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THE ROLE OF MICROTUBULES IN AXONAL GROWTH AND GUIDANCE

by

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DISSERTATION

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to my father and mother

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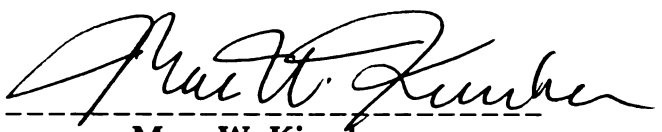
THE ROLE OF MICROTUBULES

IN AXONAL GROWTH AND GUIDANCE

by

James Henderson Sabry



Marc W. Kirschner
Chair, Thesis Committee

ABSTRACT

The mechanism by which neurons create and maintain synaptic connections lies at the center of both the development and the function of the nervous system. It has been appreciated over the past few decades that this process of axonal growth, axonal guidance and synapse formation is regulated by factors in the environment; the nature of how these factors operate is not known.

We have investigated this question by asking how microtubules, one of the prominent biopolymers in neurons, are transported down the axon, and how their arrangement changes as growth cones steer. To do this, we have used a system that allows axons to respond to their natural cues - the embryonic grasshopper limb. We have developed technology to inject the Ti1 neuron in this limb with fluorescently conjugated tubulin, and to image the fluorescently labeled microtubules in the axon and growth cone.

We have found that their is no evidence that microtubules are translocated down the axon as polymer, and assume that their principal mode of

transport is as tubulin monomer. In the growth cone, microtubules are present as a dynamic population. We have described the rearrangements of these microtubules that occur as turning decisions are made, and have found that at certain turns, the microtubules invade specific branches arrayed in the direction of future growth. This provides a framework for further studies on the role of the cytoskeleton in axonal growth, and guidance. It is our hope that these findings will also be relevant to the enormously important field of synapse formation and function.

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CHAPTER ONE - INTRODUCTION

INTRODUCTION

The modern view of nervous system function is that individual neurons make specific synaptic connections with other cells, mainly other neurons, and that the regulation of the synaptic connection underlies all behavior (Kandel et al., 1991). This theory is in part a spatial theory, that is the functioning of the nervous system is dependent on *what* connections the neurons make. The spatial nature of nervous system functioning can be appreciated on two levels. Firstly, on a large scale level, it has been known for quite some time that different brain functions (behaviors, if you wish) are localized in different areas (Geshwind, 1979). For instance, neurons that connect the retina to the lateral geniculate nucleus of the thalamus are visual neurons, and the nature of this connection (to what LGN layer, where on the LGN dendrite) defines the physiology of the anterior visual system (Hubel, 1988). This means that the connectivity of whole populations of neurons is important for the functioning of that region of the brain.

Secondly, on a smaller scale, individual neurons may rapidly change their connectivities in both the embryo and the adult in order to respond to the environment in a long term fashion (Antonini and Stryker, 1992; Recanzone et al., 1992). Although it has long been appreciated that the brain can slowly change its axonal arbors in response to injury or external stimuli, it was thought that this change was simply the removal of certain axonal branches to synapses that had long been ineffective (Hubel et al., 1977). Recent studies have been striking in that the axonal rearrangements have occurred on the same time scale as the changes in physiology. This suggests that axonal arbors bearing active synapses may be altered by environmental cues, and raises the intriguing possibility that small changes in physical

connectivity between a synapse and its target may explain certain brain plasticity events that underlie complex behaviors.

The development of nervous system, and this spatial distribution of neuronal connections, can be thought to be complex in two broad ways. Firstly, development entails the growth of the number of cells, and their differentiation into neurons. This biological event is similar to development of other organs, in that it involves growth of the number of cells, through regulation of cell proliferation and cell death (Purves and Lichtman, 1985; Raff et al., 1993). Secondly, nervous system development must involve the development of precise neuronal connections (and, indeed, as mentioned above, this may indicate that *developmental* mechanisms are important throughout life). This event includes neurite (meaning axon or dendrite) initiation, neurite growth and neurite guidance. This biology is unique to the nervous system, and, indeed, it is what allows long range signaling to occur. This thesis is about that second developmental complexity.

Historically, the appreciation that neurite growth and guidance are a central aspect of nervous system development began in the early 1800s when Theodor Schwann introduced the fundamental concept of the cell (Schwann, 1839). His idea, new at the time, was that organisms were made up of fundamental units, called cells, and that the functioning of an organ was the aggregate functioning of all the cells in that organ. This theory was well accepted at the time for all organs, except the nervous system. According to Shepherd (1991), this was for two reasons. Firstly, the histology of the nervous system at the time did not clearly show whether neurites arose from nerve cells. Secondly, it was not clear that neurites had definite terminations; they were thought to form a web of continuous protoplasm, a syncytium.

Then in the late 1800s, Camille Golgi developed a staining technique that allowed individual nerve cells to be identified (Golgi, 1873). Ramon y Cajal used this technique to study the whole nervous system, and concluded that the cell theory must apply to the nervous system (Cajal, 1894). Wilhelm Waldeyer agreed and, in a review in 1891, coined a new term to identify the cell of the nervous system (see Shepherd, 1991). He called it a neuron, and the theory that the neuron formed the functional and anatomic unit of the nervous system became known as the neuron doctrine.

The neuron doctrine was rapidly accepted by the biological community. Perhaps most importantly by a Yale biologist named Ross Granville Harrison. Harrison had pioneered the technique of tissue culture, and was interested in using his technique to examine how neurons grew (Harrison, 1969). He dissected portions of frog embryonic neural tube into a hanging drop of lymph, and watched as the neurons grew (Harrison, 1907):

> The {nerve} fibers are about 1.5-3 μ thick and their contours show here and there irregular varicosities. The most remarkable feature of the fiber is its enlarged end, from which extend numerous fine simple or branched filaments. The end swelling bears a resemblance to certain rhizopods and close observation reveals a continuous change in form, especially as regards the origin and branching of the filaments. In fact, the changes are so rapid that it is difficult to draw the details accurately. ...other tissues of the embryo, such as the myotomes, yolk endoderm, notochord, and indifferent ectoderm form{sic} the abdominal region do not give rise to structures of this kind. There can, therefore, be no doubt that we are dealing with a specific characteristic of nervous tissue.

This then was the beginning of the modern age of developmental neurobiology. Although Cajal had identified these swollen ends of nerve cells in his fixed tissue, and called them "growth cones", it was Harrison that

brought them to life. He observed their dynamics, and speculated on the nature of factors that might control where and how fast they moved. In addition, he recognized that nerve growth was a phenomenon unique to the nervous system. So, not only in an experimental way, but also in an intellectual way, Harrison set the stage for the field of axonal growth and guidance.

The Biology of Neuronal Growth Cones

Since Harrison's time, work on both invertebrates and vertebrates, in the intact embryo and in tissue culture has continued to elucidate the mechanism by which neurites form, grow and steer (Landis, 1983). In addition, much work has been done on the general mechanism of cell motility, and this has informed the axonal guidance field (Condeelis et al., 1990).

It has become clear that neuronal growth is not random. It is rather highly directed, and it is thought that environmental signals instruct the growing neuron where to and how fast to grow (Lance-Jones and Landmesser, 1980). These signals may be glycoproteins in the extracellular matrix, molecules on the surface of other cells or diffusible signals released by a target (Grenningloh and Goodman, 1992; Reichardt et al., 1989; Rutishauser and Jessell, 1988; Tessier-Lavigne and Placzek, 1991)]. These signals are all thought to affect the growth cone itself.

As Harrison noted, the growth cone is the expanded tip present at the end of axonal and dendritic fibers. It is flattened, highly motile, and can be branched. In addition, it has numerous protrusions called filopodia and lamellipodia. Growth cones are highly dynamic, and it is thought that they

play a central role in reading environmental signals that give information as to where and how fast a neurite should grow (Cypher and Letourneau, 1992).

Perhaps the best described example of this function is the Ti1 pioneer neuron growth cone in the embryonic grasshopper limb (Bentley and O'Connor, 1992). This growth cone has numerous, long filopodia, and contact of a single filopodium to certain guidepost cells can reorient the growth cone (O'Connor et al., 1990). It is thought that the many filopodia search out discrete cues in the limb environment. If one of them contacts one of these cues, the filopodium increases in diameter, and eventually the growth cone proper reorients along this chosen filopodium. Importantly, if the guidance cue (that is the guidepost cell) is ablated, then no reorientation occurs (Bentley and Caudy, 1983). This suggests that filopodial contact with the guidepost is necessary for growth cone guidance. Experimenters have tried to show directly that filopodia are required for growth cone guidance by eliminating them using the actin disrupting drug cytochalasin (Bentley and Toroian-Raymond, 1986; Chien et al., 1993). Cytochalasin treatment reduces the amount of filamentous actin in the growth cone, and causes filopodia to retract. In both the Ti1 neuron growth cone and in xenopus retinal neurons, cytochalasin treatment causes the growth cone to guide aberantly. These two types of experiments suggest that the growth cone, and its protrusions are crucial to steering the growing neurite.

It has long been thought that the mechanism of growth cone action involved the formation of physically adhesive contacts with the extraneuronal environment. A filopodia would seek out a favorable guidance cue, and would bind to it. It would then pull the growth cone proper in that direction. Evidence for this was taken from careful examination of filopodia in vitro, where it was found that they formed at the

leading edge of the growth cone, were swept back to the neck as the growth cone migrated forward, and were then resorbed (Letourneau, 1975). Further evidence for adhesion based movement came from studying neurons on patterned substrates of differing adhesivities; growth cones and filopodia preferred the more adhesive substrate (Bray and Chapman, 1985).

However, further studies muddied the waters by finding that neurons would prefer a less adhesive substrate to a more adhesive one (Gundersen, 1987). Furthermore, studies of aplysia neurons in culture revealed that turns at substrate boundaries were made by microprunning existing filopodia that have extended in the "wrong" direction, rather than by asymmetric filopodial extension postulated in earlier studies (Burmeister and Goldberg, 1988).

It is possible that neurons use many different mechanisms to turn, and that the variability seen in the above studies represents biologic variability. However, studies done with higher resolution examining the neuronal cytoskeleton have produced a more coherent picture (Smith, 1988), work in this thesis). Before discussing these studies, I will briefly discuss the biology of the cytoskeleton.

The Biochemistry of the Cytoskeleton

It is likely that the cytoskeleton is involved in mediating the effect of environmental signals on the growth cone (Mitchison and Kirschner, 1988). The growth cone cytoskeleton consists of three biopolymers, microtubules, actin and neurofilaments. There are undoubtedly unknown cytoskeletal elements in the growth cone, but ultrastructurally the three mentioned make up the vast majority. I will discuss the first two of these, as neurofilaments

are not present in every neuron, and little is known about their role in growth cone steering or neurite growth.

Actin is a polarized polymer made up of a gently leftward turning two filament helix composed of g-actin monomers (Kabsch and Vanderkerckhove, 1992). Actin, like microtubules, can bind and hydrolyze nucleotides (Mitchison, 1992). Furthermore, again like microtubules, the polymer exhibits structural and kinetic polarity with a more dynamic or barbed end, and a less dynamic pointed end. The dynamics of polymer assembly can be described as consisting of a kinetic barrier to polymer nucleation, and a rate of assembly that increases linearly with increasing monomer concentration (Oosawa and Asakura, 1975). It is thought that actin forms a cross linked gel in most cells . Furthermore, regulation of the density of the cross linking may underlie cell motility or protrusive events in the growth cone (Condeelis, 1993; Smith, 1988).

There exists a great number of actin binding proteins that may cross link, sever or cap actin filaments (Bamburg and Bernstein, 1991). In addition, myosin an actin based mechanochemical motor, binds to actin filaments and can move them relative to one another or to other cellular components (Titus, 1993). The force for protrusive activity (the forward movement of filopodia, lamellipodia and growth cone branches) may arise from many molecular sources. It can be supplied by the energy of actin polymerization itself, with the newly forming polymer pushing the cell membrane forward. Alternatively, myosin can move actin filaments relative to each other or to other cellular components. A third source of work can come from osmotic forces that accompany actin gel-sol transitions (recall that the osmotic force is a colligative property that depends on the number of particles, not their size). Which of these is important or regulated in growth cone movement or cell

motility is a topic of lively debate (Condeelis, 1993). In the neuron, actin is found as a subplasmallemmal cylinder in the axon, and as a highly cross linked gel in the growth cone (Lewis and Bridgman, 1992; Yamada et al., 1971). As will become evident during the discussion of the thesis, it is thought that actin may play a central role in growth cone steering.

The other main cytoskeletal element in the growth cone are microtubules. They are biopolymers in the form of a hollow tube approximately 24 nm in diameter (Avila, 1990). The tubes are formed from (usually) 13 protofilaments each composed of a polymer of the tubulin monomer (Wade et al., 1990). The monomer is actually a tightly coupled dimer of alpha and beta tubulin, globular proteins of approximately 50 kD molecular weight. The biology of microtubules is a direct result of two characteristics, the ability of the polymer to bind and hydrolyze GTP resulting in non-classical monomer-polymer kinetics, and the fact that many proteins bind directly to the polymer to change the monomer-polymer kinetics or to act as mechanochemical motors.

Both theoretical and experimental investigations have described the kinetics of microtubule assembly and disassembly (Dogterom and Leibler, 1993; Erickson and O'Brien, 1992). The monomers assemble into polymers in a uniform fashion, hence generating a polymer that has structural polarity, a "+" and "-" end. In the absence of nucleotide hydrolysis, one could expect theoretically that the polymer could have a fast growing (+) end, and a slow growing (-) end. However, the rate of polymer growth would be directly proportional to the monomer concentration at both ends, with the zero growth point (the critical concentration) identical for both ends (Kirschner, 1980). It is well known that tubulin can bind GTP, and that GTP hydrolysis is favoured when the monomers assemble (Penningroth and Kirschner, 1977).

Wegner hypothesized that this assembly induced nucleotide hydrolysis would allow the critical concentration to differ for the + or - ends of a polymer (Wegner, 1976). He suggested that this would allow monomers to assemble onto the + end of the polymer, and disassemble from the - end, in effect allowing monomers to "treadmill" through the polymer. This phenomenon would allow microtubule anchored at their - end (as in a centrosome) to be selectively stabilized, while suppressing growth of non-stabilized microtubules. Indeed, their has been experimental evidence for this phenomenon (Margolis and Wilson, 1978).

However, these theoretical and experimental investigations considered each microtubule as identical and that the behavior of the bulk population would reflect the behavior of the individual microtubule. This was the state of microtubule chemistry until the mid 1980s, when Tim Mitchison and Marc Kirschner observed individual microtubules from populations nucleated from isolated centrosomes (Mitchison and Kirschner, 1984b). To there amazement, it was clear that not all microtubules behaved identically even though the bath concentration of tubulin was a constant. Instead, the monomer-polymer kinetics were described more accurately by "dynamic instability" with four kinetic parameters, a growth rate, a shrinkage rate, a rate of transition from growing to shrinking called the catastrophe rate, and a rate of transition from shrinking to growing called a rescue rate (Mitchison and Kirschner, 1984a). It became evident that dynamic instability would allow non-stabilized microtubules to be rapidly disassembled. This raised the possibility that a cell could selectively stabilize certain populations of microtubules, and dynamic instability would disassemble the rest; microtubule lattices could be rapidly changed by simply changing the targets of selective stabilization (Kirschner and Mitchison, 1986).

Dynamic instability was shown to exist in live cells, including neurons (Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Tanaka and Kirschner, 1991). The rapid rearrangement of the microtubule lattice that occurs during mitosis was explained nicely by showing that kinetochores could stabilize spindle microtubules, and that most non-spindle microtubules would disassemble because of the intrinsically unstable nature of microtubule dynamics (Mitchison, 1989a).

In neurons, it is clear that microtubules in the growth cone show classic dynamic instability. However, in the neurite or in the cell body, this has not been shown. In the neurite, microtubules are found oriented with their + ends distal towards the growth cone (Hirokawa et al., 1988). In dendrites, approximately half of them are oriented with + end distal, the other half being + end proximal (Baas et al., 1988).

Microtubules are stabilized by microtubule associated proteins (MAPs) in neurons (Matus, 1990). These proteins bind to microtubules and stabilize them. In the axon, the major MAP is a series of proteins called tau proteins (Migheli et al., 1988; Weingarten et al., 1975). They stabilize microtubules by increasing growth rate, decreasing shrinkage rate and decreasing the catastrophe rate (Drechsel et al., 1992). In dendrites, the major MAP is MAP2 (Binder et al., 1986). It is not clear whether tau and MAP2 are active in the cell body or growth cone, but the fact that neurite microtubules tend to be more stable than those in the growth cone suggests that their main locus of activity is the neurite (Bamburg et al., 1986). Indeed, it is likely that a stable population of neuritic microtubules is required for neurite maintenance, as disassembly of these microtubules causes neurite collapse.

The other major class of microtubule associated proteins are the mechanochemical microtubule based motors (Vale, 1987). These enzymes

use the energy of hydrolysis of ATP to generate unidirectional force along the microtubule protofilament. These fall into two broad classes, the "+" end directed motors, such as kinesin, and the "-" end motors such as dynein. These motors play a role in axonal transport, and they may also function in growth cone guidance.

The Dynamic Biochemistry of Neuronal Growth Cones

The picture of the growth cone that emerges from the above studies is of a structure that consists of a dynamic population of actin filaments and a few microtubules, and a machinery designed to sense guidance cues, and to use them to change the cytoskeleton to effect a change in the rate or direction of growth of the neurite. The molecular events in this process are not known, but work outlined in chapter 3 directly addresses this issue.

An introduction to these events can be had, though, by examining a series of influential studies carried out by Paul Forscher and Steven Smith. By using differential interference contrast (DIC) microscopy, they studied the dynamics of aplysia bag cell neuronal growth cones in vitro. As in other neurons, there exists a constant retrograde flow of particles from the leading edge of the growth cone back towards the central region (Forscher and Smith, 1988). This flow is thought to represent the retrograde flow of actin which is polymerizing at the leading edge (the barbed end of the actin filament), and being disassembled in the body of the growth cone. If actin assembly is inhibited using cytochalasin, then the retrograde flow stops, first at the leading edge, followed by movement of the flow stoppage back to the central aspect of the growth cone. This suggests that the retrograde movement of actin is powered by motors in the growth cone rather than by actin

polymerization per se. More interesting, after the actin disassembles, there is a rapid movement of microtubules from the central region of the growth cone to the periphery. When the cytochalasin is washed out, then the actin reassembles, beginning at the leading edge, and moving backward, pushing the microtubules back to the central region as the assembly progresses.

These studies suggested that growth cone microtubules are being pushed or assembled into the distal aspect of the growth cones, but are stopped by a stearic wall of cross linked actin (Smith, 1988). As will be discussed in chapter 3, we feel that regulation of the porosity of this actin wall may be the central event in growth cone guidance.

The Transport of the Axonal Cytoskeleton

A related issue is how the growth cone is supplied with cytoskeletal proteins. Most if not all the protein synthesis in neurons occurs in the cell body and dendrites (Karlsson and Sjostrand, 1971). The proteins must then be transported from their site of synthesis to their location of action. In the case of axons, some proteins must travel long distances to reach the growth cone or synapse. The mechanism used to accomplish this task has been heartily debated (Vallee and Bloom, 1991).

Early studies were carried out by metabolically labeling all proteins in the retina, and then dissecting out the optic nerve and tract, and cutting into short segments that were solubilized and run out on SDS gels (Lasek, 1982). The findings of these studies were informative; tubulin moves as a coherent phase with persistent peak amplitude in a component designated as slow component a (SCa) at speed of about 0.25 mm/d. Interestingly, tubulin moves with exactly the same kinetics as neurofilament proteins and tau. The fact

that tubulin moves as a persistent coherent peak suggests that it is in a non-diffusible, possibly polymeric form. Furthermore, Lasek suggested that microtubules may be cross linked to neurofilament polymers, and that the whole cytoskeleton may be moving down the axon. Biochemical analysis of the tubulin transported in SCa suggested that it was mainly in a cold-stable polymeric form (Brady et al., 1984; Filliatreau et al., 1988; Tashiro and Komiya, 1989; Tashiro et al., 1984).

These studies examined tubulin transport in adult nerves using indirect means. Since then direct imaging of tubulin transport has been carried out in axons growing in culture. Two similar techniques have been used, and conflicting data has resulted with each approach (Hirokawa, 1993). One approach has been to covalently link fluorescein to tubulin and inject it into the growing neuron. The injected tubulin assembles into existing microtubules, and is thought to label all or most of the microtubules in the neuron. A mark is then made on the microtubules in the axon using a high energy pulse of light to photobleach the fluorophore. In the case of PC12 cells, it seems that the photobleach spot moves as the axon grows (Keith, 1987). However, other groups have seen no spot translocation in PC12 cells or chick DRG cells (Lim et al., 1989; Okabe and Hirokawa, 1990).

A more elegant technique has been to use a photoactivatable fluorescein analogue allowing a reduction of the energy needed to mark the microtubules. Yet this technique has also lead to conflicting results with mark translocation seen in xenopus neurons, and not in chick DRG cells (Okabe and Hirokawa, 1992; Reinsch et al., 1991). Chapter 2 of the thesis addresses this issue of axonal transport of tubulin.

Although it was hoped that the thesis would generate a cohesive model of the role of microtubules in axonal growth and guidance, it has

generated more questions than answers. It does, however, create a framework on which the next level of studies can be carried out. If it encourages further experiments in this incredibly fascinating and, indeed, clinically relevant area, then it will have value.

CHAPTER TWO

THE AXONAL TRANSPORT OF TUBULIN

ABSTRACT

Neurons are unique cells in that the majority of the cytoplasm is located in axons or dendrites. Furthermore, the protein synthetic machinery is located only in the cell soma or the dendrites. Hence, for axons, the protein synthetic machinery is located many centimeters or even metres from the location where the protein is required. As a result of this constraint, some mechanisms must exist to transport the newly synthesized protein from the cell body to its location of action in the axon. This phenomenon, axonal transport, has been studied for decades, and has been resolved into two broad kinetic phases. The fast phase, termed fast axonal transport, carries vesicles and other membranous components and their associated proteins at 3 - 400 mm/day. The second phase, termed slow axonal transport, carries cytoskeletal proteins, including tubulin, at 0.1 - 4 mm/day.

As microtubules make up the scaffolding of the axon, it is crucial to the growing neuron that tubulin be efficiently transported with kinetics that closely match those of axonal growth. Indeed, the rate and amount of tubulin transport may be one of the major regulators of the rate of axonal growth.

The nature of the transport form of tubulin, and the mechanism of its transport are not known. In this chapter, we directly mark axonal microtubules in situ in the developing grasshopper limb, and ask whether polymer transport can account for tubulin transport in a natural, in vivo setting. We find no evidence for axonal transport of the labeled proteins, and discuss this finding in light of other reports on microtubule movement in growing axons.

INTRODUCTION

Perhaps the most unique attribute of neurons is their highly polarized shape. Indeed, measurements of neuronal morphology of cat a-motoneurons have shown that 99.6% of the cytoplasm of the cell is in nerve processes, with 99.7% of this in axons (Culheim et al., 1987). Furthermore, all or most of the protein synthetic machinery is located in the cell body and dendrites (Karlsson and Sjostrand, 1971). For axons, this generates a very rigid constraint, namely that it must transport the vast majority of newly synthesized proteins from the cell body to their location of action in the axon.

This phenomenon of axonal transport was first reliably demonstrated by the elegant experiments of Paul Weiss. He carefully ligated nerve fibers, and described the histological changes that occurred at the site of the ligature (Weiss and Hiscoe, 1948). On the basis of the accumulation of axoplasm, he proposed that the axoplasm of the nerve was in continuous motion being generated in the cell body and flowing distalward. More recently, vesicular movement has been demonstrated directly (Brady et al., 1982).

Direct studies of the molecular components of axonal transport has revealed that it is heterogeneous both in the proteins transported, and in the kinetics of transport. One can resolve five kinetic classes of axonal transport (Vallee and Bloom, 1991). Fast axonal transport comprises class I and class II, with the polypeptides in class I being carried at 100-400 mm/day, and those of class II at 20-70 mm/day (Grafstein and Forman, 1980). Three slower classes exist, a poorly defined class III at 3-20 mm/day, and the better described slow component classes, slow component a (SCa), and slow component b (SCb).

These slow components are thought to be the kinetic phases where transport of the cytoskeleton, including tubulin occur (Black and Lasek, 1980).

They transport proteins at 0.1 - 4 mm/day. They were defined in rat retinal ganglion cells, where bulk proteins were labeled by injection of radiolabelled amino acids into the eye, followed by serial dissection of the axonal segments of the optic nerve and tract. It was found that tubulin was transported in SCb as a coherent, linear, non-diminishing phase of radiolabelled protein that moved over tens of days from the eye along the axon. Interestingly, tubulin was transported coherently with the three neurofilament proteins. It was proposed that microtubules and neurofilaments were transported as assembled polymers as a cohesive cytoskeletal network.

More recently, a series of experiments have taken advantage of newer technology to directly visualize microtubules in axons, and ask if they move as neurons grow. The first attempts at this visualization used photobleaching of fluorescent dye (Keith, 1987; Lim et al., 1990; Lim et al., 1989; Okabe and Hirokawa, 1990). These experiments loaded neurons with fluorescein labeled tubulin, and allowed the introduced tubulin to incorporate into existing microtubules. A photobleach mark was then made in the axon, and the movement of the mark imaged as the neuron grew. As expected the mark slowly faded, as microtubules turned over and replaced photobleached tubulin with fluorescent subunits. One study found that the mark translocated (Keith, 1987). However, the remaining studies found that there was no mark translocation, and concluded that tubulin is transported as monomer, and assembled into polymer at the growth cone or distal axon.

However, worries about the high energies used to photobleach the fluorophore lead to the development of a novel photoactivation technique (Mitchison, 1989b). This technique uses a caged fluorescein coupled to tubulin. The caged fluorophore is not fluorescent until photoactivated by a beam of ultraviolet light; the energy of light required is an order of

magnitude less than that of photobleaching. In initial experiments in xenopus motor neurons, it was found that not only did the photoactivation mark fade (as expected due to turnover), but that it also translocated proximodistally as the axon grew (Reinsch et al., 1991). In contrast, when this experiment was done in chick DRG cells, the photoactivation mark did not move (Okabe and Hirokawa, 1992).

It is unknown why these two experiments gave conflicting results, or why those experiments that do not see translocation conflict with the older radiolabelling experiments. In order to further examine this question, and the nature of axonal transport, we turned to the Ti1 pioneer neuron in the grasshopper embryonic limb. This system allows one to manipulate and image the neuron while leaving it accessible to the natural in vivo cues for axonal growth and guidance. We injected the Ti1 pioneer neuron cell body with a mixture of rhodamine and caged-fluorescein labeled tubulin, and after allowing the tubulin to incorporate into existing microtubules, photoactivated a region in the axon. We have found no evidence for microtubule polymer translocation, and discuss the findings in light of the literature on axonal transport.

MATERIALS AND METHODS

Grasshopper Embryos and Dissection

Schistocerca americana embryos were obtained from the University of California at Berkeley grasshopper colony. Eggs at the 31% - 34% stages of embryonic development were sterilized and the embryos dissected as previously described (Lefcort and Bentley, 1987). The embryos were transferred to a poly-L-lysine coated coverslip and maintained in supplemented RPMI (Condic and Bentley, 1989a). Briefly, the embryos were positioned ventral side down, thus exposing the posterior aspect of the limb bud. This surface was cut along the long axis of the limb, and the sides unrolled and flattened out onto the coverslip. The exposed interior mesodermal cells were removed using a suction pipette, leaving the basal lamina and epithelium-derived cells. The Ti1 neuronal cell bodies were visualized with differential interference contrast optics using a Nikon inverted compound microscope.

Fluorescent Labeling of Tubulin

Purified bovine brain tubulin was labeled with tetramethyl-rhodamine or bis- caged fluorescein (C2CF) as previously described (Hyman et al., 1991, Mitchison, 1989). This process involved covalently linking the N-hydroxyl succinimidyl ester of tetramethyl-rhodamine (#C-1171, Molecular Probes, Eugene, OR) or C2CF (a gift of Dr. Tim Mitchison) to purified bovine brain tubulin. The labeled tubulin was then subjected to two cycles of temperature dependent assembly/disassembly to select for assembly competent tubulin. The labeled tubulin was stored at a concentration of 20-30 mg/mL in an injection buffer (50 mM Kglutamate, 0.5 mM $MgCl_2$, pH 6.5) at -80° C.

Dextran Labeling

Aminodextrans of various molecular weights (3K, 10K, 40K, 70K,2E6K Daltons) were labeled with bis caged-fluorescein by incubating the dextrans (approx 50 mg/mL) with the label (~10 fold excess) in glycerol with $NaHCO_3$, pH 8.3 at room temperature for ~3 hours. The bis-caged fluorescein was a kind gift of Dr. Tim Mitchison. The reaction was quenched with a 100 fold excess of KGlutamate, and the labeled dextrans run over a G-25 (2E6Kd) or G-10 (3K,10K,40K or 70Kd) sephadex column (Pharmacia LKB, Uppsala, Sweden). The dextrans were collected and dialyzed into injection buffer (recipe above) overnight at 4° C. They were then concentrated using a centricon tube (Amicon division of W.R. Grace Co., Beverly, MA) to a final concentration of 2-5 mg/mL and stored at -80° C.

Neuronal Labeling

The Ti1 neuron cell body was injected with a mixture of rhodamine and C2CF conjugated bovine tubulin using a pulled, beveled borosilicate micropipette. The micropipette was pulled on a Sutter instruments P-87 horizontal puller (Sutter Instruments, Novato, CA). The Ti1 neuron was visualized under DIC optics, and the cell injected using a continuous flow pressure injector (Eppendorf Corp, Madison, WI).

Microtubule Imaging and Analysis

The imaging of the rhodamine and fluorescein labeled microtubules was carried out using a cooled charge-coupled device (CCD) camera system (Photometrics; Tuscon, AZ). The caged fluorescein tubulin was photoactivated by imaging a slit or small aperture onto the axon using the

existing microscope optics and a 100/1.4 lens, and then giving a brief (3-10 s) pulse of 360 nm light. The neuron was then viewed through either a 20/0.75, or a 60/1.4 Nikon objective on a Nikon inverted compound microscope with conventional relay optics connecting the microscope to the CCD camera. The microscope projected the image onto a 1320 x 1024 pixel chip (Kodak KAF-1400), which digitized the image and transferred the data to a bulk memory storage device (REO-650 erasable optical disc; Pinnacle Micro, Inc., Irvine, CA). The chip, light path shutters, and stage focal position were controlled by a Perceptics BioVision imaging system (Knoxville, TN) on a Mac IIx computer (Apple Computers, Cupertino, CA). Illumination was provided by a 100W mercury lamp, generally in 50-100 msec exposures for rhodamine, and 200-500 msec exposures for uncaged fluorescein. To image the full thickness of the growth cone, 1-3 optical sections were usually taken. Clusters of sections were usually taken every 15 to 45 minutes. On occasion, images were taken every 3 seconds to resolve high frequency events.

Images were transferred to OMDR (Panasonic Corp) as 8-bit TIFF files, and image analysis was carried out using Image-1/Metamorph 1.1 (Universal Imaging Corp, West Chester, PA) software running in windows (Microsoft Corp, Redmond, WA) on a 486Dx2 processor (Intel Corporation). Data analysis and curve fitting routines were carried out using Excel 4.0 (Microsoft Corp), and Origin for windows 3.0 (Microcal Software Inc., Northampton, MA) on a 486DX2 processor or a Macintosh Duo 210 (Apple Computer, Cupertino, CA). Photographic rendering of the images was carried out using a Tektronix Phaser IISDX dye-sublimation printer (Tektronix Corp, Beaverton, OR).

RESULTS

The observation of the Ti1 axons was carried out in a flattened limb bathed in culture medium. We found that neither the dissection of the limb, the preparation of the flattened preparation, the injection of the cell body or the imaging of the neuron affected the rate of axonal growth, or the guidance of the growth cone. The cells were injected with a mixture of rhodamine conjugated and C2CF-conjugated tubulin. After a waiting period of between 1 and 4 hours, we photoactivated and imaged the axon. We could not resolve individual microtubules in most of the axon, however, the CCD camera is linear over a wide range of intensities, and any major increase in microtubule labeling between 1 and 4 hours would have resulted in a measurable increase in fluorescent intensity. This was not seen. Furthermore, in certain regions of the axon, and in the growth cone, individual microtubules can be resolved. We noticed no increase in the number of microtubules in these regions between 1 and 4 hours. This suggests that most of the microtubules were labeled in the first hour after injection of the labeled tubulin. However, it remains possible that a small population of extremely long lived microtubules were not imaged. This possibility is discussed further later in the chapter.

In the case of dextrans, the cell was injected, and imaged after a waiting period of tens minutes for those dextrans between 3kD and 70 kD. For the 2E6kD dextran, the waiting period was extended to 1 hour.

The injected fluorescently labeled tubulin or dextran flowed down the axon as a homogeneous wave of fluorescence, and was found only in the injected neuron. As the Ti1 cell is linked to its sister neuron by gap junctions, this suggests that there was no appreciable free fluorophore was generated in

the cell. As detailed in chapter 3, electron microscopy coupled with careful measurement of microtubule intensities suggests that linear tracts visible in the Ti1 neuron represent individual microtubules.

Microtubules do not Translocate in Ti1 Neurons

We measured the movement of photoactivation marks in 19 neurons. The marks were made at various places in the axon, ranging from near to the cell body to near the growth cone. Figure 1 shows a pseudo-colored image of a neuron with a photoactivation mark near the growth cone. The red signal represents a measure of all labeled microtubules in the cell, labeled with rhodamine-tubulin, and the green mark represents those microtubules whose C2CF tubulin was photoactivated. The mark gradually diminished in intensity until it was indistinguishable from background fluorescence. The mark could be resolved for 30-720 minutes, depending on the neuron. Of those 19 studies, 14 had growth during the duration of imaging. The average length of growth during the study was 54 μm. The remaining 5 neurons had no net growth during the imaging, but the growth cone did change shape suggesting that the neuron was indeed alive.

In all neurons, no translocation of the photoactivation mark was seen. Figure 2 shows an example of a neuron that grew by ~40 μm during the experiment. Fig2b,d show rhodamine -tubulin images showing the extent of microtubules in the cell at the beginning (Fig. 2a) and the end (Fig 2d) of the experiment. It is clear that the neuron made two appropriate turns during the experiment. Fig 2a,c show the photoactivation spot at the corresponding times as Fig 2 b and d, respectively. It is clear that the photoactivation mark (arrowhead in Fig 2b,d) does not translocate even though ~40 μm of new axon

has formed during the imaging epoch. Fig 3 shows the intensity profiles of the photoactivation mark from this experiment, and confirms the visual impression that no translocation has occurred.

We also investigated the translocation of photoactivation marks in 5 neurons whose growth cones were active, but no axonal growth occurred. This is a common, transient occurrence in normal neuronal growth. Figure 4 shows an example of this group of neurons. Fig 4a,b show the rhodamine tubulin images at the beginning and the end of the experiment, confirming that no net growth has occurred. Fig 4c-f show the photoactivation mark at various times during the experiment. No mark translocation was noted. This visual impression is confirmed by the intensity profiles shown in Fig. 5.

Figure 1. Photoactivation of the Ti1 Pioneer Neuron Axon. The neuron is at the segment boundary, and is labeled with rhodamine-tubulin (red). A photoactivation mark on the microtubules was made, and can be visualized as a green mark on a red background in this pseudocolored image. Note that the mark represents a small fraction of the axonal microtubules. Scale bar= 20 μm.

Figure2. Photoactivation marks do not translocate in growing Ti1 axons. The axon shown in b was photoactivated, and the spot is shown in a (arrowhead). At time= 430 min, the axon has grown ~40 μm, and made two appropriate turns (d), but the photoactivation mark has not moved, indicating that the microtubules have not translocated. Scale the same as Figure 1.

Figure 3. Intensity profiles of photoactivation marks. The experiment in Figure 2 was analyzed by measuring the pixel intensity along the axon with a curvilinear line the followed the center of the axon. Time=0 corresponds to Fig 2a,b, and time =430 min corresponds to fig 2c,d. The initial intensity profile at time=0 is reiterated in the subsequent graphs as a dotted line to allow comparison. There is no evidence for translocation of the intensity profile.

Figure 4. Photoactivation marks do not translocate in non-growing Ti1 axons. This neuron did not grow, although the growth cone branches were dynamic, and changed shape (compare a,b). a. Rhodamine tubulin at time=0. b. Rhodamine tubulin at time=105 minutes. c. Fluorescein tubulin at time=0 (corresponds to a). The photoactivation mark can be seen. Fluorescein images showing no movement of the photoactivated spot at time=48 min (d), 68 min (e) and 105 minutes (f).

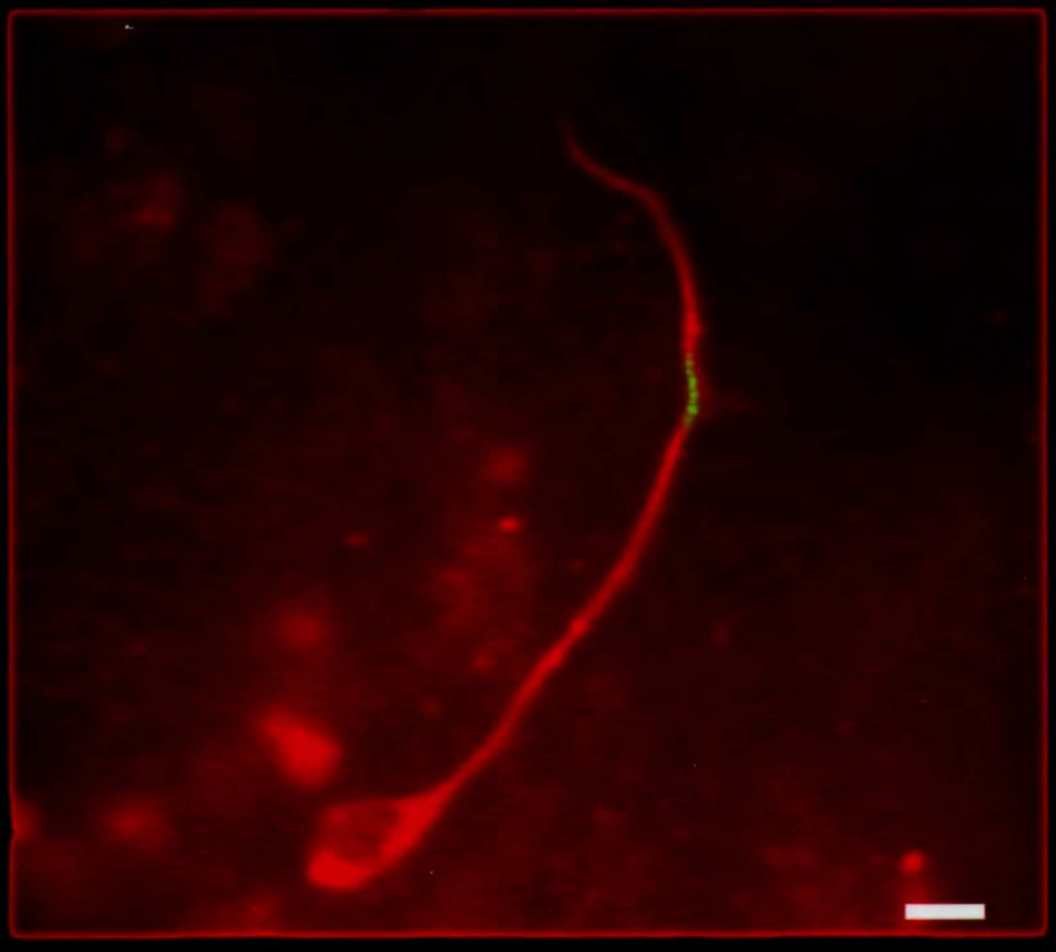


Figure 1

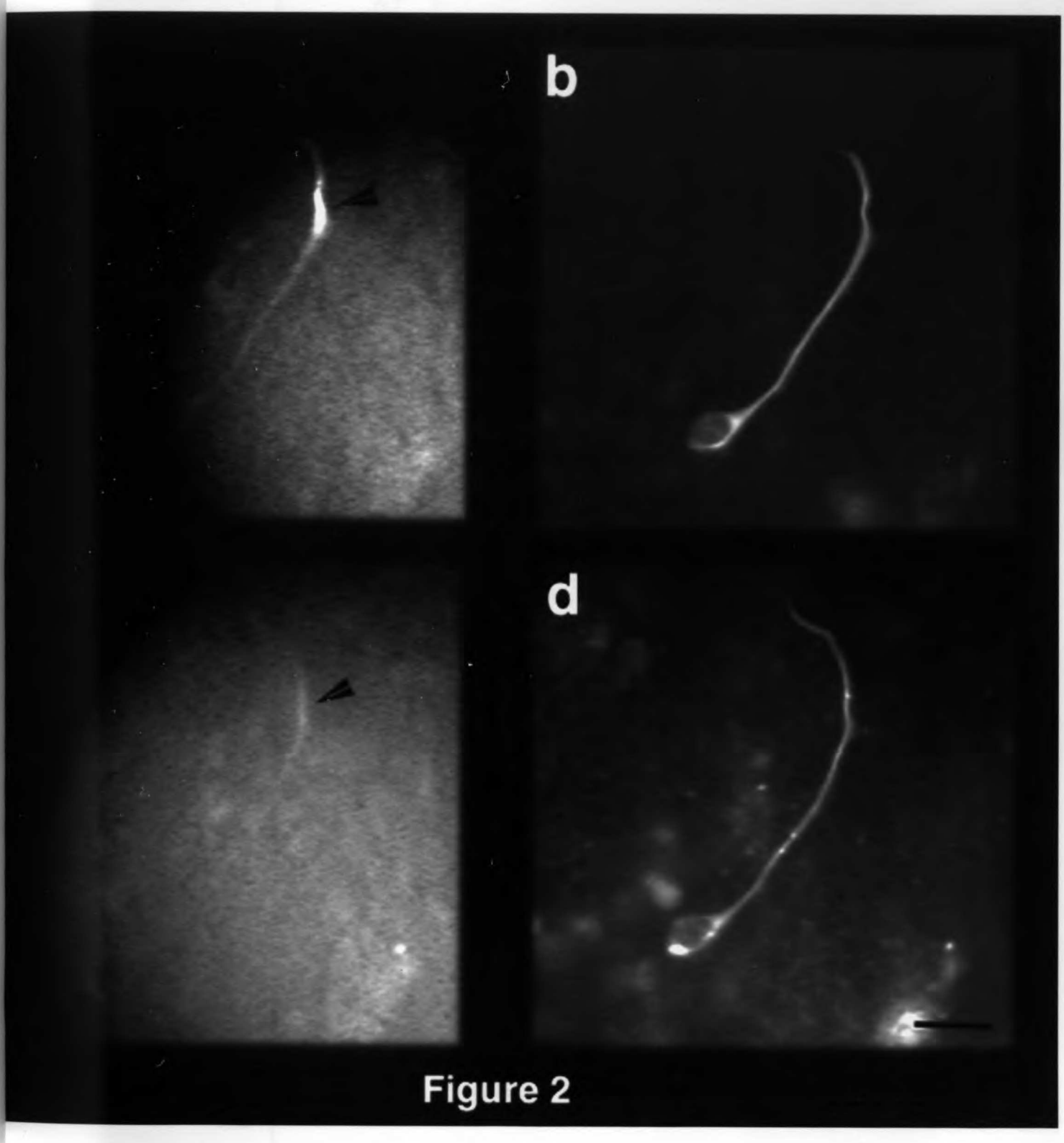
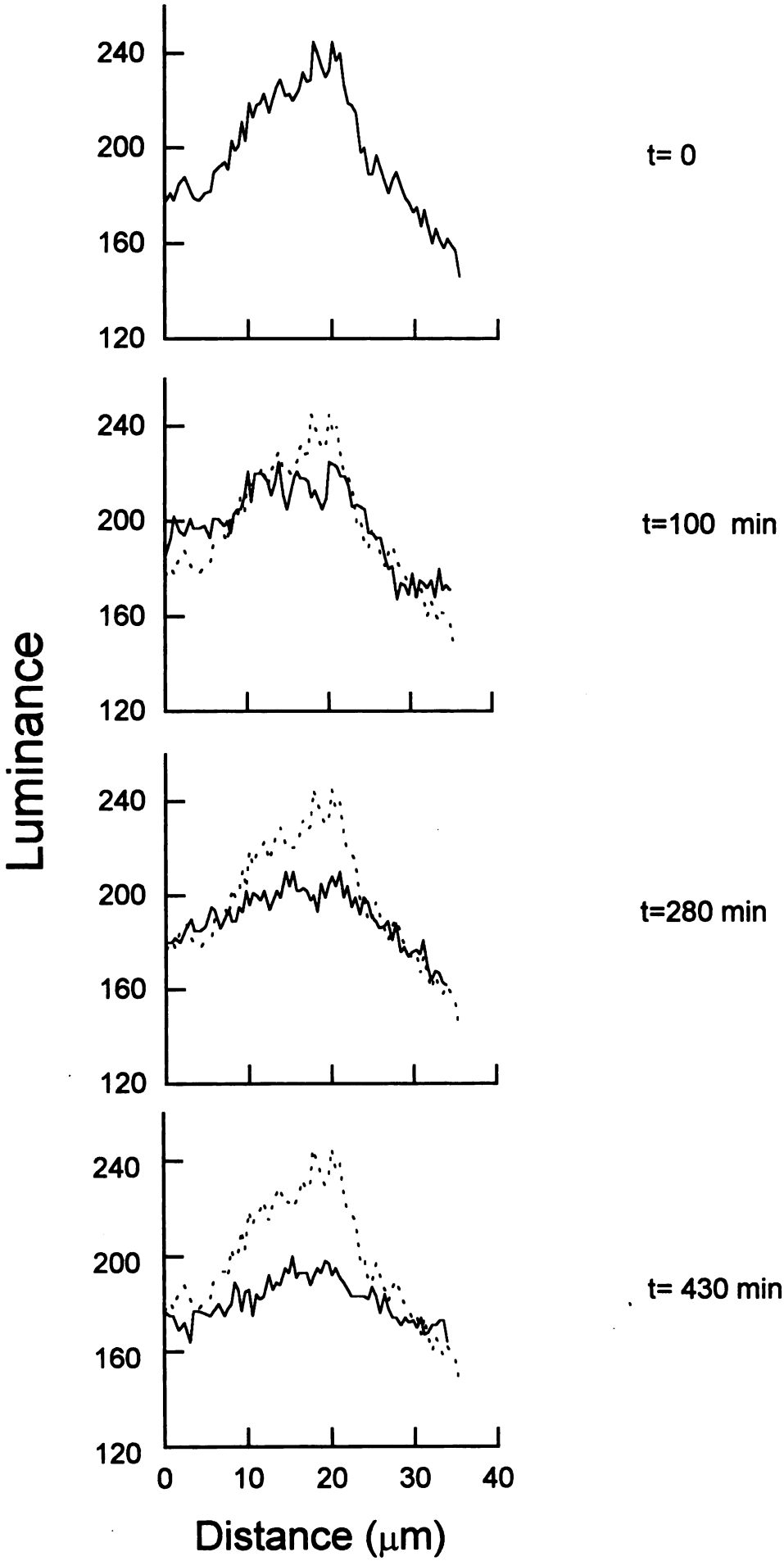


Figure 2

Figure 3. C2CF-tubulin photoactivation-migrating growth cone



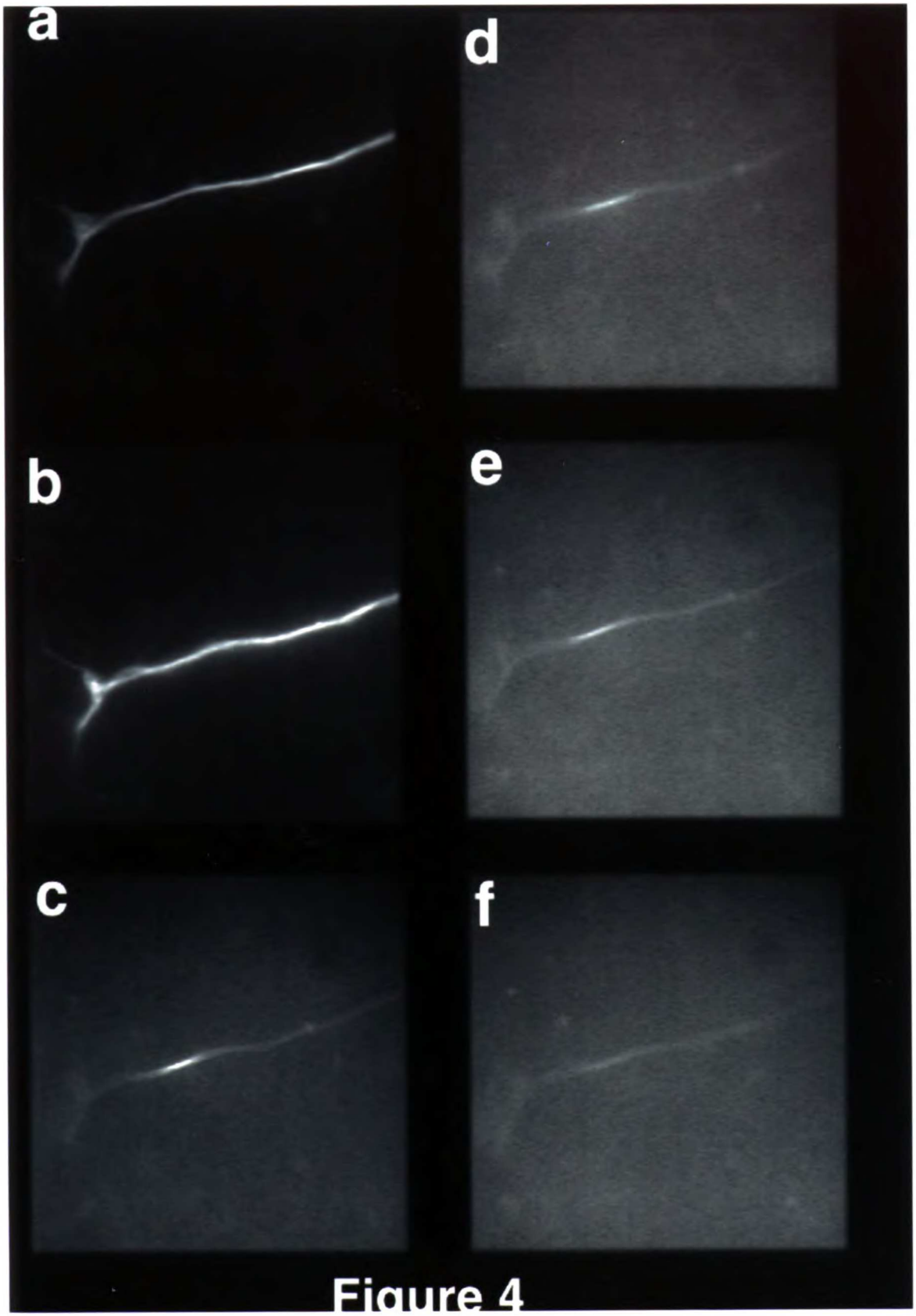


Figure 4

Caged Fluorescein Dextrans diffuse in Ti1 Neurons

In order to assess the capacity of diffusion to supply tubulin to growing neurons, we measured the diffusion coefficients of various C2CF labeled dextrans in Ti1 neurons. These experiments were so brief in duration that no net growth of the neuron could be resolved. Measurement of the diffusion of dextrans between 3kD and 40kD was impossible with the equipment used as the diffusion was faster than the fastest possible imaging frequencies. We imaged 7 neurons filled with 70kD C2CF, and 4 neurons with 2E6 kD C2CF dextran.

Figure 6 shows an example of such an experiment with a 70 kD dextran. In this experiment, the dextran was photoactivated in the cell body, and the movement of dextran down the axon imaged. The time between the top and bottom panels was 24 seconds. The apparent diffusion coefficients for a 2E6 kD dextran was 0.4 $\mu m^2/s$, and that for 70 kD dextran was 8 $\mu m^2/s$.

figure 6

DISCUSSION

The development of the nervous system is unique in that not only must cells proliferate and differentiate into a myriad of different neurons and glia cells, but neurons must then extend dendrites and axons over long distances to make synaptic connections with their targets. Indeed, it is this phenomenon of axonal (and dendritic) growth and guidance that is the cornerstone of nervous system development.

There is much evidence that the rate at which neurons grow may be regulated for biological reasons (Konishi and Akutagawa, 1985). That is, neurons may grow at different rate so as to arrive at their targets within a specific window of time. For instance, in the developing neocortex, growth cones stall in the subplate layer, and wait until target cells in the cortex proper are born (Shatz et al., 1988). In the embryonic brainstem, axons from different cranial nuclei grow out at different rates, so that they can reach their targets at the same time.

The mechanism of this regulation of axonal outgrowth is not known, but given that microtubules form the major structural components of axons, it is likely that the axonal transport of tubulin is affected either directly or indirectly by cues in the environment. We have chosen a biologic system that maintains in vivo guidance and growth cues to directly measure microtubule translocation during axonal growth.

The embryonic grasshopper limb is a tubular structure. The earliest born neurons are the Ti1 pioneer neurons that form from cells in the distal tip of the limb, delaminate from the epithelial monolayer that forms the outer surface of the tube, and then send axons proximally along a stereotyped

pathway to putative targets in the central nervous system (Bentley and Keshishian, 1982).

The environmental cues that dictate growth and guidance of this axon in vivo are preserved in the embryonic limb fillet prep that was used for these experiments (Lefcort and Bentley, 1987). As shown in chapter 3 in more detail, this allows one to inject fluorescently labeled molecules into the cell body of the Ti1 neuron, and then to image the axon or growth cone as the neuron is influenced by natural in vivo guidance cues. Given that a neuron may respond to many in vitro cues in ways that do not occur in the embryo, this approach has the advantage that the mechanisms used to control guidance and growth are likely to be very important in the developing embryo. For instance, when examining the rearrangements of microtubules in the growth cone during steering events, many different behaviors are seen when neurons turn at artificially constructed substrate boundaries in vitro (E. Tanaka, pers comm). Of these behaviors, we have found that only a select few are used in the developing embryo (see chapter 3 and Sabry etal, 1991). Hence, we feel that using this more complicating, biological system has advantages for investigating the role cytoskeleton in axonal growth and guidance.

We studied neurons that underwent no axonal growth as well as those that grew and guided during the experiment. We find no evidence for microtubule polymer translocation in any of the cells studied. Given that the intensity of the activated caged fluorescein has a certain amount of noise to it (that is, for a given intensity, i, the noise is approximated by the $\sqrt{i}$), is it possible to say with what certainty we can exclude polymer movement? Indeed, if the polymer was moving very slowly, say at 10% of the rate of growth cone movement, we could not detect it in this assay, as the

fluorescence would decay before any appreciable mark translocation would occur. However, we have a good measure of the ratio of mark translocation to growth cone movement in xenopus motor neurons, where mark translocation is know to occur (Reinsch et al., 1991). In that system, for marks made near to the growth cone, the mark translocates at approximately 0.9x the rate of growth cone movement. As the mark is made further back nearer to the cell body, this ratio decreases linearly. We applied this statistic to the marks we made on the Ti1 neuron, and asked whether we could detect a population of microtubules moving at that rate.

In order to ask whether a population of moving microtubules would be detected over the noise in the system, we artificially added a moving component to the intensity profiles shown in figure 3. The original photoactivation intensity profile approximates a single gaussian. The hypothetical moving population was made by adding a second curve (and decrementing it to the same extent as the original to account for microtubule turnover and photobleaching of the fluorophore) of the same shape, but some percentage of the area of the original and locating it distalward by the distance calculated using the measured growth cone growth rate, and the ratio statistic from xenopus neurons. We find that the noise inherent in the system makes it impossible to resolve a moving population that represents 10% of the microtubules. If the area is increased to 15% of the original area, then the resultant curve is fit closer by two gaussians (that is, the chi^2/degrees of freedom is smaller for two gaussians than for one) suggesting that there would be two populations of microtubules, one moving, the other stationery.

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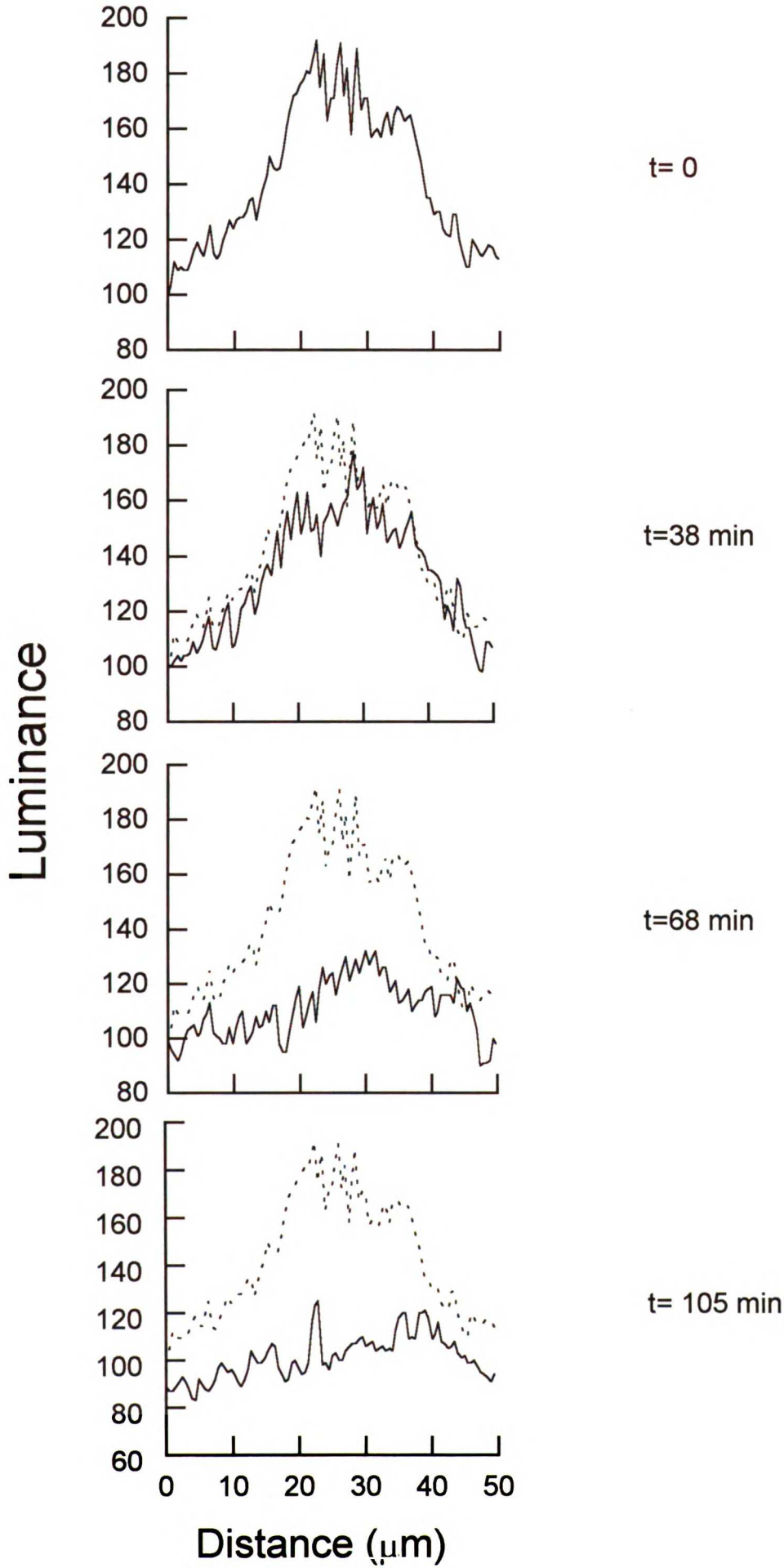
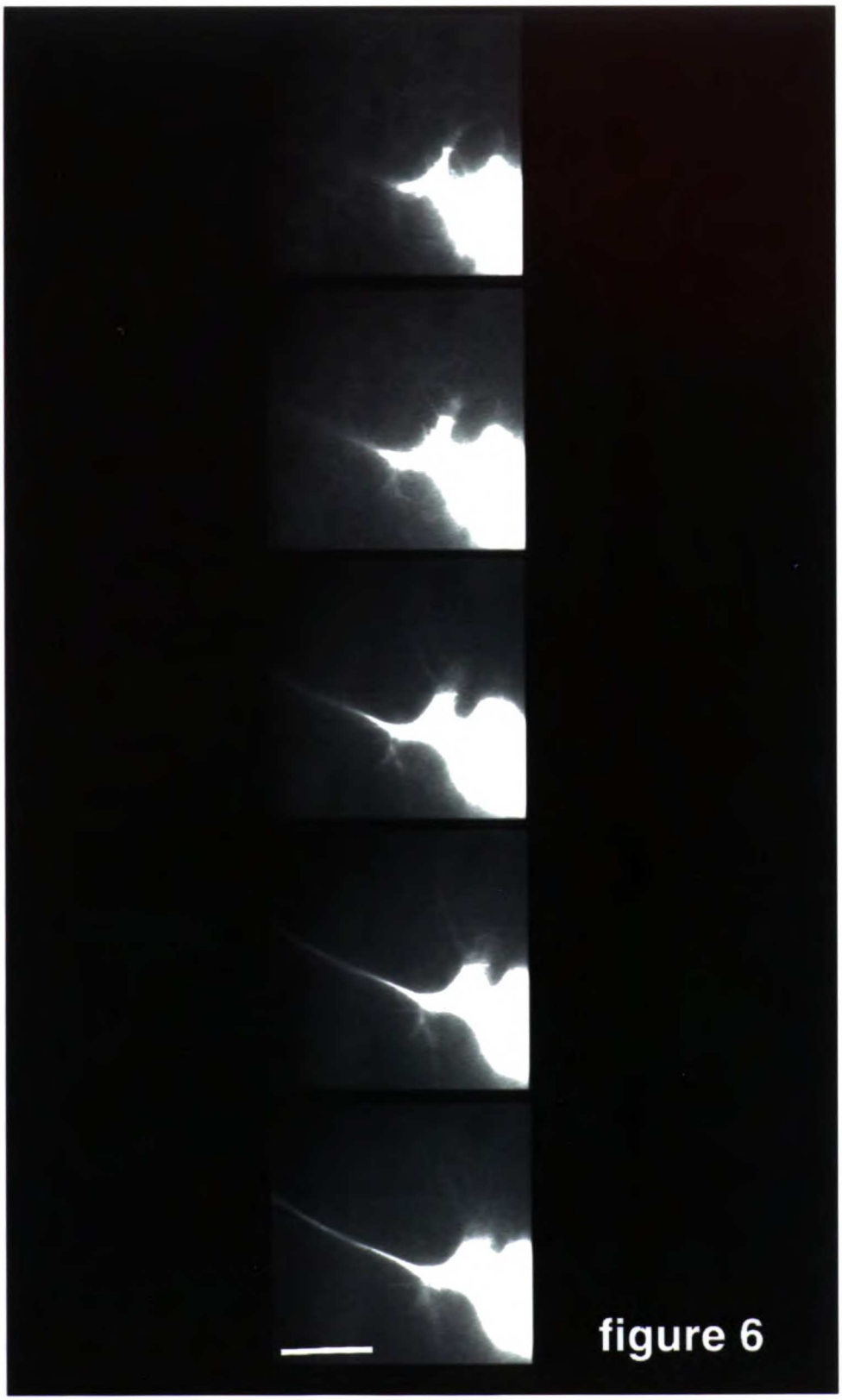
Figure 5. Intensity Profiles of photoactivated tubulin in a non-growing neuron. The experiment in Figure 4 was analyzed by measuring the pixel intensity along the axon with a curvilinear line the followed the center of the axon. Time=0 corresponds to Fig 4a,c; time=38 min corresponds to fig 4d; time= 68 min corresponds to fig 4e, and time=105 min corresponds to fig 4b,f. The initial intensity profile at time=0 is reiterated in the subsequent graphs as a dotted line to allow comparison. There is no evidence for translocation of the intensity profile.

Figure 6. Diffusion of a 70 kD dextran in a Ti1 neuron. The C2CF caged 70 kD dextran was photoactivated in the cell body, and one can see the dextran diffusing down the axon. The time between the first and last images is 24 seconds. Scale bar=10 μm.

Figure 5. C2CF photoactivation - stationary growth cone





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This result, that at least 90% of the microtubules are non-moving is in contrast to previous photoactivation studies in xenopus motorneurons, but in agreement with studies in chick DRG (Okabe and Hirokawa, 1992; Reinsch

et al., 1991). What could account for the conflicting results seen with these experiments which all use similar techniques? There are a number of possibilities. Firstly, the xenopus neurons grow 4-5 times faster than the chick DRG neurons, and 10-15 times faster than the Ti1 pioneer neuron in situ. It is possible that the faster growing neurons transport tubulin as polymer, whereas the slower growing ones do not. Secondly, it is possible that a different population of microtubules were labeled in the different experiments. For instance, in the chick DRG and Ti1 experiments, the labeled tubulin was injected into the cell, and incorporated into most of the microtubules in the axon. This has been confirmed by electron microscopy in the chick, and by fluorescence intensity in the Ti1 neuron. However, it is formally possible that a small population of microtubules was not labeled in these later experiments, and that that population is moving. This would not explain, however, why a stationery component was not seen in the xenopus experiment where all microtubules in the cell are labeled (the label is introduced to the embryo at the two cell stage and, hence, is present in the neuron from birth), or why uniform labeling was seen in the chick DRG by electron microscopy.

A third possibility is that the extreme speed of axonal growth of the xenopus neurons results in membrane stretching, and that the microtubule movement is an artifact of a conically shaped axon. Indeed, measurements of axonal diameter in this system have found that they decrease by approximately 30% from cell body to growth cone. Furthermore, this would explain why membrane and protein imbedded in the membrane would translocate during axonal growth in xenopus and not in chick DRG (Okabe and Hirokawa, 1992; Popov and Poo, 1992).

If axonal stretching or rate of outgrowth are the reasons for the discrepancy, then measuring spot translocation in slower migrating xenopus motor neurons could resolve the issue. We have begun experiments that examine mark translocation in xenopus neurons growing on poly-D- Lysine, where they grow at ~40 μm/hr to address this issue (Sabry and Evans, unpublished data).

The original definitions of axonal transport defined the rates based on the rate of crest movement after radiolabelled amino acids were introduced into the cell (Black and Lasek, 1980). It was found that the crest moved out at a constant rate, and that the amplitude of the wave did not diminish over 77 days during the movement. This was carried out in neurons that were not elongating and had already formed mature synapses. It is not clear what relation this rate has to the axonal transport of polymer seen in xenopus, or the lack of polymer movement seen in the Ti1 neuron or chick DRG. The microtubules in the radiolabelling experiments were *extremely* stable, and given that any photoactivation or photobleach mark turnover in a matter of a few hours at the most suggests that these experiments are examining two different, possibly unrelated phenomena.

Finally a few comments on what the transport form must be if it is not polymer. One possibility is that it is a rapidly diffusible oligomeric form of tubulin that was not imaged in these experiments. This form has yet to be identified. Another possibility is that, in these short growing axons, simple pipe diffusion is sufficient to supply tubulin to the growing axon as monomer.

This would predict that tubulin transport would not be the canonical transport defined by Lasek as one with constant velocity with relation to

distance from the cell body. Instead the movement of tubulin would be described by the simple one-dimensional random walk, that is that $x^2=2Dt$, where x is the distance traveled, D is the diffusion coefficient, and t is the time taken to diffuse to distance x. If this were the major form of tubulin transport then two kinetic predictions could be made. Firstly, that as a neuron grew, assuming a constant tubulin concentration in the distal axon, the concentration of tubulin in the cell body would steadily increase to drive the diffusion. Secondly, that the rate of tubulin transport would fall off linearly with distance from the cell body. Both these predictions could be tested by introducing radiolabelled tubulin into the cell body of the Ti1 neuron and carefully measuring the rate of movement its movement.

Given that diffusion does exist, it may not be surprising that these predictions might be satisfied for short neurons, however for long neurons, it seems likely that transport is necessary not because of the rate of diffusion is too slow, but rather that the concentration of tubulin in the cell body needed to drive diffusion down a long axon is too great to exist in cells. Our measured diffusion coefficients agree with those measured in xenopus neurons (Popov and Poo, 1992), so one would assume that diffusion would be similar in the two systems. It seems likely that many different mechanisms may be used to move tubulin down the axons, and that any one of these mechanisms could be regulated by outside cues.

CHAPTER THREE

MICROTUBULE BEHAVIOR DURING GUIDANCE OF PIONEER NEURON GROWTH CONES IN SITU

ABSTRACT

The growth of an axon toward its target results from the reorganization of the cytoskeleton in response to environmental guidance cues. Recently developed imaging technology makes it possible to address the effect of such cues on the neuronal cytoskeleton directly. Although high resolution studies can be carried out on neurons *in vitro,* these circumstances do not recreate the complexity of the natural environment.

We report here on the arrangement and dynamics of microtubules in live neurons pathfinding in response to natural guidance cues *in situ* using the embryonic grasshopper limb fillet preparation. A rich microtubule network was present within the body of the growth cone and normally extended into the distal growth cone margin. Complex microtubule loops often formed transiently within the growth cone. Branches both with and without microtubules were regularly observed. Microtubules did not extend into filopodia.

During growth cone steering events in response to identified guidance cues, microtubule behavior could be monitored. In turns toward guidepost cells, microtubules selectively invaded branches derived from filopodia which had contacted the guidepost cell. At limb segment boundaries, microtubules displayed a variety of behaviors, including selective branch invasion, and also invasion of multiple branches followed by selective retention in branches oriented in the correct direction. Microtubule invasion of multiple branches also was seen in growth cones migrating on intrasegmental epithelium. Both selective invasion and selective retention generate asymmetrical microtubule arrangements within the growth cone, and may play a key role in growth cone steering events.

INTRODUCTION

Developing neurons may send their processes over great distances and through complex environments to establish contact with their target cells (Caudy and Bentley, 1986b; Dodd and Jessell, 1988; Goodman et al., 1984; Harris et al., 1987; O'Leary and Terashima, 1988; Tosney and Landmesser, 1985; Westerfield and Eisen, 1988). This outgrowth is not random but, rather, is guided by environmental cues. It has long been appreciated that neuronal guidance can essentially be viewed as the process by which the growth cone, the dynamic and expanded tip of the neurite, is oriented by environmental cues (Harrison, 1910). These cues appear to be provided by a variety of factors, including substrate-bound molecules on cell surfaces and in the extracellular matrix, and diffusible chemoattractant molecules (Anderson, 1988; Elkins et al., 1990; Fessler and Fessler, 1989; Goodman et al., 1984; Reichardt and Tomaselli, 1991; Tessier-Lavigne and Placzek, 1991). The arrangement of these factors in the developing embryo presumably provides the information necessary to guide the developing neurons. In order for growth cones to reach their targets, they must alter their direction of growth in response to these factors, a process termed steering.

The process of growth cone steering first involves the exploration of the environment by growth cone filopodia, lamellipodia and branches to locate discrete guidance cues or to evaluate the spatial distribution of cues in the region around the growth cone (Bentley and Toroian-Raymond, 1986; Bray and Hollenbeck, 1988; Goldberg and Burmeister, 1989; Mitchison and Kirschner, 1988). A subset of filopodia, lamellipodia or branches are then selected for the direction of further growth cone extension. This is followed by the intrusion of cellular material into the selected process, resulting in growth cone reorientation (Aletta and Greene, 1988; Goldberg and

Burmeister, 1986; Heidemann et al., 1984). These cellular components are subsequently consolidated into a stable configuration characteristic of the axon (Hirokawa et al., 1988; Lewis, 1989).

Many cellular components, including cytoskeletal elements such as microtubules and actin filaments, and intracellular second messenger molecules, are likely to be involved in the directed growth of axons (Goldberg and Burmeister, 1989; Kater and Mills, 1991; Lankford and Letourneau, 1989; Meininger and Binet, 1989). It is not known whether environmental cues directly affect all these components, but oriented axonal growth ultimately must alter the arrangement of cytoskeletal polymers within the axon. Indeed, the generation of an asymmetric arrangement of microtubules may be a key event in growth cone steering. This can be accomplished by two mechanisms: (1) selective microtubule invasion of a limited region of the growth cone, or (2) random invasion of many regions of the growth cone followed by selective retention of microtubules extended in the direction of future growth. Recently, direct observations of microtubules in living *Xenopus* growth cones *in vitro* have identified microtubule behaviors that may underlie steering decisions (Tanaka and Kirschner, 1991). However, it is not known which if any of these behaviors play a role in pathfinding in the complex environment of the embryo.

In order to address this issue, we characterized the dynamic behavior of microtubules in response to *in situ* guidance cues by imaging fluorescently labelled microtubules in Ti1 pioneer neurons in live grasshopper embryonic limb fillets. In the grasshopper embryo, a pair of sibling neurons, termed the Ti1 pioneers are the first neurons to extend axons toward the central nervous system (Bate, 1976; Bentley and Keshishian, 1982; Ho and Goodman, 1982). They are born at the distal tip of the limb bud, emerge on the basal surface of

the epithelium, and extend growth cones proximally along the limb axis. As they migrate, the growth cones contact a complex environment consisting of epithelial cells, basal lamina and pre-axonogenesis neurons termed "guidepost cells" (Anderson and Tucker, 1988; Caudy and Bentley, 1986b; Condic and Bentley, 1989b; Lefcort and Bentley, 1987). The pathway taken by the Ti1 growth cones is illustrated in Figure 1. Key regions of the pathway, where distinctive growth cone behaviors occur, are enclosed in the series of boxes. Box 1 shows a growth cone in the femur, where it interacts primarily with intrasegmental epithelial cells and the overlying basal lamina. In this region, growth cones migrate proximally along the limb axis with frequent small course corrections. Box 2 shows the growth cones spreading on the Tr1 guidepost cell at the Tr-Cx segment boundary. At this location, the growth cones encounter two orthogonally arranged bands of limb segment boundary cells, a distal band of high adhesivity cells (Fig. 1, filled hexagons), and a proximal band of lower adhesivity cells shown by the unfilled hexagons in Fig. 1 (Bastiani et al., in press; Caudy and Bentley, 1987; Condic and Bentley, 1989a). The growth cones extend branches both dorsally (up in all figures) and ventrally on the first band of cells, but eventually always make a ventral turn. Box 3 shows the growth cones migrating ventrally along the Tr-Cx segment boundary and approaching the Cx1 guidepost cells. When a growth cone filopodium contacts the Cx1 cells, the growth cone turns along it, by a process of filopodial dilation, toward the CNS (O'Connor et al., 1990).

The Ti1 pathway in the embryonic grasshopper limb can be rendered accessible for manipulation using an opened epithelial "fillet" preparation (Lefcort and Bentley, 1987). This exposes the Ti1 cell bodies, but preserves the guidance information present in the limb, and allows the growth cones to be imaged as they migrate proximally. In the experiments reported here, we

injected the Ti1 neuron with rhodamine-conjugated bovine tubulin and imaged the labelled microtubules using a cooled charge-coupled device (CCD) camera system (Castleman, 1979; Hiraoka et al., 1987). The arrangement of microtubules in growth cones migrating in a complex environment, and how they respond during pathfinding decisions, could be examined. In particular, we determined where microtubules are present in growth cones *in situ*, and how their arrangement changes over time as steering decisions are made. We also investigated whether microtubule re-arrangements are different during steering events at different locations along the pathway. This approach revealed novel aspects of microtubule behavior that address the mechanism of growth cone steering.

MATERIALS AND METHODS

Grasshopper Embryos and Dissection

Schistocerca americana embryos were obtained from the University of California at Berkeley grasshopper colony. Eggs at the 31% - 34% stages of embryonic development were sterilized and the embryos dissected as previously described (Lefcort and Bentley, 1987). The embryos were transferred to a poly-L-lysine coated coverslip and maintained in supplemented RPMI (Condic and Bentley, 1989a). Briefly, the embryos were positioned ventral side down, thus exposing the posterior aspect of the limb bud. This surface was cut along the long axis of the limb, and the sides unrolled and flattened out onto the coverslip. The exposed interior mesodermal cells were removed using a suction pipette, leaving the basal lamina and epithelium-derived cells. The Ti1 neuronal cell bodies were

visualized with differential interference contrast optics using a Nikon inverted compound microscope.

Fluorescent Labelling of Tubulin

Purified bovine brain tubulin was labelled with tetramethyl-rhodamine as previously described (Hyman et al., 1991). This process involved covalently linking the N-hydroxyl succinimidyl ester of tetramethyl-rhodamine (#C-1171, Molecular Probes, Eugene, OR) to purified bovine brain tubulin. The labelled tubulin was then subjected to two cycles of temperature dependent assembly/disassembly to select for assembly competent tubulin. The labelled tubulin was stored at a concentration of 20-30 mg/ml in an injection buffer (50 mM Kglutamate, 0.5 mM $MgCl_2$, pH 6.5) at -80° C.

Neuronal Labelling

A Ti1 neuron cell body was injected with rhodamine-conjugated bovine tubulin using a pulled, bevelled borosilicate micropipette. Selected cells were also double-labelled with the carbocyanine dye, DiO (1,1'-dihexadecyloxacarbocyanine perchorate, $DiOC_{16}$, #D-1125, Molecular Probes, Eugene, OR) as previously described (O'Connor et al., 1990). Briefly, this involved air drying the DiO onto the tip of a pulled micropipette, and labelling the cell by gently touching it with the micropipette.

Microtubule Imaging and Analysis

Most imaging, including the images selected for the figures, was done at the Berkeley Low-light-level Video Center using a cooled charge-coupled device (CCD) camera system (Photometrics; Tuscon, AZ). This system

comprised viewing the fluorescent tissue through a 100X, 1.4 N.A. Nikon objective on a Nikon inverted compound microscope with conventional relay optics connecting the microscope to the CCD camera. The microscope projected the image onto a 1320 x 1024 pixel chip (Kodak KAF-1400), which digitized the image and transferred the data to a bulk memory storage device (REO-650 erasable optical disc; Pinnacle Micro, Inc., Irvine, CA). Image space on the chip was 0.06 μm/pixel. The chip, light path shutters, and stage focal position were controlled by a Perceptics BioVision imaging system (Knoxville, TN) on a Mac IIx computer (Apple Computers, Cupertino, CA). Illumination was provided by a 100W mercury lamp, generally in 300-400 msec exposures. To image the full thickness of the growth cone, 5-8 optical sections were usually taken. Clusters of sections were usually taken every 4 to 8 minutes. On occasion, images were taken every 3 seconds to resolve high frequency events. By tracking fluorescence through multiple image planes, out-of-focus fluorescence was identified and reduced using Focus software (Vaytek; Fairfield, IA). Microtubules traversing multiple sections were montaged with NIH Image software. Processed images were photographed directly from a 1280 x 1024 pixel video-screen (E-Machines; Beaverton, OR). Using a system developed by Drs. John Sedat and David Agard (Department of Biochemistry, University of California at San Francisco), additional images were visualized on an inverted Olympus IMT-2 microscope connected to a peltier cooled CCD (Photometrics, Tuscon, AZ) equipped with a 900 x 900 pixel chip (Texas Instruments), and controlled by a Microvax II workstation (Digital Equipment Corp., Marlboro, MA).

Electron Microscopy

After imaging, selected embryos were prepared for electron microscopy. This involved fixing them in 2% glutaraldehyde in PBS for 4 hours at room temperature. They were then reacted with a primary serum antibody (against horse-radish peroxidase) that recognizes insect neurons (Jan and Jan, 1982; Snow et al., 1987). This antibody was visualized using a 10 nm gold conjugated secondary antibody (Amersham,UK). After a second fixation in 2% glutaraldehyde in PBS, the tissue was rinsed and osmicated in 2% OsO_4 in cacodylate buffer for 30 minutes. The labelled tissue was then dehydrated rapidly through an ethanol series, and embedded in araldite (Grade CY-212 British, Polysciences, Warrington, PA). The Ti1 pioneer neuron was first identified in thick sections at the light microscopic level by silver enhancing the gold particles (Amersham, UK). Thin sections were then cut and observed in a Phillips 400 electron microscope (80 kV).

RESULTS

In order to observe microtubules in growing neurons, we flattened out the tubular limb bud, bathed it in culture medium, injected the Ti1 pioneer neuron, and exposed it to brief pulses of visible light. Under these conditions, the route taken by the Ti1 growth cone was indistinguishable from that of the neuron *in vivo* . This suggests not only that natural *in vivo* guidance cues are preserved in the fillet, but also that the incorporation and subsequent imaging of rhodamine-conjugated bovine tubulin has little effect on the pattern of Ti1 axonal growth.

The Ti1 pioneer neurons are siblings, and only one of each pair was injected for these studies. Although the two neurons are strongly coupled by

dye-passing junctions (Keshishian and Bentley, 1983; Taghert et al., 1982), no label was ever found to cross to the non-injected cell, implying that no unconjugated free rhodamine dye was generated by proteolysis of the labelled monomer. The label travelled down the axon and was found to persist as a low level of background fluorescence, thought to represent monomeric rhodamine-tubulin, and in clearly defined linear tracks in the axon and growth cone. An example of these tracks is illustrated in Figure 2 which shows a rich network of linear tracks in the growth cone. A single track invading a branch is shown by the arrowhead. We measured the width and intensity of the tracks in this and other cells. The image width of these tracks, which we will refer to as microtubules, was found to be very uniform (0.24 μm, sem=0.01 μm, mean of 15; 3 measurements/microtubule, 3 microtubules/cell in 5 cells at various stages of development). This value is similar to published widths of fluorescent images of electron microscope-confirmed single microtubules (Sammak and Borisy, 1988). More importantly, although the pixel intensity of these linear profiles varied significantly from cell to cell, within a given cell all profiles were of similar intensity. For instance, in the growth cone shown in Figure 2, the mean intensity of 5 tracks (5 measurements/track) was 91.6 units with a sem= 2.7 (2.9%). This represented a value in the middle of the dynamic range of the CCD chip which was 0 - 255 units. As detailed below, electron microscopy identified many single microtubules in the distal growth cone. These data strongly suggest that these fluorescent tracks represent single microtubules. The label persisted for approximately seven hours, and then slowly faded, probably as a result of tubulin turnover.

Figure 1. A diagram of the Ti1 pioneer neuron pathway in the embryonic grasshopper limb bud at the 35% stage. The pair of sibling Ti1 neurons arise at the limb tip and their growth cones migrate to the central nervous system (CNS) along a stereotyped route. The growth cone route reflects a series of steering decisions resulting from encounters with guidance cues. These include preaxonogenesis ("guidepost") neurons (Fe1, Tr1, Cx1), high affinity (filled hexagons) and low affinity (unfilled hexagons) circumferential bands of epithelial cells at limb segment boundaries (Ti-Fe, Fe-Tr, Tr-Cx), and segmental nerve-root glial cells (SnG). The dashed boxes (1-3) indicate limb regions where growth cone microtubule behavior is illustrated in other figures. Ti-Fe, tibia-femur boundary; Fe-Tr, femur-trochanter boundary; Tr-Cx, trochanter-coxa boundary. The length of the leg is approximately 350 μm. Dorsal, up; proximal to right.

Figure 2. Microtubule arrangements in a pioneer growth cone spreading on guidepost cell Tr1. A Ti1 pioneer neuron has been injected with rhodamine-tubulin and the growth cone is at the Tr1 cell (asterisk). This position corresponds to Fig.1, box 2. Many uniform caliber linear fluorescent tracks, which appear to be single microtubules (see text), are observed. Although the growth cone will eventually turn ventrally at this location, microtubules are present in branches (white arrows) extending both dorsally and ventrally along the Tr-Cx segment boundary. A single microtubule (arrowhead) is present in a ventrally extended branch. Scale bar=5 μm.

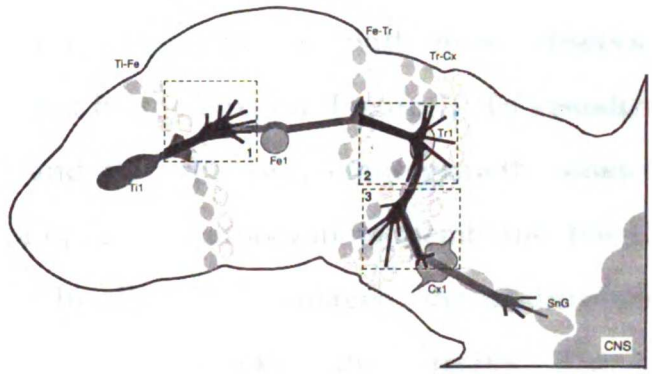


Figure 1.

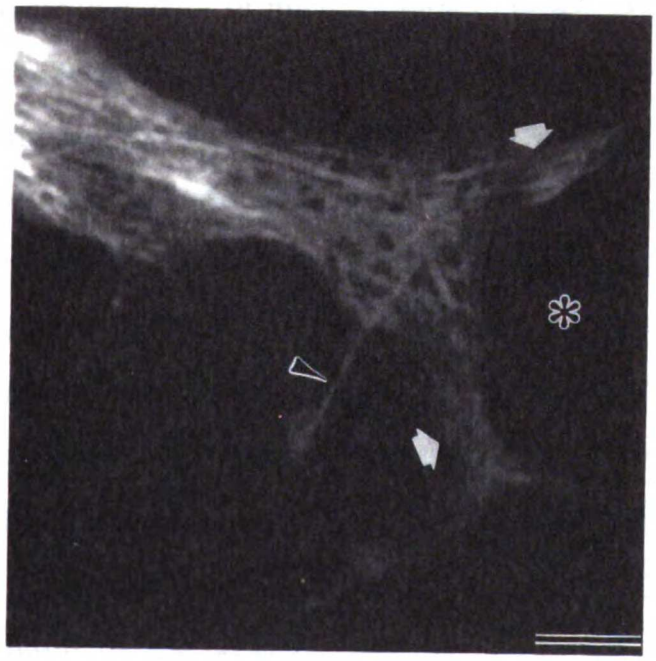


Figure 2

The Arrangement of Microtubules in Ti1 Neurons

In thirty-one embryos, thirty-one Ti1 growth cones were imaged in which microtubules were clearly resolved. These images were obtained at various places in the pathway, with most observations at the turning decisions outlined by the boxes in Figure 1. To visualize the full extent of the growth cone and its processes, eight growth cones were simultaneously labelled with a lipophilic carbocyanine membrane dye, DiO.

Axons. In all neurons imaged, the microtubules were found in the axon cylinder in closely packed linear arrays. This can be appreciated in Figure 3F, where the microtubules in the upper left of the panel were found oriented along the long axis of the neuron and were closely bundled together. The plasma membrane was closely apposed to the microtubule array. Other examples of the arrangement of microtubules in axons can be seen in Figures 2, 3E, 4, 7, and 8. Occasionally, at locations of laterally extended protrusions, the plasma membrane was not closely apposed to the microtubule bundle, and some microtubule free axoplasm could be visualized (data not shown).

The morphologic boundary between the axon and growth cone can usually be defined when neurons are viewed *in vitro* as that region where the axonal cylinder abruptly increases in caliber (Bray and Chapman, 1985; Goldberg and Burmeister, 1986). However, *in situ*, this border could not easily be identified by plasma membrane morphology in many Ti1 neurons. In these cases, no abrupt transition from the cylindrical shape of the axon to the globular shape of the growth cone was evident. However, in some neurons, the arrangement of microtubules did show an unambiguous transition from the tightly bundled arrangement characteristic of axons to a rich network of single or small bundles of microtubules characteristic of the

growth cone. For example, Figure 3F shows microtubules in a growth cone of this type; the white arrow indicates the transition from the bundled microtubule array of the axon, to the left of the white arrow, to the more dispersed arrangement of the growth cone, to the right.

Growth Cones. All growth cones were heavily invested with microtubules. In all cases, microtubules were found to inhabit the distal region of the growth cone, up to the plasma membrane. In Figure 2, for instance, a rich network of microtubules can be seen in the whole growth cone. In this figure, the edge of the growth cone was indicated by the background level of fluorescence. In Figure 3E, microtubules (indicated by the unfilled arrow) seem to have extended directly underneath the distal-most aspect of the growth cone plasma membrane. Microtubules were not restricted to the central portion of the growth cone for prolonged periods of time, as has been described for some types of neurons *in vitro* (Bridgman and Dailey, 1989; Forscher and Smith, 1988). In contrast to the axon, growth cone microtubules were not restricted to tight bundles, but frequently occurred singly or as small bundles. In several neurons, images were taken every three seconds; rapid shrinkage and re-growth of microtubules was not apparent.

In the fillet, although the cell body could be identified by differential interference contrast optics, the growth cone could only be visualized using epi-fluorescence. Many protrusions, particularly branches, could be identified by the presence of background fluorescence; however, as it was not clear that all the fine structure of the growth cone could be seen, eight growth cones were double-labelled with the lipophilic plasma membrane dye, DiO. As has been described previously (O'Connor et al., 1990), we observed extensive and varied protrusive activity from the growth cone. Types of protrusions included: (1) filopodia: fine, tubular structures of a single uniform caliber that

arise from the growth cone or from branches, (2) veils and lamellipodia: thin sheets that often extend between existing filopodia, and (3) branches: elongate processes of variable size and shape that were wider than filopodia, and often tapered from their base to tip.

Filopodia. The Ti1 growth cone is rich in filopodia, which were abundant in all double-labelled growth cones. In total, 150 filopodia were identified on DiO images. In Figure 3C, many long filopodia (arrowheads) can be seen to have arisen at the distal end of the growth cone. In the imaging plane, the mean number of filopodia per growth cone was 21 with a range from 8 to 34. The mean width was 0.31 μm, with a sem of .015 μm (4.8%). As was described by O'Connor et. al. (1990), some filopodia were very stable and unchanging in length or position over tens of minutes, whereas others were very dynamic existing for a minute or less. None of the filopodia, even those with long lifetimes, harbored microtubules. This can be seen in Fig. 3D, which is the rhodamine-tubulin image of a double-labelled growth cone (the DiO image is seen in Fig. 3C). The unfilled arrows point to microtubules in the body of the growth cone, and in a branch, but none of the filopodia identified by arrowheads in Fig 3C harbored microtubules (low intensity and lack of uniform caliber indicate that the faint signal in the filopodia in Fig. 3D represents background fluorescence).

Figure 3. Microtubule arrangements during growth cone migration on a relatively homogeneous substrate. These positions correspond to Fig. 1, box 1. **A-B**: A growth cone double-labelled with a lipophilic plasma membrane dye (DiO; A), and rhodamine tubulin (B). Bundles of microtubules are present in branches extending in different, but generally proximally oriented directions (unfilled arrows, A and B). Microtubules are absent from some branches (black arrow, A and B). Pronounced microtubule loops exist in the growth cone (white arrows, A and B). The sibling neuron was slightly labelled with DiO, and the faint image of the sibling growth cone can be seen in the lower left part of panel A. **C-D:** In another double-labelled growth cone in this region, single microtubules are present in the growth cone periphery (double arrow, D), and can extend into small branches (unfilled arrows, C and D). Many uniform diameter filopodia extend from the growth cone (arrowheads, C); these do not contain microtubules. **E-F:** In a nascent growth cone which has migrated a short distance from the cell body (E, asterisk=nucleus), microtubule bundles form loops against the growth cone perimeter (arrow, E). 34 minutes later, these loops have straightened somewhat as the microtubules enter a branch forming dorsally (unfilled arrow, F). Microtubules also enter a ventral branch which was not selected as the direction of growth cone extension. This growth cone was subsequently imaged by electron microscopy shown in Figs. 5 and 6. Within the body of the growth cone, a marked transition zone (white arrow,F) occurs where dispersed microtubules of the growth cone periphery form into a closely packed axonal array. Scale bars=5 μm. Bar in F refers to panels A-D and F.

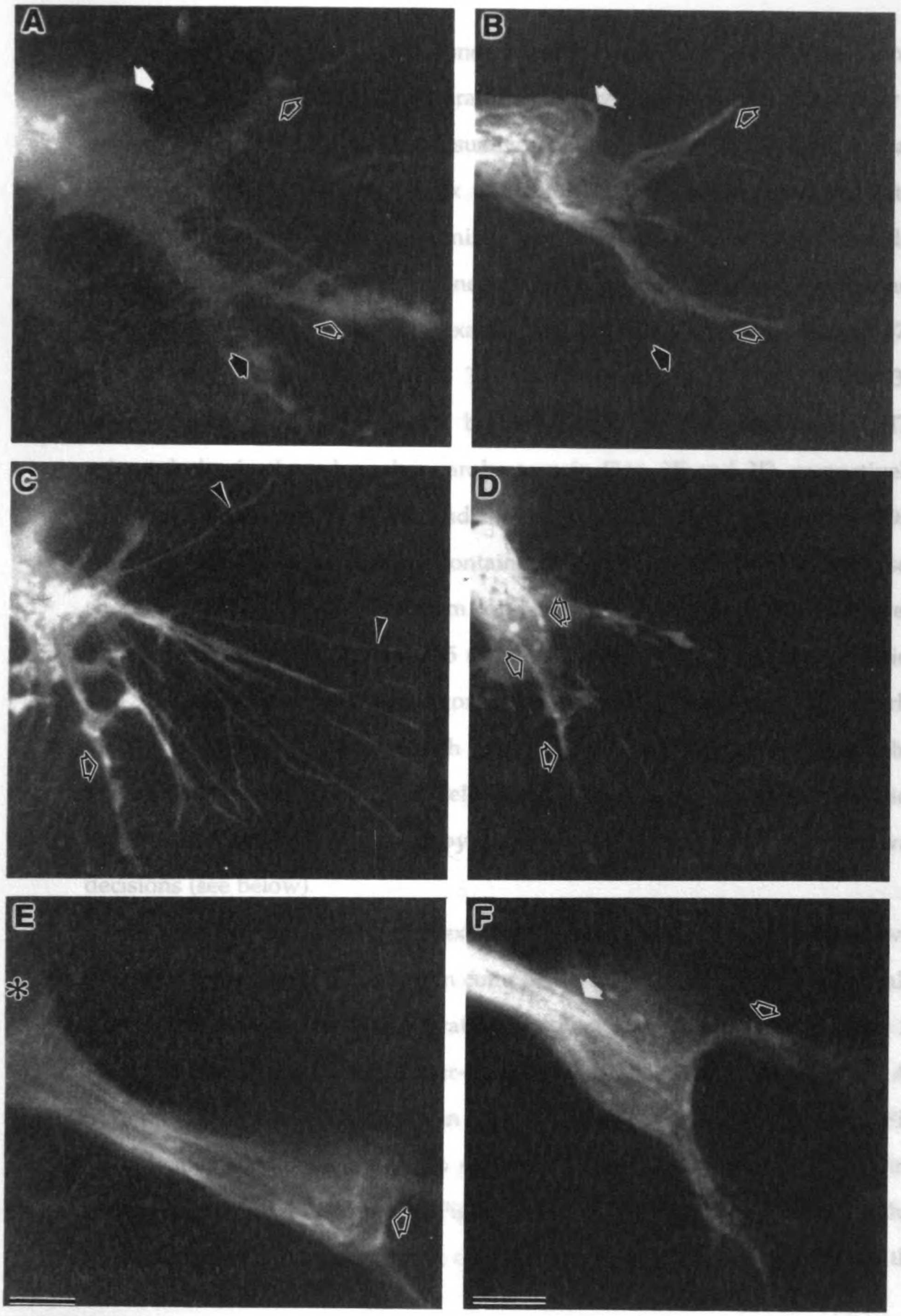


Figure 3

Branches. In all growth cones, branches were observed. As mentioned above, we operationally defined branches as any elongate protrusion larger in caliber than a filopodium. They usually had a tapering profile so that the base width was greater than the apex width. Unlike filopodia, branches often harbored microtubules. We examined the number of microtubule-invaded branches in growth cones simultaneously labelled with DiO and rhodamine - tubulin. In six growth cones so examined, microtubules were present in 57% of branches (range 40% - 100%). This is illustrated in Figures 3A and 3C, where unfilled arrows indicate branches seen by DiO labelling. The microtubules in those branches can be seen in Figs. 3B and 3D, respectively. The black arrows in Figs. 3A and 3B indicate a branch identified by DiO staining (Fig. 3A), that does not contain microtubules (Fig. 3B). In two cases, short branches were seen to form long before the microtubules selectively invaded the structure (41 and 185 minutes). This implies that the formation and maintenance of some short branches does not require nearby microtubules, and that branch existence does not imply inevitable microtubule invasion. Indeed, selective and non-random branch invasion seems to be a mechanism used by growth cones to steer at given pathway decisions (see below).

Microtubules form complex loops in the growth cone. In 14 growth cones, microtubules were found in complex loop structures. This was usually seen as the growth cone was migrating proximally in the femur (Fig. 1, box 1), or was encountering the trochanter-coxa segment boundary (Fig. 1, box 2). An example of the former is shown in Figs. 3A and 3B. The white arrow in Fig. 3B indicates the apex of a group of microtubule loops. The corresponding membrane image, illustrated in Fig. 3A, shows that these loops form within the confines of a smooth growth cone; their presence is not apparent in the

shape of the cell itself. Another example of microtubule loops can be seen in Fig. 3E. Here, the microtubules are indicated by the unfilled arrow, and can be seen to loop just under the distal surface of the growth cone. Figure 3F is an image of the same growth cone shown in Fig. 3E, 34 minutes later. The microtubule loops can still be seen, and the microtubules indicated by the unfilled arrow have invaded a branch. In many instances, the loops were found to be transient. This is illustrated in Fig. 4, which shows a temporal sequence of images of the same growth cone. The microtubule loops are indicated by the unfilled arrows. They exist in Fig. 4A, collapse in Fig. 4B, and then reform in Fig. 4C.

Electron microscopy confirms microtubule arrangements. In order to examine microtubule arrangements and loops at the ultrastructural level, growth cones were imaged, and then prepared for electron microscopy. This allowed the correlation of microtubule organization in the imaging study with an electron microscopic analysis of the same growth cone. The growth cone shown in Figure 3F was fixed shortly after this image was taken, and examined by electron microscopy (Fig. 5). The surface of the growth cone is immuno-labelled with gold particles (see Methods and Materials). The two branches seen in Fig. 3F can be seen in the upper right and lower left regions of the micrograph. The microtubules indicated by the unfilled arrow in Fig. 3F correspond in configuration and location to those indicated by the large arrowheads in Fig. 5. Note that they are found as single microtubules. Looped microtubules were also found at the electron microscopic level, and are indicated by the arrow. Figure 6 shows a higher power image of the same growth cone as in Fig. 5. Looped microtubules, indicated by the small arrowheads in Fig. 5 are indicated by the large arrowheads in Fig 6. Although the looped microtubule was not continuous at the EM level, it did show

bends with similar curvature to that in the fluorescent images. Given that each section was 0.1 μm in thickness, it was not clear whether the looped microtubule was continuous in serial sections.

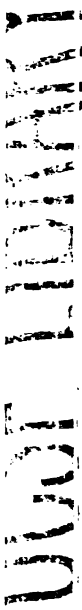


Figure 4. Transient microtubule loop formation in growth cones. This time-lapse sequence of a growth cone in the femur shows the transient formation (A), collapse (B), and re-formation (C) of microtubule loops (unfilled arrows). Time A: 0 min, B: 52 min, C: 86 min. Scale bar=5 μm.

Figure 5. Electron micrograph of a growth cone growing in the femur. The growth cone shown in Fig. 3F was prepared for electron microscopy to confirm the arrangement of microtubules seen in the imaging study. The Ti1 growth cone was identified by immuno-gold visualization of a neuron-specific primary antibody. The curved microtubules seen in Fig. 3F are located in the center of the growth cone (arrow). Several individual microtubules can be seen in the dorsal branch (large arrowheads). The microtubule indicated by the small arrowheads is shown in detail in Figure 6. Scale bar=1 μm.

Figure 6. Electron micrograph showing detail of microtubule bending. A higher magnification of the micrograph shown in Fig.5 illustrating the curvature of microtubules seen in the fluorescent images shown in Fig 3F. Scale bar =1 μm.

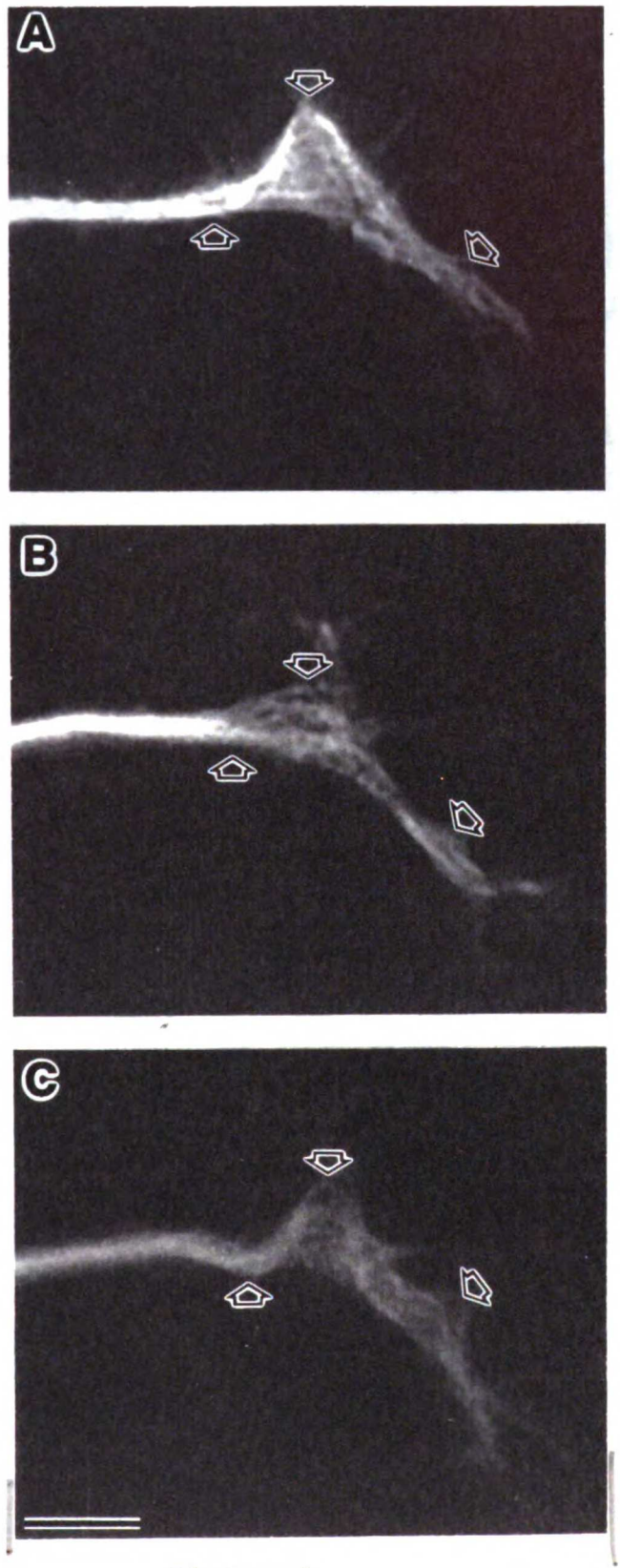


Figure 4

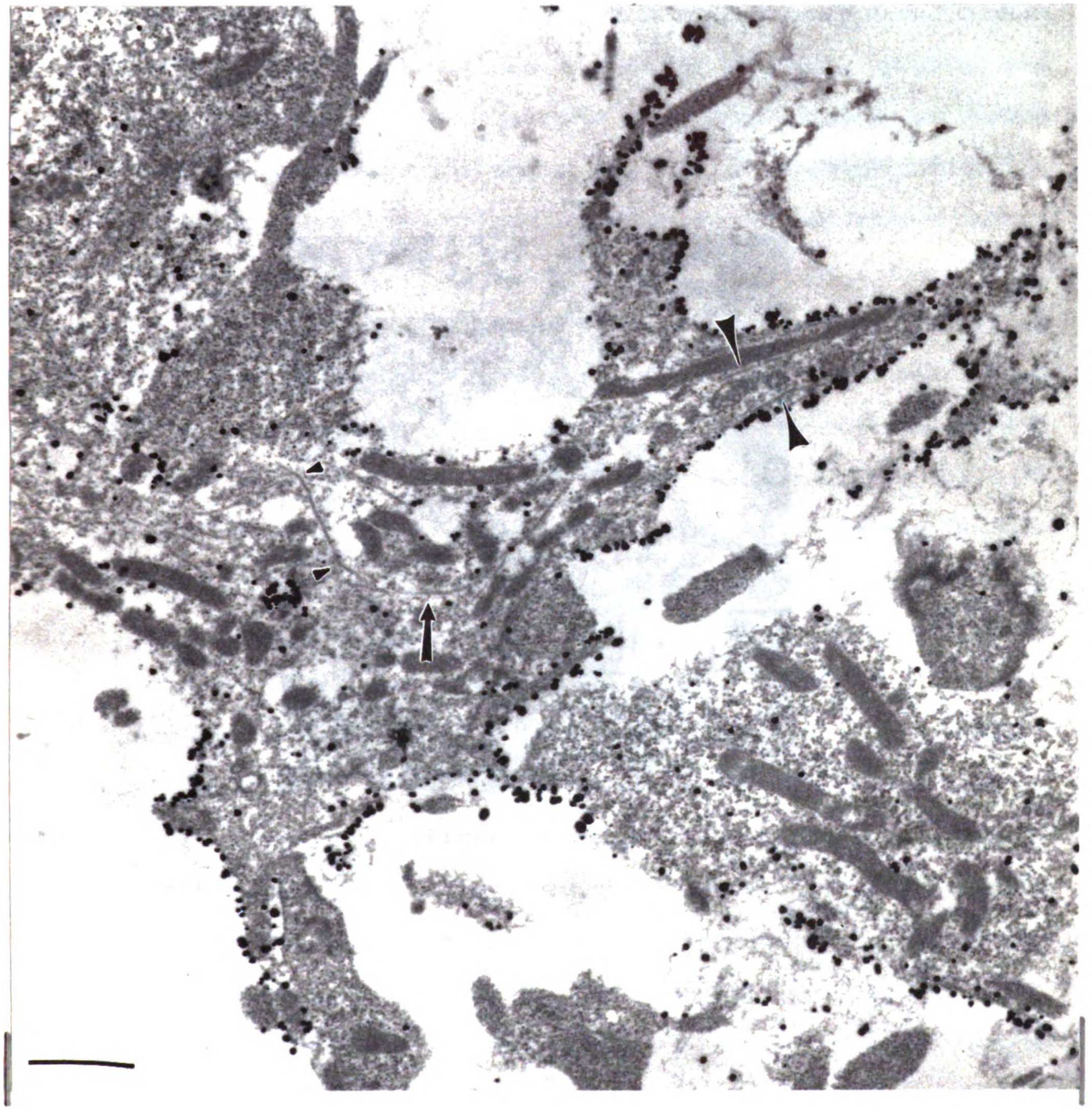


Figure 5

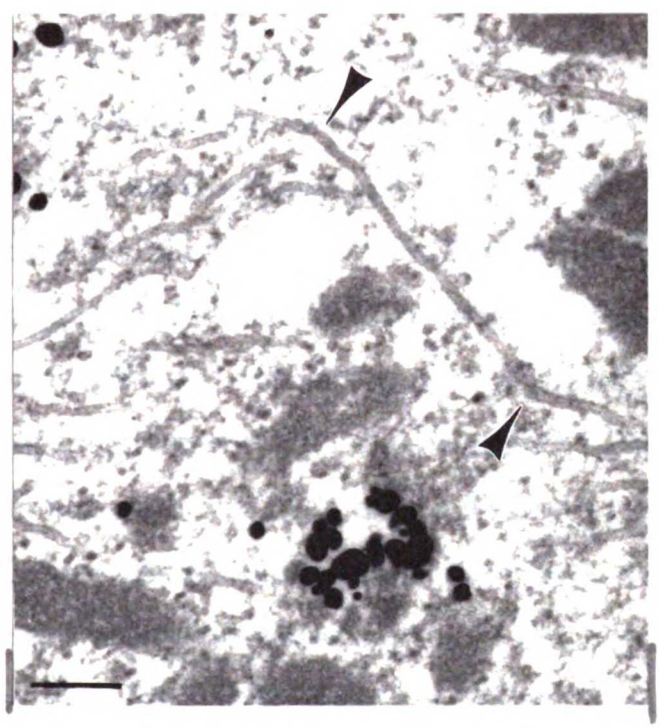


Figure 6

The Dynamics of Microtubule Movement during Steering Events

Pioneer growth cone steering behavior differs in different limb regions, depending upon the nature of the substrate with which the growth cone is in contact. Several general classes of steering behaviors, including veil extension or retraction, branch extension or retraction and filopodial dilation have been distinguished (O'Connor et al., 1990). We examined microtubule behavior at various limb locations where these three main types of steering events occur.

First, on intrasegmental epithelial cells in the mid-femur region (Fig. 1, box 1), the growth cone constantly makes small course corrections to keep it growing proximally along the limb axis (O'Connor et al., 1990). Five growth cones were imaged in this region. The major morphologic form of growth cone migration was by veil extension; initial veil extrusion between filopodia was followed by engorgement of the veil by growth cone cytoplasm. This type of growth is similar to that of PC12 cells and *Aplysia* neurons imaged *in vitro* (Aletta and Greene, 1988; Goldberg and Burmeister, 1986). Branches were normally present in growth cones in this region, and they often harbored microtubules. Figs. 3A and 3B show a growth cone in the femur, and the branches containing microtubules are indicated by the unfilled arrows. A single branch without microtubules is shown by the black arrow. Usually more than one branch harbored microtubules, although only one was oriented in the future direction of growth cone extension. In this situation, microtubules invaded more than one branch, but were retained in only one. For instance, the growth cone shown in Fig. 3F was in the femur, and microtubules were present in both the dorsal branch, indicated by the unfilled arrow, and the ventral branch, seen in the lower right of the image.

Although both branches were generally oriented proximally, that is to the right, the ventral branch was eventually withdrawn and the growth cone extended along the dorsal branch (data not shown). Hence, in this region of the limb, asymmetric microtubule arrangements were set up by selective retention of microtubules in specific branches.

Another type of steering event occurs where the Ti1 growth cone encounters the epithelial cells that form the Tr-Cx limb segment boundary (Fig. 1, box 2). The epithelial cells at the boundary are known to differ in shape and surface properties from their intrasegmental counterparts (Bastiani et al., in press; Caudy and Bentley, 1986a; Condic and Bentley, 1989a). The growth cones encounter an orthogonal interface between a distal band of highly adhesive epithelial cells (shown as a band of filled hexagons in Fig. 1) and an adjacent proximal band of lower-affinity cells (shown as a band of unfilled hexagons in Fig 1). At this interface, branches are sent in both dorsal, (up in all figures) and ventral directions. The relative sizes of these branches is quite variable and ranges from large dorsal branches and small ventral branches, through symmetric branch size, to large ventral and small dorsal branches. However, regardless of the initial branch size, the growth cone invariably makes a ventral turn along the boundary.

Five growth cones were imaged as they made this ventral turn at the trochanter-coxa segment boundary. All five represented situations where branches were sent both dorsally and ventrally. Two classes of microtubule movements were seen. In the first class (3 cases), microtubules initially invaded both the dorsal and ventral branches. This is illustrated in Fig. 2, which shows a growth cone spreading at the Tr-Cx boundary (the location of the Tr1 guidepost cell is indicated by the asterisk). Note that the ventral (upper) and dorsal branches, indicated by the white arrows, both contain

microtubules, even though the growth cone will eventually turn ventrally. With time, more microtubules were seen to invade the ventral branch (data not shown). In all cases, the dorsal branch and its microtubules persisted during this period. Other studies have shown that this dorsal branch is eventually resorbed (Caudy and Bentley, 1986b; O'Connor et al., 1990). This turning mechanism did not seem to involve selective branch invasion by microtubules, but rather selective retention of those microtubules in the ventral branch.

In contrast, the second class (2 cases) of microtubule movements at this segment boundary turn did involve selective ventral branch invasion, and is portrayed in Fig. 7. This figure shows a series of images of the same growth cone as it makes the ventral turn at the Tr-Cx segment boundary. The stage was moved slightly after the image in Fig. 7C to follow the growth cone. The growth cone has migrated to the Tr1 cell, which is indicated by the black arrow in Fig. 7A. At this point, several filopodia and small branches extend both dorsally and ventrally along the segment boundary, at the right of each of the panels. The microtubules form a closely packed bundle in the growth cone in Fig. 7C, but are not oriented dorosventrally along the boundary. In Fig. 7D, however, the bundle shows a slight ventral orientation. Note that no microtubules have entered the dorsal or proximal branches seen in the upper right region of Fig. 7D. The ventral branch, which is also void of microtubules at this time, is shown by the arrowheads in Fig. 7D. In Fig. 7E, the microtubules have clearly entered the ventral branch (indicated by the arrowheads). The dorsal branches persisted for the duration of the imaging, but they never harbored microtubules. No morphologic differences were apparent between growth cones that used this type of turning and those in the former class. Although these two classes of microtubule movements

differed in whether the dorsal branch harbored microtubules, both turns eventually involved the streaming of microtubules into the ventral branch. Hence, at the steering decision made at the Tr-Cx segment boundary, both selective invasion and selective retention were seen as mechanisms of generating asymmetric microtubule arrangements.

Figure 7. Selective microtubule invasion during a growth cone steering event at a limb segment boundary. **A:** (time: 0 min). The position of this rhodamine-tubulin labelled pioneer neuron growth cone is shown in Fig. 1, box 2. A pronounced microtubule loop is present at the Fe-Tr segment boundary (white arrow). A small bundle of microtubules (unfilled arrow) extends along the process which contacts the Tr1 cell (black arrow) located at the Tr-Cx segment boundary. **B:** (time: 2 hr, 31 min). The process extending to the Tr-Cx boundary has thickened (unfilled arrow). The loop at the Fe-Tr boundary (white arrow) has straightened, and the axon proximal to this boundary (that is, left of the white arrow) has enlarged. **C:** (time: 2 hrs, 56 min). The process extending to the Tr-Cx boundary continues to enlarge (unfilled arrow). **D:** (time: 4 hrs, 55 min). The process extending to the Tr-Cx boundary has expanded to normal axonal caliber (unfilled arrow). The Tr-Cx segment boundary is indicated by the triangle. Microtubules end abruptly at the boundary, although filopodia and branches have extended in several directions including ventrally (arrowheads). **E:** (time: 7 hrs, 9 min). Microtubules (arrowheads) have selectively invaded the branches extending ventrally, in the direction the growth cone will take. Microtubules did not invade branches extended dorsally or proximally. Scale bar = 5 μm.

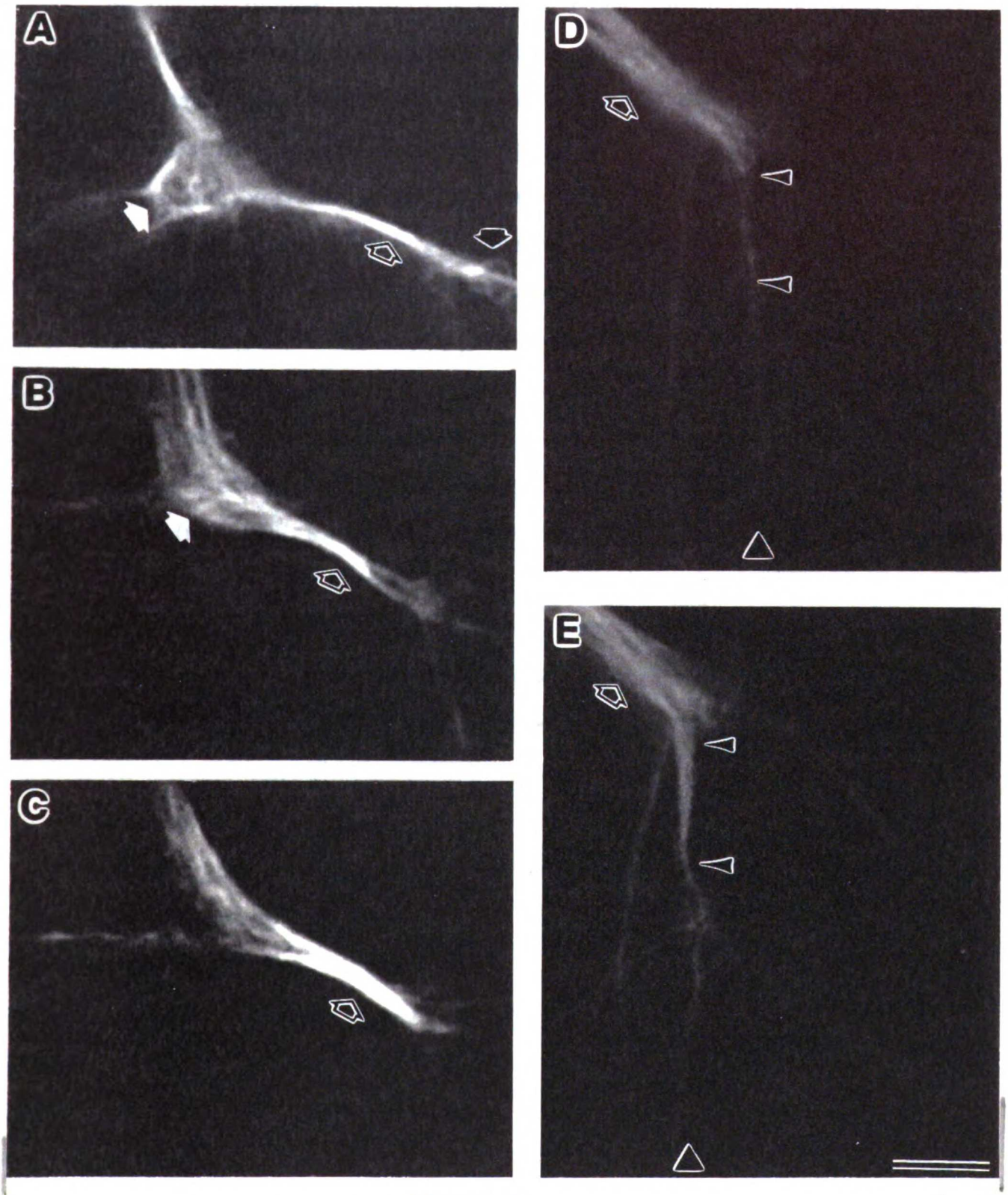


Figure 7

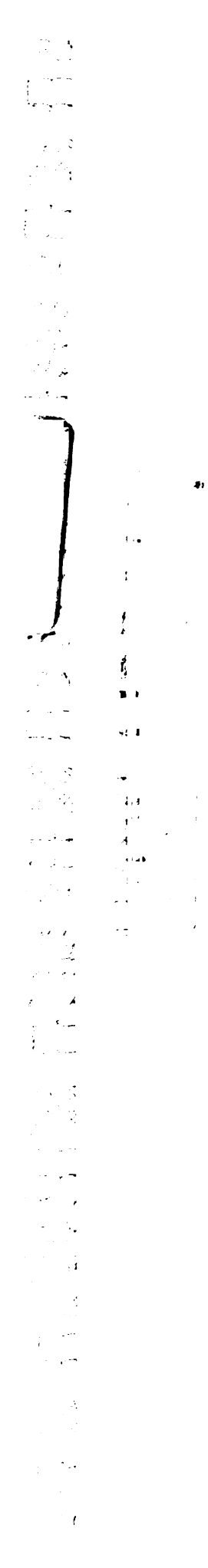
The third, and perhaps most striking, of the steering mechanisms used by the Ti1 pioneer neurons is elicited by an interaction between the Ti1 growth cone and guidepost cells. Three guidepost cells are found in the Ti1 pathway: Fe1, Tr1 and the Cx1 pair. A major growth cone re-orientation occurs at the Cx1 turn (shown in Fig. 1, box 3). This turn was imaged three times, and the smaller angle Tr1 turn was imaged twice. In all five cases, the turns were accomplished by selective invasion of a single growth cone branch by microtubules. Figs. 8A and 8B show a growth cone making the turn to the Cx1s. The growth cone has migrated ventrally along the segment boundary, which is indicated by the triangle in Fig. 8A. The location of the Cx1 cells is indicated by the unfilled arrow. In Fig. 8A and 8B, note that filopodia and branches extend from the growth cone in both proximal (to the right) and distal (to the left) directions. A single filopodium can be seen extending to the Cx1s, as shown by the white arrow. Fig. 8B shows the same growth cone 28 minutes later; microtubule(s) have selectively invaded the branch which has formed from the filopodium extending to the Cx1 cells (white arrow). Therefore, microtubules had selectively extended in the direction of growth cone migration. This selectivity occurred when there was only one branch present, extending to the Cx1 cells, and also when additional proximal or distal branches were present as in Figs. 8A and 8B. Figs. 8C and 8D show another growth cone at a later stage in the turn. The initially small number of microtubules that have entered the branch extending to the guidepost cell have become the nascent axon microtubule fascicle as the turn is completed.

Previous studies of this turn indicate that occasionally a proximally extended filopodium may not make contact with a Cx1 cell until after the leading edge of the growth cone has migrated ventrally past the cell (Caudy and Bentley, 1986b; O'Connor et al., 1990). We imaged one growth cone

where this pattern of growth apparently occurred. In this situation, the branch contacting the Cx1 guidepost cell harbored microtubules that appeared to originate directly from the main growth cone bundles. This is shown in Fig. 8E, and in higher magnification in Fig. 8F. The location of the Cx1 cells is indicated by the unfilled arrrow, and the triangle in Fig. 8E shows the location of the Tr-Cx segment boundary. Note that four microtubule bundles seem to arise from larger bundles within the growth cone, and enter the branch extending to the Cx1 cell (white arrow). The arrowhead in Fig. 8F shows microtubules in the distal region of the growth cone, which had migrated past the Cx1 cells.

Before growth cones make the turn at the trochanter-coxa boundary (Fig. 1, box 2), a guidepost cell mediated turn occurs at the Tr1 cell. A single filopodial contact with the Tr1 guidepost cell serves to reorient the growth cone by filopodial dilation. We observed two growth cones at this location where a small number of microtubules selectively entered a branch in contact with the Tr1 cell. The later stages of one such turn is shown in the first three panels of Figure 7. The Tr1 guidepost cell is indicated by the black arrow, and the branch contacting it by the unfilled arrow. Although other filopodia and small branches were present, microtubules only entered the branch extending to Tr1. Subsequently, additional microtubules accrued within this branch until it reached the caliber of the nascent axon (Fig. 7D). These results suggest that at all guidepost cells, steering by selective filopodial dilation is accompanied by selective microtubule invasion.

Figure 8. Selective microtubule invasion during growth cone steering events at the Cx1 guidepost cells. Three rhodamine-tubulin labelled growth cones (A-B, C-D and E-F) are imaged as they reorient abruptly at the Cx1 guidepost cells (Fig.1, box 3). **A:** (time: 0 min). A growth cone migrating ventrally along the Tr-Cx segment boundary (shown by triangle) extends a single filopodium (white arrow) to contact the Cx1 cells (unfilled arrow). **B:** (time: 28 min). The filopodium now has a branch morphology, and has been invaded by microtubule(s) (white arrow). Microtubules have not invaded other regions of the growth cone. **C:** (time: 0 min). Another growth cone at a slightly later stage has a broad lamellum on the Tr-Cx segment boundary (indicated by the triangle). A small number of microtubules (white arrow) are present in the process crossing from the segment boundary to the Cx1 cells (unfilled arrow). **D:** (time: 51 min). The lamellum has withdrawn from the boundary, and the nascent axon (white arrow) crosses from the boundary to the Cx1 cell (unfilled arrow). **E:** Another growth cone, extending ventrally along the Tr-Cx boundary (triangle) also has a process (white arrow) extending across the boundary to the Cx1 cells (unfilled arrow). **F:** In an enlarged view of the branch point shown in E, it appears that microtubules diverge from three different microtubule bundles present along the Tr-Cx boundary to enter the branch (white arrow) crossing to the Cx1 cells (unfilled arrow). Some microtubules (arrowhead) continue along the boundary past the branch point. In such cases, the growth cone may have advanced along the boundary past the Cx1 cells before the first filopodial contact with those cells. Scale bar = 5 μm (A-E); 1 μm (F).



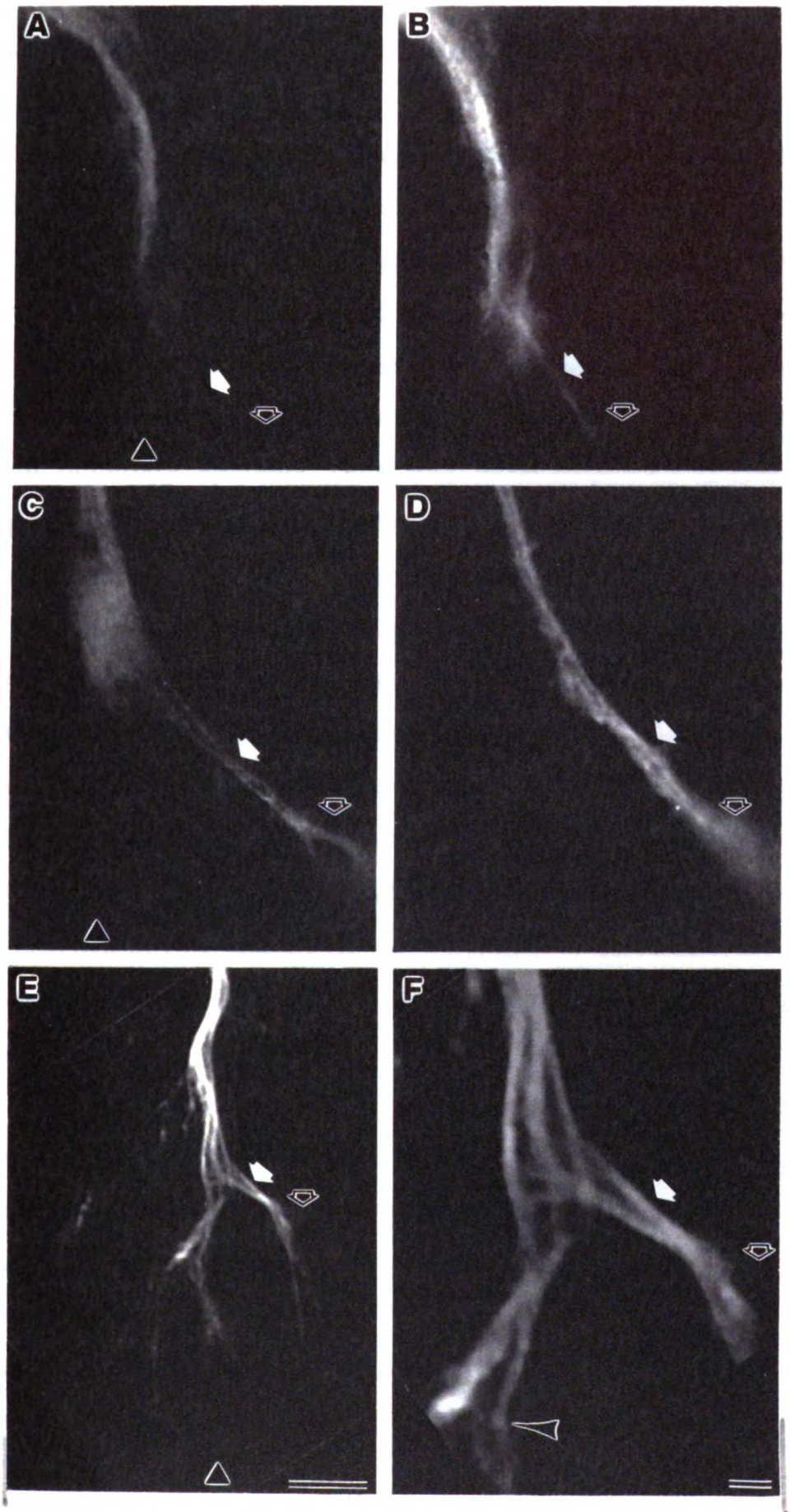
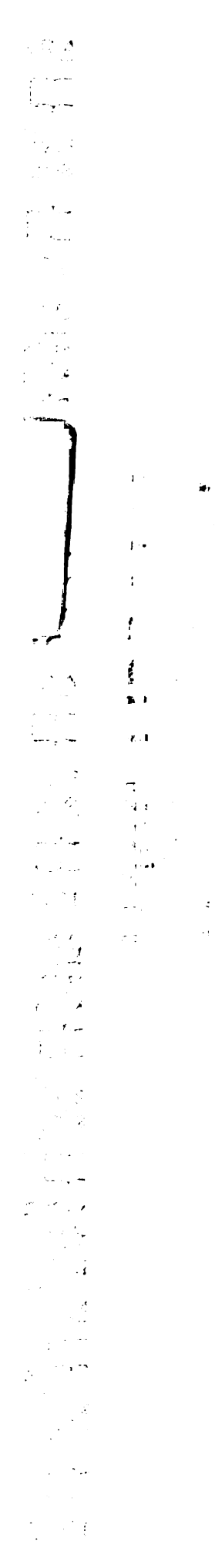


Figure 8

DISCUSSION

In the embryonic grasshopper leg, the migration of the Ti1 pioneer growth cones follows a stereotyped pathway (Fig. 1) from the tibia to the central nervous system, a path length of approximately 0.5 mm (Bate, 1976; Bentley and Caudy, 1983; Caudy and Bentley, 1986b; Ho and Goodman, 1982). Guidance over this complex pathway is accomplished by many growth cone-cell interactions including those involving intrasegmental epithelial cells, segment boundary epithelial cells, and preaxonogenesis neurons called guidepost cells (Caudy and Bentley, 1986b; Condic and Bentley, 1989b; Keshishian and Bentley, 1983; Lefcort and Bentley, 1987; O'Connor et al., 1990). Guidance features which mediate normal growth cone migration are preserved in a limb fillet preparation where individual cells are accessible for observation and experimental manipulation (Lefcort and Bentley, 1987; O'Connor et al., 1990). We observed microtubule arrangements during growth cone migration in response to these *in situ* guidance cues by injecting the Ti1 neuron with rhodamine-labeled tubulin.

Labelled bovine tubulin rapidly incorporated into linear tracks in the axon and growth cone. This is consistent with the high degree of homology between bovine and insect tubulin (Rudolph et al., 1987). Projection of the image onto a high resolution CCD chip, deconvolution of multiple image planes, and use of conventional computer image enhancement software provided adequate resolution of linear tracks. Individual tracks were uniform in width and intensity along their whole length. Furthermore, within the same growth cone, different tracks were uniform in width and intensity. Widths corresponded closely to those reported for fluorescently labeled microtubules whose unity and identity were subsequently confirmed in



electron micrographs (Sammak and Borisy, 1988). Electron micrographs of Ti1 growth cones fixed immediately after imaging revealed individual microtubules similar in location and shape to fluorescent tracks previously imaged in the same growth cone (arrowheads in Figs. 5, 6). These results strongly suggest that the unitary fluorescent tracks were individual microtubles. These are the first observations of individual microtubule disposition and behavior in growth cones migrating and steering on the *in situ* substrate.

The Arrangement of Microtubules in Ti1 Neurons

Axons. In both fixed and live neurons *in vitro*, microtubules in the axon are arranged in a closely packed, linear bundle (Black et al., 1989; Brady et al., 1984; Bray and Hollenbeck, 1988; Heidemann et al., 1984; Hirokawa et al., 1988; Keith, 1990; Meininger and Binet, 1989). This arrangement was also seen in Ti1 axons *in situ* (see Figs. 3F and 4). Although axonal microtubules were generally in this configuration, occasionally they could be displaced to one side or splay out into small bundles, especially at the locations of lateral protrusions.

In neurons viewed *in vitro*, the border between the growth cone and axon is defined as that region where the axonal cylinder abruptly increases in caliber (Bray and Chapman, 1985; Goldberg and Burmeister, 1986). At this border there is a transition in the arrangement of both actin and microtubules (Forscher and Smith, 1988; Mitchison and Kirschner, 1988). In this region, the microtubule arrangement changes from the highly bundled form in the axon to the splayed out pattern in the growth cone. In some Ti1 neurons, this change was easily identifiable. Furthermore, the location of the change in

microtubule arrangement could be displaced from the change in cell shape at the border between the growth cone and axon (Fig. 3F). It would seem that the formation of the closely-packed bundle of microtubules characteristic of the axon can occur centrally within the growth cone. This suggests that the formation of an axon may first involve the formation of tight bundle of microtubules in the growth cone, and subsequently the collapse of the plasma membrane around that bundle. This process has been seen in live *Xenopus* neurons viewed in time lapse *in vitro* (Tanaka and Kirschner, 1991). It is likely that the axonal bundling in the growth cone reflects the activity of microtubule associated proteins (Matus, 1990), suggesting that the activity of such molecules is spatially localized to the axon and central growth cone.

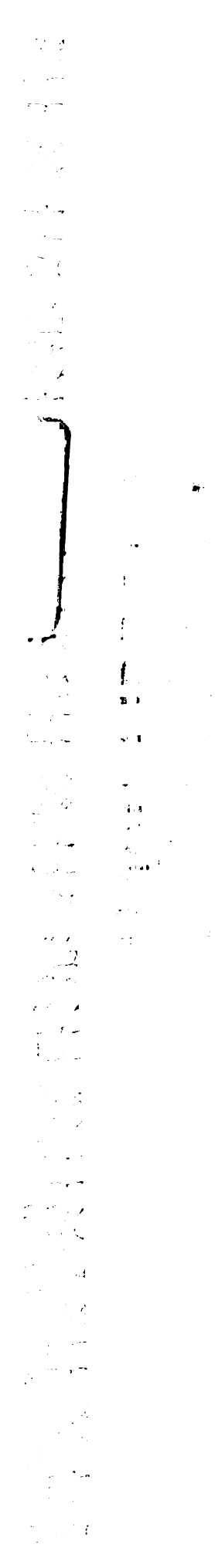
Growth Cones. The grasshoppper Ti1 growth cones *in situ* have a rich investment of microtubules. This and other features of the Ti1 growth cone are shown in schematic form in Fig 9. An issue on which previous observations have varied is the degree to which microtubules are confined to the central core area of the growth cone. In some early electron microscope studies (Bunge, 1973; Isenberg and Small, 1978), and in live *Aplysia* bag cell growth cones which are immobilized on a highly adhesive substrate (Forscher and Smith, 1988), microtubules rarely extend to the growth cone margin. In other studies, on fixed and extracted chick sensory and retinal neuron, or rat sympathetic growth cones (Bridgman and Dailey, 1989; Letourneau, 1983; Letourneau and Shattuck, 1989; Tsui et al., 1984), and live *Xenopus* growth cones *in vitro* (Tanaka and Kirschner, 1991), microtubules regularly extend to the distal margin of the growth cone. In Ti1 neurons *in situ*, the usual situation is for a dense array of microtubules to be extended right up to the leading margin of the growth cone (Fig. 2). Thus, the distal growth cone region of Ti1 pioneer neurons appears to be more heavily

invested with microtubules than has been observed in some growth cones *in vitro*.

Filopodia extending from Ti1 growth cones *in situ* never harbored microtubules (Fig. 3C, 3D; Fig. 9). These *in situ* observations are in accordance with previous *in vitro* studies, at both the light and electron microscope levels, indicating that filopodia are composed primarily of F-actin and lack microtubules (Bridgman and Dailey, 1989; Letourneau, 1983; Smith, 1988). In neurons *in vitro*, and in Ti1 neurons *in vivo*, cytochalasin-induced disassembly of filamentous actin results in the loss of filopodia, suggesting that actin is necessary for filopodial extension and maintenance (Bentley and Toroian-Raymond, 1986; Marsh and Letourneau, 1984).

Unlike filopodia, branches extending from the Ti1 growth cone often did harbor microtubules (Fig. 2; Fig. 9). However, approximately half of growth cone branches did not contain microtubules (see Figs. 3A and 3B). This is in accordance with observations of fixed neurons *in vitro*, where branches both with and without microtubules have been observed (Tsui et al., 1984). Furthermore, many short branches formed and existed without nearby microtubules. We conclude that microtubules are not necessary for the initiation of branch formation, nor for the maintenance of short branches.

Branches can be formed from pre-existing filopodia. This is perhaps best appreciated in those branches that form from filopodia in contact with guidepost cells. After the filopodium contacts a guidepost cell, it increases in caliber and eventually converts to a branch (O'Connor et al., 1990). Such branches always acquired microtubules. However, the conversion of the filopodium to a branch always occurred in advance of the acquisition of microtubules. In Fig. 8A, for example, a filopodium contacted the Cx1 guidepost cells. However, microtubules did not invade the structure until it



has undergone a conversion to a branch some 30 minutes later (Fig. 8B). Thus, filopodia did not appear to be enlarged by the process of microtubule intrusion; rather, microtubules always intruded into a space that was already present.

Microtubule Movement during Steering Events

The finding that many microtubules are normally present in the growth cone and in branches of Ti1 growth cones *in situ* raises the possibility that they may play a direct role in certain phases of steering. The process of growth cone steering can be formally divided into three phases. The first phase comprises exploration or sampling of the environment in the vicinity of the growth cone. It appears to be mediated by the random protrusion of filopodia; there is no evidence that filopodial extension is preferentially directed towards the target. Before a turn is made, however, one or a few of the branches or filopodia must be chosen as the future track of the extending growth cone. This second phase of steering is called orientation. The cytoskeletal requirements for orientation are not known. The final phase of steering is consolidation and conversion of the arrangements of cytoskeletal and other molecular features of the growth cone to the chemically and mechanically more stable configurations found in the nascent axon.

While the absence of microtubles from filopodia indicates that microtubules are unlikely to be important in the exploration phase, their disposition appears to be a key element of the orientation phase. This can be seen in Figs. 8A and 8B. At the beginning of this phase (Fig. 8A), their disposition is not oriented, and at its end (Fig. 8B) their placement defines where consolidation into the nascent axon can occur. Consolidation is shown

in Figs. 8C and 8D. Hence, we suggest that the endpoint of orientation is the generation of an asymmetric arrangement of microtubules in the growth cone. There are two general mechanisms by which this can be accomplished. One is that microtubules could invade only those branches that are found in the future direction of growth cone extension. We term this selective microtubule invasion. An alternative mechanism would be that microtubules randomly invade all extant branches, and are subsequently stabilized in those extending in the preferred direction. This we term selective microtubule retention. The microtubule imaging in this study allows one to address directly which mechanism is operating at a given turn.

At guidepost cell induced turns, such as those at Tr1 and Cx1, selective microtubule invasion was seen invariably (Figs. 8 and 9). In addition, this selective mechanism was also seen in 2 of 5 turns observed at the trochanter-coxa segment boundary (Fig. 7). In contrast to this, 3 of 5 turns at the segment boundary were made using selective retention to generate an asymmetric microtubule arrangement; microtubules invade both the ventral and dorsal branches (Fig. 2). Intrusion of microtubules into some, but not all branches was also seen in growth cones migrating proximally through the femur (Fig. 3). Therefore, both selective microtubule invasion and selective microtubule retention appear to underlie steering decisions, depending on the growth cone location in the limb.

The mechanism used to generate the asymmetric microtubule arrangement is likely to be determined by both the heterogeneity and nature of guidance information confronted by the growth cone during the exploratory phase (O'Connor et al., 1990). At guidepost cell-mediated turns, the observed selective microtubule invasion may reflect a large difference in the signal provided by a single, highly localized, high affinity, guidepost cell,

and the surrounding cells. Selective microtubule retention, on the other hand, may occur where the differences between the substrates encountered by Ti1 filopodia are not so dissimilar. Both at different locations within the femur, and along the segment boundary, the spatial rate of change of substrate affinity may be quite low. In this more ambiguous situation, the sequence from filopodial contact with an acceptable substrate, to branch formation, to accrual of successive microtubules into the branch, to consolidation of a large bundle of microtubules may proceed through several steps before one branch prevails. Thus selective microtubule invasion of a single branch and selective microtubule retention following multi-branch invasion seem likely to be extremes of a range of microtubule behavior which is dependent upon the disparity between environment-dependent intracellular signals in different growth cone regions.

What sort of signals may be involved? The generation of heterogeneous microtubule arrangements is central to many cellular processes, including yeast bud formation (Adams and Pringle, 1984), pigment granule aggregation in teleost retina (Troutt and Burnside, 1988), epithelial cell response to wounds, and spindle formation during mitosis (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1985). Of these, the mechanism by which the microtubules are arranged has been investigated in the most detail in spindle formation. Here, it appears that during formation of the spindle, microtubules randomly assemble, and are selectively stabilized by contact with the kinetochore (Cassimeris et al., 1990; Hayden et al., 1990; Mitchison et al., 1986). A similar situation could underlie selective retention of microtubules in selected growth cone regions or branches.

Where selective invasion occurs, it seems likely that certain regions of the growth cone are more receptive to microtubule invasion or assembly.

The molecular and biophysical basis for selective microtubule invasion is unknown. It appears that under baseline conditions, microtubule invasion may be prevented by the physical presence of the peripheral actin network. This is suggested by ultrastructural observations (Letourneau, 1983), experimental perturbations of actin in growth cones *in vitro* (Forscher and Smith, 1988; Smith, 1988), and by biophysical considerations of diffusion coefficients in actin gels (Luby-Phelps et al., 1988). If this is the case, then contact with high affinity cues must locally alter this situation. This alteration could be effected by spatially restricted changes in tension (Bray and Hollenbeck, 1988; Heidemann et al., 1990; Letourneau, 1975), and/or the concentration of second messengers molecules (Bentley et al., 1991; Bixby, 1989; Forscher, 1989; Kater and Mills, 1991; Lankford and Letourneau, 1989; Letourneau and Shattuck, 1989) . It is also possible that these effector molecules change a chemical interaction between actin and microtubules that promotes microtubule invasion (Goslin et al., 1989; Letourneau, 1983; Morales and Fifkova, 1989).

Another issue raised by our findings is the nature of the mechanism by which microtubules invade growth cone branches. This invasion may represent new microtubule assembly from the monomeric tubulin pool onto the distal, plus ends of the microtubules (Baas and Black, 1990; Bamburg et al., 1986). Alternatively, microtubules could be translocated into branches. One approach to distinguishing these alternatives would be to make a small fluorescent mark on the microtubules, and then observe the movement of the mark as microtubules invade the growth cone. This type of study has been carried out; it was found that microtubules marked in the axon near the growth cone move distally into the growth cone, even in the absence of

axonal elongation (Reinsch et al., 1991). This suggests that microtubule translocation may contribute to branch invasion.

One of the most striking features of the Ti1 growth cones *in situ* was the regular occurrence of transient microtubule loops. Looping microtubules have been described before in fixed, permeabilized growth cones *in vitro* (Lankford and Klein, 1990; Tsui et al., 1984). They have also been seen to form and collapse in real time imaging of live *Xenopus* neurons *in vitro* (Tanaka and Kirschner, 1991). Their presence in time-lapse images of migrating Ti1 growth cones, subsequently confirmed by electron microscopy, indicates that they are normal features in process outgrowth. Although microtubule polymers have been described as biophysically rigid when compared to other cytoskeletal polymers (Mizushima-Sugano et al., 1983), the forces required to bend microtubules could be supplied by the GTP hydrolysis associated with assembly, or by the ATP hydrolysis associated with microtubule based motors (Hill, 1987). Whatever the source, this force could provide the energy needed to allow microtubules to invade selective regions of the growth cone.

A schematic showing the major types of microtubule arrangements found in the Ti1 pioneer growth cone *in situ* is shown in Fig. 9. Many of these features may contribute to strategies underlying growth cone steering. Further studies on the molecular nature of microtubule stabilization and interaction with other cytoskeletal and second messenger elements in the growth cone will increase our understanding of how neurons steer toward their target, and generate the connections of the adult nervous system.

Figure 9. A diagram of features of microtubule arrangements in pioneer growth cones *in situ*. 1: bundled microtubules in the axon. 2: microtubule loops seen at axonal branch points and in the growth cone. 3: bundles of microtubules extending into a branch. 4: absence of microtubules in filopodia. 5: regions of the growth cone periphery can be devoid of microtubules. 6: where a filopodium expands into a branch, microtubule invasion is often observed. 7: microtubules often extend to the growth cone periphery. 8: microtubules selectively invade a branch formed from a filopodium which has contacted a guidepost cell. 9: some branches are devoid of microtubules.

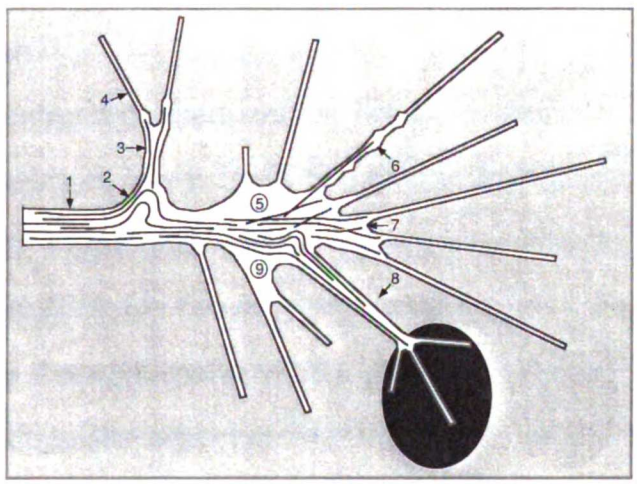


Figure 9

CHAPTER FOUR - HETEROLOGOUS mRNA EXPRESSION AS A TOOL FOR STUDYING THE CYTOSKELETON IN AXONAL DEVELOPMENT

ABSTRACT

The characteristics of an ideal system for studying the biology of axonal growth and guidance would combine ease and quality of imaging with the ability to manipulate the important molecules both in the growth cone proper and in the environment. For instance, it would be important to be able to do high resolution imaging of the cytoskeleton, *and* to manipulate the cell surface receptors and their downstream regulatory molecules. At the present time, certain systems have certain advantages, but an ideal system has not arisen.

The xenopus explant system has been used for high resolution imaging of the cytoskeleton, but it lacks the genetic manipulability of other organisms (Tanaka and Kirschner, 1991). We have begun to ameliorate this by introducing mRNA for the serotonin receptor into the 2 cell stage blastomere, and examine the expression of the protein at neurulation. We find that the embryo tolerates the presence of the receptor, and antigenic receptor can be found on neurons using immunocytochemistry. We propose that this system may be ideal for further studies on the biology of axonal growth and guidance.

INTRODUCTION

The amphibian xenopus laevis has been used extensively for many studies on early germ layer induction, gastrulation and neurulation (Amaya et al., 1991; Gerhart, 1980; Lamb et al., 1993). It has also been used as a system to study the mechanism of axonal guidance and growth (Reinsch et al., 1991; Tanaka and Kirschner, 1991). It has many advantages for these more cellular studies. Firstly, because the neurula stage embryo is approximately the same size as the two cell stage blastomere, fluorescently labeled proteins microinjected into the blastomere are inherited into all cells of the neurula without significant dilution . This has two main advantages: (1) fluorescently labeled molecules can be "introduced" into any cell regardless of its size, and (2) in the case of cytoskeletal proteins, all polymers are labeled, as the cells have undergone many rounds of polymer assembly and disassembly during development. A second advantage of this system is that the neurons that grow out from explanted neural tubes (presumably motoneurons) can be cultured in anoxic conditions which drastically reduces the phototoxicity that is inherent in imaging fluorescently labeled proteins in live cells.

These characteristics make the xenopus neural tube one of the finest systems for cytoskeletal imaging. However, little is known about the receptors and effector molecules present in the growth cones, and few reagants are available to study the effects of eliminating or enhancing the activity of these cell signaling systems. It has become apparent, however, that the xenopus egg and blastomere are efficient machines for the expression of heterologous mRNAs introduced by cell injection. This has been used to clone molecules with many different activities including ion channels,

neurotransmitter receptors and neural inducing factors (Krafte and Lester, 1992; Masu et al., 1987)K. Lustig and M. Kirschner, pers comm).

We have investigated the use of this system to express molecules of interest in neurons. In particular, we show that injection of mRNA for the serotonin 1c receptor at the two cell stage generates a receptor that is present on the surface of neurons explanted from stage 20 embryos. This will allow one to examine the effects of known signaling system molecules on the spatial distribution of the cytoskeleton, and to screen cDNA libraries for novel proteins that are important for growth cone guidance and axonal growth.

MATERIALS AND METHODS

RNA preparation

Plasmids containing either the entire rat 5HT1c receptor (pSR1c) or the entire rat 5HT1c receptor with an influenza hemagglutinin (HA) tag on the amino terminus (pCR8) were a generous gift of David Julius (Dept. of Pharmacology, UCSF). The plasmids were linearized with BamH1 (pSR1c) or Xho (pCR8), and extracted with phenol/chloroform. Transciptions were carried out using T3 (pCR8) or T7 (pSR1c) RNA polymerase, and the resultant transcipts extracted and stored.

Xenopus blastomere injections and development

Xenopus eggs were obtained, fertilized and dejellied using previously published techniques (Newport and Kirschner, 1982). Approximately 90 minutes after fertilization at the two cell stage, the blastomeres were placed in 1X MMR with 6% ficoll (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, 0.1 mM EDTA, pH 7.8), and were positioned under a dissecting

microscope (Wild instruments). Both blastomeres were injected with 1-10 pg of RNA. The injection needles were autoclaved prior to use, and the injections were carried out with latex gloves to avoid RNAse contamination. The embryos were placed at 18° C for approximately 1 - 2 hrs after which they were placed in 0.1 X MMR with 6% ficoll. They were allowed to develop at this temperature until approximately stage 20 (approximately 36-40 hours).

Neuron Cultures

The normally developing embryos were transferred to Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$, 4.6 mM Tris, pH 7.8), and the dorsal half of the embryo dissected into 1 mg/mL collagenase in Steinberg's solution (type 1A, Sigma Chemical Co., St. Louis, MO). After incubation in collagenase for approximately 30 minutes, the embryo halves were placed in a dissecting dish containing fresh Steinberg's solution, and the neural tube dissected away from the adjacent tissues. The neural tubes were placed in plating media (20% Leibovitz L-15 media, 58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$, 4.6 mM Tris, pH 7.8, 10 mM $MgCl_2$, 0.1 % gentamycin) on a coverslip that had been acid washed (1 M HCl for 2 hours at 70° C), rinsed copiously , and then coated with matrigel (Collaborative Research, Bedford, MA) for 2 hours. After approximately 8 hours at 18° C, axons had grown away from the explant onto the coverslip.

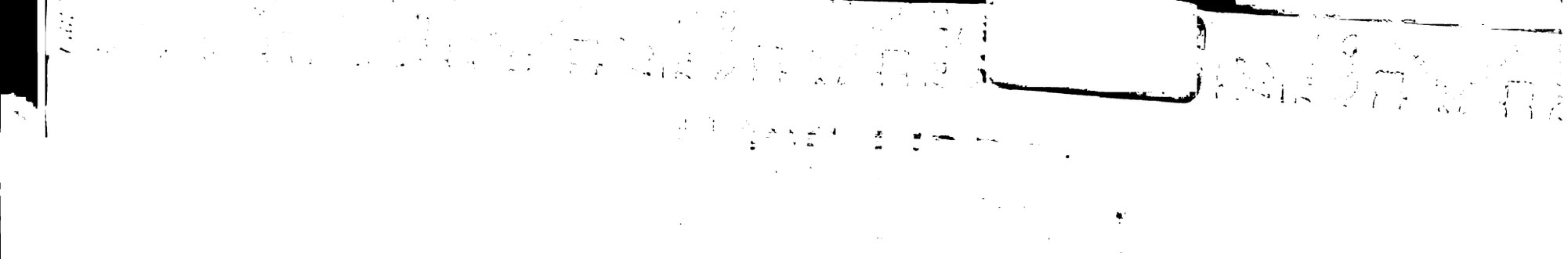
Neuron Imaging

The neuron containing coverslip was carefully placed in a stage chamber and immersed in plating media and topped with Silicone fluid to prevent media evaporation (Serva DC 200 fluid, 10 cst, Accurate Chemical and Scientific Corp, Westbury, NY). The chamber was placed on a Zeiss

IM405 inverted microscope, and the growth cones were imaged with either a 40/0.75 or 63/1.4 objective. Images were captured using standard relay optics and a video rate CCD (Charge-coupled device) camera (Hammamatsu C2400, Hammamatsu Photonics, Japan). The images, shutters and image analysis was carried out with a 386 processor running Image-1 software (Universal Imaging Corp, West Chester, PA).

Immunocytochemistry

Coverslips containing neural tube explants were transferred to light tight boxes and placed on parafilm trays. They were rinsed with PBS multiple times, and then fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. The fixative was removed, and the samples rinsed, and blocked with PBS containing bovine serum albumin 2 mg/mL (fraction V, Sigma Chemical Corp, St. Louis, MO) for 1 hour. They were incubated with a mouse monoclonal IgG anti-HA for 1 hour (gift of David Julius, UCSF). After rinsing, and BSA blocking for 30 min, the cells were incubated with Rhodamine conjugated Goat anti-Mouse polyclonal serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. The coverslips were then rinsed, and mounted on glass slides in 2 mg/mL PPDA. They were examined using a Zeiss axiophot microscope (Carl Zeiss Inc., Thornwood, NY).



RESULTS

Approximately half of the embryos receiving 1-10 pg of RNA lived through stage 20. There was no difference in viability between those embryos that had received the 5HT1c receptor and those that had received the HA tagged version. Water injected controls had similar viabilities as those injected with RNA, hence, it is unlikely that the presence of the mRNA itself had any effect on development up to the neurulation stage. The addition of 1-10 μM mianserin, a specific 5HT1c blocker had no effect on the development of the embryos.

Injection of 5HT1c Receptor mRNA Does Not Affect Axon Outgrowth

Of the 55 explants of neural tubes from embryos whose blastomeres had been injected with the 5HT1c receptor (5HT1cR), 38 generated more than one neuron in culture. This was identical to the water injected control embryos (40/65 injected with water grew axons). Those explants not generating axons were usually poorly adherent to the coverslip or were surrounded by many non-neuronal fibroblastoid appearing cells.

When only those explants generating axons were examined, the mean number of axons/explant was similar for both water and 5HT1cR injected embryos (17 ± 6.5 axons/explant for the 5HT1cR injected versus 19 ± 7.2 for the water injected). There was no apparent difference in the shape of the axon or growth cone between the groups. It is difficult to precisely measure the length of axons in an explant as the cell body cannot be seen, however the length of axon outside the explant was no different for the two groups.

5HT1c Receptor is found on explanted neurons of injected embryos

After outgrowth, cultures of neurons from embryos injected with epitope tagged 5HT1cR were fixed and stained for the presence of the receptor. As shown in Figure 1a, the epitope was present throughout the axon and the growth cone. Indeed, it was found on most neurons and other non-neuronal cells that could be visualized in the culture. There was no staining seen in those cells whose embryos had been injected with the non-tagged version of the 5HT1c (Figure 1b), or those injected with water (data not shown). Note that the embryos were not permeabilized, and that as expected for its location at the amino terminus of the receptor, the epitope is extracellular.

Serotonin does not affect growth cone morphology or growth rate of 5HT1c motoneurons

Given that the receptor for 5HT was present on these neurons, we asked what would be the effect of added serotonin to growth cone morphology or axonal growth rate. Addition of 1-10 μM 5HT had no effect on the morphology of the growth cone in either 5HT1c or water injected embryo neurons. Furthermore, the rate of axonal growth was identical in both groups (78± 15.2 μm/hr (n=24) for 5HT1c injected versus 74± 17.8 μm/hr (n=30) for water injected). When the same 5HT1c receptor was expressed on the surface of xenopus oocytes, addition of 1 μM 5HT caused a rapid rise in intracellular calcium consistent with activation of the receptor.

Figure 1. Serotonin receptor message expression in neuronal cultures. Neural explants from embryos injected with the HA tagged 5HT1c receptor. a: immunocytochemical staining reveals the receptor in every cell. b: control culture from embryos injected with the non-tagged 5HT1c receptor.

A

B

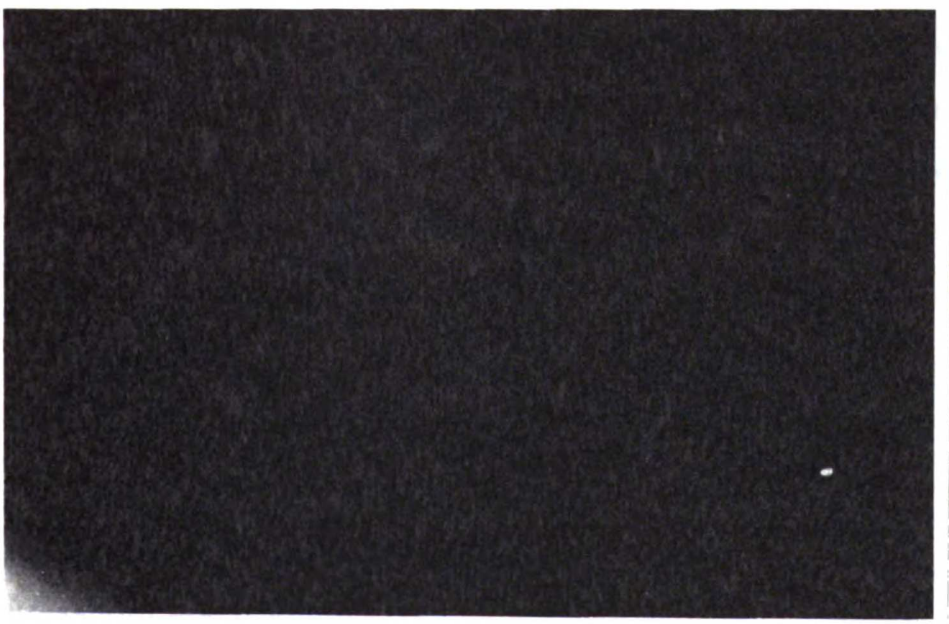


Figure 1

Discussion

The xenopus oocyte has been used as an expression system for heterologous mRNAs introduced by microinjection (Krafte and Lester, 1992). We show here that this system can be extended as a longer term expression system; messages introduced at the two cell stage produce protein that can be detected at neurulation (Figure 1). We feel that this may make this system an important one in which to carry out detailed studies of the biology of axonal growth.

The use of high resolution imaging of live cells and individual proteins inside those cells has lead to the discovery of the cytoskeletal events underlying growth cone guidance (Lin and Forscher, 1993b; Sabry et al., 1991; Tanaka and Kirschner, 1991). However, none of the biological systems used to date combine ease and quality of imaging with genetic manipulability. The grasshopper embryonic limb has many advantages for imaging, and leaves the neuron in its natural environment, but little is known about the guidance cues present in the limb, and even less about the signaling molecules in the neuron itself. Chick dorsal root ganglion sensory neurons in culture have been used extensively for morphologic studies of growth cone guidance, and much is known about the signaling pathway in the neuron (Letourneau and Shattuck, 1989). However, they are extremely light sensitive making it difficult to image fluorescently labeled proteins (unpublished observ). Aplysia neurons provide superior imaging possibilities, and can be injected with fluorescently labeled proteins (Lin and Forscher, 1993a). Like grasshoppers, though, they suffer from lack of manipulability of the important signaling molecules. The xenopus system described here has the possibility of ameliorating these difficulties.

The 5HT1c receptor is not usually found on motoneurons. Its only location in spinal cord is in the dorsal lamina V and VII, consistent with its proposed role in modulating nociceptive perception (Molineaux et al., 1989). Not surprisingly then, we found no effect of adding 1-10 μM 5HT to the growth rate or growth cone morphology of water injected controls. However, the neurons expressing the 5HT1c receptor also showed no response. This concentration of 5HT had a rapid effect on calcium levels in xenopus oocytes expressing the same message. It is possible that the receptor is not active in neurons, even though it is active in oocytes. Alternatively, the effector machinery - G_o or calcium mobilizing machinery - may not present in xenopus motoneurons.

Although the effect of 5HT1c activation on neuronal growth cones has not been studies, 5HT has profound effects in Helisoma buccal and pedal ganglion neurons. In adult cells, addition of 5HT caused growth cone collapse and arrest of axonal growth (Haydon et al., 1984). Conversely in embryonic neurons, the response is complex with some neurons showing enhanced growth rates, and others showing growth arrest(Goldberg et al., 1991). Both these effects are thought to be mediated through modulation of intracellular calcium levels.

Although the above experiments are inconclusive, the system has potential for use in many ways. For instance, as for the 5HT1c receptor, one could examine the effect of receptors for factors known to affect axonal growth and guidance such as trk-the neurotrophin receptor. The ability to image the cytoskeleton allows one to directly ask what effect activation of these receptors has on cytoskeletal arrangements.

A second potential use is in the screening for novel molecules involved in signal reception and transduction. This takes advantage of the

ability to sib select a specific messages from a pool of messages. For instance, collapsin, an environmental factor thought to modulate axonal growth does not affect motoneuron growth cones (Luo et al., 1993). One could inject mRNA from a given cDNA library and screen using neuronal collapse of explanted neural tube neurons as a bioassay for the receptor. Furthermore, one could screen for effector molecules important in signal transduction by injecting library mRNA together with a known receptor, such as the 5HT1c receptor. If a member of the signal transduction cascade is missing in the motoneurons, then one would expect no effect of added serotonin unless the missing activity was provided by the library .

We hope that these approaches will help in the identification of those molecules important for growth cone guidance and axonal growth, and that combining this information with what is known on cytoskeletal rearrangements would generate a comprehensive model of this biology.

CHAPTER FIVE - CONCLUSION

CONCLUSION

During embryogenesis, the vertebrate central nervous system matures through a number of well defined developmental stages (Easter et al., 1988; Patterson and Purves, 1982). The earliest stages are characterized by the induction of the neural tube by underlying mesoderm (Schoenwolf and Smith, 1990). This specialized ectodermal derivative undergoes many complex morphogenetic changes to generate the diversity of neurons seen in the mature organism. Up to this point, the development of the nervous system resembles that of other organs, albeit somewhat more complex. However, the next stage of neural development is unique to the nervous system, and, as such, may require biologic events and factors that are unique to the nervous system or used for non-related events in other systems. These stages are those of neurite outgrowth, axon and dendrite morphogenesis, and synaptogenesis. The early part of this stage seems to involve the use of a complexity of cell attached and secreted factors, while the later parts probably rely on electrical activity in the neuron for signaling (Jessell and Kandel, 1993). I would like to discuss some aspects of this development before addressing the more specific issues that constitute this thesis.

Firstly, although the last paragraph began with the phrase "vertebrate nervous system", it is becoming evident that factors and mechanisms are liberally shared between invertebrates and vertebrates. For instance, the same extracellular glycoproteins and cell surface adhesion molecules that are so important in vertebrate development are present in the invertebrate where they play a similar, if not identical, role (Montell and Goodman, 1988). Furthermore, the receptors and intracellular signaling mechanisms used by cells to sense the external environment are similar (Zusman et al., 1990). Indeed, it is common to find a factor using vertebrate bioassays, and then to

have its role in development defined using the genetic power of some of the invertebrate systems.

Secondly, it is becoming difficult to say when development ends, and mature function begins. This is especially true of the field of axonal growth and growth cone guidance. During development, neurites grow out to their selected targets, and make synapses. However, as a mature nervous system, the details of this fine anatomy is most likely very plastic with synapses being formed and eliminated constantly (Goodman and Shatz, 1993). It is also likely that these constant synaptic renovations may underlie certain adaptations of the adult nervous system, by allowing the circuitry of the system to respond in a physical way to the external world. One predictions of these adaptations is that they would martial the same machinery that had been previously used in development to set up the synapse in the first place. This machinery would include the cytoskeleton and its regulators. It would be interesting to examine microtubule and actin dynamics in synapses, and to look at whether they changed with electrophysiolgic plasticity. The imaging technology used in this thesis may be useful for these types of experiments.

The Nature of Microtubule Transport during Axonal Growth

Early studies by Paul Weiss and colleagues suggested that in adult nerves, there exists a proximodistal flow of cytoplasm that supplies material to the end to the neuron (Weiss and Hiscoe, 1948). This idea was biochemically formalized by Raymond Lasek in an elegant series of experiments where he bulk radiolabelled proteins in the cell body of retinal ganglion cells, and then examined how fast they moved down the axon (Lasek, 1982). He did this by sectioning the optic nerve and tract into small

segments, and then examining each segment by SDS-page. He found that tubulin traveled as a wave in a kinetic phase he termed slow component a (SCa). There are a number of remarkable characteristics of this transport. Firstly, the label moved at a constant velocity regardless of where in the axon the label was. This suggests that the material was being transported rather than diffusing, as the rate of diffusion would have fallen off linearly with distance from the cell body (see chapter 2). Secondly, the amplitude of the wave crest did not diminish, nor the intensity profile widen over 77 days. This suggests that the tubulin was being transported as a form that was not only extremely stable, but itself was not diffusing as it transported. Later experiments identified that the transported component consisted of extremely cold stable microtubules (Brady et al., 1984; Filliatreau et al., 1988; Tashiro and Komiya, 1989; Tashiro et al., 1984).

More recent studies have tried to directly visualize the translocation of microtubules in living neurons. Two related, but technically different approaches have been taken. In the first approach, tubulin was labeled with fluorescein, and a fiduciary mark made on the microtubules using a photobleaching pulse of light (Lim et al., 1990; Lim et al., 1989; Okabe and Hirokawa, 1990). This makes a black mark on a fluorescent background. The second approach has been to label tubulin with a caged fluorescein that fluoresces only after photoactivation with a pulse of light (Okabe and Hirokawa, 1992; Reinsch et al., 1991). This generates a fluorescent mark on a black background. Both approaches mark microtubules (some monomeric tubulin may be marked, but given the high concentration of microtubules in the axon, this represents a very small signal). The majority of these studies have failed to find translocation, and did the studies outlined in chapter 2. Two studies did, however, find that polymer translocated as a coherent phase

at rates similar to Lasekian SCb (Keith, 1987; Reinsch et al., 1991). What accounts for these conflicting results?

It is clear that the transport species identified in SCb represent a highly stable population of microtubules. In no recent study of developing neurons, has such a population been identified. Stable microtubules become more prevalent as neurons mature, and it is possible that the translocation of stable microtubules occurs only in adult tissue. Furthermore, they could represent a small fraction of the existing microtubule pool, and hence their transport would be sufficient to supply tubulin to a mature non-growing neuron, but would not be so for a growing cell. Indeed, it seems that the transport form of neurofilaments, another SCb transported molecule represents an extremely small part of the total axonal amount (Nixon and Shea, 1992). If the transported form represented 10% or less of the total microtubule population, then the imaging experiments described in chapter 2, and in the literature may not be able to detect it.

However, the xenopus motor neuron study represents an imaging study in growing neurons that does detect polymer translocation. How does one reconcile this experiment with the remainder of the imaging literature. As mentioned in the discussion of chapter 2, it is possible that xenopus neurons use a different mechanism than other cells because they grow so quickly. Alternatively, it is possible that the growth of xenopus neurons at this rate results in some mechanical stretching of the axon so that the movement of microtubules is an apparent rather than real result. These possibilities are presently being investigated by growing xenopus neurons on substrates that support slower growth.

Finally, what are the regulators of axonal growth. Imaging experiments in both grasshopper and xenopus suggest that new axon is

formed by the collapsing of membrane around existing microtubule bundles in the growth cone (Sabry et al., 1991; Tanaka and Kirschner, 1991). Furthermore, that growth requires the presence of a dynamic population of microtubules, probably those in the growth cone (E. Tanaka, pers comm). It is likely that local factors in the growth cone that regulate microtubule bundling and actin based membrane collapse are important for regulating how fast axons grow. Given this, it may be that axonal transport simply supplies monomer to the growth cone (either as polymer or monomer), and that the transport responds to the need that is defined by the growth cone itself. It is likely that the growth cone then plays a central role in axonal growth, and indeed, as outlined below, also in growth cone guidance.

A Model of Growth Cone Guidance

The presence of microtubules in the growth cone as shown in Figure 2 of chapter 3 suggests that they may play a role in growth cone guidance. When the arrangement of microtubules is examined in turning growth cones, it is found that an asymmetric array of microtubules is set up with microtubules in those branches contacting guidepost cells. There are two ways in which this asymmetry could be generated. One method is that the microtubules would sample the whole growth cone and its branches, and become stabilized in those branches that were in contact with favorable guidance cues. This method is similar to that used in setting up the mitotic spindle, where the kinetochore captures centrosomally nucleated microtubules (Mitchison and Kirschner, 1985). The second method is that

microtubules would directly invade those branches in contact with the favorable cue without sampling the other branches.

These two methods would predict different regulated events in the growth cone would be responsible for setting up the asymmetric array. In the first method, contact with a favorable cue could activate some local stabilizing activity that could bind microtubules at their end (like the kinetochore) or along their shaft (like tau). Importantly, this activity would be local, present only in the growth cone branch contacting the favorable cue. The second method would suggest that microtubule movement *itself* is regulated. This could result from regulation of microtubule based motors or of microtubule assembly. Alternatively, it is possible that the local stearic environment would be modified so that microtubules would only have room to invade the chosen branch.

Time lapse imaging of microtubules allows us to distinguish between these two possibilities. In the case of guidepost cells, it seems that microtubules selectively invade the branch in contact with the guidepost cell while never sampling the remainder of the growth cone. Interestingly, this same mechanism is used by aplysia neurons in vitro turning onto other growth cones (Lin and Forscher, 1993b).

This advances the question of growth cone guidance to the nature of the regulated event. In both the grasshopper and aplysia, it seems that a change in the actin cytoskeleton precedes the microtubule invasion (Lin and Forscher, 1993b; O'Connor and Bentley, 1993). In both systems, there is an accumulation of F-actin in those branches in contact with favorable guidance cues. The reason for the actin buildup is not known, but in aplysia there is preliminary evidence that the rate of retrograde actin flow is slowed in those chosen branches.

The current model, then, divides the turning decision into four stages, each with specific cytoskeletal requirements (Figure 1). The first step is the sensing of the environment by the growth cone and filopodia. Indeed without these structures, guidance seems to be perturbed (Bentley and Toroian-Raymond, 1986; Chien et al., 1993). The movement of growth cone protrusions seems to be random, but restricted to the semisphere ahead of the axon end (Letourneau, 1975). The constant formation and elimination of filopodia requires filamentous actin. This aspect of growth cone guidance is probably very similar to cell motility and chemoattraction in non-neuronal systems (Condeelis, 1993).

When contact with a favorable cue is made, a signaling cascade is put into effect in that branch of the growth cone, and the second stage of turning is initiated. The first event in this stage is an accumulation of F-actin in the distal aspect, and a loss of actin from the proximal regions of the chosen branch. This has been associated with a slowing of the retrograde actin flow in turning Aplysia neurons. One guess is that this event has to do with a slowing of the actin based motor myosin, or a switch from one myosin to another slower isoform, or perhaps even a decrease in the total amount of actin-myosin interactions. However, it is also possible that the loss of actin from the proximal branch is a result of activation of actin severing activities or the inhibition of actin nucleating activities.

Figure 1. A Schematic Model of Growth Cone Steering. As outlined in the text, the steering of growth cones can be divided into four stages. The microtubules are shown as brown thick lines oriented longitudnally in the axon. Actin is shown as red cross linked filaments. The blue ellipse represents a discrete guidance cue such as a guidepost cell. See text for details.

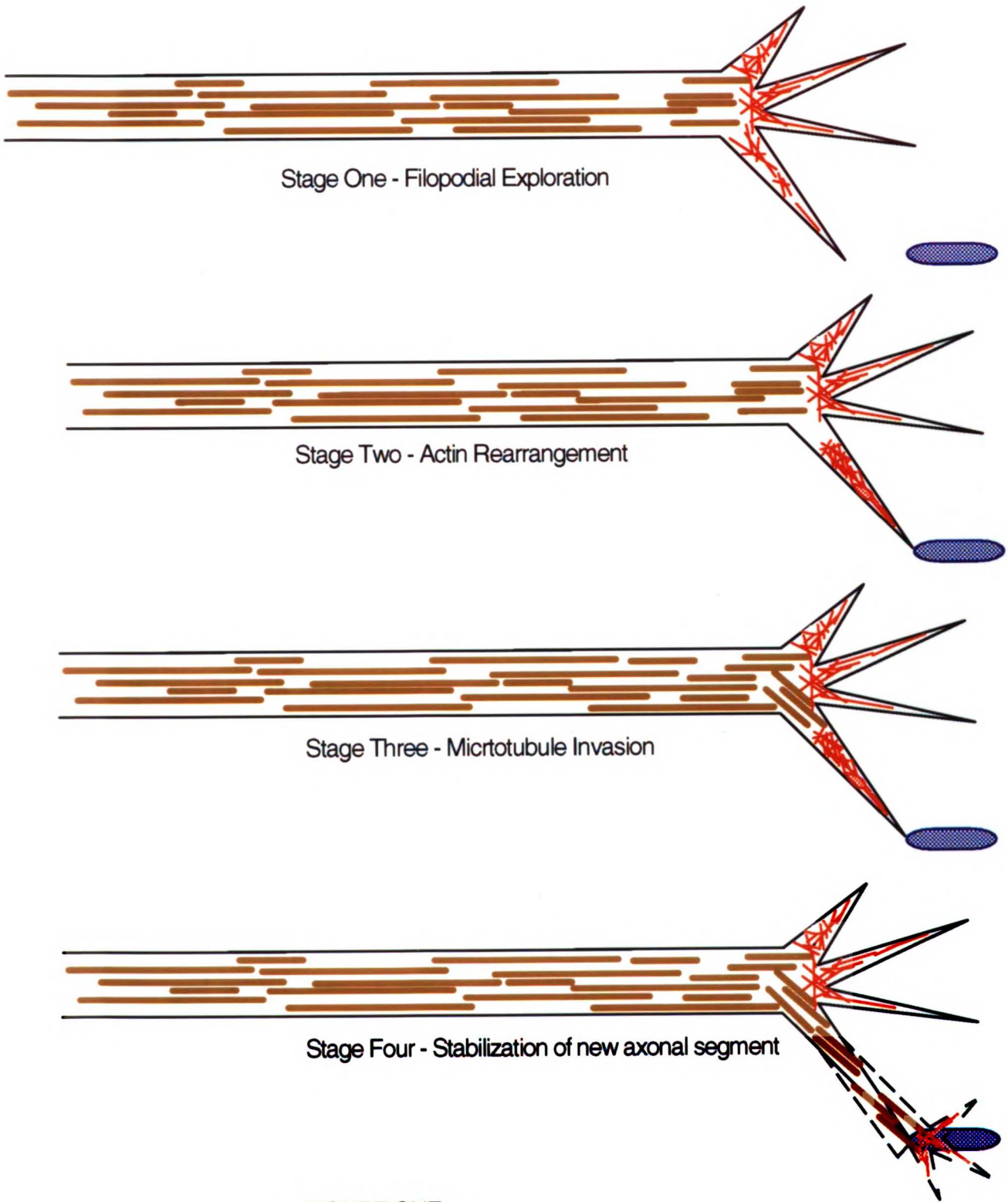

Stage One - Filopodial Exploration
Stage Two - Actin Rearrangement
Stage Three - Micrtotubule Invasion
Stage Four - Stabilization of new axonal segment

FIGURE ONE

The change in actin arrangements initiates the third stage of turning, the movement of microtubules into that branch. At this time, it is not known whether the movement of microtubules is one of assembly onto existing polymer, or simple translocation of polymer into the branch. High resolution of microtubule movement in xenopus neurons suggests that translocation may be the important event. However, more recent studies have examined the effect of low dose vinblastine on the arrangement of microtubules in growth cones (E. Tanaka, pers comm.). At low doses, vinblastine inhibits microtubule dynamics without affecting existing polymer. When applied to xenopus neurons in vitro, this drug prevents the invasion of some areas of the growth cone by microtubules, suggesting that in turning, the important event may involve assembly of polymer. It will be important to try to give vinblastine at specific times in the turning decision to address this question with more temporal resolution.

Alternatively, one can ask whether microtubules translocate into the chosen branch is to adapt the photoactivation techniques used in chapter 2 to the growth cone. The experiment would be to photoactivate a mark on the microtubules in the proximal growth cone, allow microtubule invasion to occur and to ask whether the photoactivation mark translocates into the branch. This was attempted many times, however, the poor signal to noise ratio of the photoactivated fluorescein resulted in a loss of signal even when imaged with either an intensified silicon-intensified-target (ISIT) camera or a cooled CCD camera. In collaboration with Tim Mitchison at UCSF, we have been synthesizing novel caged "antigens" with an intent to use the signal amplification inherent with immunocytochemistry to increase the signal to noise ratio and allow detection of single microtubules labeled with photoactivated compounds. In this approach, one would photoactivate, and

wait a certain amount of time, then fix the cell and use immunocytochemistry with an antibody that would bind only the uncaged antigen to label the photoactivation mark. Preliminary results suggest that small haptens such as coumarin and serotonin can be successfully caged and conjugated to tubulin.

After an asymmetric microtubule array is organized in the turning growth cone, then more microtubules invade the chosen branch (chapter 3, Figure 8), and the choice is cemented. This is the fourth and final stage of turning and basically involves turning the chosen branch into a de facto axon. It is expected that this stage involves the stabilization of these "young axonal" microtubules with specific MAPs such as tau.

This model is highly speculative, but is based on careful observations of the cytoskeleton in living neurons as turning decisions are made. The nature of this type of biology requires that experiements be done so as to preserve the spatial relationships in the cell. These types of spatial biologic investigations rely heavily on optics and high spatial and temporal resolution imaging, and therein lies their beauty and their difficulties. The problems inherent in observing fluorescent molecules inside a cell make it clear that one must be meticulous about ensuring the health of the cell, and avoiding photodamage. Neurons, in particular, are extremely sensitive to light, and photoactivation itself produces toxic adducts.

However, I believe that there exists a whole realm of biology for which this is the appropriate mode of investigation. This realm of biology is characterized by events that alter the spatial relationships between molecules in the cell. In this case, the free energy requirements are for entropy rather than the energy of biochemical synthesis ($T\Delta S$ rather than ΔH in the equation $\Delta G = \Delta H - T\Delta S$). These types of biologic events occur in every cell, but they are

more common in the nervous system because of the great asymmetry of the cells there. For this reason alone, these types of experimental approaches are required for addressing many questions in developmental neurobiology.

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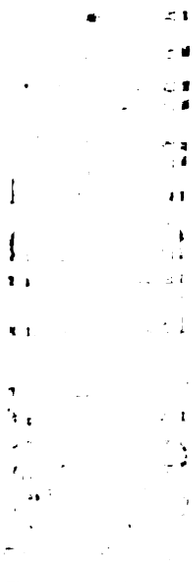

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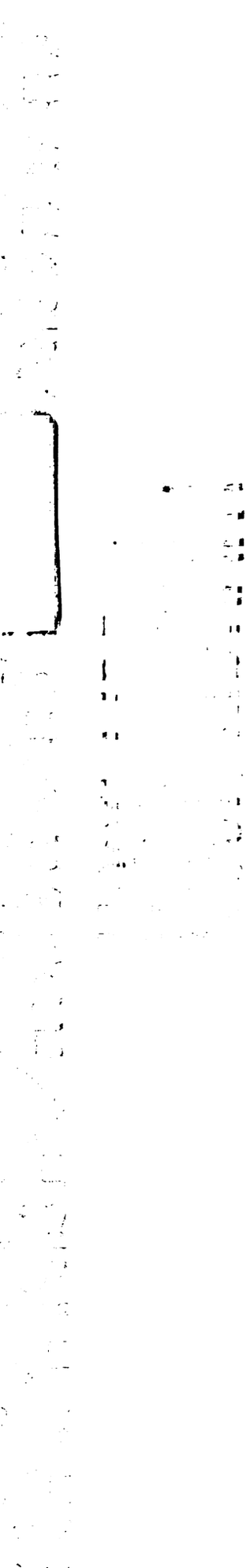

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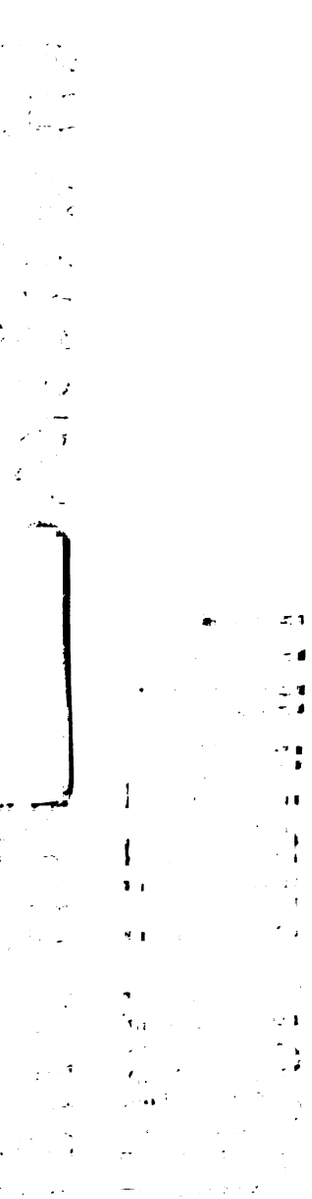

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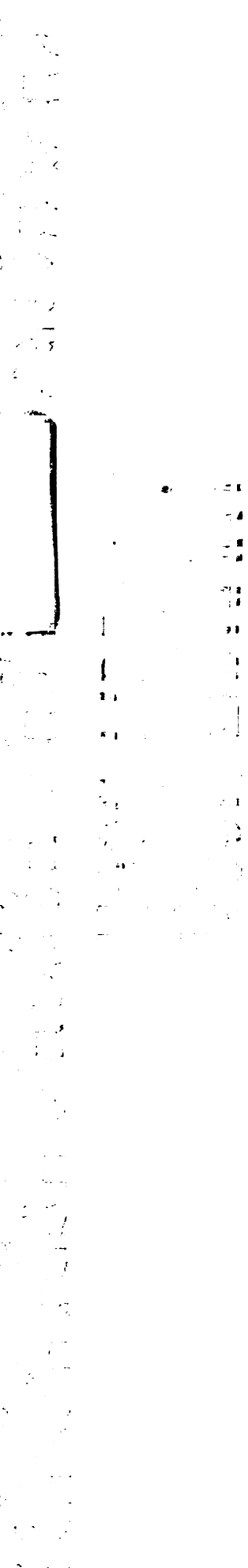

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