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By

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ABSTRACT

INVESTIGATION OF TENSILE REGULATION OF AXONAL GROWTH

By

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This dissertation represents an investigation of the role of tension in neurite initiation and elongation. Neurites of cultured chick sensory neurons were attached by their growth cones to glass needles and were subjected to increasing tensions. This experimental process resembles "towed growth" normally occurring *in situ*. The growth sensitivity of neurites to tension was surprisingly high: an increase in tension of 1 μdyn increased the elongation rate an average of 1.5 μm/hr. Neurite elongation rate increased in proportion to tension magnitude greater than a tension threshold, which varied among neurons with most between 50-150 μdyn. The linear relationship between growth rate and tension provides a simple control mechanism for axons to accommodate tissue expansion in growing animal.

The size and pattern of surface addition during the experimental "towed growth" regime was also examined using microspheres to label the surface of neurites. New membrane is added interstitially throughout the neurite, but different regions of neurite vary widely in the amount of new membrane added.

We confirmed that neurites can be initiated *de novo* by application

of tensions above some thresholds. Initiated neurites developed growth cones capable of normal motility and axonal elongation. As assessed by electron microscopy and immunofluorescence, tension-induced neurites containing an array of MTs indistinguishable from control neurites. Like growth cone-mediated neurites, MTs of tension-induced neurites are extensively acetylated, axially oriented and present in large numbers. This normal microtubule array suggests that tension stimulates a rapid reorganization of cell body microtubules including microtubule bundling and/or *de novo* assembly of microtubules. To decide the role of reorganization and assembly, we investigated tension-induced neurite initiation in the presence 4nM vinblastine, which poisons further assembly of microtubules without depolymerizing extant polymers. We found that only 10% of poisoned neurons would form neurites in contrast to 53% in unpoisoned group. Almost 25% of poisoned neurons that failed to form neurites succumbed in a type of the "stretch-and-break" that was structural failure never observed in unpoisoned neurons. An abnormally low density of poorly oriented microtubules was also found in vinblastine-poisoned towed neurites. These data suggest that tension application rapidly stimulates significant levels of new MT assembly concomitant with neurite initiation, the reorganization of existing MTs plays little or no role.

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List of Abbreviations

MT:	Microtubule
MTs:	Microtubules
MTSB:	microtubule-stabilized buffer: 100 mM Pipes, 2 mM EGTA, 4% (w/v), polyethylene glycol (WM ca. 8000), 0.025% sodium azide, pH 6.8
PBS:	Phosphate Buffered Saline
mAb:	Monoclonal Antibody
Buffer I:	Hanks' buffered salt solution containing 5 mM Pipes, 2 mM $MgCl_2$, and 2 mM EGTA, pH 6.
TBS I:	10 mM tris, 140 mM NaCl, pH 7.6.
TBS II:	20 mM tris, 140 mM NaCl, pH 8.2.

Introduction

The fundamental tasks of the neuron are to receive, integrate conduct, and transmit signals. To perform these functions, neurons establish their connections by extending neuritic processes (axonal or dendritic) away from the rather fixed position of the cell body. The growth of the axon is a particularly complex and interesting developmental process. Despite intensive study, the mechanism that regulates axonal development is still poorly understood and surrounded by controversy. Axonal development has been studied largely by observing and examining naturally occurring neurite elongation [24, 26, 46, 55, 67, 74, 76, 84]. Accumulating evidence has suggested that mechanical tension is an important intrinsic regulator of axonal development [36]. The evidence that tension is a normal regulator of axonal development stimulated the development of methods allowing experimental intervention using tension. This thesis represents a new approach to the study of axonal development, one in which the experimenter can control neurite initiation and elongation through application of mechanical force.

The growth of axons includes axon/neurite initiation and elongation. By 1941, Paul Weiss had categorized axonal elongation into three successive stages that remain entirely relevant today [94]. The first two stages "pioneering" and "application" are mediated by growth cone activity. In contrast, the growth cone is quiescent in the final "towing" stage of growth: once the growth cone has contacted its target, the growth cone transforms into a nonmotile synaptic terminal. Migrating target cells pull on their attached axon, causing "towed" axonal growth. This was the first evidence that tension might regulate axonal elongation.

The role of tension in axonal development was largely ignored until

Dennis Bray [13] showed indirectly by a vectorial analysis of neurites' geometric pattern on the dish that neurites of culture neurons were under tension. There is now widespread agreement that growth cone-mediated elongation also reflected tension stimulated growth via a pulling growth cones [15, 16, 49, 51, 56, 75, 92]. The best evidence came from Lamoureux et al [49]. They reported direct measurements of neurite tension as a function of growth-cone advance. They showed that growth cone advance and neurite tension are linearly related and accompanied by apparent neurite growth. No increase in force occurs in neurites whose growth cone fails to advance. So all three stages of axonal elongation may be mediated by mechanical tension, whether the tension is provided by the advance of the growth cone or through the movement of the target tissue [21]. The question is, "How does the mechanical tension regulate the process?"

One of the important underlying questions concerns the cytoskeleton. The cytoskeleton is the major internal structure of axons [30, 56, 58]; it consists principally of microtubules (MT), neurofilaments (intermediate filaments in neurons), and actin filaments. Actin filaments form a cortical network below the axon plasma membrane. In growing axons, actin filaments extand into the motile leading edge termed the growth cone [20, 48, 51, 96]. Microtubules and neurofilaments are present in parallel array within the shaft of the axon [20, 51, 96]. It is generally appreciated that neuronal elongation is critically dependent upon the assembly of microtubules [66]. This raises the question of how mechanical tension regulates the chemical reactions of cytoskeletal assembly.

One possible answer is the thermodynamic model postulated by Buxbaum and Heidemann [21]. That is, mechanical force is a source of (thermodynamic) free energy that can affect the monomer/polymer

assembly equilibrium of any assembling polymer [21, 22, 37]. Compression of MTs raises their free energy relative to no force load; more compression shifts the tubulin/microtubule equilibrium toward disassembly, less compression favors assembly [21, 22, 37]. The model assumes that traction force (tension) exerted by the growth cone on the underlying substratum shifts tensile support away from the compressed MTs onto the substratum. The shift of compression away from the MTs to the substratum with the advance of growth cone lowers the critical concentration of tubulin required for assembly. Consequently, tubulin that a moment previously was in equilibrium with the high free energy, compressed polymer now adds into the low free-energy, compression-relieved polymer. This MT assembly continues until equilibrium compression on the MTs is again reached. If Buxbaum and Heidemann scheme's is right , we would expect that there should be a threshold tension for axonal growth at which monomer/polymer reaches equilibrium, we would also expect a reproducible relationship between neurite tension and neurite growth.

Neurite initiation appears to be different from neurite elongation. Initiation involves a localized protrusion of plasma membraneand formation an axial array of MTs that is previously absent [61, 62, 86, 87, 94]. The mechanism that regulates neurite initiation is poorly understood. For example, the factors that determine the side on a cell body from which a growth cone will emerge remain unidentified. That is, why does an axon grow out on one side of the cell body but not at another?

Interestingly, Bray found that, using a "towing motor," long neurites could be initiated simply by pulling the margin of previously rounded neuronal cell bodies. The neurites produced by microelectrode towing had a normal appearance with abundant, longitudinally aligned MTs and

neurofilaments [16]. This data strongly suggests that mechanical tension also can regulate neurite initiation. The process of MTs events accompanying neurites initiation is of particular morphogenetic and cell biological interesting because: First, the centrosome, which has been postulated to play a major role in determination of cell morphology by inducing an asymmetry in the distribution of MT [18, 65, 60] has not typically been found at the base of a neurite [81, 86]. How MTs organize into an axial array after application of tension is an important and unanswered question. Second, axons differ from the cell body by the absence of protein synthetic machinery [53] and the presence of a dense array of high ordered cytoskeletal elements [14, 20, 52, 62, 69, 70, 96]. Whether MTs assemble principally in the cell body or/and at the growth cone and how MTs or tubulin are transported in the axon has been controversial for a decade and is still unclear [7, 6, 52, 54, 57, 68, 76]. An investigation of MTs assembly and/or reorganization during neurite initiation, especially at the very early stages, should be of fundamental importance to answering these questions.

Another important question is that of membrane addition during neurite elongation. Based on normal, growth cone-mediated growth, elongation rates of 40-100 μm/hr [11, 12, 59], as much as 3 μm^2 of surface may be added per minute [90]. New membrane is added at the growth tip in growth cone-mediated growth. Labeling studies with fluorescent lectin or carmine particles [11, 31], pulse-chase experiments with ferritin-labeled lectins and with phospholipid precursors [71, 72], and autoradiography of radiolabeled membrane proteins [88, 34] support this concept. In contrast, the assumption in towed growth is that mass addition is interstitial [38], a possibility that is supported by indirect experimental results of Campenot [23]. Where is new membrane added when the growth cone is

not actively involved in elongation?

This dissertation represents an attempt to address the questions mentioned in this introduction. In chapter 1, an experimental process similar to "towing" growth *in situ* was used to investigate the relationship between mechanical tension and neurites growth rates, as well as membrane addition pattern accompanying neurite towed growth. This method is also used to quantitatively investigate the relationship between tension and neurite elongation or initiation predicted by the thermodynamic models of Buxbaum and Heidemann [21, 22].

Chapter 2 focuses on MTs events accompanying the neurite initiation induced by experimentally applied tension, especially at the very early stages, about which little is known [29, 55]. To assume that the neurite initiation by tension resembles growth cone-mediated initiation, I compared the density, orientations, and acetylation pattern of microtubules in tension-induced and "normal" growth cone mediated neurites. With the aid of MT "kinetic stabilizer": vinblastine [40, 41], the relative roles of reorganization and assembly of MTs in neurite initiation was also assessed.

1 Tensile Regulation Of Axonal Elongation And Initiation

1.1 Introduction

Evidence is accumulating that mechanical tension is an important regulator of axonal development [36]. Tension applied to neurites of chick sensory neurons and to PC12 cells stimulates their elongation [16, 28]. This mirrors instances of tensile regulation of axonal elongation *in vivo*. Growth cones pull on neurites, stimulating their elongation [49]. In later stages of axonal development, migrating target cells pull on their attached axons, causing "towed" axonal growth [94]. Balice-Gordon and Lichtman [5] reported that a similar mechanical coupling between target cells and neurons is responsible for the growth of motor nerve terminals on mouse muscle fibers, thus integrating the size of the synapse with the size of the muscle fiber. Experimentally applied tension can also initiate new neurites from chick sensory cells [16]. Conversely, sudden declines in neurite tension cause chick sensory neurites to develop tension actively and to retract [28], providing a potential mechanism for axonal retraction *in vivo* [77]. Growth and retraction of neurites stimulated by supplying or withdrawing NGF to/from different region of rat sympathetic neuron also appeared to be regulated by neurite tension [23]. Both axonal growth and retraction must involve assembly/disassembly of cytoskeletal elements. A complementary force interaction between compressed axonal microtubules supporting tensile axonal actin may regulate microtubule assembly in the axon by a thermodynamic mechanism, integrating microtubule assembly with growth cone advance [21, 27].

We report here further investigations of the relationship between axonal growth and mechanical tension. We wish to test the preliminary

finding of Dennerll et al. [28] that axonal elongation rate is linearly correlated with tension magnitudes. The procedure used for this experiment is similar to towed growth; for example, the active role of the growth cone is eliminated. New membrane is added at the growing tip in growth cone-mediated growth [11, 31, 71]. Where is new membrane added when the growth cone is not actively involved in elongation? Finally, we wish to extend the work of Bray [16] on neurite initiation by measuring the force required to induce neurite initiation to determine if initiation differs mechanically from elongation of extant neurites.

1.2 Material and methods

1.2.1 Materials

Polystyrene microspheres and Polybed 812 embedding resin were obtained from Polysciences, Inc. Phosphate-buffered saline (PBS) and fetal calf serum were purchased from Gibco. L-15 medium, Collagen IV, poly-L-lysine, trypsin, and polyethyleneimine were all purchased from Sigman Chemical Co. Laminin was obtained from Collaborative Research. A mouse monoclonal antibody against α-tubulin was the kind gift of Dr. David Asai of Purdue University.[1] Fluorescei-labeled goat anti-mouse IgG was purchased from Kirkegaard and Perry Lab. Inc.

1.2.2 Cell Culture

Chick sensory neurons were isolated from lumbosacral dorsal root ganglia of 10-12 day old chick embryos, as described by Sinclair et al. [83]. These dorsal root ganglia were rinsed in supplemented L-15 medium (L-15 supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, and 136 μg/ml steptomycin), and treated with 0.25% trypsin for 30 minutes at 37^{o}C. The trypsin was then removed, the ganglia were rinsed 3 times with the supplemented L-15 medium containing 10% fetal calf serum. The ganglia were triturated with pipette into a single cell dispersion, and cultured in the supplemented L-15 medium containing 10% fetal calf serum and 100 ng/ml 7S nerve growth factor isolated from mouse salivary glands [93]. Cells were grown on untreated tissue culture dishes at a density low enough to render non-neuronal contamination inconsequential. Neurons were cultured for 16-24 hr prior to experimentation.

1.2.3 Neurite Elongation And Direct Axial Force And Length Measurement

The axial tensions of neurites were measured with force-calibrated glass needles, and their length was determined as previously described [28]. The bending moduli of the experimental needles were between 5.1 and 15.4 μdyn/μm. A calibrated needle was attached to a neuron's growth cone, and the neurite was pulled in 30-60 minutes "steps" of constant force; that is, a tension magnitude was chosen, beginning at 50-100 μdyn, and this tension was held constant for 30-60 minutes by moving the micromanipulator to maintain the appropriate deflection of the calibrated needle. Subsequently, the same technique was used to apply 30-60 minutes periods of higher tension to the neurite, each level typically 25-50 μdyn higher than the previous value. The experiments were recorded on videotape at 24X time lapse and were subsequently analyzed for neurite length and tension as described previously [28].

In addition to an expected towed growth response [16], chick sensory neurons have a passive viscoelastic solid response to applied tension [28]. In order to determine accurately (inelastic) the growth parameters from neurite length measurements, initial analysis subtracted the elastic stretching component of neurite length using a previously described the mechanical model for passive viscoelastic behavior [28]. Individual neurite values for the stiffness of the mechanical elements were obtained as described by Dennerll et al. [28]. The elastic response of a neurite to tension over time was calculated, and this value was subtracted from total neurite length to give a value for growth. These analyses showed that the rather small stretching increases for each tension step were complete after the first 10-15 minutes. Consequently, in later experiments growth rates could be calculated from length data omitting the first 15 minutes

following tension increases.

1.2.4 Neurite Initiation

Neurons without extant neurites were attached to calibrated experimental needles at the cell margins. The response of the cell to the application of various tension levels was recorded on videotape and analyzed for tension and neurite length as above. Some experimentally initiated neurites were examined by immunofluorescence or by transmission electron microscopy for the presence of microtubules.

1.2.5 Indirect Immunofluorescence

Following initiation, neurites were subjected to tension to allow continued elongation to various lengths. The distal ends of such neurites were then micromanipulated from the needle back onto the culture substrate. A diamond-tripped "objective" was used to mark the experimental neuron by circling the dish beneath. Immunofluorescent staining of microtubules was carried out by a method similar to that of Thompson et al. [89]: Medium was carefully removed, and the culture was permeabilized in 0.5% Triton X-100 in the MTSB (microtubule-stabilized buffer: 100 mM Pipes, 2 mM EGTA, 4% (w/v), polyethylene glycol (MW ca. 8000), 0.025% sodium azide, pH 6.8), then fixed in 3.7% formaldehyde solution in MSTB, all at 37°C, followed by extraction in methanol at -20°C. The neurites were then incubated with the primary antibody to α-tubulin (kindly provided by Dr. David Asai), rinsed then incubated with fluorescein-labeled secondary antibody goat anti-mouse IgG. The prepared samples were viewed with a Nikon inverted microscope equipped for fluorescence equipment.

1.2.6 Transmission Electron Microscopy

Ultrastructural observations of experimentally initiated neurites were made using standard methods previously described [2, 42]: as soon as the distal end of neurite was micromanipulated from the glass needle back onto the culture substrate, concentrated glutaradehyde was added. The neuron was fixed for 30 minutes with 2% glutaradehyde in supplemented L-15 medium, postfixed for 5 minutes with 1% OsO_4 in 0.1M Cacodylate, dehydrated in ethanol series, and embedded in Polybed 812. Thin sections cut parallel to the substrate were stained with uranyl acetate and lead citrate, and observed with a Phillips 300 transmission electron microscope.

1.2.7 Neurite Membrane Addition And Subtraction

Polystyrene microspheres with diameter of 1.6 μm were treated with 5% polyethylenimine, then washed 5 times in distilled water and 1 time in L-15 medium, and stored in L-15 medium at 0.8% (w/v). Ten microliters of microspheres suspension were added to the cell cultures containing 3-4 ml culture medium. After incubating for 30-60 minutes at 37°C to allow the microspheres to attach to neurons, excess microspheres were removed by rinsing the culture with fresh L-15 medium. Calibrated needles were attached, as above, to the growth cones of neurites judged to be appropriately labeled with microspheres, and the neurites were pulled or slackened. The experimental process was videotaped and the distances between cell body and microspheres were measured by the same procedure used to measure neurite length.

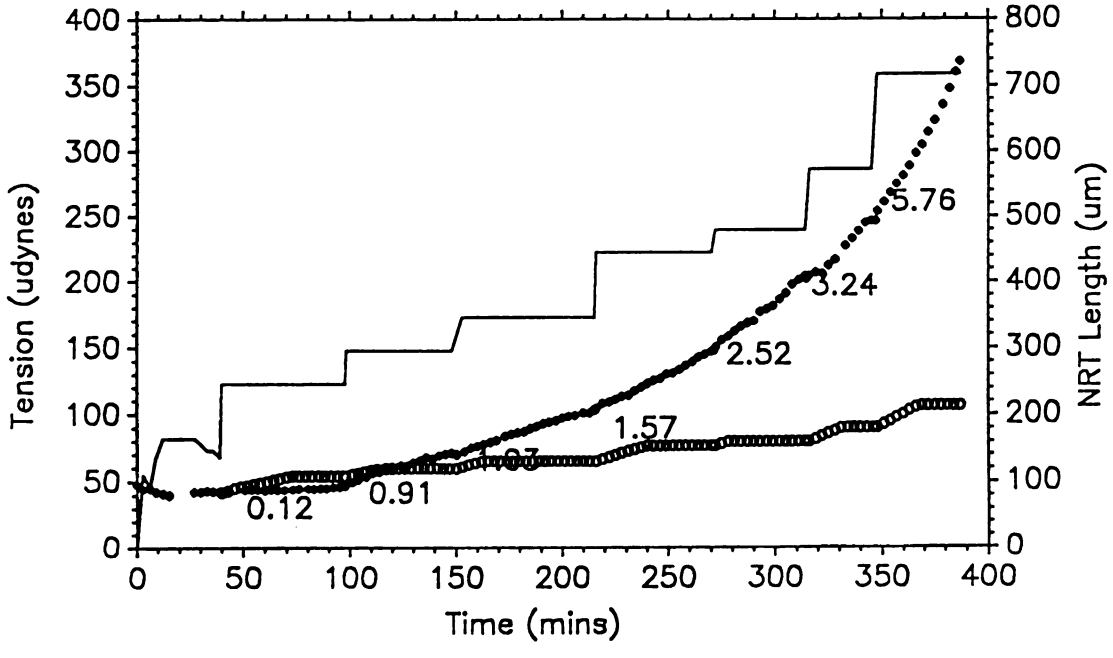
1.3 Results

1.3.1 Control Of Neurite Elongation By Tension

PC12 neurites subjected to experimentally applied tensions of less than 100 μdyn elongated as simple viscoelastic solid showing no long term extension. However, neurites showed plastic, long term elongation, interpreted as growth, when subjected to tension >100 μdyn. The long term elongation rate above this threshold was proportional to the magnitude of the initially applied tension [28]. These experiments, however, suffered from a technical limitation, applied force declined as the neurite lengthened such that both neurite length and tension were changing at all time. We wish to confirm the findings of Dennerll et al. [28] using a better-controlled method on authentic neurons.

Fourteen chick sensory neurites were attached to the calibrated glass needles at their growth cones and subjected to increase steps of tension. Each step was a period of constant force of 30-60 minutes duration, and steps differed from each other by 25-50 μdyn. Figure 1 summarizes one such neurite-lengthening experiment. As for PC12, chick sensory neurites lengthened continuously above a threshold tension, here 43 μdyn. Also, neurite lengthening increased with increasing tension level. However, not all of this length increase was growth. Some portion of this neurite lengthening was elastic stretching because chick sensory neurites, like PC12 neurites, have a viscoelastic solid component to their extension behavior [28]. The lower, broad line of Figure 1 plots the elastic response of the neurite predicted by a mechanical (spring and dashpot) model for the viscoelastic behavior of chick sensory neurons [28]. As shown by the dotted line, this component of the behavior equilibriums a given tension by a given change in length (i.e. as an elastic solid) over a period of 10-15

Figure 1. Neurite lengthening in response to increasing steps of tension. Chick sensory neurons were pulled from their distal ends by a calibrated glass needle. *Bar graph*, The deflection of the needle was stabilized by micromanipulation to exert various levels of force (*left ordinate*) on the neurite, which were maintained for 25-60 minutes before increasing the force to a new level. *Upper, dotted line*, Overall neurite length (*right ordinate*) from cell body to needle; *lower, broad line*, passive viscoelastic response of neurite predicted from neurite mechanical constants and the mechanical model for neurite response as described by Dennerll et al. [28].



minutes. Yet the neurite continues to elongate after this time, apparently at constant rate, and we interpret this neurite elongation that follows the elastic equilibration period to be towed growth [16, 28]. Like Bray [16], we found that this lengthening occurred with an apparent increase in volume of the neurite, that is, in the absence of neurite thinning expected of stretching except at the very highest elongation rates, and in the absence of cell body shrinkage (see Figures 8 and 9 for visual confirmation in a different context). We found, as shown in Figure 2, that this growth rate (i.e. corrected for elastic stretching) is proportional to tension magnitude above a tension threshold. That is, plots of growth rate versus tension for all 14 neurites showed a simple linear relation between tension above a threshold and growth rate. Correlation coefficients were between 0.83-0.99 and were greater than 0.9 for 10/14 neurites. As seen in Figure 2, this relationship hold even at quite high, non-physiological rates of elongation, up to 400 μm/hr. The application of uniformly increasing steps of force with accompanying neurite lengthening gave rise to the possibility that growth rate was proportional to neurite length, rather than tension. To check this, decrement tension steps were applied among the incremental steps in a few experiments. In all case, as shown in Figure 2C, growth rate declined with decreasing tension. This indicates that growth rate was a function of tension, not of neurite length.

The relationship between growth rate and tension, the slopes of the lines in Figure 2 reflects the sensitivity of neurite growth rate to tension, that is, the growth rate per μdyn of tension. Figure 3 is a frequency distribution of this sensitivity for the 14 neurites. Neurites' tension sensitivity varied, most neurites elongated between 0.22-2.0 μm/hr/μdyn. However, some neurites were more sensitive, one elongated 5.5 μm/hr/μdyn. Indeed, the sensitivity of sensory neurites to tension was surprisingly high;

Figure 2. Growth rates of neurites(elongation rate corrected for elastic stretching) as a function of applied tension for three typical neurites. The slope of the lines is the sensitivity of neurite elongation to tension (see Figure 3). The zero growth intercept is the tension threshold for growth (see Figure 4). The experiments shown in (A) and (B) consist of incremental steps of tension only, as in Figure 1. The experiment shown in (C) includes 2 decrement steps of tension(*triangles*) among the incremental steps. Growth rate declined during decrement steps as expected.

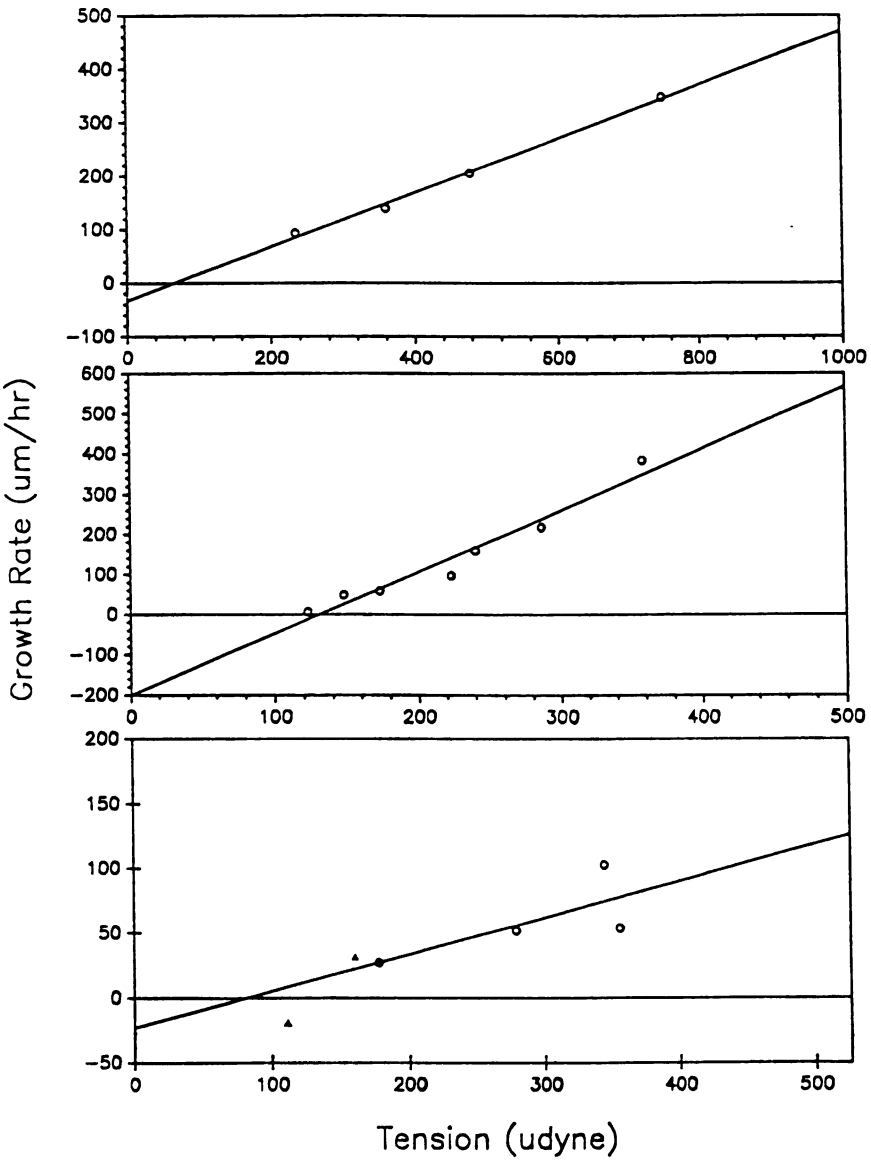
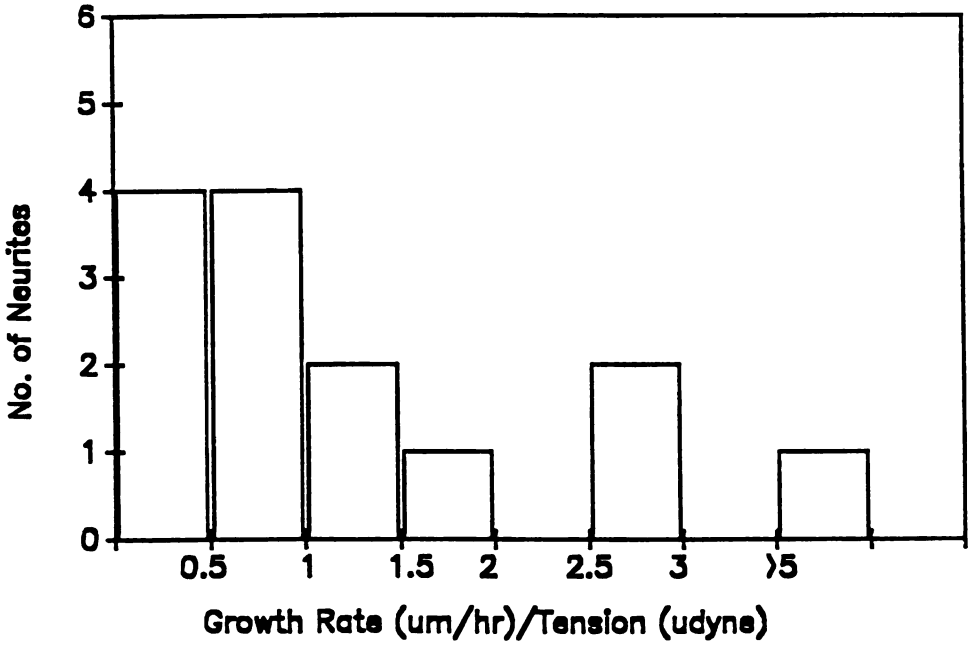


Figure 3. Frequency distribution of the tension sensitivity of neurite elongation for 14 experimental neurites. Tension sensitivity is given as the growth rate per unit tension.



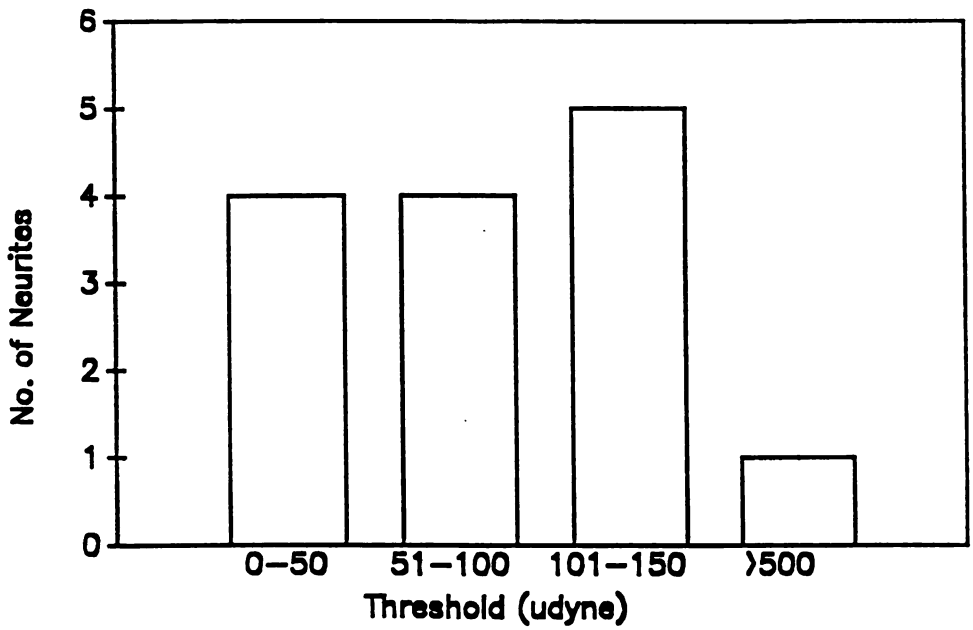
that is, differences in force at our method's detection limit (approx. 5 μdyn) would cause a moderate difference in growth rate in most neurites. We found no correlation between a neurite's initial length and its sensitivity to tension.

Dennerll et al. [28] found that PC12 cells had a surprisingly reproducible tension threshold for growth of 100 μdyn. Figure 4 summarizes the tension threshold (the tension intercept at zero growth rate as in Figure 2) for all 14 neurites. Thresholds for chick sensory neurites are seen to vary between 35-560 μdyn with the majority (9/14) between 50-159 μdyn. This is similar to but more variable than the 100 μdyn threshold for the clonal PC12 cells. An important confirmation of the tension threshold for growth was that 8 of 14 neurites that were subjected to initial tensions lower than their threshold either retracted or showed no growth, shown in Table 1. For example, a neurite whose zero growth intercept was later found to be 140 μdyn retracted at 7 μm/hr when subjected to 115 μdyn of force. Two of these eight neurites also showed retraction when subjected to force slightly above their threshold; for example, one neurite with an intercept of 120 μdyn retracted at 138 μdyn. The remaining 6 neurites were not subjected to force less than their threshold. Also, we found no correlation between the neurite's initial length and its threshold tension.

1.3.2 Surface Addition In Towed growth

It was of interest to determine where membrane addition occurs in experiments similar to the above, which resemble towed growth, particularly in view of work by Campenot [23], who found evidence for "intercalated" growth and retraction, that is, mass addition and subtraction occurring in the middle of neurites. Microspheres treated with polyethylenimine sticked aggressively to the neuronal membrane and appear to be

Figure 4. Frequency distribution of the tension thresholds (tension value of zero growth intercept from plots as in Figure 2) for experimentally stimulated neurite elongation from 14 experimental neurites.



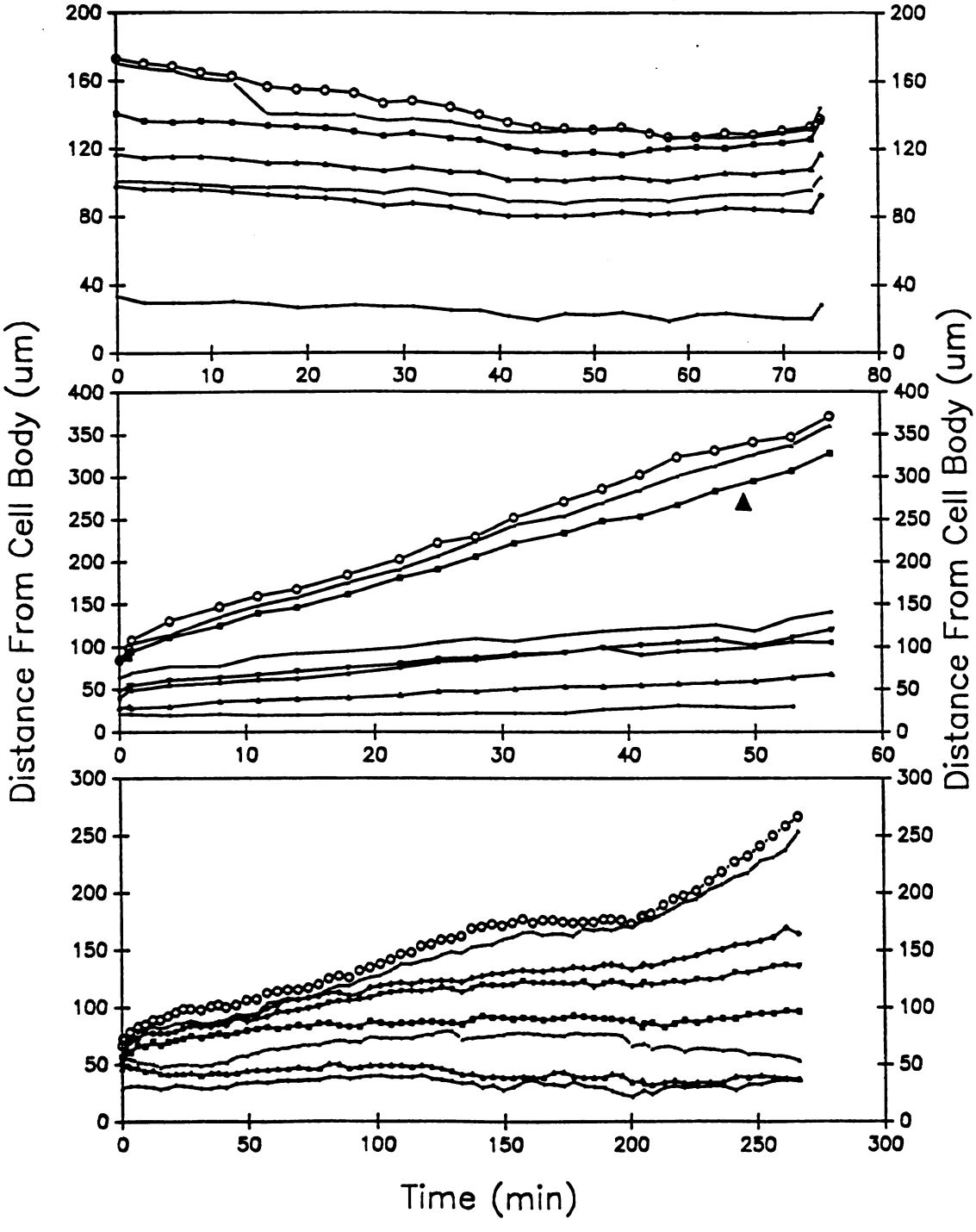
No. of Trials	Analyzed Threshold	Tension Applied	Elongation Rates (μm/hr)
1	3.46	2.84	-7.88
2	98.04	83.72	-6.45
3	52.39	34.96	-72.28
4	139.43	115.37	-7.68
5	105.73	46.60	-23.58
6	105.73	74.60	-7.50
7	120.00	138.00	-7.50
8	109.69	111.24	-18.00

Table 1: Comparison of analyzed threshold with experimentally applied tension

markers for membrane regions per se, similar to the carmine particles used by Bray [11]. Labeled chick sensory neurites were elongated by pulling from the growth cone with glass needles, as above, and the videotape was analyzed for the changed distances of microspheres from the cell body. Figure 5 shows the results for 3 of the 7 neurites analyzed in this way. As shown in Figure 5a, microspheres remained essentially stationary on neurons that were not being pulled. In this experiment, there was some small drift in the position of the micromanipulator, allowing for a small amount of neurite retraction observable over the long term. Like Bray [11], we found that microspheres were not moved by neuronal motility processes. However, microspheres did oscillate slightly when observed at time-lapse speed (24x), as described by Bray [11] for carmine particles. In pulled neurites, as shown in Figures 5, b and c, the distance between microspheres and between most microspheres and the cell body increased. However, different regions of neurites clearly varied in the extent of change. For example, in Figure 5b, a substantial change in the distance of the microspheres symbolized by squares (arrowhead) contrasts with the nearly stable position of the microspheres nearest the cell body. There was also some tendency for the greatest separation to occur in the distal half of neurites as shown in Figure 5, b and c.

A similar analysis for marking membrane subtraction during neurite retraction is shown in Figure 6 and 7. We previously found that slackening neurites initiated active contraction and tension generation [28]. Six neurites tethered at their growth cone were slackened in a series of short steps to cause retraction, as showed in Figure 6. An effort was made to hold the neurite close to zero tension so that it was under neither tension nor compression. As shown in Figure 6, this gave rise to small, local sinusoids, but the entire neurite was not permitted to slacken into a sinusoid,

Figure 5. Surface movements of three neurites subjected to tension-induced neurite elongation. Neurite surfaces were labeled with polyethyleneimine-treated vinyl microspheres. The position of these microspheres relative to the cell body was recorded as the neurite was lengthened by pulling with a glass needle. The *open circles* represent the position of the growth cones. All *other symbols* represent particular microspheres, with their time course of position change given as a connecting *solid line.* (a). Control neurite tethered onto a glass needle but not pulled. Some drift in the position of the micromanipulator allowed some neurite retraction during the course of the experiment. (b). A neurite that showed particularly variable surface movement. As seen here, most of the surface movement occurred between the second microspheres behind the growth cone (*arrowhead*) and the microspheres behind it. As seen at t=0, these microspheres were originally located in the distal third of the neurite. (c). A neurite that showed more evenly distributed surface movements as elongation proceeded.


Distance From Cell Body (um)
Distance From Cell Body (um)
Time (min)
200
160
120
80
40
0
0
10
20
30
40
50
60
70
80
400
350
300
250
200
150
100
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10
20
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300
250
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200
250
300

Figure 6. Phase images of neurite retraction, with polyethyleneimine-treated microspheres marking the neurite surface, as photographed from the videotape of the experiment. (a). Neurite decorated with microspheres immediately prior to the initial slacking. (b). Five minutes later, a small sinusoid typically seen during these experiments can be seen distal to a microsphere group. (c). Ten minutes later, the neurite has again been slackened, and small sinusoids can be seen. (d). Ten minutes later, the sinusoids seen in (c) have been resorbed. The neurite retracted 50 μm during the 25 minutes of this sequence. Bar, 20 μm for a-d.

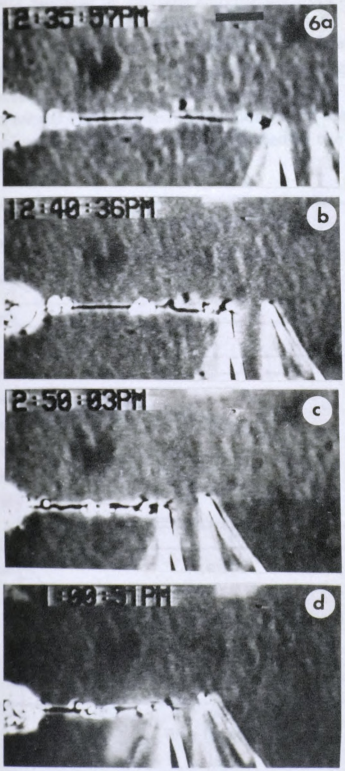
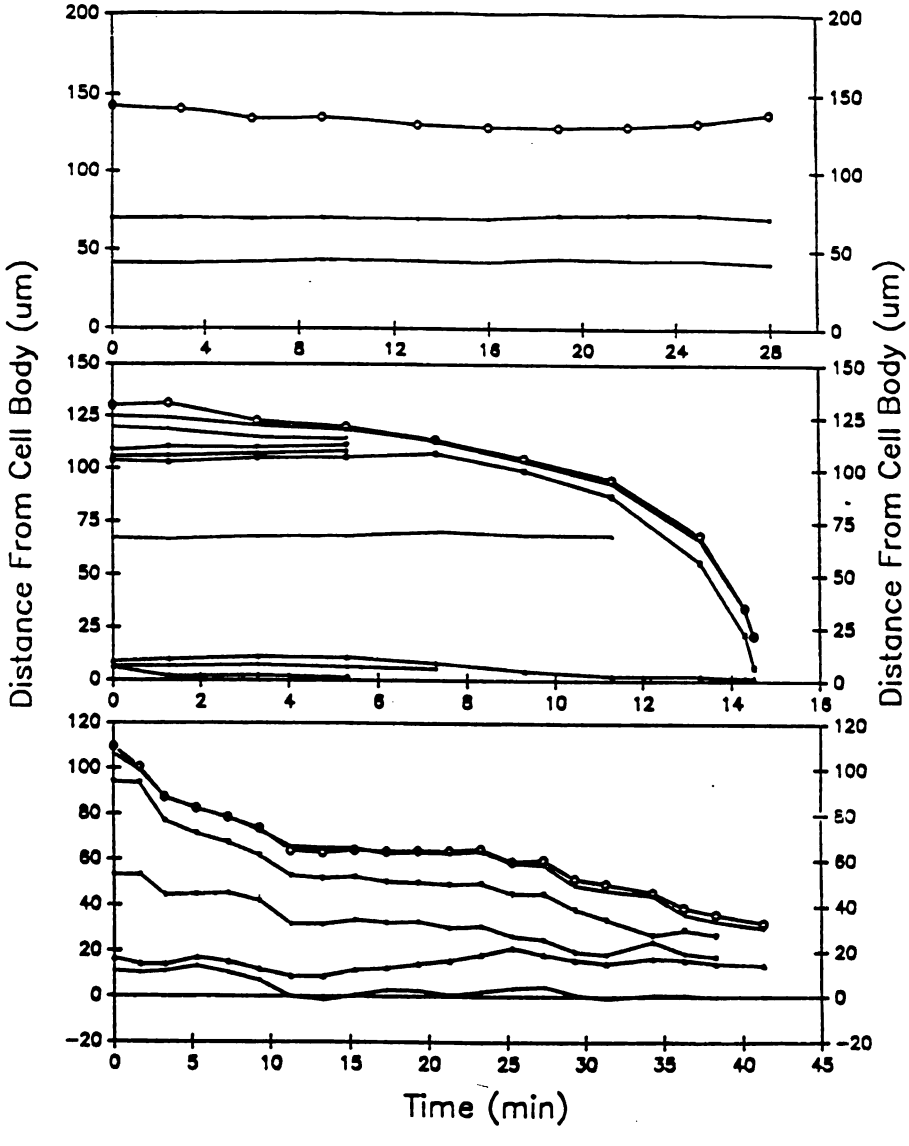

12:35:57PM
6a
12:40:36PM
b
2:50:03PM
c
d

Figure 7. Surface movements of three neurites allowed to retract in response to neurite slackening. Surface movements were marked with polyethyleneimine-treated microspheres as for Figure 6. *Symbols* are as in Figure 5. (a). Control neurite tethered to a glass needle but no slackened. (b) and (c). Position of microspheres and growth cones of neurites retracting in response to slackening. In some experiments microspheres converged due to neurite shortening such that they could no longer be resolved as separate. This is reflected in the termination of several lines at various times in Figure 7b.

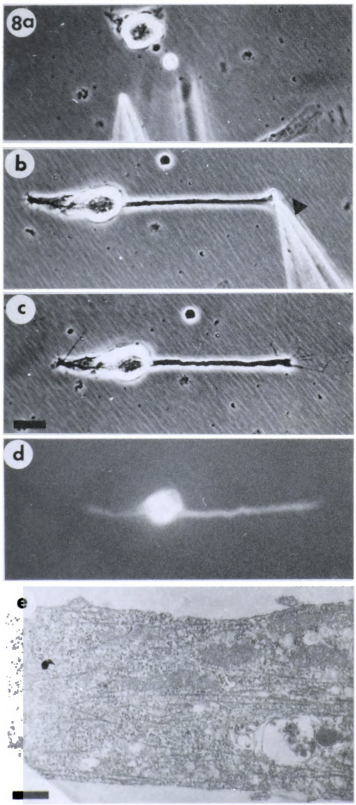


as occurs upon axotomy [2]. The videotape of such experiments was analyzed for the decrease in the distance of microspheres from the cell body, data shown in Figure 7. Figure 7a shows the data from a control neurite that was not slackened and did not retract, microspheres maintain their position about each other and to the cell body. In slacked neurites, the distances between microspheres decreased all along the neurite but, As for elongation, to a highly variable extent. For example, the neurite shown in Figure 7b lost surface primarily from the most distal regions. Analysis of retraction was complicated by the fact that, as the neurite retracted, some microspheres came too close together to resolve. This is apparent in Figure 7b, in which some lines representing microspheres position terminate at various time points. In contrast, the neurite shown in Figure 7c lost surface proportionately throughout its length.

1.3.3 Neurite Initiation

Bray [16] showed that neurites could be initiated *de novo* by experimentally applied tension. We wished to confirm and extent this results on neurons without extant neurites. Before experimental intervention, some experimental neurons were completely devoid of extensions from the rounded cell margins while others (Figure 8a) had motile lamellipodial extensions extending from the cell margins as described by Collins [25]. Calibrated glass needles were applied tangent to the margin of cells in random locations. No differences were noted among various regions of the margin for the capacity to initiate neurites, with the single exception of margins near asymmetrically located nuclei; that is, cell margins overlying a thin layer of cytoplasm between the plasma membrane and the nuclear membrane could not be induced to form and elongate. Applied tensions of 60-1000 μdyn caused a cell process to form and elongate

Figure 8. *De novo* initiation of neurites from chick sensory neurons by experimentally applied tension. (a). Neuron prior to a glass needle attachment. (b). Same cell as in previous frame after 96 minutes of experimentally applied tension. In this cell, neurite initiation occurred both at the site of needle attachment to the cell margin and at the opposite cell margin. The *arrowhead* points to filopodia on the distal end of the neurite attached to the needle. Note also the growth cone-like appearance of the distal end opposite the site of needle attachment. (c). Eight minutes later, the distal end of the neurite formerly attached to the glass needle has been manipulated onto the culture dish. The growth cone-like appearance of this end is apparent. (d). The same cell as in frame (c) fixed and processed for immunofluorescence with a monoclonal antibody against α-tubulin (see Materials and Methods). (e). Transmission electron micrograph of a neurite initiated *de novo* by experimentally applied tension (not the same cell as shown in frame a-d). Microtubules are readily apparent. Scale bar, a-d, 20 μm. e, 0.5 μm.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e

Figure 9. *De novo* initiation of neurites from chick sensory neurons by tension experimentally applied to the cell body. These micrographs were taken from the videotape of the experiment. (a). Prior to the beginning of tension application. A neuron with no outgrowth is attached to a glass needle. (b). A neurite formed on the margin of the cell opposite the pulling needle. This micrograph was taken 22 minutes after tension was first applied to the cell margin. Note the growth cone-like appearance of the distal end and the attachment point of this end relative to the cell below. (c). Fifteen minutes later, the neurite elongated by towing of the cell body, i.e., without a change in the position of distal end of the neurite. This micrograph reflects the maximum extent of towed growth for this neurite; i.e., no additional movements of the micromanipulator were imposed after this time. (d). Thirty-three minutes later, additional neurite elongation was caused during this period by the advance of the growth cone, as seen by the change in the position of the neurite growth cone relative to the cell below. Bars, 10 μm in a-d.

9a
b
c
d

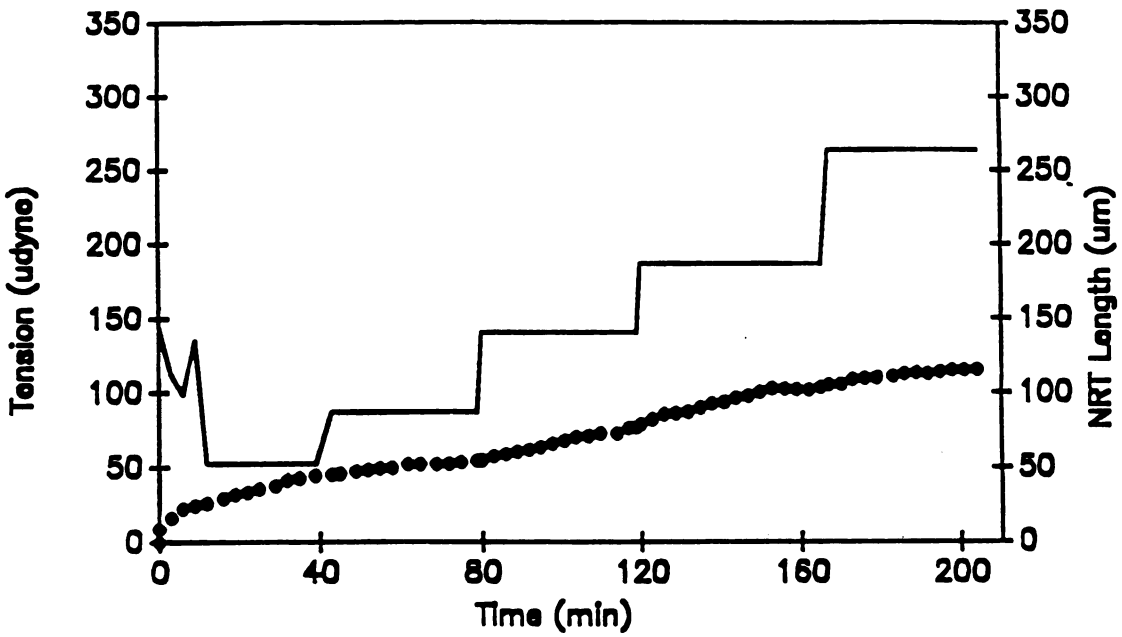
(Figure 8b) in 80% of the cells to which tension was applied. In the remaining 20% of cells, applied tensions only caused the cell body to elongate but failed to cause process formation, even tensions > 1000 μdyn. As shown in Figures 8 and 9, the elongation of initiated neurites occurred without obvious thinning of the lengthening neurite, or shrinking of the cell body.

In total, we initiated neurites to varying lengths from 41 neurons and obtained some force measurements from 35 of these. We generally found no lag time for neurite formation after force application, 33 of 41 neurons responded immediately with changes of some type. In some of these cases, however, the cell elongated before an obvious process formed. In other cases, an obvious processes formed immediately. Process formation generally occurred at the cell margin attached to the glass needle. In 7 cases, the process formed at the cell margin opposite the needle (Figures 9a-c). That is, micromanipulation pulled the cell body, and the process remained attached to the dish at its distal end, suggesting that tension per se, not the needle or its surface treatment, stimulated the initiation process. As for elongation of extent neurites, there was evidence that neurite initiation required tension above some threshold; that is, in 10 neurons we found that initial applied forces were insufficient to initiate neurites, but that subsequent higher tensions were immediately successful. For example, one neuron failed to respond to tensions of 73 and 98 μdyn applied for 5 minutes each, but rapidly produced a neurite at 123 μdyn. However, we have no information about the thresholds of most cells because initiation is a singular occurrence (one cannot "go back" and try a lower tension if the initial tension is successful), not a continuous variable (one also cannot extrapolate a graph back to a zero intercept). The forces at which neurites were successfully initiated are as follows:

The majority (21/35) required less than 200 μdyn; in five cases forces < 100 μdyn initiated neurite formation. Four neurons initiated neurites in response to forces between 200-300 μdyn for process formation, seven neurons formed processes between 300-600 μdyn and three neurons initiated neurites in response to forces greater than 800 μdyn. We do not understand the basis of this wide variability in tension requires to initiate neurites. Cell body size, for example, was not a factor. The only systematic difference was that more force was generally required for those neurites "towed" by the cell body, as in Figure 9, than for those towed by their distal end, as in Figure 8. Although the variability is poorly understood, sensory neurons are a heterogeneous group of cells, so the differences are not entirely surprising.

In many cases neurite initiation appeared to consist of two phases. Initially, a "bump" or "nubbin" formed, and this was easily drawn out from the cell margin for a period of some 10-20 minutes. During this period, too high a force frequently caused the process to break or caused a loss of attachment to the needle. Subsequent elongation from the nubbin was somewhat slower and frequently involved a higher force, though still less than 200 μdyn generally. Unlike elongation from extant neurites, elongation of neurites initiated *de novo* was not linearly related to force. Rather, neurites appeared to elongate at a particular rate despite increase in applied tension (Figure 10). Nevertheless, neurites initiated *de novo* developed microtubules and growth cones, two important characteristics of "normal" neurites. Motile filopodia were noted on seven neurites while still attached to the needle (Figure 8b). As Shown in Figure 8c, if the distal ends were manipulated off the needle back onto the dish, these ends strongly resembled growth cones. As shown in Figure 9, growth cones also formed on all 7 neurons that were towed by the cell

Figure 10. The elongation of a neurite initiated by experimentally applied tension in response to a step function of increasing tension. The *solid line* shows the tension magnitudes applied to a neuron as 30-40 minutes periods of constant force. The *solid circles* show the net length of the neurite. In contrast to self-initiated neurites (Figure 1), experimentally initiated neurites did not show a dependence of elongation rate with tension magnitude.



body rather than the neurite. This indicates that the presence of the needle was not responsible for growth cone formation. Most importantly, the growth cones formed by tension engaged in motile activity such as filopodial extension/retraction and could advance along the dish, causing neurite elongation, as shown clearly in Figure 9c and d. We did not attempt to assess the existence of growth cones on the distal ends of neurites that did not show filopodia while still attached to the needle because of competing experimental demands on initiated neurites, some for electron microscopy, some for cytomechanical analysis of long duration, and so on. In addition to functional growth cones, experimentally initiated neurites were found to contain microtubules. We examined ten cells for neurite microtubule content by either immunofluorescence or transmission electron microscopy. All eight of the initiated neurites longer than 10 μm were found to contain microtubules as shown in Figure 8d and e. As shown in Figure 8d, the tubulin-immunofluorescence image in initiated neurites showed a bundle of microtubules in which individual polymers could not be resolved. This is typical of neurons [3, 35, 57]; and of cells experimentally induced to express τ, a major microtubule-associated protein of axons [44]. Of two neurites less than 10 μm long, one was found to contain microtubules by immunofluorescence and one was found to be devoid of microtubules by electron microscopy.

1.4 Discussion

In towed growth, an axon elongates after attachment to its target as a result of tension exerted on the axon by target movements [94]. The experimental technique used here, attaching a glass needle to the distal end of a neurite and pulling, is an *in vitro* analog of this growth phase. Recent reports suggest an important role for towed growth in the extension of retinal ganglion cell dendrites of fish [39] and cats [63], in neuronal pathway formation within the visual system of *Drosophila* [85], and in size matching of pre- and postsynaptic elements of the neuromuscular junction of rats [5]. Despite this and considerable older evidence concerning towed growth, studies of tension induced axonal growth have been very limited; our work can be regarded as an extension of that of Bray [16], who fixed the elongation rate of neurites by towing with a specially designed towing motor. This allowed investigation of towed growth at slow rates, but provided no information about forces. We, in contrast, fixed the force and observed the elongation. This allowed an analysis of the relationship between force and growth rate. We found that chick sensory neurons are surprisingly sensitive to tension. Some neurites have a growth rate (μm/hr) tension (μdyn) ratio of 5:1 (Figure 3). An aspect of this sensitivity is that the elongation rates we observed were often higher than the most rapid growth cone-mediated elongation rates reported for these neurons in culture (approximately 100 μm/hr; [16]). However, we found no difference in the growth/tension relationship at elongation rates above and below this benchmark (e.g., Figure 2B).

We found that the growth rate of chick sensory neurites is proportional to tension above a threshold. This result confirms our earlier interpretation of tension stimulated growth in PC12 cells and, from a cytome-

chanical perspective, confirms a fundamental similarity between axonal growth and Bingham fluid behavior, that is, a linear relation between rate of length change and force above a threshold. The linear relationship between tension and growth rate is attractive heuristically in that it accounts both for axons accommodating small forces without growth and for large- scale tissue expansion without progressive changes in the steady-state tension on axons. If growth amount was proportional to tension, for example, very long axons would be under much greater tension than short axons and would require increasingly higher tensions as the organism grew. We observe that tensions below a threshold produced a zero growth rate. Thus, axons can accommodate tissue growth precisely if the level of tension on the axon increases with the rate of tissue expansion, an intuitively reasonable situation. Tension regulation of towed growth may then resemble typical physiological control mechanisms characterized by "set points." In this case there is an axonal tension set point: Axons bear some tension as shown for cultured neurons [13, 28]. Tissue growth exerts additional tension on associated axons stimulating their elongation. This axonal elongation dissipates the tension because the axon is adding mass to accommodate the increase in length rather than stretching elastically (imagine a spring that added gyres when pulled). As tissue expansion slows to a halt, tension on the axon declines with the addition of mass until it declines below threshold where it remains unless the tissue again expands. Thus, moderate steady state values for tensions on axons are maintained.

New membrane is added at the growth tip in growth cone-mediated axonal growth [11, 31, 71]. Microtubules are also added at the distal end in growth cone-mediated elongation [7, 57]. In contrast, the assumption in towed growth is that mass addition is interstitial [38], a possibility

that is supported by experimental results of Campenot [23]. We labeled the surface of towed neurites with polycationic microspheres, similar to Bray's [11] use of carmine particles, to determine whether new surface was added interstitially and to observe the pattern, if any, to the addition. The polyethyleneimine-treated microspheres did not translocate significantly relative to the cell body in the absence of pulling. Most importantly, the sum of the increased distances between microspheres during towed growth equaled the increased length of the neurite. These observations provide strong evidence that the movement of microspheres relative to the cell body during towed growth represents regions of new surface addition. As expected, we found that new surface is added interstitially along the entire length of the neurite (Figure 5). However, new surface addition did not occur uniformly throughout the neurite. There was some tendency for the new surface to be added preferentially at the distal half of neurites, as shown in Figure 5b and c. Also, as shown in Figure 5b, new surface tended to be added in particular regions of a given neurite, while other regions added little or no new surface; that is, towed growth was similar to growth cone-mediated elongation in that some regions of the neurite were favored for membrane insertion relative to other regions. New membrane arises from the fusion of membranous vesicles with the axolemma following the synthesis of the vesicles in the cell soma and rapid transport down the axon (for review, see [73]). The nature of the signal that specifies sites for vesicle off-loading from the transport system and/or for vesicle fusion are completely unknown. However, our results complement previous findings on growth cone-mediated growth by providing evidence for regionalization of axolemmal growth during towed growth. Also, the differing sites of addition in growth cone-mediated and towed elongation imply that this regionalization is sensitive to the mode

of growth.

Initiation of axons/dendrites appear to be a process distinct from elongation. Motile, "amoeboid" activity is initially characteristic of most, if not all, of the cell margin of cultured neurons [25, 29, 95]. Motility is then restricted to particular regions, and ultrastructural observation of such incipient outgrowths, both in culture and *in situ*, show cytoskeletal elements concentrated at these sites [61, 62, 86, 87]. However, a cause and effect relationship between spatial restriction of motility and the concentration of cytoskeletal elements remains elusive. Our evidence that mechanical force can both initiate neurites and regulate microtubule assembly suggests the possibility that tension may be a link between motile activity and the cytoskeletal assembly in process outgrowth. Mechanical tension as a regulator of axonal microtubule assembly has been discussed previously [21, 66]. The apparently normal density of microtubules within neurites initiated and elongated by experimentally applied tension (Figure 8e) provides strong additional evidence that mechanical force can regulate axonal microtubule assembly both spatially and temporally. More unexpected was the finding that tension stimulated the formation of functional growth cones at the ends of tension-induced neurites. In neurons with an appropriately differentiated state, tension appears to be sufficient to regulate spatially and initiate the formation of two crucial structures characteristic of sprouting neurons: an axonal microtubule array and a growth cone. The forces required to initiate neurites, less than 200 μdyn in most case, were in the same range as those for elongation of extant neurites. It is somewhat surprising that initiation required no greater tension than did elongation; the initiation and spatial organization of cytoskeletal assembly might be expected to require an additional energy input. However, the force magnitudes required for initiation and elonga-

tion were entirely consistent with the tension (generally above 200 μdyn, up to 500 μdyn) these neurons exert on themselves by their pulling growth cone [49], and by active neurite retraction [28]. We speculate that the restriction of motility observed in "normal" neurite initiation in culture reflects the first mitile region to exert more tension than the threshold for neurite initiation. Possibly, this effective pulling growth cone then inhibits motility elsewhere by mechanical effects on the cytoskeleton as described by Kolega [47] for inhibition of fish epidermal cell motility by experimentally applied tension. The tension exerted by the newly formed growth cone may induce neurites by concentrating cytoskeletal elements or stimulate cytoskeletal assembly in the region [21].

The available evidence on mechanical stimulation of axonal elongation raised the possibility that tension serves as general regulator, acting as a kind of "second messenger" [66]. It will be of interest to test whether the effects of various extrinsic regulators of axonal elongation such as NGF [23], Ca^{++} levels [33, 45, 50, 64], and properties of the growth substrate [56]) act through this "second messenger" to stimulate elongation.

2 Investigation Of Microtubule Assembly And Organization Accompanying Tension Induced Neurite Initiation

2.1 Introduction

Accumulating evidence suggests that mechanical tension is an important intrinsic regulator of normal axonal development, including the microtubule (MT) assembly and organization accompanying neurite elongation [16, 28, 97]. For example, growth cones, which are primary source of tension, pull on neurites, stimulating their elongation [49]. In later stage of axonal development, migrating target cells pull on their attached axons, causing "towed" axonal growth [94]. Growth of the neuromuscular junction during development also appears to be the result of mechanical force inputs [5].

In a previous study, we confirmed that neurons without neurites responded to tension application by forming of functional neurites within minutes [16, 97]. That is, the tension-induced cell processes developed two crucial characteristics: a functional growth cone and axial MTs array of normal density [97]. Since functional neurites could be initiated within minutes by tension, MT events accompanying neurite initiation must be occurring in the same short period.

Here, we focuse on two aspects of the MT organization and assembly in tension-induced neurites. First, is the new assembly of MTs required for neurite formation or are existing MTs from cell body reorganized or translocate into a cell process? Second, this is the only experimental system, to our knowledge, in which the initiation of neurites can be controlled by the investigator over the time scale of minutes. As such, it may prove

an attractive system for the investigation of the very early cytoskeletal events of neurite initiation, about which relatively little is known [29, 55]. This would require that neurite initiation by tension resembled growth cone-mediated initiation. Consequently we compared the density, orientations, and acetylation patterns of microtubules in tension-induced and "normal" growth cone-mediated neurites.

2.2 Material and methods

2.2.1 Materials

Triton X-100 and vinblastine sulfate were purchased from Sigma Co. Mouse monoclonal anti-β-tubulin, 5nM colloidal gold-labeled goat anti-mouse IgG were purchased from Amersham Inc. A mouse monoclonal antibody (mAb) specific for acetylated-α-tubulin (1-6.2 1) was the kind gift of Dr. David Asai of Purdue University. Fluorescein-labeled goat anti-mouse IgG was obtained from Kirkegaard and Perry Lab. Inc.

2.2.2 Cell Culture

Embryonic chick sensory neurons were cultured as previously described [97].

2.2.3 Effects Of Vinblastine

In all experiments, vinblastine sulfate was used at a concentration of 4 nM. To assess the effect of this drug concentration on growth cone-mediated elongation, we identified regions of poisoned and unpoisoned cultures and circled them with a diamond "objective" prior to the beginning of the experiments. These regions were then photographed at one hour interval for two hours. We measured the distance moved by each growth cone and divided by the time over which the movement occurred to obtain advance rates.

Some cell cultures containing both neurons and fibroblasts were allowed to incubate for 4 hours in 4 nM vinblastine, and then prepared for tubulin immunofluorescence microscopy as described below.

To assess the effect of vinblastine on tension-induced neurite initiation and elongation, we divided culture dishes into two groups: unpoisoned

group and poisoned group. In the poisoned group, 1 uM vinblastine in PBS was added into culture dishes to achieve a final concentration 4 nM. After incubating for 1 hour, neurons without extant neurites were attached to calibrated glass needles [27] at the cell margins. The response of cells to the application of tension was recorded on videotape and analyzed for tension and neurite length as previously described by Zheng et al. [97]. Some experimentally initiated neurites were examined by immunofluorescence or by immunoelectron microscopy. The unpoisoned groups were treated similarly except that vinblastine was omitted.

2.2.4 Immunofluorescence Microscopy

A diamond-tipped microscope "objective" was used to mark the experimental neuron by circling the dish beneath. Immunofluorescence staining of microtubules was carried out as previously described [97]. The neurites were incubated with the one of two primary anti-bodies: (i). A mouse monoclonal antibody that strongly recognizes acetylated-α-tubulin at 1:10,000 dilution (Keating and Asai, unpublished). (ii). A monoclonal mouse β-tubulin antibody used at 1:50 dilution. After incubation with primary mAb, neurites were rinsed, incubated with fluorescein-labeled secondary antibody. The prepared samples were viewed with a Nikon inverted microscope equipped for fluorescence observation.

Some cells were observed through an Odyssey confocal microscope (Noran Instruments). This instrument allows measurements of fluorescence intensity along a line chosen by the investigator. We used this to visualize and compare fluorescence intensity in the cell body and neurites of poisoned neurons (see Figure 21).

2.2.5 Immunoelectron Microscopy

The same two primary antibodies used for immunofluorescence localization were also employed for antigen localization at the ultrastructural level, using the method of Joshi et al. [43] and Geuens et al. [32]. Briefly, neurites were fixed and permeabilized simultaneously for 1 minute with 0.5% glutaraldehyde and 1.0% triton X-100 in buffer I (Hanks' buffered salt solution containing 5 mM Pipes, 2 mM $MgCl_2$, and 2 mM EGTA, pH 6). Neurites were subsequently taken through the following treatments: 0.5% glutaraldehyde in buffer I for 10 minutes; 0.5% triton X-100 in buffer I for 30 minutes; 0.5mg/ml $NaBH_4$ in buffer I for 15 minutes; TBS I (10 mM tris, 140 mM NaCl, pH 7.6) for 5 minutes. Cells were incubated at room temperature overnight with primary antibody against anti-acetylated-α-tubulin at 1: 10,000 dilution. Then cells were rinsed with TBS I and incubated for 6 hours with 1:2-1:4 dilation of secondary antibody labeled with 5 nm gold-conjugated second antibody. Cells were washed again with TBS II (20 mM Tris, 140 mM NaCl, pH 8.2) and postfixed with 1% glutaradehyde in buffer I for 10 minutes, 2% OsO_4 in 0.1 M Cacodylate for 20 minutes, stained with 0.5% uranyl acetate in 1% phosphotungstic acid for 30 minutes, dehydrated with an ethanol series, embedded in Polybed 812, and ultrathin sections were examined using an electron microscope.

2.3 Results

2.3.1 Acetylated Microtubules In Tension-Induced Neurites

The MT array of normal cultured neurites contains a post-translationally acetylated form of α-tubulin [9, 10, 78]. In rat neurons, these acetylated MTs are abundant in the neurite shaft but largely absent from the growth cone assessed by immunofluorescent staining [3, 78]. We wished to compare the pattern of acetylation of MTs in neurites initiated by growth cone advance and in neurites initiated by the application of tension, that is, by towed with a glass needle, in chick sensory neurons.

Neurites are initiated from neurons without extant neurites (Figure 11a) by attaching a force-calibrated glass needle to the cell margin and pulling [16, 97]. Typically, a pulling force of about 200 μdyn causes a process to form and elongated as shown in Figure 11. Elongation occurs without obvious thinning of the lengthening neurite, or shrinking of the cell body. Motile filopodia develop on the tips of the induced neurites (Figures. 11b and 11c). Tension-induced neurites that had been towed for periods between 10-60 minutes were micromanipulated to detach them from the needle and affix them to the dish surface. After this, the cultures were prepared for immunofluorescence with an antibody to acetylated α-tubulin (see Materials and Methods). We were successful in this delicate task with six cells and found that all the induced neurites stained brightly for acetylated α-tubulin (Figure 11e). Even at the earliest time (10 minutes), tension-induced neurites were observed to stain with anti-acetylated α-tubulin antibody in a pattern similar to that of normal, growth cone-mediated neurites on the same dish (Figure 12). Both normal and tension-induced neurites fluoresced brightly along the neurite shaft but not at the growth cone. Robson and Burgoyne [78]

Figure 11. *De novo* initiation of neurites from chick sensory neurons by applied tension. (a). A neuron prior to a glass needle attachment. (b). Same cell as in previous frame after 20 minutes of experimentally applied tension. (c). The distal end of the neurite formerly attached to the glass needle has been manipulated onto the culture dish. The growth cone-like appearance of this end is apparent. (d). Phase image of the same cell as in (c) fixed and processed for immunofluorescence. (e). Immunofluorescence image of the neuron as in (d) stained with a monoclonal antibody against acetylated α-tubulin(see Materials and Methods). Bar, 10 μm.

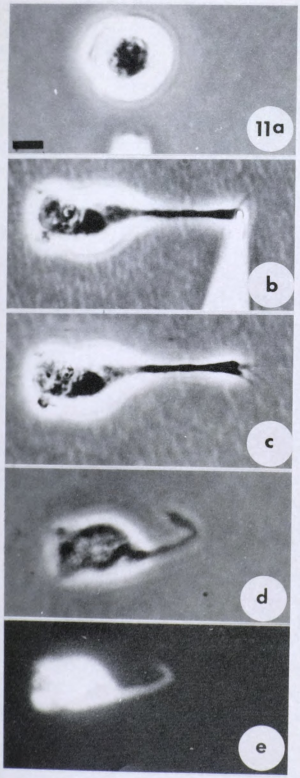

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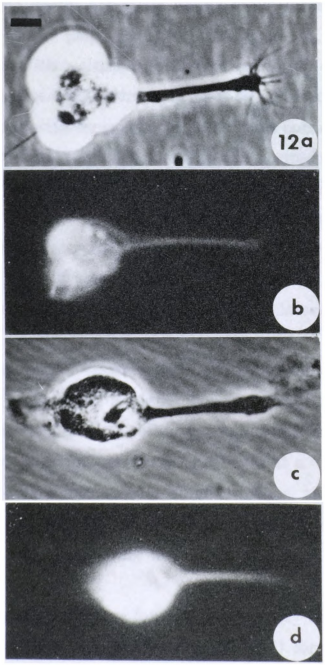
Figure 12. Both growth cone-mediate and tension-induced neurites contain similar array of acetylated MTs. (a). Phase image of an unmanipulated , cultured neuron that initiated neurites via growth cone activity. (b). Immunofluorescence image of same cell as in panel (a) stained with 1-6.2 1, the monoclonal antibody against acetylated α-tubulin (1:10,000 dilution). (c). Phase image of a towed neuron whose neurite was experimentally initiated as in Figure 11 and fixed following 57 minutes of tension. (d). Immunofluorescence image of the same neuron as panel (c), stained with a monoclonal antibody against acetylated α-tubulin. Notice the lack of staining in the growth cones region of both cells. Bar, 10 μm.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reported a similar result in cultured rat neurons.

The density and pattern of acetylated MTs in these neurons was also examined using the immunoelectron microscopic method of Joshi et al [43] and Geuens et al. [32]. As shown in Figure 13, acetylated MTs of different lengths with a similar pattern of randomly orientations were found in the cell bodies of all neurons. This is to say that neurons without neurites and neurons with tension-induced neurites contained a MT array similar to that in the cell bodies of neurons with normal neurites. None of the 11 cell bodies we observed had regional concentrations of MTs near the cell margins. With respect to individual MTs, some were densely and uniformly decorated with gold particles, while on other MTs the gold particles occurred in distinct clusters or domains. This differential labeling of MTs may represent distinct stability classes of MT polymer [3].

In the transition region between cell bodies and tension-induced neurite shafts, there is generally a higher density of MTs in the proximal neurite than in the neighboring cell body region (Figure 14). The MTs in this initial segment of the neurite do not begin at a well defined point, or an organizing center like a centrosome. Rather, the MTs appeared to radiate from many different directions, similar to the "initial segment funnel" found in cells undergoing neurite initiation *in situ* [55] and in PC12 cells [86].

In the neurite shaft itself, we found a low density of acetylated MTs even in very short, incipient processes ("nubbin") formed very shortly (3.5 minutes) after tension application to the cell margin (Figure 15a). By ten minutes, tension-induced neurites of approximate one cell body length contained a normal array of acetylated MTs (Figure 15b), confirming the light microscopic results. The antibody for acetylated α-tubulin

Figure 13. Acetylation of neuronal cell body microtubules. All panels are transmission electron micrographs of cell bodies immunostained with the monoclonal antibody against acetylated α-tubulin (1-6. 2 1 at 1:10, 000 dilution), and an appropriate second antibody conjugated to 5 nm colloidal gold particles(see Material and Methods). In all panels the image is of a region midway between the nucleus and the plasma membrane. Insets of all three panels show individual MTs at higher magnification from each group respectively. (A). A neuron lacking neurites. (B). A neuron with a tension-induced neurite. (C). A neuron with normal growth cones mediated neurites. Bars, 0.5 μm.

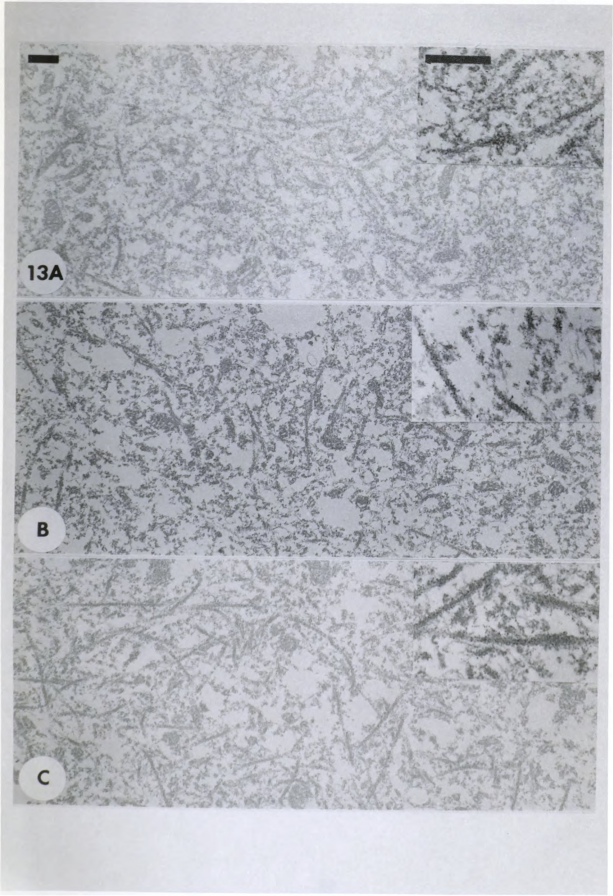

13A
B
C

Figure 14. Transition region between the cell body and tension-induced neurite stained for acetylated α-tubulin as described. Bar, 0.5 μm.

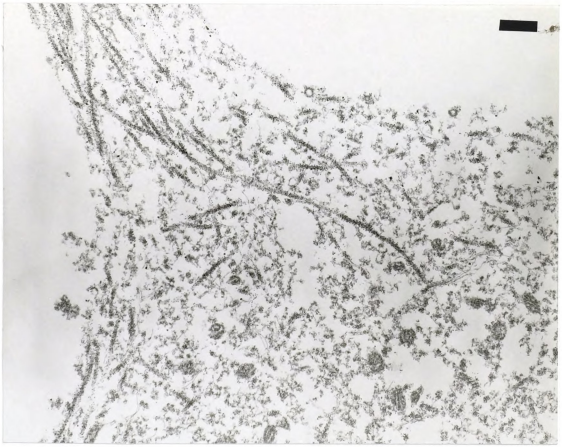
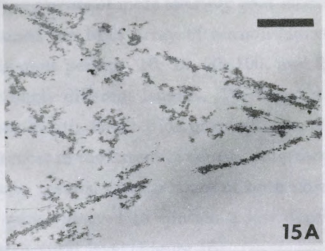


Figure 15. Density of acetylated microtubules in tension-initiated and normal neurites. All panels were stained for acetylated tubulin as described in Material and Methods (a). A low density of acetylated MTs appears in "nubbin" induced by experimental force applied for 3.5 minutes. (b). Acetylated MTs in neurite shaft 10 minutes after application of tension. (c). Acetylated MTs in neurite shaft elongated by growth cone-mediated processes for 24 hours prior to fixation. Bar, 0.5 μm.


15A


B


C

stained the MTs of both normal and tension-induced neurites intensely and, in most profiles, along their entire length (Figures 15b and 15c). On occasion, short segments of more sparsely gold-labeled MTs were observed. We examined the MTs array of tension-induced neurites that were pulled for different periods, 10, 15, 50, 100, and 230 minutes, and were of correspondingly different lengths. We found no detectable differences in apparent length distribution or orientation of acetylated MTs despite large differences in the extent of neurite outgrowth. In contrast to the light microscopic results, growth cones of both normal and tension-induced neurites were observed to contain a low density of acetylated MTs at the ultrastructural level (Figure 16).

A similar difference in the ability to observe acetylated MTs by light and electron optics was also noted in fibroblasts, which contaminate our primary neuronal cultures (Figure 17). Immunofluorescence observations of fibroblasts frequently indicated a lack of acetylated microtubules, particularly when fibroblasts were in the vicinity of neurons (Figures 17a and 17b). When fibroblasts were isolated on the dish, however, or at the electron microscopic level, they were found to have low levels of tubulin acetylation (Figure 17c).

2.3.2 Effects Of Kinetic Stabilization Of MTs On Tension-Induced Neurite Initiation

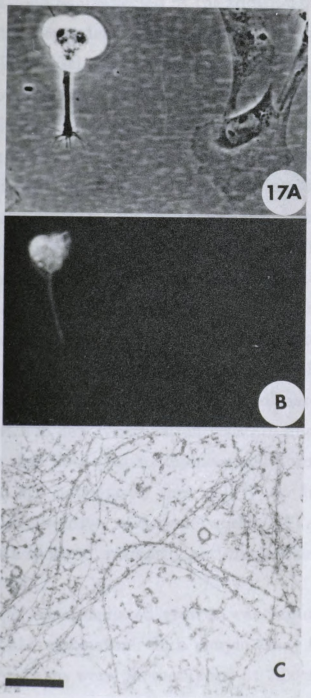
The data above indicate that tension stimulates a rapid reorganization of cell body MTs to accommodate neurite formation and/or a tension-induced assembly of axonal MTs that are then very rapidly acetylated. To assess the relative roles of these two processes, we exploited the recent discovery that nanomolar concentrations of vinblastine stabilize MTs by inhibiting both the "on" and "off" reactions of tubulin exchange [40, 41].

Figure 16. Acetylated microtubules in the growth cones of tension-initiated and normal neurites. All panels were stained for acetylated α-tubulin by immunogold method described in Materials and Methods. (A). Electron micrograph from the distal end of a neurite elongated by a growth cone. (B). Electron micrograph of the distal end of a neurite elongated by experimentally applied tension for 10 minutes. At the time this neurite was micromanipulated from the glass needle for fixation, the distal end showed filopodia, similar to Figure 11b and 11c. Bar, 0.5 μm.


16A


B

Figure 17. Comparison of apparent microtubule acetylation in fibroblasts by immunofluorescence and immunoelectron microscopy. (a). Phase image of dish region containing a neuron with growth cone-mediated neurite and contaminating fibroblasts. (b). Immunofluorescence image, stained for acetylated α-tubulin, of the same region of the dish as in panel α. (c). Immunoelectron microscopic image, again stained for acetylated α-tubulin, of a different fibroblast showing the low staining level seen at this higher resolution. Bar, 1 μm.

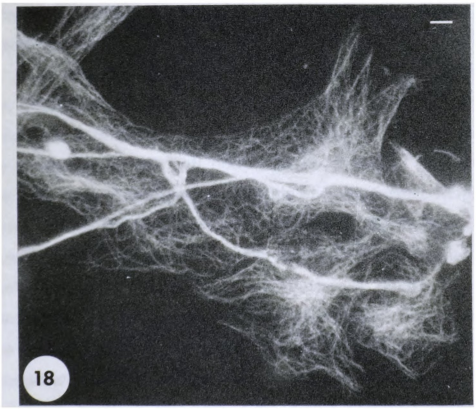

17A
B
C

This "kinetic stabilization" of microtubules prevents both polymer disassembly and new assembly [41]. Thus, events depend upon re-organization of existing MTs should be unaffected by the addition of the vinca alkaloid, while MT assembly-dependent events should be inhibited.

Jordan et al. [41] found the treatment of Hela cells with 4 nM vinblastine for 18-20 hours arrested cells in mitotic metaphase without serious spindle disorganization or detectable loss of MT polymer. We wished to confirm that vinblastine at these low concentrations did not cause appreciable MTs disassembly in our cell types during the typical durations of our pulling experiments. We examined anti-β-tubulin immunofluorescence in both chick sensory neurons and the chick fibroblasts that typically contaminate the neuronal cultures. Cells were incubated for 4 hours in the presence of 4 nM vinblastine, then fixed and processed for immunofluorescence microscopy with anti-β-tubulin antibody. Fibroblasts were of interest because reports of relatively rapid MT-turnover [79, 80] suggested that they would be more sensitive to disassembly than neuritic MTs, which are rich in depolymerization-resistant microtubules [4, 8, 42]. Figure 18 shows that both vinblastine-poisoned fibroblasts and neurons contain a normal MT network for their cell types. In particular, the fibroblasts show the extensive, spidery network of MTs that extend to the cell margins, not a reduced, primarily peri-nuclear network indicative of only stable MTs [80].

Normal, growth cone-mediated growth was rapidly inhibited by incubation with 4 nM vinblastine. In a simple assay for growth, marked regions of culture dishes were photographed at 20x magnification at the beginning of the experiments and two hour after drug or sham addition (control). The change in length of neurites during this two hours period was compared and expressed as an hourly elongation rate. In

Figure 18. Microtubules in fibroblasts and neurons following 4 hours of incubation in medium containing 4 nM vinblastine sulfate. Cells were immunofluorescently stained for β-tubulin as described in Materials and Methods. Bar, 10 μm.



observations of 6 neurites from 3 unpoisoned neurons, the average growth rate was 6.6 μm/hr per neurite. In contrast, 12 neurites from 7 poisoned neurons were found to remain essentially stationary over 2 hours period with a growth rate of -0.5 μm/hr per neurite.

Tension-induced neurite initiation and growth was seriously compromised by the presence of 4 nM vinblastine as shown in Table 2. Of the 11 unpoisoned neurons studied, more than 50% initiated and elongated lengthy neurites. (In previous experiments of this kind, a higher percentage, 70-80% of all neurons, were induced to form neurites [97]). In contrast, only 4 of 38 poisoned neurons initiated neurites that subsequently elongated for two cell body lengths or more. The "successful" neurites were normal in appearance and in one case formed filopodia at its distal end. However, these poisoned neurites did not behave like unpoisoned neurites in their response to the manipulations intended to attach the "towed" neurite onto the dish surface, that is, remove the needle for immunofluorescent and ultrastructural observations. First, the poisoned neurites had a tendency to stretch and break along their length during the manipulation. This occurs rarely in normal neurites but 2 of 4 poisoned neurites broke in this way. Also, 2 of 4 poisoned neurites rapidly retracted to less than half length when their distal end was freed from the needle. One neurite retracted 38 μm within 20 seconds, while the other retracted 40 μm in 100 seconds. In contrast, unpoisoned tension-induced neurites typically elongate a bit during needle removal because of the tugging required for detachment. In no case did we observe more than a 2% decrease in the length of unpoisoned neurites after detachment from the needle.

Among the 38 poisoned neurons, we classified 34 as having failed to initiate neurites. One type of failure was particular interesting. In about

	Control Group (11)	Vinblastine group (38)
Failure*	27%	29%
Nubbin Only	18%	36.8%
Stretch and Break While Towing	0%	23.7%
Success	54.6%	10.5%

Table 2: Effects Of vinblastine on *de novo* initiation of neurites

* (I) **Failure**. No process formation. For unknown reasons, a neurite could not be initiated ***de novo*** by experimentally applied tension.

(II) **Nubbin Only**. A initial nubbin drawn out from the cell margin by experimentally **applied** tension failed to elongate.

(III) **Streak and break while towing**. Elongation only last for a short period. The elon**gated** length from a formed nubbin was shorter than 1-2 cell body length.

(IV) **Success**. A neurite could elongate as long as a properly tension applied to the **neurite**, that is, cell process is larger than 2 cell body length.

25% of the poisoned neurons tested, a process formed that elongated for 1-2 cell body lengths, but suddenly ceased elongating and began to stretch out and break. The break could occur all along their length, in the middle of neurite shaft or at the distal end (Figure 19). This stretching process was not the result of any increase in applied tension, it generally occurred during periods of steady tension. In some instances, neurite tension was reduced at the first sign of stretching, nevertheless the neurite continued to thin out and break. Such stretch-and-break failure has never been observed in unpoisoned cells in this or previous studies [97]. Once initiation is well underway in unpoisoned cells, the neurite continues to elongate for long periods, several hours at least.

2.3.3 Neurites Formed In The Presence of 4nM Vinblastine Lack Microtubules

We were able to successfully prepare vinblastine-poisoned neurons for analysis of their MTs content. Figure 20 shows transmission electron micrographs of one neuron in which neurite formation failed in the manner described in the previous paragraph. We were able to fix this cell before the neurite broke. Figure 20a shows that the cell body of this neuron contains an apparently normal array of MTs. Yet, as shown in Figure 20b, the neurite shaft is nearly devoid of MTs, containing only unusually short fragments that often not axially oriented. Apparently, the somatic MTs did not invade the transition region between soma and neurite. Also, the application of tension to the cell margin did not serve to orient the existing MTs.

Figure 21 shows phase and quantitative immunofluorescence images of neurons stained for β-tubulin in poisoned and unpoisoned cells subjected to tension application. Figure 21a are images of tension-initiated neurite

Figure 19. Two examples of stretch-and-break failure observed in cell poisoned with 4 nM vinblastine, but never observed in unpoisoned cells. See results for description. Bar, 10 μm.

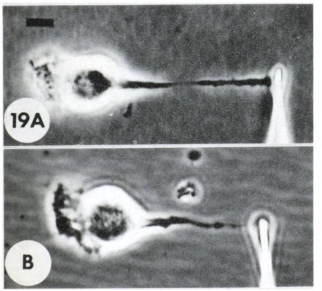


Figure 20. Electron micrograph of a vinblastine poisoned neuron fixed while undergoing stretch-and-break failure of the sort shown in Figure 19. (A). Low magnification view of neuron. Note the normal density of MTs in the region midway between the nucleus and cell margin. (B). Higher magnification view of neurite shaft showing abnormally low density of MTs, compare with Figure 15b. Bars, 1 μm.

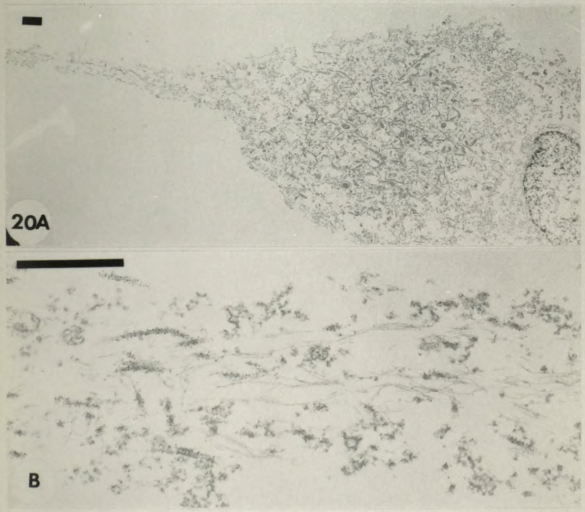
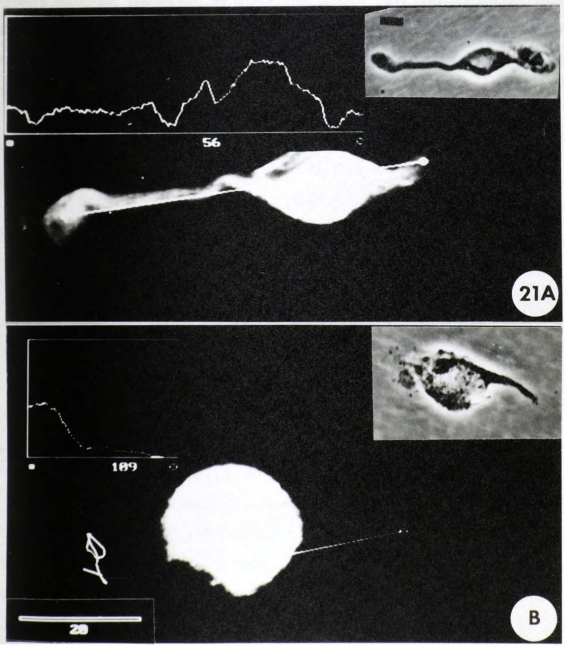


Figure 21. Phase images and quantitative confocal light microscopic images of poisoned and unpoisoned neurons with tension-induced neurites stained for β-tubulin as described in Materials and Methods. (A). Tubulin immunofluorescence in an unpoisoned neuron. The graph in the upper left corner is a measure of light intensity along the bright line shown across the image of the cell. Inset is a phase image of the cell. (B). Tubulin immunofluorescence in a poisoned neuron that initiated a "successful neurite," i.e. longer than two cell body lengths, but which broke during attempting to free the glass needle from the neurite's distal end. Once again, the graph in the upper left is a measure of the light intensity along the bright line shown across the image of the cell using the same instrument setting as in panel (A). Inset is a phase image of the cell. Inset bar, 10 μm, main panel bar, 20 μm.


56
21A
109
20
B

in the absence of vinblastine. Both neurite and cell body are brightly fluorescent. Figure 21b are images of a vinblastine-poisoned neuron that was "successfully" initiated in that the neurite was originally more than 2 cell body lengths. However, as described above, this neurite broke during manipulations to release the needle. Little or no tubulin immunofluorescence is visible in this "successful" neurite, while the cell body is brightly fluorescent.

2.4 Discussion

In previous studies, we confirmed that functional neurites can be initiated and elongated by experimentally applied tension [16, 97]. Here, we report that the organization of MTs accompanying tension-induced neurite initiation is very similar to growth cone-mediated axonogenesis reported by Lefcort and Bentley [55] in an identified neuron of the grasshopper limb, and by Stevens et al. [86] in PC 12 cells. At the earliest times after the applying tension to margin of chick sensory neurons, relatively short MTs are located in the "nubbin" that form (Figure 15a). These microtubules do not begin at a well-defined point organizing center. Rather, they radiate in all direction within the funnel-shaped transition region between the cell body and the incipient neurite (Figure 14), similar to the "initial segment funnel" reported by Stevens et al. [86], and consistent with the accumulation of tubulin seen by Lefcort and Bentley [55] as the growth cone emerges. By the time the towed neurite is one cell body length, as early as 10 minutes after tension application, the microtubules have taken on the dense axial arrangement typical of these neurites. Clearly, applied tension either induced significant new MT polymerization and/or cause significant reorganization of existing MTs.

The majority of α-tubulin in axonal neurites of cultured neurons is acetylated [4, 9, 10]. We found that the pattern of acetylated tubulin in tension-induced neurites is very similar to that reported for growth cone-mediated neurites. The axial array of neuritic MTs is densely and uniformly labeled with anti-acetylated α-tubulin antibody at very early times after neurite initiation, 10-20 minutes (Figures 11e and 15b). At the light microscopic level, fluorescence is observed throughout the cell body and the neurite shaft except the growth cone region, as also reported

by others [3, 78]. However, at the electron microscopic level, we found a low density of MTs that were acetylated in both tension-induced and "normal" growth cones (Figure 16). The data suggests two possible explanations for the apparent absence of anti-acetylated α-tubulin staining in growth cones at visible light resolutions. One is that the low density of MTs within the growth cone does not produce enough fluorescence intensity to see. The other possibility is that the level of acetylation of growth cone MTs is sufficiently low that, like fibroblasts (Figure 17), it produces too weak an immunofluorescence signal.

Although the function of tubulin acetylation is unknown, it has been suggested that it can be as a marker for MT age. That is, microtubule persistence, over the time scale of tens of minutes. For example, in African green monkey kidney cells, the timing of reappearance of a steady-state level of acetylated MTs lagged 30 minutes behind MT repolymerization after drug induced disassembly [19]. In neurite initiation experiments, in contrast, acetylated tubulin was apparent in cells fixed at earliest time after tension application, 3.5 minutes (Figure 15a). As noted above, the acetylation pattern was indistinguishable from normal neurites by 10-20 minutes (Figures 11e and 15b). The substrate for acetylation is known to be the MT polymer [10]. The degree of labeling of tension-induced neurite MTs appears to represent an extensive level of acetylation judging by (1) the much lower levels of antibody staining of fibroblasts on the same dish and (2) the similarity of labeling of both neuritic and somatic MTs. The neuronal soma were found to be heavily acetylated in this and previous studies [3, 4, 78].

To determine the relative roles of MT reorganization and *de novo* assembly in tension-induced neurite initiation, we compared the effect of "towing" in the presence or absence of MT assembly. We exploited the

recent discovery that very low concentrations of vinblastine act as a "kinetic stabilizer," suppressing both "on" and "off" reactions at MT ends. The overall effect is that additional MT assembly is poisoned with little or no loss of extant polymer [40, 41, 91]. We confirmed that 4 nM vinblastine causes no observable MT depolymerization in either fibroblasts or neurons of chick dorsal root ganglia (Figure 18). Further, the vinca alkaloid at this concentration inhibited growth cone-mediated elongation. Vinblastine poisoning appeared to arrest growth cone advance, poisoned neurites did not significantly advance or retract during a two hour period of observation.

The response of neurons to tension application in the presence of 4 nM vinblastine argues strongly in favor of tubulin assembly underlying tension induced neurite initiation. First, neurite initiation by pulling was seriously inhibited by the presence of the poison. Those few neurites/cell processes that did form were found to be abnormal. Cell processes both longer and shorter than two cell body lengths formed in the presence of vinca alkaloid were abnormally prone to breaking under tension. The appearance of the failures, and the instances in which stretching and breakage continued despite reduced tension loads are consistent with lack of an internal structural support from MTs [22, 27]. Although such failure might also occur from lack of new membrane, which is normally supplied by microtubule-based transport [73, 82]. Those few poisoned neurites we were able to examine were found to be nearly devoid of MTs.

Both electron microscopic and immunofluorescence images indicated that apparently normal MT arrays in the cell body of poisoned neurons failed to invade the neurite or even form an initial segment funnel. That is, we find no clear evidence for reorganization of existing MTs. We are unable to draw any conclusions about the few MT fragments that are ap-

parently a feature of poisoned neurites (Figure 20). These may be examples of tension-induced tubulin assembly severely limited by the poison. Alternatively, the fragments may reflect very limited MT reorganization of cell body MTs. If this latter is the case, it is difficult to understand why the MTs are so short and poorly aligned unless some fragmentation process occurs in poisoned cells that is normally suppressed or quickly ameliorated.

We conclude that application of tension to the margin of chick sensory neurons rapidly stimulates significant levels of new MT assembly concomitant with neurite initiation. Our data also suggests that tubulin acetylation in these neurons occurs very rapidly after assembly. Baas et al. [4] also concluded that tubulin acetylation occurs soon after assembly and our results show extensive acetylation can occur within a very few minutes. The ability to experimentally control neurite initiation, elongation and the accompanying MT assembly by tension application suggests an attractive method for the study of the questions and controversies that currently swirl through the field of axonal MT transport and assembly.

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