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LOCAL CONTROL OF THE AXONAL CYTOSKELETON

By

Peter William Baas

A DISSERTATION

Submitted to Michigan State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

LOCAL CONTROL OF THE AXONAL CYTOSKELETON

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Peter William Baas

The proximal segment of amputated neurites routinely regrows while the distal segment only regrows if first it collapses to a "bead" of axoplasm. Heidemann et al have proposed that stable MT fragments may serve as nucleating elements for the local control of MT dynamics in the axon (J. Cell Biol. 99:1289-1295). To test this proposal, regrowth of "beads" was examined after recovery from treatments (cold or 0.1 ug/ml nocodazole) that depolymerized the MTs in the bead to fragments too short to "unfurl" during regrowth. Regrowth ensued immediately after rewarming or rinsing out the nocodazole at the same rate as unarrested regrowth. Beads treated with 1.0 ug/ml nocodazole in 11 of 13 cases were devoid of MT fragments, failed to regrow, and never reassembled MTs even hours after rinsing. These data indicate that regrowth requires MT nucleation sites suggesting that tubulin in the bead and perhaps in the neurite has a limited capacity for self-nucleation.

The polarity orientation of MTs in intact neurites was uniform with [+] ends facing the growth cone. In uncollapsed amputated neurites, [+] ends faced the cut in regrowing proximal segments, while [-] ends faced the cut in non-regrowing distal segments. Regrowth of collapsed distal segments was accompanied by a MT polarity reversal that re-established the characteristic relationship between the new growth cone and [+] ends



of MTs. These results suggest that [+] ends of MTs are a requirement for growth cone formation, and that axonal MTs are organized by an interaction between MT assembly and growth cone motility.

Intact neurites treated with various MT poisons lost the cytoplasmic demarcation between the ribosome rich soma and the ribosome deficient neurite. Treated neurites showed many ribosomes in the first 100 um and a decreasing gradient with increasing distances from the soma. Ribosomes appeared in proximal but not distal segments of neurites amputated prior to nocodazole treatment. These results suggest that MTs in the neurite prevent the movement of ribosomes into the axoplasm, and thus may play an early role in the cytoplasmic compartmentation of neurons.



dedicated to my father



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LIST OF ABBREVIATIONS

MT	Microtubule
IF	Intermediate Filament
PBS	Phosphate Buffered Saline
GEP Buffer	0.1 M PIPES, pH 6.9, with KOH, 1 mM EGTA, 2 mM GTP
Bead	Collapsed Distal Segment of Amputated Neurite
CW	Clockwise
CCW	Counterclockwise
distal segment	amputated neurite fragment freed from cell body
proximal segment	amputated neurite fragment attached to cell body

x



INTRODUCTION

The cytoskeleton is a set of structural proteins that are critical for the development and maintenance of cell shape and cytoplasmic organization in eucarvotic cells (1. 28). The cytoskeleton consists principally of microtubules (MTs), intermediate filaments (IFs), and actin filaments, but also involves many regulatory and linking elements (1, 28). The factors that regulate, organize, and integrate the cytoskeleton in eucarvotic cells are poorly understood. An especially useful model for studying cytoskeletal functions underlying cellular morphology has been the neuron. The neuron shows a remarkable anisotropic geometry, and is arguably the cell type whose function is most intimately related to its shape. Neurons consist of a rounded cell body that extends axons over distances many times the diameter of the cell body (77). The cytoplasm of the axon is differentiated from that of the cell body by the absence of protein synthetic machinery (57) and the presence of a dense array of highly ordered cytoskeletal elements (15, 16, 54, 63, 75, 77, 96). Actin filaments form a cortical network below the plasma membrane of the axon, and extend in growing axons into a motile leading edge termed the growth cone (16, 52, 53, 96). MTs and IFs are present in parallel array within the shaft of the axon, but terminate at the base of the growth cone (16, 53, 96). MTs are dynamic structures generally regarded to provide plasticity to the axon during axonal outgrowth, while IFs are thought to provide stability as the axon



matures (54). There is widespread agreement that axonal growth depends on both MT-based and actin-based cytoskeletal networks (49, 53, 97). In this regard, the axon is a system uniquely suited for studying the integration of different elements of the cytoskeleton.

MTs in the axon are uniform in their polarity orientation (19, 32, 39, 41), and yet do not extend from a centriole or any observable MT organizing structure (64, 81, 98). McIntosh has argued that traditional MT organizing centers function as if they were localized regions of MT fragments available for elongation (67). Morris and Lasek (70) and Brady et al (12) have speculated that the spatial organization of axonal MTs may be locally regulated by nucleation sites woven throughout the the axon. Heidemann et al (41) and others (9, 12, 70) have proposed that especially stable regions of otherwise labile MTs may serve as the proposed nuclei. It is known however that the concentration of tubulin in the axon is well above the concentration that permits de novo initiation of MT assembly in vitro (71). If Heidemann's scheme is correct, how is the local assembly of MTs in the axon limited to the elongation of existing MTs?

The genesis of MT organization in the axon is an issue not addressed by the local nucleation hypothesis. Lasek and his colleagues have argued that MTs are assembled in the cell body and then transported in orderly fashion down the axon as intact polymers where they are ultimately degraded in the axon terminal (54, 55). This proposal has recently been challenged, however, by evidence that the growth cone is a major site of MT assembly in growing axons. Bamburg et al reported that axonal elongation is 100 times more sensitive to MT poisons applied at the growth cone than applied at the cell body or axonal shaft (5).



Bamburg's scheme is consistant with the local nucleation hypothesis in that MT assembly in the axon would occur via the elongation of existing MTs at the base of the growth cone. Joshi et al have suggested that the elongation of MTs at the growth cone is regulated by the motility of the growth cone releasing compressive force on the MTs (49). Such an intricately regulated system would likely demand that the MTs be uniformly oriented in the axon in that the two ends of a MT have different assembly kinetics and equilibria (2, 6, 7, 25, 65, 66). Might the genesis of MT organization in the axon be the result of an integration between MT assembly and the actin-based motility of the growth cone?

The controversy of whether MTs are assembled principally in the cell body or at the growth cone is one of a number of issues that question the relative contributions of the cell body and local (in the axon) cues for the regulation of the axonal cytoskeleton. Solomon has argued that the pattern of outgrowth and branching of axons is dictated by "endogenous determinants" from the cell body (85), whereas others have argued that the growth cone is more responsive to environmental cues once it crawls away from the cell body (13, 14, 22, 47, 59). Shaw and Bray reported that the collapsed distal segments of amputated neurites, although physically separated from the cell body, are able to regrow neurites entirely similar to normal neurites on the light microscopic level (82). Do the MTs within the regrown neurites enjoy the same degree of spatial organization as do MTs in normal neurites? If so, what does this say about the relative contributions of the cell body and local influences to MT organization in axons?

Lasek and Brady have argued that the organization of cytoplasm in



the neuron is not a special case, but rather is an exaggeration of themes common across eucaryotic cells (57). Protein synthetic machinery tends to be localized to the perikaryon, while the differentiated cytoplasm specific to the cell type tends to be found at the periphery of the cell. In the case of the neuron, the long distances traversed by axons demand the existence of a complex system for the transport down the axon of substances synthesized in the perikaryon. Although axonal transport has been extensively studied (33, 36, 43, 54, 55), to my knowledge no experimental evidence exists for the mechanism by which the cytoplasm in neurons is compartmented into "translational" and "expressional" types of cytoplasm. Lasek has proposed that ribosomes are localized to the perikarya of mature neurons by attachments to cytoskeletal elements within the perikaryon (54). Lasek and Katz have pointed out that the "granular" cytoskeleton of the perikaryon is devoid of MTs (56). Might the exclusion of ribosomes from axoplasm during neuronal development be attributable in part to the cytoskeleton. specifically the MTs, within the axon rather than (or in addition to) the cytoskeleton of the perikaryon?

The research presented in the next three chapters represents an attempt to address the questions put forth in this introduction. Chapter 1 exploits the collapse/regrowth model to test the validity of the local nucleation hypothesis. Chapter 2 similarly exploits the collapse/regrowth model to explore the proposed relationship between MT organization in the axon and the actin-based motility of the growth cone. Chapter 3 examines the role of MTs within the axon in the exclusion of ribosomes from axoplasm during early neuronal development.


CHAPTER 1. Microtubule Reassembly from Nucleating Elements During the Regrowth of Amputated Neurites

INTRODUCTION

Microtubules (MTs) are dynamic structures that are critical for the growth and maintenance of axons (16, 53, 95, 96). Axonal MTs enjoy a high degree of spatial organization (15, 39, 41, 75), yet are apparently not under the influence of a traditional MT organizing center (64, 81, 98). Indeed, a growing body of evidence suggests that local and environmental cues play an important role in the control of the axonal cytoskeleton (13, 22, 47, 59). Lasek and his colleagues (12, 70) have speculated that cytoskeletal nucleation sites may be woven throughout the axon for the local maintenance of cytoskeletal morphology. Heidemann et al have reported that MTs of cat sympathetic nerves are oriented in polar fashion (39), and are able to recapitulate their polar organization during recovery from cold or drug induced depolymerization (41). Heidemann et al proposed that MT fragments that resist depolymerization may play a role in MT organization by acting as nucleating "seeds" for MT elongation (41). Morris and Lasek (70) and Brady et al (12) have similarly suggested that stable polymers in the cytoskeleton may play an important role in cytoskeletal organization. Biochemical evidence (45) suggesting that stable MTs exist in vitro as short, disassembly-resistant regions of longer, otherwise labile MTs has



been confirmed by observations <u>in vivo</u> (50, 79). Stable MTs have been shown to be biochemically distinct from the labile polymer (9, 12, 42, 45, 48, 92). Nucleated assembly from stable fragments seems attractive in that elongation from an existing MT is faster and more energetically favorable than <u>de novo</u> initiation (2, 73, 74). A functional role for stable fragments as nucleation seeds would lend substance to speculations on individual nucleating elements for each MT (84, 90). Stable fragments may also provide the physical "cap" against treadmilling proposed by Kirschner (51), and may be of particular importance in MT organization if axonal MTs share the kind of "dynamic instability" recently reported of MTs in vitro (69).

Shaw and Bray reported that distal segments of amputated neurites from cultured chick sensory neurons collapse to shortened segments or "beads" of axoplasm that subsequently regrow neurites entirely similar to ordinary neurites on the light microscopic level (82). Wessells et al (93) demonstrated the same phenomenon in ciliary ganglion neurons and provided evidence for the importance of growth cone motility in cytoskeletal organization during regrowth. George and Lasek have suggested that the collapse of amputated neurites may involve the same kinds of regulatory mechanisms that coordinate the cytoskeleton during slow transport (35). Although completely divorced from the cell body, the amputated neurites clearly contain all the informational cues required for cytoskeletal reorganization underlying collapse and regrowth. In an effort to understand this cytoskeletal reorganization we have examined the disassembly and reassembly of MTs during the collapse and regrowth of amputated neurites.



MATERIALS AND METHODS

Cell Culture

Embryonic chick sensory neurons were cultured using a procedure slightly modified from that of Shaw and Bray (82). Dorsal root ganglia were dissected from the lumbosacral regions of 12 day embryos, and placed in L-15⁺ medium (L-15 purchased from Gibco Laboratories, Grand Island, NY supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin. and 100 mg/ml streptomycin). The ganglia were rinsed twice. then treated with 0.25% trypsin for 25 minutes at 37°C. The trypsin was removed. L-15⁺ with 10% fetal calf serum (Hazleton Dutchland. Inc., Denver, PA) was added, and the ganglia were triturated with a pipette into a single cell dispersion. The cells were rinsed twice, and plated in L-15⁺ with 10% fetal calf serum. 0.6% methyl cellulose (Methocel A4M. Dow Chemical Company, Midland, MI), and 100 ug/ml nerve growth factor from mouse saliva (17). The cells were plated into 35 mm Corning tissue culture dishes at a density sufficiently low so as to render non-neuronal contamination inconsequential. Cultures were kept in a humid. 37[°]C incubator for 18-30 hours prior to experimentation.

Amputation Experiments

Cultures were covered with a thin layer of mineral oil to prevent evaporation and maintain pH, and placed on a microscope stage warmed to 37° C with a Sage air curtain incubator. Temperatures of warming cultures were monitored by submerging the feedback thermister of the air



curtain incubator directly into the culture medium, and taking the cooling mode as an indicator that the set temperature had been reached. Control experiments using larger volumes of fluid showed the air curtain incubator to be accurate within 1°C. Amoutations were performed with a Leitz micromanipulator using glass needles made on a microelectrode puller. The needles were often broken to a diameter of about 10 microns, and neurites were cut from directly above to minimize lateral displacement. In an effort to maximize the number of amputated neurites that collapsed completely to beads of axoplasm, i.e. with no visible processes remaining after collapse, unbranched neurites with modest growth cones were selected for amputation and cut approximately 100 um from the growth cone. Regrowth of collapsed neurites was arrested in three sets of experiments using conditions known to depolymerize MTs. Cultures were cold treated by incubating the dishes in a -5° C bath for 2 hours, or treated with either 0.1 ug/ml or 1.0 ug/ml nocodazole (Aldrich Chemical Company, Milwaukee, WI) for 15-20 minutes at 37°C. After these treatments, regrowth properties were examined by rewarming the cold treated beads on the microscope stage, or rinsing the nocodazole-treated beads twice with PBS then returning them to undrugged medium.

In another set of experiments, cold treated beads were permeabilized under conditions slightly modified from a procedure reported by Cande (23) to depolymerize spindle MTs in PtK1 cells. In these experiments cultures were gently lysed after cold treatment for 4-5 minutes at 37° C in a buffer containing 0.1 M PIPES adjusted to pH 6.9 with KOH, 1 mM EGTA, and 2.5 mM GTP (GEP buffer), with 0.08-0.1% Brij 58. Normal neurites were also lysed in this buffer with or without added 2M glycerol. The latter conditions have been found to stabilize assembled



MTs against dissassembly by dilution while allowing diffusion of free tubulin in a variety of cells (8, 86).

Regrowth properties of collapsed neurites treated with 1.0 ug/ml nocodazole were examined 15 and 30 minutes after rinsing by exposing them to either 2.3 x 10^{-5} M or 4.6 x 10^{-8} M taxol. Taxol, a drug reported to promote MT assembly (80), was a gift from the Developmental Therapeutics Program of the National Cancer Institute.

Electron Microscopy

Cultures were fixed for electron microscopy using 1 of 2 methods. Neurites and untreated collapsed neurites were fixed by replacing the medium with a solution containing 0.1 M cacodylate, 1 mM MgCl₂, and 2\$ glutaraldehyde for 20-30 minutes. Because collapsed neurites treated under MT depolymerizing or lysis conditions tended to lift from the dish surface during exchanges of fluid, these cultures were fixed by the addition of an equal amount of the same medium or buffer containing 4\$ glutaraldehyde. The cultures were then rinsed twice in 0.1 M cacodylate with 5\$ sucrose, treated with 0.15\$ tannic acid for 5 minutes, rinsed twice, postfixed in 1\$ $080_{\rm H}$ for 5 minutes, Marrington, PA). Thin sections cut parallel to the substratum were stained with uranyl acetate and lead citrate, and observed with a Phillips 300 transmission electron microscope.



Serial Reconstruction of Beads

In order to determine whether the MTs left in collapsed neurites after cold treatment were in the form of fragments, a modification of the method of Nicklas (72) was used for serial reconstruction. Outer boarders and MTs together with many membranous vesicles within the collapsed neurite were traced from the micrographs onto transparent plastic sheets. The tracings were aligned first by the membranous registration markers, then adjusted to maximize the matches of MT ends in consecutive sections by moving the tracings within a range of two MT diameters. MT length and orientation were then depicted in the form of a composite drawing.



RESULTS

Collapse of Amputated Neurites

Our studies confirmed the observations of Shaw and Bray (82). Distal segments of amputated neurites collapsed to beads of axoplasm (hereafter "beads") which then regrew neurites almost immediately. Because we were interested in examining collapse and regrowth separately, we selected for amputation unbranched neurites with modest growth cones (Figure 1a), and amputated distal segments approximately 100 microns in length. We found such neurite segments most likely to completely collapse to beads before beginning regrowth. Figure 1 shows a typical sequence of amputation, collapse, and regrowth. Fifty three amputations were performed in a set of experiments to monitor regrowth. In 16 cases, regrowth began prior to the complete collapse of the neurite. In 37 cases, however, the amputated neurite collapsed to a flattened sphere or "bead" of axoplasm with no visible processes prior to the onset of regrowth. Initially, the growth cone flaired and began to "melt" almost immediately upon severing the neurite. Then, as reported by Shaw and Bray (82), the distal segment coiled (Figure 1b) as it collapsed to a bead (Figures 1c and d). Electron micrographs of distal segments at various times after amputation show varying degrees of MT depolymerization roughly correlated to the degree of coiling suffered by the neurite.



Figure 1: Phase-contrast micrographs of a sequence of collapse and regrowth of neurites after amputation. (a) Neurite before amputation. (b) 3 minutes after amputation, growth cone is flaired and neurite is coiled and retracted. (c) 12 minutes after amputation, collapse to a bead is almost complete. (d) 13 minutes after amputation, collapse is complete and minor regrowth has begun. (e) 16 minutes after amputation, multipolar regrowths are apparent. (f) 18 minutes after amputation, regrowths have lengthened. Bar, 20 um.





<u>Ultrastructure of Collapsed Neurites with and without MT</u> Depolymerization

We examined the ultrastructure of 10 amputated, untreated neurites collapsed to beads. All ten clearly showed many long MTs in addition to multiple cross-sections and apparent fragments (Figure 2). Electron micrographs of 4 beads that were cold treated for 2 hours in a -5° C bath (Figure 3a) show the marked absence of any long MTs similar to those found in the untreated beads. In order to confirm that the MTs left in the cold treated bead were indeed short fragments, we reconstructed 9 consecutive serial sections. approximately 75%, of one typical bead. The reconstruction (Figure 3b) shows many cross sections of MTs and 158 longitudinal fragments. In this particular bead all MTs were found to be shorter than 0.5 microns in length while over 80% were shorter than 0.2 microns in length. Treatment of collapsed neurites with 0.1 ug/ml nocodazole for 15-20 minutes likewise resulted in substantial MT depolymerization. We examined 4 such beads and found short fragments as well as some longer MTs in the peripheral regions (Figure 4a). However. treatment with a higher dose of 1.0 ug/ml nocodazole appeared to completely depolymerize MTs. We examined sections from 3 such beads and could find no unambiguous MTs (Figure 4b).

We wished to determine if the MT fragments that persisted in the cold treated bead were merely "mass action fragments" that could not depolymerize in the high tubulin concentration of the cold treated bead, or if they were in some way distinct from the majority of MT mass that had depolymerized. We reasoned that detergent lysis would release soluble tubulin dimer allowing depolymerization of any "mass action



Figure 2: Transmission electron micrograph of a distal segment of an amputated neurite collapsed to a bead and fixed before regrowth. Many MTs of varying lengths are apparent. Bar, 0.5 um.





Figure 3: (a) Transmission electron micrograph of a cold treated bead, (b) Serial reconstruction of 9 consecutive sections through the cold treated bead. Arrows mark MT fragment longitudinal sections, Reconstructions shows MT longitudinal sections as line segments and MT cross sections as dots, Bar, 0.5 um.







Figure 4: Transmission electron micrographs of beads treated with (a) 0.1 ug/ml and (b) 1.0 ug/ml nocodazole. MT fragments remain in the lower dosed bead (arrows), while deplymerization is complete in the higher dosed bead. Arrow in (b) marks ambiguous vesicular element. Bar, 0.5 un.





fragments." The efficacy of this treatment in depolvmerizing MTs in neurites was examined by comparing the MT array left in intact neurites lysed in a MT reassembly buffer ("GEP" see Methods section) with the array left in neurites lysed in the same buffer supplemented with 2 M glycerol. The latter conditions have been found to stabilize assembled MTs against dissassembly by dilution while allowing diffusion of free tubulin in a variety of cells (8, 86). Light microscope observations of neurites lysed in GEP without glycerol showed a transient beading within 1 minute of adding lysis buffer, an effect symptomatic of MT depolymerization in neurites (26, 43, 50). Neurites lysed in GEP with glycerol showed no such beading. Electron micrographs of neurites lysed in GEP with glycerol show a dense array of long MTs (Figure 5a). while electron micrographs of neurites lysed in GEP alone show substantial MT depolymerization (Figure 5b). Electron micrographs of cold treated beads lysed in GEP without glycerol (Figure 5c) show MT fragments similar to those MT fragments in unlysed, cold treated beads. That is, lysis of cold treated beads caused no major additional MT disassembly.

Regrowth from Collapsed Neurites

Regrowth of neurites from beads began either within the first 30 seconds after collapse or often before collapse was complete. We followed regrowth in some cases for 45-60 minutes after collapse. In these cases the total length of the regrown neurites approximated that of the original distal segment, although the bead never entirely disappeared. The fastest and most robust regrowth typically occurred



Figure 5: Transmission electron micrographs of (a) a neurite lysed in GEP buffer supplemented with 2 M glycerol, (b) a neurite bundle lysed in GEP buffer alone, and (c) a cold treated bead lysed in GEP alone, lysis in GEP alone permitted substantial MT disassembly by dilution in normal neurites but apparently permitted no further depolymerization in the cold treated beads. Bar, 0.5 um.





over the first 15 minutes. Out of 53 amputations, 16 began regrowth prior to the complete collapse of the neurite. In these cases, the remnants of the growth cone or neurite elongated to form the regrown neurite(s). In the 37 cases in which the amputated neurite collapsed completely there was no relationship between the number and/or orientation of regrown neurites with the orginal neurite/growth cone. In all cases regrowth ensued at rates of 3.5-4.5 microns/minute per regrown neurite averaged per bead. These rates were at least double those reported by Shaw and Bray (82), perhaps due to our use of plastic instead of glass substrata. Electron micrographs of regrown beads show paraxial MT arrays in the regrown neurites (Figures 6a and b) entirely similar to those in ordinary neurites, and fewer MTs in the bead than before regrowth (Figure 6a).

After rewarming, regrowth from cold treated beads was very similar to regrowth from untreated beads. Cold arrested regrowth resumed within 30 seconds of the cultures reaching a narrow temperature range within $1-2^{\circ}$ C of 37° C. The rates of regrowth from cold treated beads were in the same range of 3.5-4.5 microns/minute as regrowth from untreated beads.

The regrowth of nocodazole treated beads was more difficult to study in that the rinsing required to remove the nocodazole often caused detachment of the bead from the dish surface. Both cold and nocodazole treated beads were less adherent to the substratum than untreated beads. However, cold treated beads did not require a medium change and thus could be regrown with minimal disturbance. Only 6 of 40 beads remained attached after rinsing of the lower dose (0.1 ug/ml) of nocodazole and only 5 of 50 beads survived rinsing of the higher dose (1.0 ug/ml). All









6 of the beads treated with the lower dose began regrowth within 30 seconds of their return to undrugged medium at a rate of 3.5-4.5 microns/minute. However, the beads treated with the higher dose of nocozole showed very poor regrowth. Only 1 of the 5 beads recovering from the higher dose regrew and this was only after a 20 minute delay. The other 4 never regrew even after 4 hours of observation.

We wished to determine whether MT assembly was occurring without regrowth in beads rinsed after treatment with 1.0 ug/ml nocodazole. Eight beads were treated with 1.0 ug/ml nocodazole, rinsed twice, and returned to undrugged medium. Seven of the 8 failed to regrow. Electron micrographs of 2 such beads fixed after 15 minutes in undrugged medium and 3 such beads fixed after 30 minutes in undrugged medium show no MTs (not shown). In 2 of the 30 minute cases we were able to obtain sections covering approximately 90% of the bead and all were devoid of MTs. The single bead that regrew did so after a 15 minute delay, its regrown neurite contained MTs as expected (not shown) and the bead was on the same plate as a non-regrowing bead found to be devoid of MTs. In order to determine whether the apparent inability of such beads to initiate MT assembly was due to the incomplete removal of nocodazole by our rinsing method. we examined the recovery of normal neurites treated with a 20 fold higher dose (20 ug/ml) of nocodazole. These unamputated neurites retained a very few MT fragments (Figure 7a) and reassembled a dense array of MTs after 15 minutes in undrugged medium (Figure 7b).

In order to determine whether beads treated with 1.0 ug/ml nocodazole were inviable and/or whether MT reassembly was no longer possible, we treated such beads with taxol. All 4 beads treated with 1.0 ug/ml nocodazole for 15 minutes, rinsed and treated with 4.6 x 10^{-8}



Figure 7: Transmission electron micrographs of (a) an intact neurite treated with 20 ug/ml nocodazole, rinsed with PBS, and returned to undrugged medium for 15 minutes before fixation. An apparently normal MT array has been recovered. Bar, 0.5 um.




M taxol rapidly regrew after a delay of about 3 minutes (Figure 8). Such regrown neurites contained an array of MTs similar to those of unarrested regrown beads (not shown). Six of 6 beads treated with nocodazole, rinsed and treated with 2.3 x 10^{-5} M taxol failed to regrow but showed substantial MT reassembly in the beads.



Figure 8: Phase-contrast micrograph of a bead treated with 1.0 ug/ml nocodazole for 15-20 minutes, rinsed twice with PBS, then treated with fresh medium containing 4.6 X 10^{-8} M taxol. The bead began robust regrowth 3 minutes after adding the taxol. Arrow marks bead, Bar, 20 um.





DISCUSSION

We report here an investigation of the role of MT assembly and disassembly in the regrowth of neurites from the collapsed, distal segments of amputated neurites of chick sensory neurons. Fully collapsed beads contained many long MTs (Figure 2). One possibility was that these MTs might simply "unfurl" into the axon during regrowth in a manner roughly analogous to Lasek's proposal for slow axonal transport of MTs in the form of assembled polymers (55). However, beads treated at -5°C for 2 hours (Figure 3) or with 0.1 ug/ml nocodazole for 15-20 minutes (Figure 4a) contained only short fragments of MTs, and yet regrew with no delay after reaching 37°C or rinsing out the nocodazole in the absence of MTs long enough to "unfurl". The rates of regrowth in all 3 cases were indistinguishable and in the range of 3.5-4.5 microns/minute. The rapidity of regrowth and lack of a delay prior to the onset of regrowth argue in favor of nucleated reassembly of MTs and against the the possibility of de novo initiation (2, 46, 73, 74). Indeed, beads treated with a higher dose of 1.0 ug/ml nocodazole were devoid of MTs (Figure 4b), and failed to regrow in 11 out of 13 cases. Although the poor adhesion of the treated beads to the substrata might also disable regrowth by not allowing a growth cone to form, beads treated with cold or with the lower dose of nocodazole were equally precariously attached yet regrew without delay. Taxol, not known to increase cell adhesion, enabled beads treated with 1.0 ug/ml nocodazole to regrow. Incomplete removal of nocodazole by our rinsing method also seems an unlikely factor in that a very high dose of 20 ug/ml was rapidly reversible in normal neurites (Figure 7). Further, neurites



treated with the lower dose of nocodazole began regrowth within 30 seconds of rinsing. The rapidity of this reversal would not be expected if nocodazole was sequestered by some peculiarity of the bead. Nocodazole poisoning has been found to be immediately reversible in a variety of cell types (27, 83). We conclude that rapid regrowth of axonal fragments is dependent upon the presence of MTs or MT fragments that serve as nucleating elements for MT elongation.

In the absence of nucleating elements, the expectation on the basis of MT assembly in vitro would be a delay in regrowth while initial nucleation occurred, after which assembly would occur rapidly (2, 46, 73, 74). In 2 out of 13 cases, beads treated with the higher dose of nocodazole apparently sufficient to completely depolymerize MTs indeed responded in this fashion, while the majority, 11 of 13, did not. One possibility is that in the 2 cases of regrowth the 1.0 ug/ml nocodazole was not sufficient to completely depolymerize bead MTs. More likely. given the 15-20 minute delay before regrowth, is that in these cases the bead tubulin managed self-nucleated MT assembly after complete depolymerization. However, the failure of 11/13 to regrow even after extended periods suggests a limited capacity of bead tubulin for self-nucleation. This suggestion is supported by the observation that beads that failed to regrow after 15 or 30 minutes in undrugged medium were still devoid of MTs. Treatment of beads rinsed of 1.0 ug/ml nocodazole with taxol, a drug that promotes MT assembly (80), was sufficient to stimulate bead tubulin into initiating assembly de novo. In 4 out of 4 cases, beads treated with 4.6 x 10^{-8} M taxol after being rinsed of 1.0 ug/ml nocodazole regrew after a 3 minute delay (Figure 8). In 6 other cases a higher dose of 2.3 x 10^{-5} M taxol failed to stimulate



regrowth, but did cause substantial MT reassembly in the beads. Letourneau and Ressler had previously reported that neurite outgrowth from chick sensory neurons was limited to low levels of taxol and that higher doses inhibit neurite outgrowth (60). The ready regrowth when MT assembly is stimulated by taxol suggests that it was indeed the lack of MT assembly that inhibited regrowth previously, rather than inviability of the bead or loss of competent tubulin. It is unclear from our experiments whether the limited capacity of tubulin to initiate assembly is characteristic of neuronal tubulin or of the bead only. Morris and Lasek (71) have found in souid axoplasm that the amount of tubulin in subunit form is higher than what would be predicted by in vitro studies given the total tubulin concentration. They concluded that there are factors in the cell that inhibit the polymerization of tubulin. Our evidence suggests that such factors may function in part by inhibiting de novo initiation. On the other hand, we found that the bead had some MT disassembly properties not shared by the intact neurite. Nocodazole was much more effective at completely depolymerizing MTs in beads than in intact neurites (Figures 4b and 7a). Lasek's model for the slow transport of cytoskeletal elements maintains that MTs are selectively degraded in axon terminals (54). Burton has recently reported evidence of MT disassembly at the distal ends of intact frog axons (18). During collapse of the neurite, its terminal growth cone becomes incorporated into the bead. If a degradation factor similar to that in synaptic terminals exists in the growth cones of advancing neurites, it might account for both the increased sensitivity of bead MTs to higher nocodazole concentrations and their limited ability to self-nucleate.

If neuronal tubulin has only a limited capacity for self-nucleation,



the importance of MT nucleating elements would be paramount for cytoskeletal reorganizations occurring during axonal growth. Abundant evidence suggests that certain neuronal MTs are especially stable (9. 12. 42. 45. 48. 92), and that these MTs exist in the form of short regions of otherwise labile MTs (45, 50, 79). We wondered whether the fragments left in the cold treated bead were merely "mass action fragments" maintained because of a high tubulin concentration in the bead, or if they were intrinsically stable regions of longer MTs. We reasoned that if the fragments were "mass action fragments" they would depolymerize if the free tubulin were allowed to escape from the bead by lysing. If, however, the fragments were intrinsically stable, they would not depolymerize upon subunit dilution. Indeed, as shown in Figure 5. MT fragments persisted in cold treated beads lysed under conditions that caused substantial MT depolymerization in normal neurites. All available evidence indicates that the lysis buffer we employed causes MT disassembly by dilution since it supports MT assembly in vitro while allowing soluble tubulin to diffuse from lysed cells (8. 23, 86). We interpret this as indirect evidence that the fragments in the cold treated beads were different from the majority of the MT mass. Job et al (45), Brady et al (12), and others have provided evidence that cold stable MTs differ in either MAP or tubulin composition. The fragments left in the cold treated beads might similarly vary from the solubule MT fraction for these reasons.



CHAPTER 2. Microtubule Polarity Reversal Accompanies Regrowth of Amputated Neurites

INTRODUCTION

Microtubules (MTs) are intrinsically polar structures. The two ends of the MT differ in the concentration of subunits needed for assembly and their rates of elongation (2, 6, 7, 25, 65, 66). The role of MT polarity in regulating cellular processes that are dependent upon MT assembly, such as axonal growth (15, 75), is largely unexplored. Axons contain dense paraxial arrays of MTs that are uniform in their polarity orientation; the [+] or "fast growing" ends of MTs are found in the axon terminal (19, 32, 39, 41). The cellular and molecular factors contributing to this uniform polarity orientation are unknown. The uniform polarity orientation of MTs extending from centrosomes or other MT organizing centers indicates that such structures regulate MT assembly in a polar fashion (30, 31, 37). However, unlike most interphase MT arrays, axonal MTs do not extend from a centrosome or any observable organizing structure (64, 81, 98). Although some studies have postulated that MTs are first assembled in the cell body and then transported down the axon as intact polymers (10, 55), others have reported persuasive evidence that the growth cone is the major site of MT assembly supporting axonal growth (5). Consequently, there is a controversy whether the growth cone or cell body is the principle site



of MT organization in axonal growth.

Amputated neurites of cultured chick sensory neurons are a useful system in which to investigate the principle site of MT organization in neurons and the role of MT polarity in axonal growth. We previously reported that collapse and subsequent regrowth of the amputated neurite segment freed from the cell body (the distal segment) involves MT disassembly and reassembly (3). Here we wished to determine whether neurites regrown from the collapsed distal segment contain a uniformly polar MT array despite the absence of the cell body. Also of interest is the difference in the regrowth of the neurite segment attached to the cell body (hereafter proximal segment) and the distal segment in neurites that remain extended after amputation. Extended proximal segments routinely form new growth cones at their cut ends. However. distal segments that remain extended after amputation do not form new growth cones or show signs of regrowth at their cut ends (14). This contrasts with the routine formation of new growth cones and robust regrowth by distal segments that first collapse to "beads" of axoplasm (3, 82). Do differences in the ability of neurite segments to regrow correlate with differences in MT polarity?



MATERIALS AND METHODS

Cell Culture

Embryonic chick sensory neurons were cultured by two methods. For determination of MT polarity in intact neurites, dorsal root ganglia were dissected from the lumbosacral regions of 12 day embryos and cut into two or three pieces with forceps. The chunks were cultured (using a modification of the method of Vlodavsky et al, Ref. 91) on polylysine treated tissue culture dishes in L-15⁺ medium (L-15 purchased from Gibco Laboratories, Grand Island, NY supplemented with 0.6\$ glucose, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin) containing 10\$ fetal calf serum, 20 ug/ml 7s NGF, and 5 ug/ml laminin (Polysciences, Inc., Warrington, PA). The chunks were allowed to grow neurites for 48 hours prior to experimentation. For amputation experiments, dissociated cells were cultured as previously described (3) on untreated or polylysine treated tissue culture plastic. Dissociated cells were allowed to grow neurites for 12-30 hours prior to experimentation.

Amputation Expriments

Amputation experiments were performed on a microscope stage prewarmed to 37° C with a Sage air curtain incubator (as previously described in Ref. 3). Unbranched neurites with modest growth cones were selected and amputated ca. 100 um from the growth cone in an effort to



promote complete collapse to "beads" of axoplasm.

MT Polarity Determination

The polarity orientation of MTs in the neurites of cultured neurons was determined by the "hook method" previously described (19. 32. 39. 40. 41). The ganglia chunk preparation was used to increase the number of neurites sectioned at one time. The polarity of MTs in amputated and regrown neurites was determined using modifications designed to decrease the tendancy of the neurite segments to loosen and wash away from the substratum during hook treatment. The modifications reported here permit the routine polarity determination of MTs in even these very delicate poorly adhered cell processes. After amputations, cultures were rinsed briefly in PBS, then exposed to a MT assembly mixture containing 1.2 mg/ml beef brain MT protein (twice cycled in the presence of glycerol) in 0.5 M PIPES, pH 6.9, 2.5% DMSO, 0.05 mM EDTA, 0.5 mM EGTA, 0.5 mM MgCl₂, 0.5 mM GTP, and 0.06-0.08% Brij 58 for 2 minutes at 37° C. The mixture was then removed and replaced for 30 minutes at 37° C with an identical mixture not containing the Brij 58 detergent. An equal quantity of 4% glutaraldehyde in 0.1 M cacodylate was then added to stabilize lysed neurites from being rinsed away. After 2-3 minutes cultures were rinsed twice in 2% glutaraldehyde in 0.1 M cacodylate in order to remove excess polymerized tubulin. Neurites were then allowed to fix for 30 minutes after which cultures were again gently rinsed (5 times) in 0.1 M cacodylate with 5% sucrose. This procedure resulted in ca. 75% of the lysed neurites remaining attached to the substratum.



After fixation, cultures were treated for 5 minutes in 0.15% tannic acid. rinsed twice in 0.1 M cacodylate with 5% sucrose, postfixed in 1% OsO, for 5 minutes, dehydrated in ethanol series, and embedded in Polybed 812 (Polysciences, Inc.). Samples were then relocated and circled with a diamond marker objective. Orientation of the neurite was marked by a series of circles colinear with the length of the neurite. Thin sections were cut perpendicular to the substratum in order to obtain cross-sections of the neurites. Blockfaces were cut in asymmetric shapes so that samples could be quickly relocated on the electron microscope, and to make clear whether any sections had been flipped. Sections were picked up on 0.75% formvar coated 75 mesh grids (Ernest F. Fullam, Inc., Schenectady, NY), stained with uranyl acetate and lead citrate, and observed with a Phillips 300 transmission electron microscope. Care was taken to relocate the regrown neurite on thin sections with great certainty (even though the rinsing procedure was quite effective in removing exogenous polymerized tubulin) in that much of the membrane was extracted in many cases. Hooks were analyzed as previously described (40).



RESULTS

Polarity Orientation of MTs in Neurites of Cultured Neurons

Heidemann and others have reported that in axons of nerve fibers from intact animals MTs are oriented with their [+] or "fast growing" ends pointed away from the cell body toward the axon terminal (19, 32, 39. 41). In order to determine whether neurites of cultured neurons share this uniform polarity orientation, we performed MT-polarity revealing "hook" experiments on bundles of neurites grown out from cultured chunks of embryonic chick dorsal root ganglia. In this method "hooks" of MT protofilaments are formed on the sides of MTs. Hooks with a clockwise (CW) orientation indicate that the [+] end of the MT is pointed toward the observer, while hooks with a counterclockwise (CCW) orientation indicate that the [-] end of the MT is pointed toward the observer. The results of 3 separate experiments are recorded in Table 1. Of those MTs that were unambiguously hooked, 97% had clockwise hooks as seen from the growth cones looking toward the ganglia (Figure 9). That is, like MT in axons from intact animals, neurite MTs were found to have their [+] ends in the distal terminal of the neuronal process.



Sample	Neurites Counted	<u>cw</u> *	<u>ccw</u> *	%CW
1	50	152	3	98
2	100	296	13	96
3	65	242	8	97
Total	215	690	24	97

Table 1: Polarity Orientation of MTs in Intact Neurites

*-----

as seen by an observer standing at the growth cone.



Figure 9: Transmission electron micrograph of a cross section through a neurite mass grown from a cultured ganglion chunk treated under the MT polarity revealing "hock" procedure. Hocks are predominately CW (arrows) indicating MTs are oriented with their [+] ends pointing away from the cell body toward the growth cone. Bar, 0.8 um.





Regrowth and MT Polarity Orientation in Amputated Uncollapsed Neurite Segments

Following amputation, neurite segments either remain extended or collapse depending on how firmly they adhere to the substratum. On the highly adhesive substrate of polylysine treated tissue culture plastic, in over 15 cases we observed little or no collapse of either neurite segment within the first 15-20 minutes after amputation. While new growth cones routinely formed at the cut ends of the segments proximal to the cell body, growth cones never formed at the cut ends of the distal segments severed from the cell body. This finding is similar to that of Bray et al on neurites amputated under the highly adhesive condition of serum free medium (14).

All further amputation experiments were performed on the moderately adhesive surface of untreated tissue culture plastic. Approximately 9 out of 10 distal segments of neurites amputated on this substratum collapsed, while roughly 1 out of 10 remained extended. This allowed us to examine regrowth and MT polarity in both collapsed and extended neurite segments under the same substrate conditions, which are known to affect growth cone formation (14, 59, 93). We observed 7 amputated neurites whose proximal and distal segments remained extended after amputation. For these uncollapsed amputated segments, again the cut ends of the proximal segments routinely formed new advancing growth cones usually within 15-20 minutes, while the cut ends of the distal segments never formed new growth cones (Figure 10). Some cut ends of proximal and distal segments retracted a few microns and formed rounded varicosities of cytoplasm (retraction bulbs) on their cut ends. These



Figure 10: Phase-contrast micrograph of a firmly adherent neurite whose proximal and distal segments did not collapse 20 minutes after amputation. The cut end of the segment proximal to the cell body has formed a new growth cone (thin arrow), while the cut end of the segment distal to the cell body remains crushed in appearance. Thick arrow marks scar from amputation with glass needle. Bar, 20 um.





retraction bulbs formed new growth cones on cut ends of proximal segments, while retraction bulbs on cut ends of distal segments never formed a growth cone. In a very few (less than 5%) cases a distal retraction bulb formed one or two wispy filopodia. We previously reported that retraction bulbs are sites of MT disassembly (3), and MT disassembly is known to stimulate formation of filopodia (14, 50).

We examined the polarity orientation of MTs in both the proximal and distal segments of uncollapsed neurites 15 minutes after amputation using the very gentle conditions for MT-polarity revealing hook formation described in Materials and Methods. These conditions permitted the determination of MT polarity in even these delicate neurite segments. Although the degree of hooking was low (generally less than 30%), the results were very consistant in all samples we analyzed. We used the original growth cone of the neurite prior to amputation as the reference point for both proximal and distal segments. In all 4 proximal and all 4 distal segments we analyzed, over 90% of the unambiguously hooked MTs were clockwise (Table 2, Figure 11). That is, in both segments [+] ends were pointed away from the cell body toward the original growth cone. Plus ends of MTs faced the cut end of the regrowing proximal segments, while [-] ends of MTs faced the cut end of the non-regrowing distal segments.



Sample	Proximal/Distal	<u>cw</u> *	CCW	1 CW
1	Proximal	26	2	93
1	Distal	38	2	95
2	Proximal	51	1	98
2	Distal	32	3	91
3	Proximal	38	2	95
3	Distal	38	1	97
4	Proximal	11	0	100
4	Distal	18	0	100

<u>Table 2: Polarity Orientation of MTs in Amputated</u> <u>Uncollapsed Neurites</u>

as seen by an observer standing at the original growth cone of the neurite.


Figure 11: Transmission electron micrograph of a cross section through the distal segment of an amputated neurite that failed to collapse 15 minutes after amputation subsequently treated under HT-polarity revealing "hock" conditions. Hooks are predominately CW (arrows) indicating (+) ends of MTs pointing toward the growth cone, ie. [-] ends of MTs pointed toward the cut. The polarity of MTs was similarly [+] ends toward the original growth cone in proximal segments, ie. [+] ends pointed toward the LBar, 0.8 um.





<u>Polarity Orientation of MTs in Neurites Regrown form Collapsed Distal</u> Segments

As previously reported (3, 82, 93), distal segments of neurites that underwent significant collapse to beads of axoplasm after amputation routinely regrew neurites (Figure 12). We found a close correlation between the degree of collapse and the ability to regrow. Distal segments that retracted less than approx. 60% of their initial amputated length did not regrow. Distal segments that did collapse and regrow formed at least two new advancing growth cones in all cases. In 62 collapse/regrowth events we monitored. 18 "beads" began to regrow before completely retracting all remnants of the original neurite. In all these cases, the remaining short stub of neurite always gave rise to a regrowing neurite (Figure 13). In the 44 cases of regrowth from a bead with no visible processes, only 7 regrew a neurite colinear with the original. In 23 of the 44 cases the bead regrew tripolar or tetrapolar neurites (Figure 1f). In 14 other cases, the bead regrew bipolar neurites but at a significant angle to the original neurite (Figure 12). Except in the cases in which stubs of the original neurite remained, we could find no correlation between the pattern of regrowth and any aspect of the original neurite, growth cone, or neuron.

MT polarity orientation in neurites regrown from beads of axoplasm was determined by the same method used for uncollapsed, amputated neurites. We used the bead of axoplasm as the point of reference for MT polarity because the bead occupied the position of the original growth cone on the unamputated neurite. Table 3 shows that 85% to 100% of the hooked MTs were counterclockwise (as seen from the bead, see Figure 14)



Figure 12: Phase-contrast micrograph of an amputated neurite whose distal segment collapsed and then regrew neurites. The regrown neurites regrew bipolar at a sharp angle to the orientation of the original neurite. The cut end of the proximal segment formed a retraction bulb, then a new growth cone (thin arrow). Thick arrow marks the scar from amputation with glass needle. Bar, 20 um.





Figure 13: Phase-contrast micrographs of an amputated neurite whose distal segment (a) incompletely collapsed to a bead that (b) regrew a neurite from the remaining stub of the original neurite. The proximal cut end formed a new advancing growth cone. Bar, 20 um.





regardless of the direction or pattern of regrowth in all 9 regrown neurites we were able to examine. That is, [+] ends of MTs were now in the newly formed growth cone and [-] MTs ends were located in the position of the original growth cone.



Table 3: Polarity Orientation of MTs in Regrown Neurites

as seen by	v an observer	standing at	the bead	(original	growth cone).
9	bipolar	incomplete	10	0	100
8	bipolar	incomplete	13	0	100
7	multipolar	complete	15	0	100
6	bipolar	complete	46	8	85
5	bipolar	complete	4	0	100
4	multipolar	complete	21	0	100
3	bipolar	complete	100	14	88
2	bipolar	complete	29	2	94
1	bipolar	complete	18	0	100
Sample	Pattern	Collapse	CCW	CW	1 CCW



Figure 14: Transmission electron micrographs of a cross section through a regrown neurite treated under MT-polarity revealing "hook" conditions. Hooks are predominately CCW (arrow) indicating [+] ends of MTs pointed away from the bead. The polarity of MTs was [+] ends away form the bead regardless of the pattern of regrowth. Bar, 0.8 wm.





DISCUSSION

We report here that MTs in the neurites of cultured neurons have the same uniform polarity orientation as previously reported for MTs in the axons of mature neurons (19, 32, 39, 41). The [+] or "fast growing" ends of MTs are found in the growth cone or axon terminal. As expected from this MT polarity orientation in intact neurites. MTs in amputated neurites that did not collapse were oriented with [+] ends facing the cut end of the proximal segment, and [-] ends facing the cut end of the distal segment. Our data show that MTs reorganized and reversed their polarity during the regrowth of neurites from collapsed distal segments of amputated neurites. Prior to amputation or collapse [+] MT ends face an observer standing at the growth cone, while after collapse and regrowth the stationary observer sees [-] ends of MTs. This finding is similar to that of McNiven et al who showed that arms of fish melanophores severed from the cell body form a new pigment migration center from which MTs are organized with a uniform polarity reversed compared to the original orientation (68). In our experiments [+] ends of MTs were closely correlated with the formation of new growth cones in amputated neurites both at the proximal segment of uncollapsed neurites and at the growth cones newly formed from collapsed distal segments. Neither neurite elongation nor growth cone formation was observed at the cut ends of the distal segments that remained extended. Distal segments regrew neurites only if they initially collapsed to a signifcant degree. Since MT disassembly accompanies neurite collapse (3). this finding suggests that MT reorganization is a requirement for growth cone formation in the distal segment. After reorganization [+] MT ends faced



the growth cones. Although axonal growth has long been thought to depend upon MT elongation (15, 54, 75), our results suggest that assembly at the [+] ends is required for growth. This interpretation is consistent with the finding of Bamburg et al that axonal extension is 100 times more sensitive to colcemid applied to growth cones than applied elsewhere (5). The formation of growth cones has previously been shown to be stimulated after experimentally induced MT disassembly and reassembly (14). Wessells et al speculated that growth cone formation requires cytoskeletal ends (93). Our data support this hypothesis and suggest that growth cone formation requires [+] ends of MTs in the immediate vicinity of the axonal surface/cortex.

We find particularly interesting the ability of beads of axoplasm completely separated from the cell body to reorganize MTs so that regrown neurites share the same uniform MT polarity orientation characteristic of intact neurites and axons. We found that the number and spatial orientation of regrown neurites was independent of any observable quality of the original neurite. This suggests that the potential for forming neurites is widespread around the margin of the axoplasmic bead and that neurite formation is occurring de novo. The collapsed distal segment can apparently reorganize a uniformly polar MT array and support neurite growth without contributions from the cell body such as centrioles (81, 87), endogenous determinants (85), or slow axonal transport (33. 55). At the very least, our experiments show that local, ie. in the neurite, processes are sufficient for the task of organizing MT assembly during neurite growth. Based on the ultrastructural similarity of regrown and normal neurites (3) and the similarity of regrowth to normal growth. it is our view that the same



mechanism of MT organization obtains in both normal neurite outgrowth and the regrowth studied here. We suggest that organization of MT assembly at the growth cone is crucial for axonal growth. No organizing structure other than MTs or MT fragments were observed in this or previous ultrastructural study (3) of axoplasmic beads and their regrown neurites. We speculate that MT organization accompanying neurite outgrowth is not the result of a MT organizing structure but of a process, the interaction of MT assembly with the motility of the cell cortex. Neurite outgrowth begins with motile activity such as filopodia formation and ruffling by the cortex of the cell/bead (93). We suggest that in order for the typical growth cone structure to form and neurite elongation to begin, a region of the motile margin must "recruit" [+] ends of MTs. Joshi et al have proposed that MT elongation at the growth cone is then regulated by the actin-based advance of the growth cone releasing compressive force on the MTs, thus lowering their critical concentration for assembly (49). We speculate that [+] ends of MTs at the growth cone are an important element in this scheme in that they would have a competitive assembly advantage over [-] ends at any given tubulin concentration and degree of compression.

The small fraction of MTs that displayed polarity revealing hooks in amputated neurites is a regretable but probably unavoidable shortcoming of these experiments. Conditions for forming hooks on MTs always involve changing the fluid surrounding the cells and at least one fluid must have detergent (40). These requirements were nearly incompatible with the study of amputated neurites because of their precarious attachment to the culture substratum (3). Despite a great deal of trial and error, we were unable to define conditions that produced a high



fraction of MTs with hooks and preserved the attachment of the neurite segments. It is possible that neuronal MTs are difficult to decorate with hooks; in all previous studies of MT polarity in neuronal tissue by ourselves and others the extent of "hooking" was low (19, 32, 39, 41). Nevertheless, we would point to the consistency of our results and the finding in cat sympathetic nerve that the degree of hooking does not affect the conclusion with respect to MT polarity (41).



CHAPTER 3. Role of Microtubules in the Cytoplasmic Compartmentation of Neurons

INTRODUCTION

The exaggerated geometry of the neuron has made it a uniquely suitable model in which to study the organization of cytoplasm (16, 54, 57, 75, 77, 89, 96). Neurons display a high degree of regional specialization (20, 36, 38, 57, 76, 77). Lasek and Brady have argued that the cytoplasm of neurons can be compartmentally divided into the ribosome rich "translational" cytoplasm of the perikaryon and the ribosome deficient "expressional" cytoplasm of the axon (57). This kind of organization can also be noted in pancreatic endocrine cells (57). certain protozoa (11), fibroblasts (34), and HeLa cells (24. 44. 58). but is pronounced in neurons due to their extension of translational cytoplasm over long distances in the form of axons. The restriction of protein synthetic machinery to the perikaryon in neurons demands the existence of a complex system of axonal transport to nourish the axoplasm. Axonal transport has been extensively studied (36, 43, 54, 55), but to our knowledge there has been no experimental work examining how ribosomes are localized to the perikaryon. Lasek (54) has proposed that "free" ribosomes in neurons are restricted from entry into axons by cytoskeletal attachments in the perikaryon. Most ribosomes in immature and growing neurons however appear not to be immobilized around the



nucleus, nor are they anchored to endoplasmic reticulum (16, 21, 26, 62, 63, 89, 96), and yet they are still restricted from freely diffusing into the axoplasm.

The cytoplasm of the axon is further distinguished from the soma by the presence of dense paraxial arrays of cytoskeletal elements critical for axonal transport (15. 16. 36. 43. 54. 57). Lasek and Katz have noted that the "agranular" cytoskeleton of the axon is unique from the "granular" cytoskeleton of the perikaryon by the marked absence of microtubules (MTs) in the latter (56). The restriction of ribosomes to one compartment and MTs to the other suggests that the selective exclusions may be coordinated. We previously reported that the ultrastructure of PC12 neurites becomes very similar in appearance to that of the soma after treatments that depolymerize MTs (49). We recently reported preliminary evidence that the number of visible ribosomes in the axon-like neurites of cultured embryonic chick sensory neurons is substantially increased after MT depolymerization (4). These results suggested that MTs in the neurite may play a role in the restriction of ribosomes to the soma. We report here studies designed to determine the role of MTs in cytoplasmic compartmentation during early neuronal development.



MATERIALS AND METHODS

Cell Culture

Embryonic chick sensory neurons were cultured as previously described (3).

Experimental Treatments

Cultures were treated with various drugs by replacing the medium with fresh medium containing the drug. Cultures were treated for 15-20 minutes with 1.0 ug/ml nocodazole (Aldrich Chemical Company, Milwaukee, WI), 10 ug/ml vinblastine sulfate, 100 ug/ml griseofulvin, or 2 ug/ml cytochalasin D (Sigma Chemical Company, St. Louis, MO). Other cultures were partially submerged for 2 hours in a -5° C circulating cold bath. In some experiments, neurites were amputated at various distances from the soma before drug treatment using our modification (3) of the method of Shaw and Bray (82).

Electron Microscopy

Samples were prepared for electron microscopy as previously described (3). In one set of experiments cultures were incubated after fixation in 0.5 mg/ml ribonuclease (Sigma Chemical Company, St. Louis, MO) in 0.13 M cacodylate for 20 hours prior to osmication (to confirm



dense particles as ribosomes as described in Ref. 88). As an estimate of ribosomal density, ribosomes were counted and averaged in random 0.5 x 0.5 um regions of longitudinal thin sections ca. 50 nm thick. At least 5 sections of each sample were examined in this fashion.



RESULTS

Ultrastructure of Control Neurons

Although the ultrastructure of embryonic chick sensory neurons has been examined in some detail (26, 96), we wished to further examine the ultrastructure of control neurons with particular emphasis on the density of ribosomes in the soma, hillock, and neurite regions. Most neurites were 1-1.5 um in width and over 200 um in length. Figure 15 shows a typical control neuron of these dimensions. The somata of these control neurons contained dense populations of ribosomes most of which appeared to be unattached to endoplasmic reticulum as has been previously reported of ribosomes in the somata of growing neurons (21. 29, 61, 62, 63, 78, 89). The ribosomes appeared in densities of ca. 150-300 per square um in longitudinal thin sections (see Materials and Methods section). Ribosomes were identified by their diameter and tendency to cluster into rosettes. Unlike mature neurons (57) but typical of embryonic neurons (62, 63, 89), ribosomes extended to the periphery of the soma and sometimes into the hillock region. In the region of the hillock the cytoplasm of the axon became markedly different in appearance from that of the soma. Dense paraxial arrays of MTs and neurofilaments filled the axoplasm. The sharp demarcation between the soma and neurite was also characterized by what appeared to be a limitation of the free communication of mitochondria and other organelles between compartments. Ribosomal density quickly dropped past the hillock region. The density of ribosomes in the first 15-20 um of neurites was less than than 15 ribosomes per square um. Neurites that







were particularly short and stout (greater than 1.5 um in width and less than 200 um in length) were less consistant in their ultrastructural characteristics. Some of these neurites contained lower densities of MTs, more ribosomes, and less clear cytoplasm demarcations between compartments. Due to these inconsistancies, we selected for our compartmentation studies neurites with the more typical dimensions.

Ultrastructure of Nocodazole Treated Neurons

Neurites grown under the conditions described above (see Materials and Methods section) were sufficiently adherent to the substratum that treatment with 1.0 ug/ml nocodazole for 15-20 minutes caused minimal if any retraction of the neurites. Longer exposure times generally caused marked retraction, lifting of the neurites from the substratum, or fragmentation of the "beads" along the length of the neurite that often accompany MT depolymerization (26, 43, 50). Neurites treated for 15-20 minutes showed substantial MT depolymerization, but were also markedly different from control neurites in other aspects of their ultrastructure. Electron micrographs of the soma, hillock, and proximal neurite show the apparent dissolution of the clear compartmental demarcation observed in control neurites (Figure 16). Ribosomes and other organelles appeared to be in free communication between the soma and neurite in this region. Ribosomes populated the hillock region and proximal neurite in densities typical of the soma (150-300 per sqare um). Also as previously reported of PC12 neurites treated under MT depolymerizing conditions (49, 50), many regions of the nocodazole


Figure 16: Transmission electron micrograph of a neuron treated for . 15-20 minutes with 1.0 ug/ml nocodazole showing loss of demarcation between compartments, Bar, 0,5 um.





treated neurites contained a large number of small internal non-mitochondrial vesicles. We have reported preliminary evidence that the additional vesicles are attributable to an increase in membrane internalization that accompanies MT depolymerization in neurites (4).

Ribosomes in neurites were identified by their diameter and similarity in appearance to the electron dense particles found in control somata. In order to confirm that the "nocodazole induced" particles were indeed ribosomes, we used a method previously employed by Steward (88) for the identification of ribosomes in dendrites. After nocodazole treatment and fixation with glutaraldehyde, cultures were treated for 20 hours with 0.5 mg/ml ribonuclease. Steward reported that such treatment causes a partial digestion of ribosomes that is clearly detectable under the electron microscope. We found the treatment to be very effective in digesting the nocodazole-induced particles in proximal regions of neurites (Figure 17a), and equally effective at digesting the particles that were almost certainly ribosomes in the soma (Figure 17b).

<u>Are Ribosomes Invading the Neurite from the Soma during MT</u> Depolymerization?

In order to examine whether the ribosomes that appeared in nocodazole treated neurites were arising due to decompartmentation from the soma, or from elements already comprising the neurite, we examined the ultrastructure of treated neurites at various distances from the soma. Six of 6 neurites examined in regions past the hillock but within the first 100 um from the soma were found to contain ribosomes in



Figure 17: Transmission electron micrograph of (a) neurite, and (b) some that were treated with nocodazole, fixed, then treated for 20 hours with 0.5 mg/m l ribonuclesse. The ribonuclesse dissolved the "nocodazole induced" particles in the neurite as well as the ribosomes in the some. Bar, 0.5 um.





densities typical of the soma (150-300 per square um, Figure 18a). Four of 4 neurites examined in regions 100-200 um from the soma contained many fewer ribosomes than in the first 100 um, but generally still higher than in control neurites (20-60 per square um, Figure 18b). Distal regions of each length range contained fewer ribosomes than proximal; Figure 18c shows a neurite ca. 200 um from the soma with only sparse somewhat ambiguous ribosomes. In four of 4 neurites examined in regions beyond 200 um, we found no unambiguous ribosomes (Figure 18d).

In order to further test whether the ribosomes were moving down the neurite after MT depolymerization, we amputated neurites at distances between 50-150 um from the soma prior to nocodazole treatment. In both cases in which we were able to observe both proximal and distal fragments under the electron microscope, we found a dense population of ribosomes (ca. 300 per sqare um) in the retraction bulb and proximal segment still attached to the soma (Figure 19a), but few or no ribosomes in the collapsed distal segment (Figure 19b).

Specificity of the Phenomenon to Microtubule Depolymerization

In order to determine whether these ultrastructural alterations were specifically attributable to MT depolymerization, and not to either a specific side effect of nocodazole or to general insult, we examined the ultrastructure of at least 2 neurites at each of the 3 distances described above (under 100 um, 100-200 um, and beyond 200 um from the soma) given other experimental treatments. Neurites treated with 10 ug/ml vinblastine, a MT poison with somewhat different properties than



Figure 18: Transmission electron micrographs of regions of different neurites treated for 15-20 minutes with 1.0 ug/ml nocodexcole at various distances from the soma. Regions ca. (a) 50 um, (b) 150 um, (c) 200 um, and (d) 500 um from the soma. Ribosomes occupy proximal regions at ca. the same density as in control somata, and more distal regions with decreasing densities at increasing distances from the soma. Arrows mark sparse somewhat ambiguous ribosomes at 200 um. Increases in small vesicles varied in different regions with no correlation to distance from the soma. Bar, 0.5 um.





Figure 19: Transmission electron micrographs of (a) the proximal retraction bulb, and (b) the collapsed distal segment of a neurite amputated 50-150 um from the some prior to nocodazole treatment. Ribosomes densely populate the proximal segment still attached to the some, but are absent or nearly so in the distal segment. Bar, 0.5 um.





nocodazole or colchicine (28), or cooled to -5° C for 2 hours displayed the same ultrastructural changes as those treated with nocodazole (Figure 20a). Treatment with 100 ug/ml griseofulvin, another MT poison with a different mechanism of action than nocodazole (28), was less reliable in depolymerizing MTs. The ultrastructure of those neurites in which griseofulvin was ineffective in depolymerizing MTs was entirely similar to control neurites, while that of the neurites in which griseofulvin was effective in depolymerizing MTs clearly showed the same axoplasmic alterations as nocodazole treated neurites. Neurites treated with 2 ug/ml cytochalasin D, a potent actin disruptor (49), did not display increases in ribosomes over control levels (Figure 20b). Neurites suffering general insult by amputation alone also did not show increases in ribosomes.



Figure 20: Transmission electron micrographs of (a) a cold treated neurite, and (b) a neurite treated with 2 ug/ml cytochalasin D for $15{-}20$ minutes both at ca. 15 um from the soma. The cold treated neurite shows substantial MT depolymerization and a density of ribosomes similar to that of the soma. The cytochalasin treated neurite is nearly devoid of ribosomes. Bar, 0.5 um.





DISCUSSION

Our evidence indicates that ribosomes are able to freely move from the soma into the axon-like neurites of embryonic chick sensory neurons after treatments that depolymerize the dense MT arrays normally present in the neurites. We found densities of ribosomes similar to that of the soma in the first 100 um of the neurites, and a decreasing gradient with increasing distance from the soma (Figures 16 and 18). It is unlikely that we were simply unmasking ribosomes already present in the neurite in that ribosomes are not masked by MTs in dendrites (88) nor in initial segments of immature axons (16, 21, 26, 62, 63, 89, 96). Distal segments of neurites severed prior to nocodazole treatment dispayed no increase in ribosomes, while proximal segments still attached to the soma displayed increases typical of unsevered nocodazole treated neurites (Figure 17). The increase in ribosomes was reproducible with various MT poisons with different mechanisms of action, but was not reproducible by actin disruption with cytochalasin, nor by general insult caused by amputation alone. We interpret these results as suggesting that MT depolymerization specifically liberates ribosomes from an influence that normally limits their entry into axons during early neuronal development.

It is unlikely given the rapidity of the response that the movement of ribosomes observed here can be attributable to the same mechanism underlying the classic "chromatolytic" release of ribosomes from the Nissl substance caused by axotomy (78). Furthermore, very few ribosomes are bound to endoplasmic reticulum ("Nissl substance") even prior to MT depolymerization in immature and growing neurons (21, 29, 61, 62, 63,



78, 89). Lasek (54) has proposed that "free" ribosomes are actually attached to cytoskeletal elements of the perikaryon in a fashion similar to that observed in the perikarya of other cell types (24, 34, 44, 58, 94). Ribosomes in embryonic neurons however appear not to be immobilized around the nucleus as in mature neurons (57). Furthermore, in direct contrast to our results, perikaryal attachments of ribosomes to microtrabeculae in other cell types have been shown to be sensitive to actin disruption but not MT depolymerization (44, 58).

We would argue that the maintenance of cytoplasmic compartmentation in embryonic neurons is primarily dependent upon the cytoskeleton within the neurite rather than the soma. We suggest that the presence of a dense array of MTs in neurites provides a physical barrier to the entry and movement of ribosomes through the axoplasm. This suggestion is substantiated by our finding that shorter stouter neurites with lower MT densities less consistantly contained sharp cytoplasmic demarcations between compartments than did the more typical, thin neurites with densely packed MTs. The finding that ribosomes extend to the periphery of the soma in immature neurons but are restricted, even those not membrane bound, to the perikarya of mature neurons suggests that the proposed perikarval attachments may be developmentally established. We propose that the assembly of a dense array of MTs in axons during early neuronal development provides an impediment to the movement of ribosomes into the axoplasm. This may be an an initial measure toward cytoplasmic compartmentation until perikarval attachments to endoplasmic reticulum and microtrabeculae can be secured. On the other hand, it may be the principal mechanism by which cytoplasmic compartmentation is maintained throughout the life of the neuron.



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