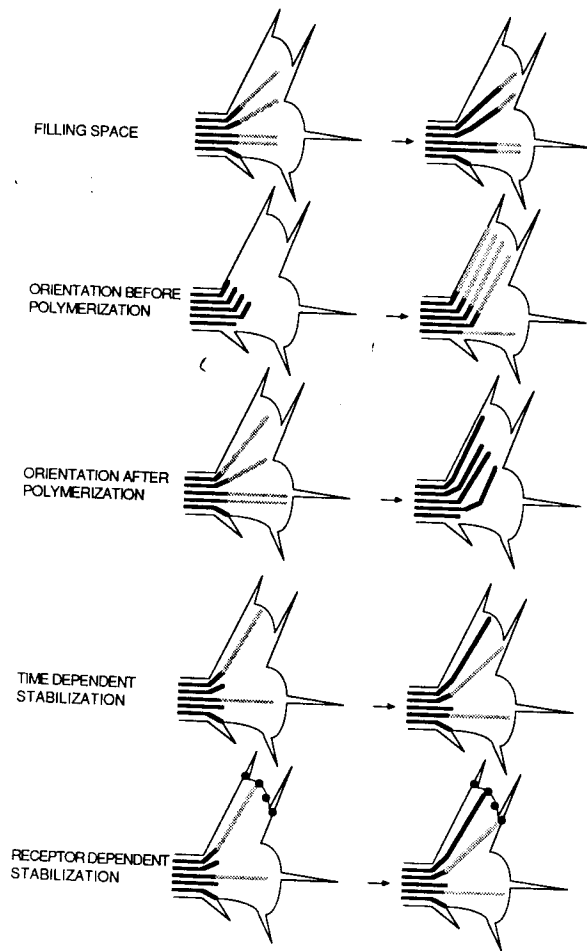


Figure 8. Consolidation of the Preferred Direction of Axonal Growth by Microtubules

In this figure the axon is attempting to turn toward the top of the page. The orientation phase has led to the stabilization of filopodia in this direction. Unstable microtubules shown as light lines are being converted to stable microtubules, shown as dark lines. The various situations are described, going from top to bottom. In (1) microtubules grow into the available space; in (2) microtubules are oriented to grow in a certain direction; in (3) microtubules first polymerize and then are reoriented into the preferred direction; in (4) microtubules grow randomly and are stabilized according to how long the filopodia persist; in (5) microtubules are stabilized by specific receptors, shown as filled circles.

guished by experiments looking at the basic dynamics and mechanical parameters of the system; for example, do filopodia exert tension during pathfinding decisions?

### Consolidation

Following orientation, microtubules must be polymerized in the preferred direction of growth. We term this process consolidation to reflect the fact that the initially transient orientation of the growth cone will be consolidated by microtubule growth. Given the inherent uncertainties of the orientation mechanism, it will often be the case that a choice must be made during consolidation. Thus consolidation is likely to follow directions established by orientation, but also to make decisions based

on new information. We can divide mechanisms for consolidation into two classes. One class (the first, second, and third mechanisms described below) is *instructive* and involves directing the polymerization or final placement of microtubules along directions specified by the orientation phase. The other class (the fourth and fifth mechanisms described below) is *selective* and involves the stabilization of a subset of the dynamic population of microtubules that initially polymerized in random directions (see Figure 8) (Kirschner and Mitchison, 1986).

– Physical pulling of the growth cone toward the target. This model depends on tension being generated by oriented filopodia to orient the growth cone. Tension generated by filopodia could cause a general flow of cytoplasmic volume in a particular direction. In this model microtubules play no informational role.

– Orientation of microtubule growth by actin. Actin filaments at the base of filopodia could direct microtubule assembly using myosin or microtubule-associated proteins to orient the growing ends (Letourneau, 1985). In support of this idea, myosin II in the growth cone tends to be found in the neck region where the majority of axonal microtubules terminate (Letourneau, 1981), and there are indications of myosin–microtubule interactions in other systems (Antin et al., 1981). It is also possible that the growth of microtubules is not oriented by direct interaction with actin or myosin, but rather that mechanical tension in the growth cone produces tracks in the actin cortex that provide channels to allow polymerization; some evidence for the existence of tracks of preferred microtubule growth has been found in fibroblast cells (Schulze and Kirschner, 1988).

– Reorientation of microtubules after polymerization by actin and myosin. This model has an effect like the second model. The mechanisms, however, would be different. Already polymerized microtubules would be dragged by the actin cytoskeleton into new positions.

– Time-dependent stabilization of randomly grown microtubules. In this model microtubules grow randomly into the base of filopodia. Usually they retract, but there is a certain probability of their being stabilized by capping or some other mechanism. The longer the filopodium persists, the more chance there would be for stabilization of a microtubule by microtubule-associated protein binding or posttranslational modification.

– Receptor-dependent stabilization of randomly grown microtubules. This model operates in the same way as the fourth mechanism, depending on random, rather than directed growth. In this model we postulate that guidance cue receptors actively cause local microtubule stabilization by capping or other mechanisms. Recently in fibroblast cells the location of microtubule ends has been correlated with the insertion point of actin filaments on the cytoplasmic side of the plasma membrane and the accumulation of matrix receptors on the outside of the plasma membrane (Rinnerthaler et al., 1988).

The last two models put more emphasis on dynamic instability of microtubules playing an active role in decision making. The important factor in both of these cases

is the selective conversion of kinetoplasm into axoplasm, mediated by outgrowth and stabilization of microtubules.

### Conversion

As long as actin and microtubules are in their dynamic state, the pathfinding decisions are transient. Following consolidation, both actin filaments and microtubules are in their proper location for directed axonal growth and both systems have been stabilized to some extent. To maintain the initial choices permanently, some additional change must take place in the polymers so that kinetoplasm can be permanently converted into axoplasm. Inhibition of protrusive activity in actin may be a response to the presence of microtubules. Further stabilization of microtubules may occur as a result of posttranslational modification or binding of other proteins. The actin cortex, inhibited in its protrusive activity, can now exert isotropic compressive tension on the microtubule array leading to bundling of the microtubules in the axoplasm. This bundling may also be promoted by microtubule-associated proteins. At this time, the axon tube also loses its adhesiveness for the substratum. A new segment of axon has been formed.

### The Future

Early observers of nerve cell motility must have doubted the possibility of a molecular explanation for a process that seemed both so random in detail, while being so purposeful in ultimate design. Fortunately, today we are no longer without molecular models for generating wide diversity in structure, whether it occurs through random motility or dynamic processes of assembly and disassembly. We have, however, not yet succeeded in understanding the purposefulness in ultimate design.

Part of the design may be found in the extracellular signals that direct nerve growth. Since the cytoskeletal content of all neurons is the same, it seems very likely that external signals direct the growth of the individual neurons in the nervous system. However, for a single neuron, in the elaboration of its morphology, the greatest complexity must lie not in the signals, but in the cell response. Most likely the capacity to generate any morphology already exists within the cell, and the external signal either stimulates some latent program or, just as likely, stabilizes a morphology that is generated randomly by the cell itself.

Therefore, the future challenge for neurobiology and the principal challenge for all of developmental biology is to determine how the diverse morphology of multicellular systems is generated and how this diversity is harnessed by simple extracellular signals. We have seen that an important locus for generating diversity of structure is the cytoskeleton. Not only is the cytoskeleton complex, but the basic components have the capacity to generate further complexity. Other materials, including the membrane and the extracellular matrix, must act on this complexity-generating machinery to determine the final structure of the neuron. Therefore, a satisfactory

knowledge of neuronal morphogenesis will require knowledge from all fields of cell biology. Central to this understanding must be an elucidation of the control of the dynamic properties of actin and tubulin.

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