

Experimental Modification of PC12 Neurite Shape with the Microtubule-depolymerizing Drug Nocodazole: A Serial Electron Microscopic Study of Neurite Shape Control

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Abstract. The microtubule-depolymerizing drug Nocodazole has been used to experimentally manipulate the form of PC12 neurites. Both time-lapse photography and serial electron microscopy demonstrate that microtubule depolymerization leads to varicosity formation due to a clustering of membranous organelles in young neurites (nerve growth factor activated within 7 d). Neurites that have been nerve growth factor activated 7 or more d before Nocodazole application are resistant to microtubule depolymerization. These data and data from previous papers has

been combined in an attempt to predict quantitatively the volume and the shape of a neurite. The relationship is described mathematically by $V_n = 4.52 V_o + 0.0054 MTL$, where V_n is local neurite volume, V_o is organelle volume, and MTL is MT length (the constant, 0.0054 is μm^2), and 4.52 is the obligatory volume constant derived from serial electron microscopic studies. The equation predicts the total volume of neurites despite alterations of morphology due to Nocodazole and despite changes in morphology during development.

FOR over a century anatomists and physiologists have assumed that neuronal form and function must be related, but little was known about the actual cellular mechanisms responsible for the control of form. It has only become clear in the last decade that an internal "cytoskeleton," consisting of neurofilaments, microtubules (MTs),¹ and a subcellular matrix was in some way responsible for neurite shape control (Lasek and Hoffman, 1976; Ellisman and Porter, 1980; Brady et al., 1984; Hirokawa, 1982; Schnapp and Reese, 1982; Yamada et al., 1971; Berthold, 1978). In a previous set of papers we have refined this cytoskeleton concept by studying three-dimensional structure of dendrites and axons (Sasaki et al., 1983, 1984; Jacobs and Stevens, 1983). We have suggested that the axial shape and neurite volume is largely controlled by four intracellular, variables: (a) the number and placement of organelles within the neurite; (b) an obligatory volume constant associated with these organelles; (c) the number and distribution of MTs within the neurite; (d) the MT exclusion zone or what we believe corresponds to the size of the microtubule-associated proteins (MAPs).

Other cytoskeletal components, such as intermediate filaments, undoubtedly contribute to shape control as well, particularly in large peripheral axons (Hoffman et al., 1984), but much of our own past work suggests that in general, these four factors can be used to predict the shape and volume of

most central nervous system neurites with a great deal of accuracy. We examine this possibility experimentally in the present paper.

It is well known that the drug Nocodazole depolymerizes MTs in neurites (Solomon, 1980) and can be used to acutely alter the number of MTs in PC12 cells (Jacobs and Stevens, 1983). We use this drug to "perturb" the cytoskeletal shape control system of nerve growth factor (NGF)-activated PC12 neurites. First, via time-lapse photography we have recorded the redistribution of neurite volume we predict should occur from acute MT depolymerization. Second, the treated material was fixed and through serial electron microscopic (EM) reconstruction, we quantified the relationship which the four key variables listed above have to these observed changes. These data and data presented in previous papers are then used to generate a comprehensive, quantitative model of the axial control of neurite shape that predicts axial volume quite accurately.

Materials and Methods

Materials and methods are as described in the previous paper (Jacobs and Stevens, 1986). To depolymerize MTs, Nocodazole (gift of Janssen Pharmaceutica, Beerse, Belgium) was used at a final concentration of 5 $\mu\text{g}/\text{ml}$, diluted from stock solution of 1 mg/ml in dimethyl sulfoxide. Threshold for gross morphological response was judged by the generation of varicose neurites visible with the phase-contrast microscope. The threshold with Nocodazole varied from 0.5 to 5.0 $\mu\text{g}/\text{ml}$. Final dimethyl sulfoxide concentration was 0.5% or lower, well below threshold for disruption of axonal transport and ultrastructural effects (Donoso et al., 1977). The MT-depolymerizing properties of Nocodazole are well documented. It was cho-

1. *Abbreviations used in this paper:* AR, agranular reticulum; EM, electron microscopic; MAP, microtubule-associated protein; MT, microtubule; NGF, nerve growth factor.

sen because it is as effective as other agents (Colchicine, Colcemid), while showing greater consistency and reversibility of effect (DeBrabander et al., 1981; Jacobs and Stevens, 1983). Linear regressions are based upon Model one assumptions. These assumptions are as follows: (a) the independent variable is measured without error; (b) the dependent variable varies linearly with respect to the independent variable; (c) values are normally distributed; and (d) variance is independent of the magnitude of the variable. There were not enough data to fully test all these assumptions. Confidence limits for slopes and testing of hypotheses for equivalence of slopes are based on the *t* test statistic (Sokal and Rohlf, 1969). Volume data on all membranous organelle types were treated identically, based upon the results of Sasaki et al. (1984), which established the equivalence of all membranous organelle types in volume contribution to neurites. Serial EM and computer methods used are described in Stevens and Trogadis (1984).

Results

Nocodazole Treatment and Changes In Neurite Morphology

When Nocodazole is added to NGF-activated PC12 cultures it induces neurites of uniform caliber to develop varicose ex-

pansions connected by thin necks (Jacobs and Stevens, 1983) within a very short period of time. The threshold for this effect varies between 0.5 and 5 $\mu\text{g/ml}$. Varicosities appear after 1–2 h at threshold doses, and sooner at higher doses. The Nocodazole effect on gross neurite shape is very pronounced with young (<7-d-old) neurites. This is shown in Fig. 1 (*bottom*).

In contrast, neurites that have been in NGF for >7 d seem to be less sensitive to Nocodazole. The gross morphology of mature neurites seems to be unaffected by Nocodazole treatment (Fig. 1, *top*). Consistent with the observations of the previous paper (Jacobs and Stevens, 1986), we will refer to the cells that have been in NGF for <7 d as immature and those that have been in NGF >7 d as mature. While this difference in Nocodazole sensitivity is of direct interest, we will examine it here primarily as a fortuitous control system for fixation, changes in osmolarity, and any other possible artifacts from drug treatment that might lead to neuritic shape changes.

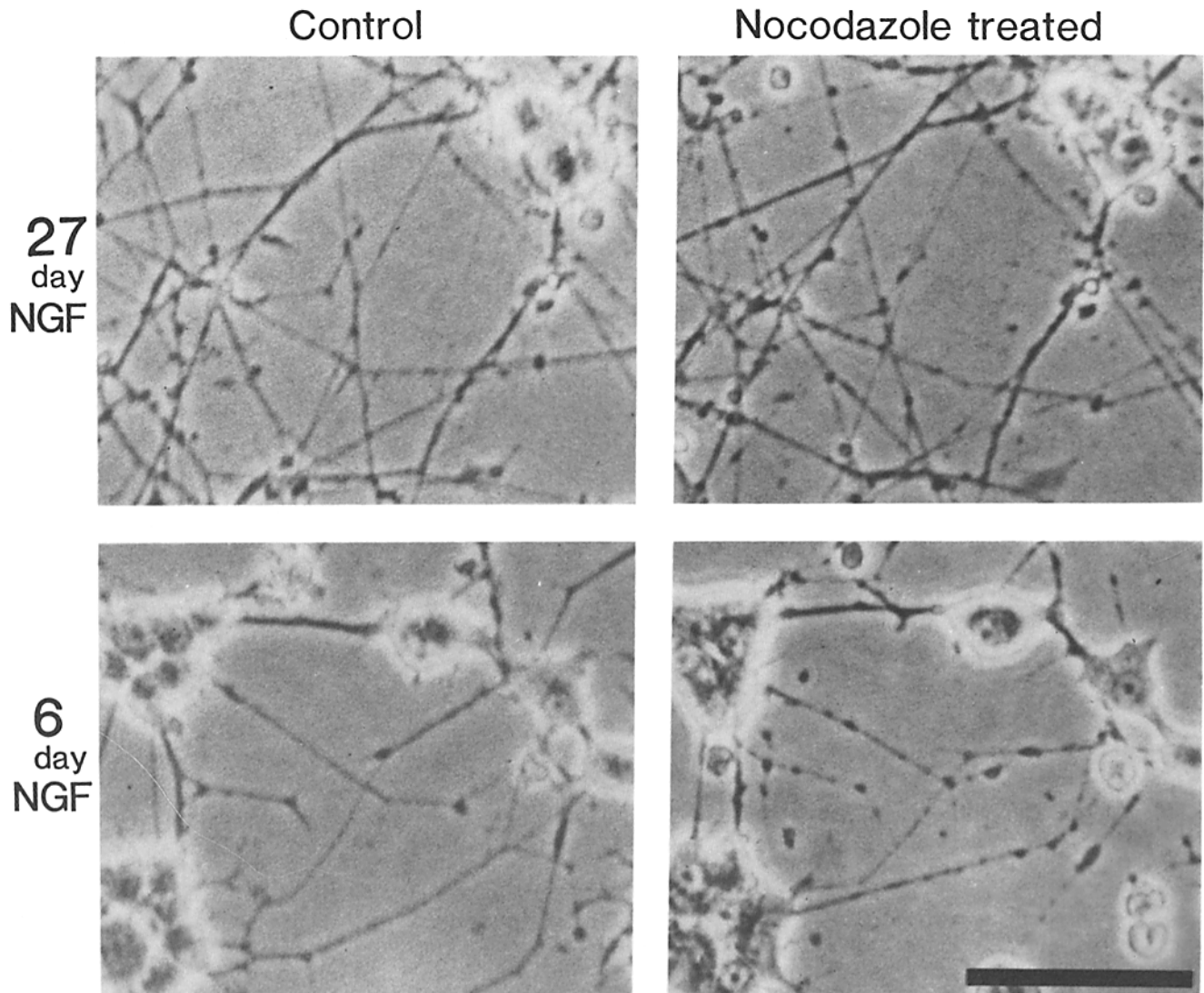


Figure 1. The effect of Nocodazole on gross neurite morphology. Light micrographs at left are typical views of differentiated PC12 cells 27 (*top*) and 6 (*bottom*) d after addition of NGF. The same region is shown at right after 2 (*top*) or 4 (*bottom*) h in 5 $\mu\text{g/ml}$ Nocodazole. Bar, 100 μm .

Direct EM Observations

Fig. 2 illustrates the ultrastructure at the EM level of young neurites after Nocodazole treatment in longitudinal sections, and Fig. 3 compares immature and mature neurites in cross section after Nocodazole treatment. Young neurites contain numerous varicosities, which are filled with randomly oriented membranous organelles. The agranular reticulum (AR) is broken into an atypical wispy cobweb-like lattice (see especially Fig. 2 *b* and Fig. 3, *a* and *b*). Necks of young Nocodazole-treated neurites have few, if any, membranous organelles (Fig. 2). They contain many 10-nm filaments and occasionally a few MTs. In contrast, the ultrastructure of mature neurites that have also been treated with Nocodazole appears normal (Fig. 3, *c-e*). Neurite caliber is uniform, numerous MTs are found regularly placed within the neurite, and organelles are longitudinally oriented within the neurite.

Quantitative Analysis of Nocodazole-treated MT Exclusion Zones

The MT exclusion cylinder is present in Nocodazole-treated neurites (Fig. 3). Histograms similar to those shown in the preceding paper (Fig. 6 in Jacobs and Stevens, 1986) are virtually identical to the normal. The average inter-MT distance for Nocodazole-treated neurites after 22 d in NGF (67 nm) or 6 d in NGF (71 nm) is not statistically different from normal (69 nm) (approximate *t* test for means assuming unequal variance).

Organization of Membranous Organelles

MT depletion in Nocodazole-treated neurites is associated with massive spatial reorganization along the neurite length. Fig. 4 shows a serial EM reconstruction and complete volume distribution analysis for a Nocodazole-sensitive neurite after 6 d in NGF. The necks between varicosities contain only 10-nm filaments, MTs, and very little AR. Most AR, and all other membranous organelles, are now found in vari-

cosities. This is in contrast to untreated PC12 neurites (cf. Fig. 8 in Jacobs and Stevens, 1986) where AR is nearly uniformly distributed. A complete reconstruction of a Nocodazole-insensitive neurite (22 d in NGF, Fig. 5) shows a volume distribution indistinguishable from control neurites of similar age. MT volume is the major component of neurite volume and AR volume is uniformly distributed.

Obligatory Volumes

In the normal PC12 and other neurites we found a direct relationship between organelle volume and varicosity volume. We call this excess volume associated with the organelles an "Obligatory Volume." In Nocodazole-treated neurites, large clusters of membranous organelles that result from MT depletion in PC12 neurites are associated with the creation of unusually large varicosities.

We measured the total organelle volume and neurite volume for seven Nocodazole-treated varicosities and carried out a regression analysis between these two variables. Varicosities with obvious breaches in the plasmalemmal membrane were excluded from this analysis. Each micrometer of MT was attributed 0.0054 cubic micrometers of volume, which is 1.42 times the volume of a 1- μm length of MT exclusion cylinder (assumed diameter = 69 nm). A regression analysis was performed with this data and the membranous organelle volume for each corresponding neurite region.

The regression analysis (least squares method) is summarized in Fig. 6. Neurite volume (V_n) is 4.43 times organelle volume (V_o) ($r = 0.96$, $n = 7$, 90% confidence limits are 4.43 ± 0.51). In Nocodazole-treated neurites, clusters of organelles are associated with an additional neurite volume that is 4.4 times that needed to enclose the organelles. As in the normal PC12 neurites there is a very tight correlation between the local neurite volume and the local organelle volume. The normal neurite obligatory volume (V_n) is 4.52 times organelle volume (V_o) ($r = 0.998$, $n = 17$, 90% confidence limits are 4.52 ± 0.37). The slope for the volume

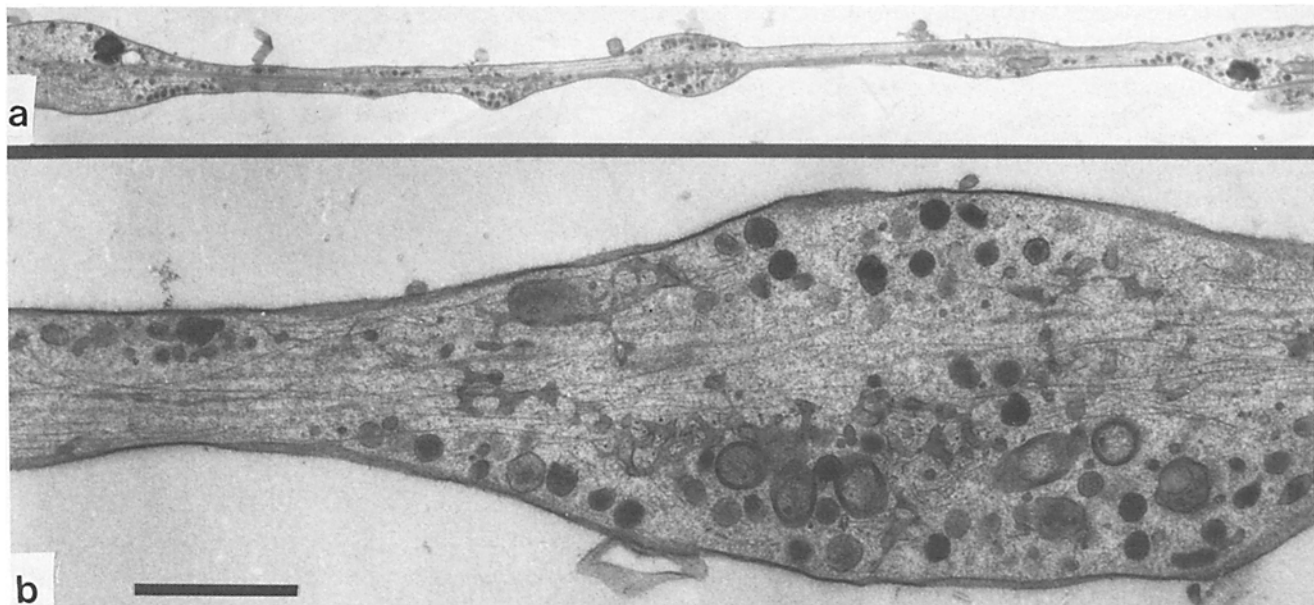


Figure 2. Nocodazole-sensitive neurites in longitudinal section. *a* and *b* are from neurites after 6 d of NGF exposure, treated with 5 $\mu\text{g/ml}$ Nocodazole for 1 h. Bar, (*a*) 2 μm ; (*b*) 0.2 μm .

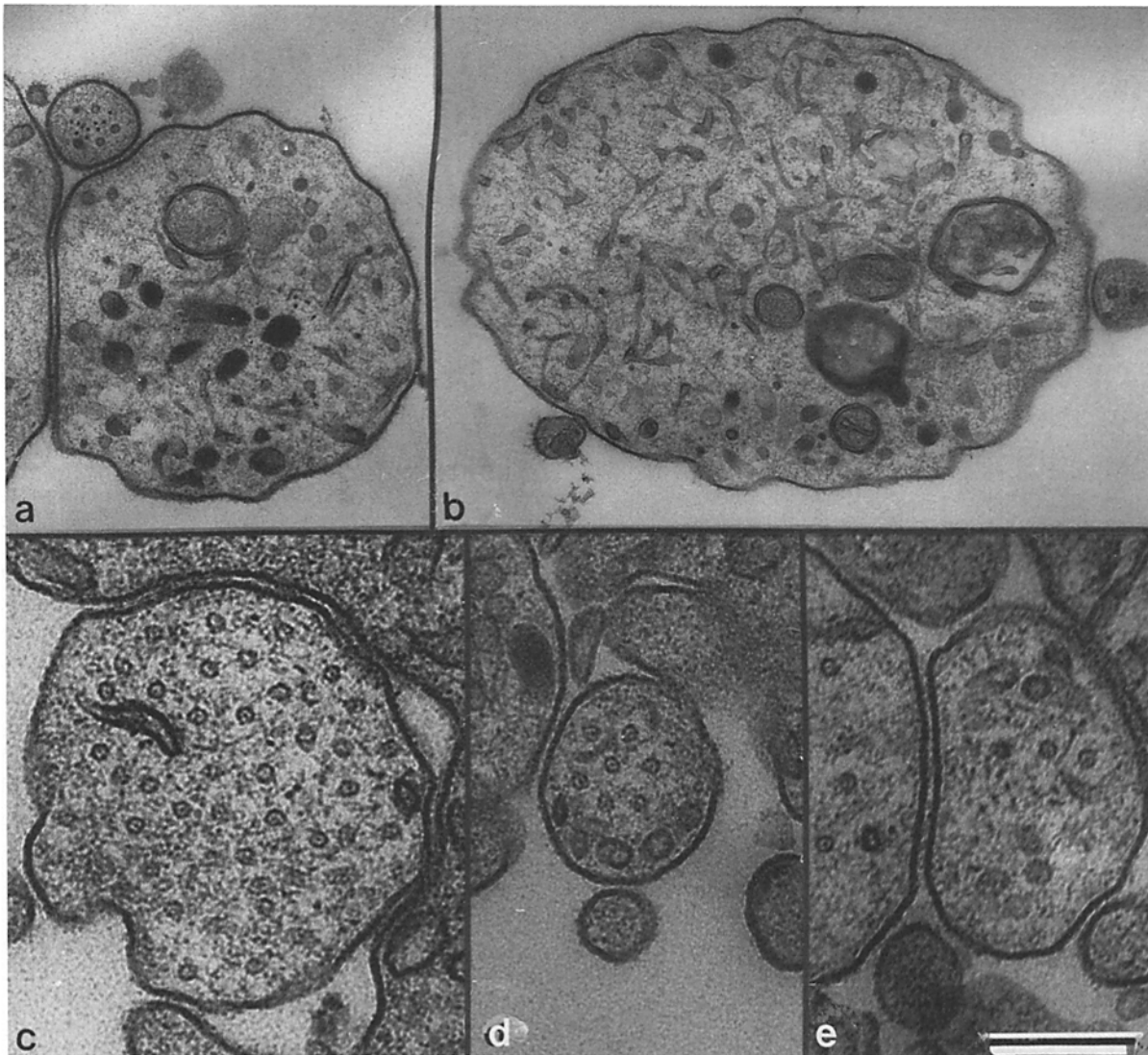


Figure 3. Typical cross sections of Nocodazole-treated neurites. *a* and *b* are neurites treated for 1 h with 5 µg/ml Nocodazole, 6 d after NGF induction. *a* contains cross sections through both a neck and a varicosity of MT-depleted neurites. (*c-e*) Nocodazole-treated neurites 22 d after NGF induction, showing a normal complement of neuritic MT. Bar, (*a* and *b*) 0.5 µm; (*c-e*) 0.2 µm.

regression from Nocodazole-treated neurites (4.43) is not statistically different from the slope for normal neurites (two-tailed *t* test, $P > 0.5$).

Discussion

Microtubule Contribution to Neurite Shape

Many workers have suggested that MTs are a direct contributor to axon caliber (Friede and Samorajski, 1970; Zenker and Hohberg, 1973; Nadelhaft, 1974; and Berthold, 1978) and dendrite caliber (Sasaki et al., 1983, 1984). As discussed in previous papers (Sasaki et al., 1983, 1984; Jacobs and Stevens, 1986), MTs are associated with a cylindrical volume component which other organelles do not penetrate. This is called the MT exclusion cylinder. The size of the cylinder does not vary in size during development. MTs that survive Nocodazole treatment seem to have an unaltered MT exclusion cylinder. As MT distribution is uniform along neurite

length, MT volume is also distributed uniformly along the length of the neurite. Regions of neurite which contain only MTs are regions of minimum neurite caliber. Though MT exclusion cylinders are likely the physical basis of MT contribution to neurite caliber, the real volume is 42% beyond the summed MT exclusion cylinder volumes. This is attributable to volume trapped by packing of MT exclusion cylinders. Ideally, if the MTs were straight tightly packed cylindrical tubes, it would represent the maximal packing configuration and would trap only an extra 16% of volume. We suspect that since the MTs are not straight cylinders, and in fact tend to weave between each other, the remaining extra volume is simply due to deviation from this ideal packing conformation.

In the preceding paper (Jacobs and Stevens, 1986), it was noted that young neurites with few MTs contained organelles with variable shape and orientation, while mature neurites, containing many more MTs, have membranous organelles with a longitudinal orientation along the same axis as the

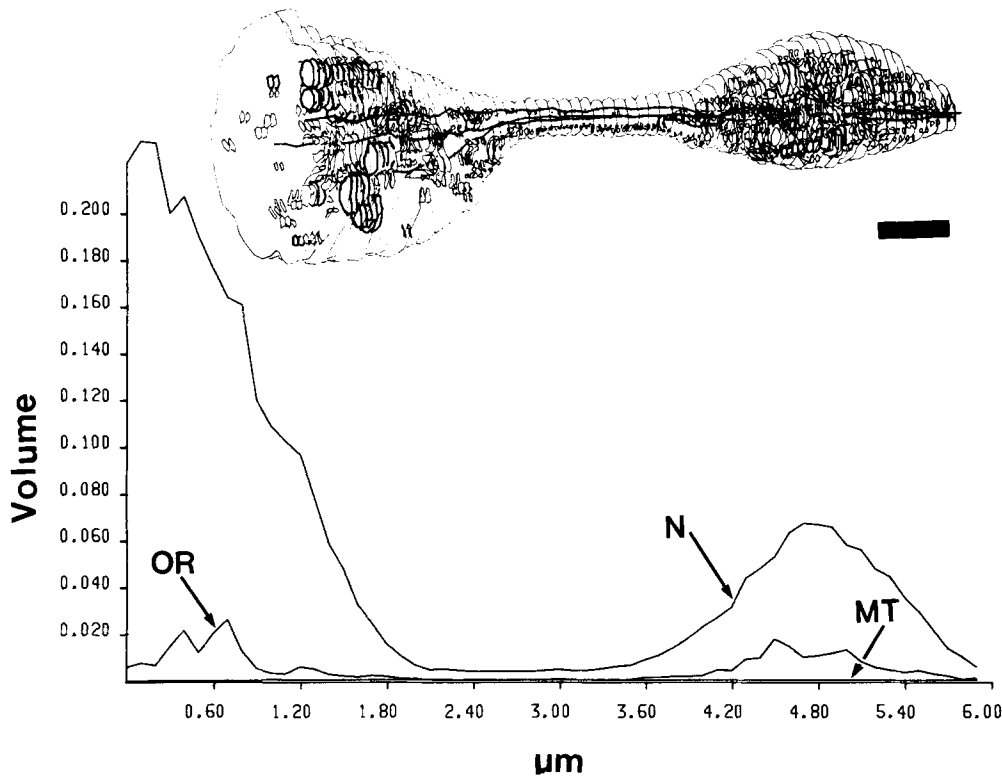


Figure 4. Regional volume distribution in a Nocodazole-sensitive neurite. (*Top*) Computer plot of all neuritic components of a complete 6- μm reconstruction of a neurite from a PC12 cell after 6 d of NGF induction and 1 h of 5 $\mu\text{g/ml}$ Nocodazole treatment. (*Bottom*) Cumulative plot of neurite contents, beginning with microtubules (MT), followed by all membranous organelles (OR), and finally plasmalemmal volume (N). Abscissa: length along the reconstruction. Ordinate: total volume of reconstructed elements for each section in the series.

MT. When MT number is depleted with Nocodazole, the longitudinal orientation of membranous organelles is lost. Without MTs, the organelles will cluster together and form large irregular varicosities similar to those in the immature neurites but larger with many membranous organelles found in any shape or orientation.

Developmental Microtubule Stability and Morphological Stability

MTs from different cytoplasmic regions, or even within the same region differ in their stability to cold or Colchicine (Jones et al., 1980; Black and Greene, 1982; Brady et al., 1984). MTs in young neurites are vulnerable to depolymer-

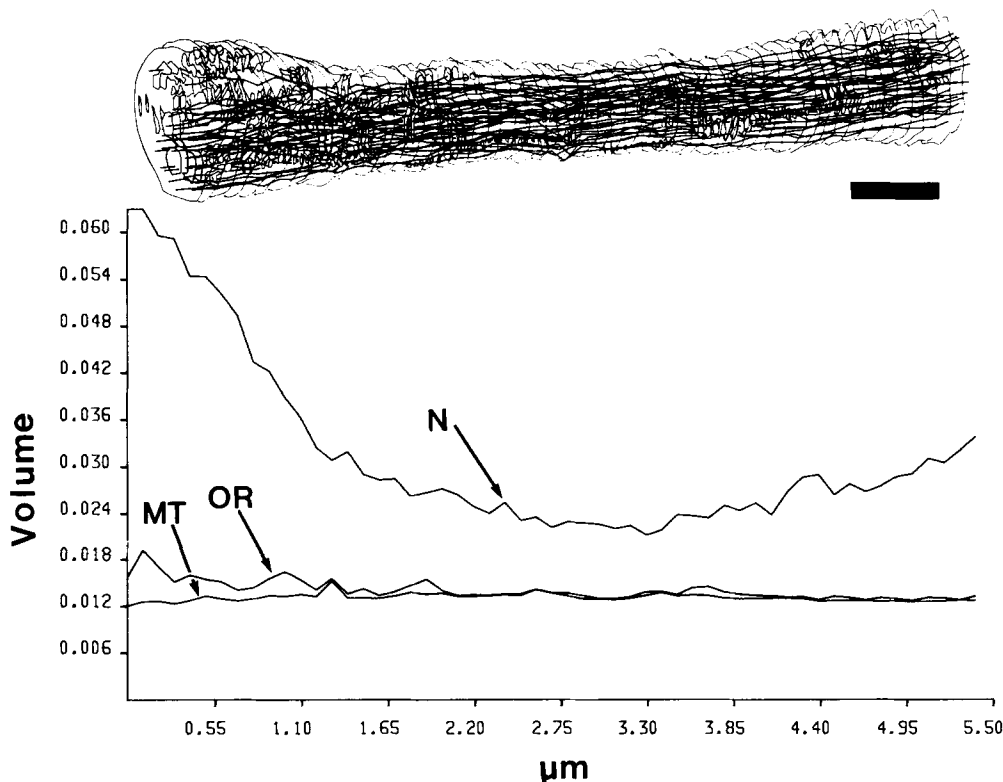


Figure 5. Regional volume distribution in a Nocodazole-insensitive neurite. A complete reconstruction of a neurite after 22 d in NGF and 1 h in 5 $\mu\text{g/ml}$ Nocodazole is shown on top. Below is a plot of the regional distribution of plasmalemmal and organelle volume over neurite length. Membranous organelle volume graph has been added to MT volume. Axes as in previous figure.

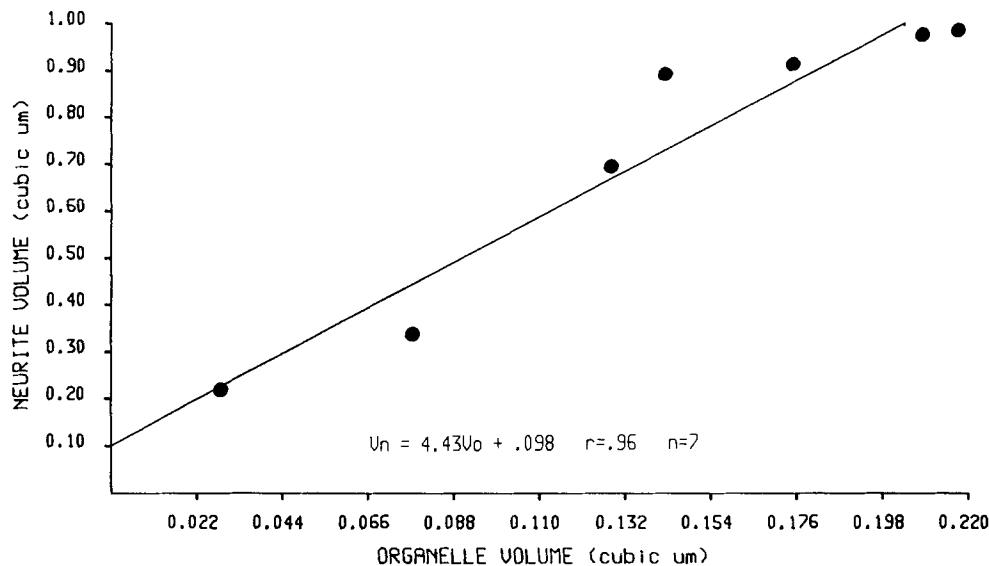


Figure 6. Linear regression of neurite volume to membranous organelle volume. This graph presents the regression results from the varicosity and organelle volume data from all reconstructed Nocodazole varicosities ($n = 7$). Varicosity volume is 4.43 times local organelle volume ($r = 0.96$). This data is already corrected for MT volume (see text).

ization by Nocodazole, while MTs in mature neurites are not. Other workers have noted that MTs stable to cold or Colchicine are not present early in neuronal differentiation but do appear later in development (Daniels, 1975; Mareck et al., 1980; Black and Greene, 1982). Our own results also show that the MTs found in mature neurites are far less sensitive to Nocodazole depolymerization than immature neurites. It is interesting that this change in sensitivity correlates well with changes in the behavior of the neurites as seen with time-lapse photography (Jacobs, 1985). In the early stages of differentiation, the neurites are highly labile and mobile, undergoing frequent extension and retraction. In the later stages of differentiation, (>8 d) they become quite stable with greatly reduced mobility.

Quantitative analysis at the serial EM level shows no change in MTs themselves or the MT exclusion cylinder that correlates with a change in MT stability. Yet, these results and the results of others show clear developmental differences. One possibility is that the MTs in mature neurites are stable simply because they are packed more closely in arrays. A second possibility is that some direct biochemical changes take place. It has been demonstrated that the presence of MT-associated proteins (MAPs) on MTs confer stability to depolymerization (Schliwa et al., 1981). Some MAPs preferentially associate with segments of MTs that are more labile to cold (Tytell et al., 1984). Cold insoluble MTs from retinal ganglion cell axons are composed of different tubulin isotypes than cold labile MTs (Brady et al., 1984). Different isotypes of MAPs and tubulins are expressed at different times in development (Francon et al., 1982; Ginsberg et al., 1983) and are produced in different amounts during differentiation (Feinstein et al., 1984). There is growing evidence, therefore, that regional specialization in MT or MAP structure can be a variable in the development of axons. It is possible that a change in composition of the MT or the MT exclusion cylinder (e.g., a change in MAPs), not detectable from electron micrographs, will modify the stability of MTs during development. This is a subject for further study at the biochemical level.

The Cytoplasmic Matrix

These results may at first seem inconsistent with other reports of a structural cytoskeletal matrix of actin filaments filling the spaces between organelles (Ellisman and Porter, 1980; Schnapp and Reese, 1982; Stossel, 1984). A view consistent with these data is simply that these actin filaments are not structural in the sense that they create or control neurite shape, but rather they may represent an active fill that occupies the volume controlled by and surrounding the organelles.

Important to this point is the observation that our obligatory volume differs from the exclusion zone of the MT in a fundamental way. The obligatory volume is a volume associated with the organelle—it is not a region or zone surrounding the organelle. For example, two organelles often come into close contact with each other (e.g., Figs. 2 and 3), and in many cases membranes may actually touch. When this occurs, their obligatory volumes simply add to create a larger total obligatory volume. This total obligatory volume simply surrounds the two organelles and is freely penetrated by MTs and intermediate filaments as well as other organelles. In contrast, the MT exclusion zone represents an absolute region surrounding the MT that can never be impinged upon by another organelle, or another MT.

A second important point comes from recent work with enhanced video methods. Several workers have demonstrated that organelles are moved along MTs (Brady et al., 1985; Schnapp et al., 1985). Additionally, time-lapse observations of non-experimental living material show that over a period of several hours, varicosities often move along the length of a neurite (Jacobs, 1985; and our personal observation). Upon fixation we find without exception, organelles associated with these moving varicosities. In the present study we have observed (Fig. 1) the active redistribution of volume as the MTs depolymerize and as the organelles move to form varicosities.

Thus, in simple mechanical terms, it would appear as if the cytoplasmic matrix that presumably occupies this obligatory volume is being moved or dragged along with the or-

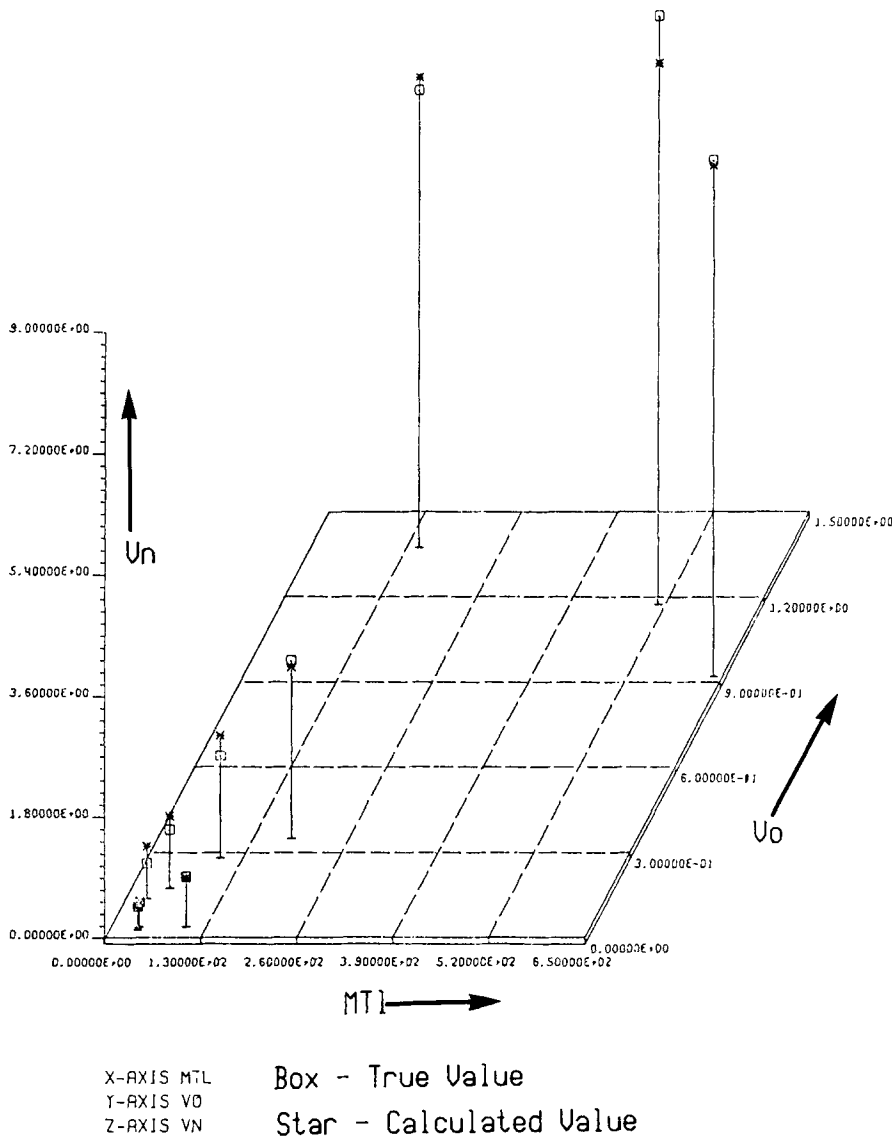


Figure 7. Three-dimensional plot of neurite volume predictions. Actual values of total neurite volume (V_n) from reconstructions (boxes) and the predicted value for the same reconstruction based upon the equation $V_n = 4.52 V_o + 0.0054 MTL$ (stars) are plotted together in this three-dimensional graph. Microtubule length (MTL) is the X-axis, organelle volume (V_o) is the Y-axis (depth), and neurite volume is the Z-axis (height).

ganelle. The interesting question raised by this suggestion is how do the organelles actually control the total volume and the distribution of the matrix? We discuss one possibility in the following section.

Membranous Organelle Contribution to Neurite Shape

The Nocodazole-treated neurites make it possible to examine the influence of membranous organelles on neurite shape in isolation from the MT contribution. In neurites without any MTs, or reduced numbers of MTs, neurite volume is exclusively associated with clusters of membranous organelles. As we have shown previously (Sasaki et al., 1983, 1984; Jacobs and Stevens, 1984, 1985), the neurite volume is much higher than that necessary to enclose the organelles. There is, in addition, a tight correlation ($r = 0.959$) between neurite varicosity volume and the total volume of organelles in the varicosity. The slope of the correlation for normal neurites of 4.52 is not statistically different from the slope of the regression for Nocodazole-treated neurites of 4.43. Addi-

tionally, as demonstrated in the preceding paper, the volumes of mature neurites and immature neurites show identical correlations with organelle volume.

Sasaki et al. (1984) demonstrated that the cytoplasmic volume associated with membranous organelles was always present in proportion to the volume of organelle. The basis of this quantitative dependence was suggested to be osmotic, because it seems to be rapid (i.e., it moves with organelle) and ubiquitous, and because membranous organelles are known to sequester ions such as calcium (Alnaes and Rahamimoff, 1975; Blaustein et al., 1978). It is well known that other systems such as the red blood cell (Ganong, 1971) go through very rapid, major shape changes due to simple shifts in distribution of ions that result in osmotic swings. Release of a solute from membranous organelles might result in a local influx of water, to maintain osmotic equilibrium. This might generate a swelling of the neurite around the organelle. One possibility is that the actin filaments contained in the cytoplasmic matrix expand and contract to fill this osmotic volume. These suggestions require further testing before serious conclusions may be drawn.

It is interesting that the relationship between membranous organelle volume and its associated neurite volume does not change in PC12 neurites, with Nocodazole treatment or development. Sasaki et al. (1984) encountered different slopes for the relationship between organelle volume and neurite volume for different amacrine cell types. Thus, it appears that this relationship might be a cell-specific phenotype that is invariant within a given cell type.

A Simple Model

In our original concept that the four basic factors, MT number, MT exclusion zones, organelles, and organelle obligatory volume are responsible for shape control in neurites is valid, we should be able to create a simple linear equation that accurately predicts total neurite volume under all conditions. Specifically: $V_n = OC \times V_o + MTc \times MTl$ where, V_n is the volume of the neurite, OC is the obligatory constant for the organelles or 4.52, V_o is the total organelle volume, MTc is the equivalent cylinder of the microtubules computed as $0.0054 \mu\text{m}^2$ per unit length, and MTl is the total length of microtubules in micrometers, or: $V_n = 4.52 V_o + 0.0054 MTl$. As a test of this equation we took organelle volume and MT length data from all complete reconstructions of normal neurites from this study and the data from the preceding paper and these were used to calculate a predicted total neurite volume. The results were then plotted and compared to the actual volumes in a three-dimensional graph (Fig. 7). As Fig. 7 illustrates, predicted values are very close to the original data. The calculated error from the predicted values ($S_{\text{diff}}/n-1 = 0.1863$) can be compared to the error term from the linear regression ($s = 0.2213$) in a standard F test statistic. The error terms are not statistically different ($P > 0.5$), which strongly supports the suggestion that the neurite volume equation adequately describes the real data.

Thus, given these constants for a specific cell type it is possible to predict cell volume and shape, given only the number and distribution of microtubules and the organelle volume. It is important to point out that this model will work only for small neurites that do not contain intermediate filaments. A more general equation would also include a term for intermediate filaments.

Summary

Using the MT-depolymerizing drug Nocodazole, we have experimentally manipulated the form of PC12 neurites. Both time-lapse photography and serial EM demonstrate that MT depolymerization leads to varicosity formation due to a clustering of membranous organelles in young neurites (NGF activated within 7 d). Tests on older neurites do not lead to pronounced varicosities. These data and data from previous papers has been combined in an attempt to predict quantitatively the volume and the shape of a neurite. The relationship is described mathematically by $V_n = 4.52 V_o + 0.0054 MTl$, where V_n is local neurite volume, V_o is organelle volume, and MTl is MT length (the constant, 0.0054 is μm^2), and 4.52 is the obligatory volume constant derived from serial EM studies. The equation predicts the total volume of neurites despite alterations of morphology due to Nocodazole and despite changes in morphology during development.

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