Microtubule Dynamics in Nerve Cells:
Analysis Using Microinjection of Biotinylated Tubulin into PC12 Cells

Shigeo Okabe and Nobutaka Hirokawa
Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, Hongo, Tokyo, 113, Japan

Abstract. To study microtubule (MT) dynamics in nerve cells, we microinjected biotin-labeled tubulin into the cell body of chemically fused and differentiated PC12 cells and performed the immunofluorescence or immunogold procedure using an anti-biotin antibody followed by secondary antibodies coupled to fluorescent dye or colloidal gold.

Incorporation of labeled subunits into the cytoskeleton of neurites was observed within minutes after microinjection. Serial electron microscopic reconstruction revealed that existing MTs in PC12 neurites incorporated labeled subunits mainly at their distal ends and the elongation rate of labeled segments was estimated to be <0.3 μm/min.

Overall organization of MTs in the nerve cells was different from that in undifferentiated cells such as fibroblasts. Namely, we have not identified any MT-organizing centers from which labeled MTs are emanating in the cell bodies of the injected cells. Stereo electron microscopy revealed that some fully labeled segments seemed to start in the close vicinity of electron dense material within the neurites. This suggests new nucleation off some structures in the neurites.

We have also studied the overall pattern of the incorporation of labeled subunits which extended progressively from the proximal part of the neurites toward their tips. To characterize the mechanism of tubulin incorporation, we have measured mean density of gold labeling per unit length of labeled segments at different parts of the neurites. The results indicate access of free tubulin subunits into the neurites and local incorporation into the neurite cytoskeleton.

Our results lead to the conclusion that MTs are not static polymers but dynamic structures that continue to elongate even within the differentiated nerve cell processes.

The neuronal cytoskeleton provides the framework that maintains the elaborate shape of the dendrites and the axon. The recently developed quick-freeze, deep-etch technique has revealed the cytoskeletal architecture of the neurites (11-16, 32, 33). The main cytoskeletal elements in nerve cells are microtubules (MTs) and neurofilaments which run longitudinally within the neurites (4, 36) and are cross-linked with each other by numerous short fibrils (11, 12, 14, 16a, 32). This static image of the extensive cross-linker system might be a snapshot of a dynamic structure and it is necessary to know the molecular dynamics of cytoskeletal proteins to further understand the morphogenesis and function of neuronal cytoskeleton.

Recent studies using the method of the microinjection of haptenic tubulin into living cells have revealed dynamics of MTs in vivo (23, 26-31, 34). Namely, incorporation of subunits occurs by the elongation of existing MTs and new nucleation off the centrosome (30, 34). The spatiotemporal pattern of MT turnover suggests that an in vitro model of MT dynamics called dynamic instability is consistent with the observed turnover in vivo (28, 30). These results are obtained from undifferentiated fibroblast cells. The dynamics in the terminally differentiated cells such as nerve cells and epithelial cells are completely unknown.

In this study we have microinjected biotinylated tubulin into fused PC12 cells which were differentiated into nerve cells by the administration of nerve growth factor (NGF) and followed the kinetics of incorporation using immunocytochemical probes and subsequent visualization in the light and electron microscope.

PC12 cells are tumor cells derived from rat adrenal chromaffin cells. They develop neuron-like processes upon exposure to NGF and differentiate into cholinergic sympathetic neurons within a few days (6, 7). In general, mammalian nerve cells in culture have small round cell bodies and thin perikarya which makes it difficult to microinject a sufficient amount of labeled proteins. To overcome the problem of small cell volume, we have fused PC12 cells chemically, treated them with NGF, and obtained large multinucle-
ate neuron-like cells (24). The large cell body, >100 μm in diameter, elevated the efficiency of microinjection and improved the images of labeled MTs after visualization with the immunocytological probes.

We found that existing MTs in the neurites incorporated labeled subunits at their distal ends and elongated at a considerably slower rate than that of fibroblast cells. However, we did find MT organization in neurons different from that of fibroblasts. The MT-organizing centers from which new MTs were emanating was not observed in the nerve cells. Instead, the results showed the possible existence of an MT-organizing structure within the neurites. The results also indicated the possibility of direct access of free tubulin subunits into the neurites and local incorporation into the cytoskeleton.

**Materials and Methods**

**Preparation of Biotinylated Tubulin**

Biotinylated tubulin was prepared according to the method of Kristofferson et al. (30). For microinjection, the stored sample was diluted with an injection buffer (50 mM potassium glutamate, 1 mM MgCl₂, pH 6.8) to 2.8 mg/ml, except where noted, clarified by centrifugation and used immediately.

**Cell Culture and Fusion**

PC12 cells (a kind gift of Y. Kuroda, Tokyo Metropolitan Institute for Neuroscience) were grown in DME supplemented with 5% precolostrum newborn calf serum (Mitsubishi Chemical Industries, Tokyo, Japan) and 5% heat-inactivated horse serum. Under the condition of constant flow rate of the sample solution, 25-cm² tissue culture flasks were seeded with 1 × 10⁵ per cm² and grown for ~24 h. The cells were washed briefly with serum-free DME and polyethylene glycol (PEG-2000, Wako Pure Chemical Industries, Osaka, Japan; 50% [wt/vol] in DME) was added and incubated for 1 min. The cells were washed four times with serum-free DME and incubated at 37°C for 30 min in DME plus 5% precolostrum newborn calf serum and 5% heat-inactivated horse serum.

Efficiency of fusion was checked under phase contrast microscope and Hank’s balanced salt solution containing 0.5% trypsin was added. The cells were incubated for 3 min, harvested, resuspended in DME containing 5% precolostrum newborn calf serum and 5% heat-inactivated horse serum, and plated onto polylysine-coated glass coverslips for immunofluorescence and electron microscopy. Cells were grown in the presence of 50 ng/ml NGF for 7-20 d before microinjection. In some cases, the staining process with tubulin antibody was omitted. The cells were examined with a Zeiss standard 14 epi-fluorescence microscope.

**Microinjection**

Microinjection was performed using the technique of Graessmann et al. (5). Glass capillaries were drawn out to ~1.0 μm diam and were filled with the sample from the tip. Cells were placed on a Nikon Diaphot inverted microscope and were pressure injected using a micromanipulator (Narishige Scientific Laboratory, Tokyo, Japan). During the injection procedure, the cells were videotaped on a time-lapse videotape recorder to determine exact incubation time after injection.

We estimated the volume injected per cell by the method of Graessmann et al. (5). Under the condition of constant flow rate of the sample solution, we recorded the movement of the meniscus within the pipette and determined the flow rate of the sample solution. Under the determined flow rate and the mean injection time per cell, we estimated that the volume injected per cell was <10⁻⁶ ml. Because the smallest cell we have used is ~50 μm diam and its height is ~2 μm, the calculated cell volume of this cell is ~1.4 × 10⁻⁸ ml. From these data, the injected volume was estimated to be <10% of the cell body volume.

**Immunofluorescence**

Cells were washed in a stabilizing buffer (80 mM KPi, 1 mM MgCl₂, 1 mM EGTA, 4% polyethylene glycol 6000, pH 6.8) containing 1 mM GTP and then permeabilized with 1% Triton X-100 in a stabilizing buffer plus 2.5 mM GTP at 37°C for 5 min.

The cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PEM buffer (100 mM KPi, 1 mM MgCl₂, 1 mM EGTA) containing 1 mM GTP for 10 min, treated with 1 mg/ml NaBH₄ in PBS for 7 min to quench the glutaraldehyde, and then placed in an antibody buffer (PBS containing 1% Triton X-100) for 30 min.

For double-label immunofluorescence, the cells were incubated successively with the following antibodies: rabbit anti-biotin (Enzo Biochem. Inc., New York, NY), fluorescein-conjugated goat anti-rabbit IgG, fluorescein-conjugated goat anti-goat IgG, mouse monoclonal anti-β-tubulin (35), and rhodamine-conjugated goat anti-mouse IgM. All antibodies except anti-tubulin were diluted 1:100 in an antibody buffer. Anti-tubulin antibody was a generous gift of D. J. Asai (Purdue University, Lafayette, IN) and diluted 1:500 in the same buffer. Rhodamine- or fluorescein-conjugated second antibodies were from Cappel Laboratories Inc. (Cocranville, PA).

In some cases, the staining process with tubulin antibody was omitted. The cells were washed in a stabilizing buffer and fixed with 0.3% glutaraldehyde in PEM buffer at 37°C for 10 min. After fixation, cells were permeabilized with 1% Triton X-100 in PBS for 5 min and unreacted glutaraldehyde was reduced with 1 mg/ml of NaBH₄. The staining process was the same except the rabbit anti-goat–goat antibody was omitted.

**Immunocytochemistry for Electron Microscopy**

The extraction process was the same with that for immunofluorescence and after extraction the cells were fixed with 1% glutaraldehyde in PEM buffer plus 1 mM GTP and 10 μM taxol at 37°C for 10 min. After fixation the cells were treated with 1 mg/ml of NaBH₄ for 7 min and then with TBS, pH 7.4, plus 1% Triton X-100 for 30 min. They were incubated for 2 h at room temperature with rabbit anti–biotin diluted 1:100, rinsed with TBS, pH 8.2, and then incubated with goat anti–rabbit IgG conjugated with 5 nm or 10 nm colloidal gold (Janssen Pharmaceutica, Beerse, Belgium) diluted 1:10 in TBS, pH 8.2, plus 1% BSA for ~12 h at 4°C. After incubation with the secondary antibody, the samples were washed with TBS, pH 8.2, fixed for 15 min in 0.1 M Na-cacodylate pH 7.4 with 2% glutaraldehyde, postfixed with 1% OsO₄ at 4°C for 1 h, dehydrated with an increasing concentration of ethanol, and embedded in Epon 812.

**Electron Microscopy**

Epon-embedded samples were excised and sectioned. For serial thin sections, the sample was thin sectioned and a ribbon of consecutive sections was picked up on a single-slot grid. Sections were stained with uranyl acetate and lead citrate and observed with a JEOL 1,200 EX electron microscope at 80 kV. For stereo electron microscopy, the sample was thick sectioned (250-500-nm sections). Sections were stained with 4% uranyl acetate for ~1 h and observed with a JEOL 1,200 EX electron microscope at 130-160 kV with a tilt of ±10°.

**Two-dimensional Reconstruction of Labeled Segments**

We have reconstructed labeled MTs according to the method of Joshi et al. (19). Labeled MTs and the neurite outline were traced on transparent sheets from the electron micrographs and alignment of the sheets were determined to maximize the number of matches between the labeled segments in the consecutive sections. After alignments between all consecutive sections were determined, all segments were traced to another transparent sheet and matched MTs were retouched to be continuous.

**Measurement of the Mean Labeling Density of the Labeled MTs within the Neurites**

The thin sections of injected cells were scanned and labeled neurites whose whole view could be obtained in one section were chosen for the measurement of the labeling density. About 1.0 × 1.0-μm regions were selected along the neurites and photographs were taken. Using these electron micrographs, labeled MTs that were separated unambiguously from other MTs...
Figure 1. Chemically fused and differentiated PC12 cells microinjected with biotin-tubulin, permeabilized shortly after injection and stained with anti-biotin antibody. (a) Rhodamine anti-tubulin staining showing total MTs of the cell permeabilized 61 s after injection. The neurites are stained homogeneously. (b) Fluorescein anti-biotin staining of the same cell in a showing incorporation of biotin-tubulin. A fibrous pattern of fluorescence is observed at the cell body. (c) Anti-tubulin staining of the cell permeabilized 134 s after injection. (d) The same cell in c showing anti-biotin staining. Labeled segments are observed in the peripheral region of the cell body and the base of the neurites. Bar, 20 μm.
and ran parallel to the section plane were picked up. The number of gold particles within 40 nm from the center of the MTs were counted and the labeling density was determined as number of gold particles/length of counted area along the MT.

**Results**

**Fusion and Cell Culture**

After treatment with polyethylene glycol >30% of the cells appeared to be multinucleate. The fused cells frequently contained at least 10 or more nuclei and were 100–200 μm in diameter. The response of these fused PC12 cells to NGF was similar to that of unfused cells and these cells began to extend the neurites within a few days. By several weeks of exposure to NGF, the fused PC12 cells formed long branching processes that extended for hundreds of micrometers (Fig. 2 c). For microinjection we have used cells that had a cell body >30 μm in diameter and long extending neurites.

**Indirect Immunofluorescence of Microinjected PC12 Cells**

When cells were permeabilized shortly after injection, anti-biotin staining was restricted to the cell body and the base of the neurites (Fig. 1). Cells shown in Fig. 1 were injected with biotin-tubulin at 7.0 mg/ml to obtain unambiguous images of fibrous staining with anti-biotin antibody. The fused PC12 cells usually had thick cell bodies and the fibrous pattern of staining with anti-biotin was unverifiable when biotin-tubulin was used at a low concentration. Fig. 1, a and b show a cell permeabilized 61 s after injection. This cell incorporated labeled subunits into short segments. Labeled segments were distributed randomly and emanation of labeled segments from a certain structure, such as a centrosome in fibroblast cells, was not identified. Fig. 1, c and d show a cell permeabilized 134 s after injection. Segments stained with anti-biotin existed in the peripheral region of the cell body and the base of the neurites. The uninjected cell stained with anti-tubulin was devoid of immunofluorescence with anti-biotin (Fig. 1 c, arrow).

As incubation time lengthened, the staining of the cell body increased and images of single MTs were obscured even in flat cells. At later times after injection, the cytoskeleton of neurites began to incorporate labeled subunits. When we compared the images of the anti-biotin staining of the cells permeabilized at different time-points, labeled subunits seemed to be incorporated into the cytoskeleton progressively from the proximal portion of the neurites (Fig. 2 b, d, and e). Boundaries between the labeled and unlabeled portions were not sharp and staining decreased gradually toward the tips of neurites. We could not identify individual MTs stained with anti-biotin at this transitional zone. Frequently, we observed neurites stained all along their length and shorter neurites tended to be labeled fully sooner after injection.

To quantitate the spreading of the incorporation of labeled subunits along the neurites, we determined the point where the staining of each neurite decreased to the level of background and measured the length along the neurite from the determined point to the base of the neurite. Fig. 4 a shows a plot of mean lengths of labeled portions for each of the injected cells versus time after injection. The mean length increased with time and the slope calculated from a least squares line constrained to pass through the origin was 6.9 μm/min.

To characterize the mechanism of the spreading of labeled subunits, we have fixed injected cells first and permeabilized them after fixation. Because free subunits would not be extracted, staining of these cells could be the superposition of that of incorporated subunits and unassembled subunits. Fig. 3 shows a cell fixed 194 s after injection. Labeled subunits spread within the neurites for ~50 μm within 200 s. We have also measured the length of the labeled part of neurites in the same manner with the extracted cells. The data are represented in Fig. 4 b and the rate of the spreading of whole labeled subunits is 12.3 μm/min. This rate is faster than that of incorporated subunits and it is likely that the spreading of unassembled subunits is a faster process than that of incorporated subunits.

When cells were permeabilized >1 h after injection, labeled subunits were evenly distributed throughout the cytoplasm except at the tips of neurites which are stained less prominently (Fig. 2 e). However, cells incubated for a few days began to show uneven distribution of labeled subunits again. This time, labeled subunits in the cell body progressively decreased and the staining of the neurites became stronger than that of the cell body (Fig. 2 f).

**Immunoelectron Microscopy of Microinjected PC12 Cells**

Fig. 5 shows a proximal portion of a PC12 neurite. This cell was permeabilized 223 s after injection. Gold particles adhered specifically on numerous MTs running parallel within the neurite. Heavily labeled MTs coexisted with unlabeled MTs and some heavily labeled MTs were continuous with unlabeled ones with a sharply defined boundary.

When we examined cells permeabilized within 3 min after injection, the distal part of the neurites frequently contained few labeled segments (Fig. 6 a). In agreement with the result of immunofluorescence, the distal part of the neurites contained more labeled segments at later times after injection (Fig. 6 b). There was no sharp boundary which separated the labeled and unlabeled areas of the neurites and the distribution of labeled segments became gradually sparse toward the distal ends. One additional point observed in immunoelectron microscopy was that the gold labeling per unit length of MTs always decreased toward the distal ends of neurites and MTs at the cell body were always labeled most heavily. This gradient of labeling density along the neurites became less prominent as the incubation time after injection increased.

To quantitate the above observation, we have measured the mean labeling densities of labeled segments at different parts of the neurite within one section. Fig. 7 shows plots of the mean labeling density per unit length versus distance from the cell body at different time points. As incubation time became longer, mean labeling density at the distal part of the neurites increased and the difference between the proximal and distal part of neurites became insignificant.

Fig. 7 also indicates that the region 60 μm away from the cell body already contained labeled segments at 223 s after injection. We calculated the expected spreading of labeled subunits within neurites from the results of immunofluorescence to be 20 μm away from the cell body at 223 s after in-
Figure 2. Immunofluorescence micrographs of fused PC12 cells injected with biotin-tubulin and incubated for various periods. Progressive incorporation of labeled subunits into the cytoskeleton of neurites was observed. (a) Rhodamine anti-tubulin staining showing total MTs of the cell permeabilized 450 s after injection. (b) Fluorescein anti-biotin staining of the same cell in a. (c) Phase-contrast image of the cell permeabilized 900 s after injection. (d) Incorporated biotin-tubulin of the same cell as in c. (e) Anti-biotin staining of the cell permeabilized 90 min after injection. The cell body and the neurites are stained homogeneously except at the tips of the neurites where staining is less prominent (arrow). (f) Anti-biotin staining of the cell permeabilized 48 h after injection. The staining of the cell body is less intense than that of the neurites. Bars, 50 μm.

Projection. (Fig. 4 a). This suggests that labeled segments with low labeling density could not be identified with immunofluorescence and the spreading of staining observed with immunofluorescence would be due to the increase of labeling density of individual MTs in the distal region.

Serial Electron Microscopic Reconstruction of Microinjected PC12 Cells

We cut serial sections of microinjected PC12 cells and undertook a two-dimensional reconstruction of labeled MTs in the neurites. Fig. 8 shows a part of the two-dimensional recon-
Figure 3. A fused PC12 cell injected with biotin-tubulin and fixed before permeabilization. (a) Phase-contrast image of the cell fixed 194 s after injection. (b) Anti-biotin staining of the same cell showing the distribution of assembled and free subunits together. Bar, 50 μm.

The construction of labeled MTs in a neurite of PC12 cell permeabilized 223 s after injection. Labeled segments that started and ended within the reconstructed region were represented and those that ran out of the reconstructed region were omitted. In this reconstructed space, we have identified 22 MTs that were labeled with colloidal gold along part of their length. 20 of them had labeled segments on the distal side and only two MTs incorporated labeled subunits at their proximal ends. The ends of the other reconstructed segments overlapped other structures in the neurite and it was unclear as to whether they were connected to unlabeled MTs.

It should be possible to know the dynamics of MT elongation in PC12 neurites from the distribution of length of the reconstructed labeled segments at a series of incubation times after injection. Because the staining of neurites spread toward the distal end with time, we chose the most proximal part of the neurites for reconstruction to minimize the difference of labeling density among the injected cells. Fig. 9 a shows histograms of reconstructed segment length at 111, 145, 223, and 509 s after injection, respectively. At earlier times after injection, labeled segments <0.4 μm predominated and as the incubation time lengthened, the peak of the histogram gradually shifted toward the right. It was also clear that some fast growing MTs also existed and elongated >1 μm within 2 min; but these MTs seemed to be a minor population.

Fig. b shows a plot of the mean segment lengths of reconstructed labeled segments versus time after injection. The mean segment length increased with time and the elongation rate roughly estimated from the plot was between 0.1 and 0.24 μm/min. To test whether long labeled segments could be reconstructed properly by our procedure, the lengths of the longest labeled segment in each cell were plotted against the time after injection (Fig. 9 c). Although the increase of the longest segment length between 223 and 509 s was smaller than the expected change estimated from the first three time points, it is clear that the longest segment length increased with time during first 500 s after injection.

Stereoelectron Microscopy of Microinjected, Gold-labeled, and Thick-sectioned PC12 Cells

To further characterize the three-dimensional organization of labeled and unlabeled segments, we have thick sectioned the injected cells and undertaken stereoelectron microscopy.

Figure 4. Mean length of the labeled portion of the neurites vs. time of incubation after injection with biotin-tubulin. We have measured the length along the neurite from its base (the point where the width of the neurite became <5 μm) to the point where the staining decreases to the level of background and determined the mean length for each injected cell. (a) Each point represents the average length of labeled portions of the neurites of a given cell. The cells were permeabilized after injection, fixed, and stained with anti-biotin. The line is a weighted linear least square fit constrained to pass through the origin. The slope is 6.9 μm/min. (b) The same plot as in a but the cells were first fixed and then permeabilized. The slope of a weighted linear least square line is 12.3 μm/min.
Fig. 5. Electron micrograph of the proximal neurite of fused PC12 cell permeabilized 223 s after injection with biotin-tubulin. Labeled subunits polymerized after injection were recognized by an antibody to biotin and colloidal gold. Both heavily labeled MTs and unlabeled MTs running in one direction are observed. Arrow indicates a juncture between labeled and unlabeled segments of an MT. The left side of this figure is distal to the cell body. (Inset) Higher magnification view of the juncture between the labeled and unlabeled segments of a MT. Bar, 0.1 μm.

Fig. 10 shows a neurite of the cell permeabilized 614 s after injection. Most of the labeled and unlabeled segments were oriented in the same direction. We have identified MTs that appeared to start within the section and run toward the distal end. Some of these MTs were fully labeled with colloidal gold along their length (Fig. 11 b), but the others were not labeled at all (Fig. 11 a). Their putative beginnings seemed to be in the close vicinity of electron dense material. Although this material was likely to be membranous organelles, permeabilization using a nonionic detergent obscured the fine structure of this material and precise characterization was difficult.

We have also examined the endings of MTs using stereo-electron microscopy. We observed some MTs that were fully labeled and appeared to terminate within the section (Fig. 11 c), but we have not seen any unlabeled MTs that appeared...
Figure 6. Electron micrographs of the neurites of PC12 cells injected with biotin-tubulin and stained with 5 nm colloidal gold anti-biotin showing progressive incorporation of labeled subunits into the neurite cytoskeleton. (a) Distal part of the neurite 31 μm away from the cell body. The cell was permeabilized 111 s after injection. Few labeled segments are observed and the labeling density of individual MTs is low. (b) Distal part of the neurite 60 μm away from the cell body. The cell was permeabilized 223 s after injection. The proportion of labeled MTs to unlabeled ones and labeling density of individual MTs are higher than those in a. Bar, 0.1 μm.

to end within the section. This suggests that most MTs in the neurites incorporated labeled subunits at their distal ends within several minutes after injection.

Discussion

Experimental Design

To reveal MT dynamics in nerve cells, we have microinjected biotin-labeled tubulin into the cell body of chemically fused and differentiated PC12 cells and visualized incorporated labeled subunits using immunocytochemical probes. Analysis of fused PC12 cells using the microinjection of haptenized cytoskeletal proteins seems to be a good model system for the study of the neuronal cytoskeleton.

Fused PC12 cells are found to express various neuronal properties identical to that of unfused cells. The fused cells respond to NGF within a few days and grow processes that extend for hundreds of micrometers (24). They contain catecholamines and express action potential mechanisms for Na⁺, Ca⁺, and K⁺ conductances. Furthermore, fused PC12 neurites, like those of unfused cells, have many MTs as a main cytoskeletal component and a small amount of intermediate filaments (Figs. 5, 6, and 10).

We have used biotinylated tubulin as a probe of MT dynamics in vivo. Previous studies indicated that biotinylated subunits retained the ability to assemble in vitro and did not diminish the assembly rate when biotin-tubulin was used in mixture with unlabeled tubulin (21). Because microinjected biotinylated subunits are mixed with a free tubulin pool of living cells, the effect of biotinylation on the assembly properties could be neglected in our experiments.

The microinjection procedure would be a possible step that interferes with the native dynamics of subunit incorporation. We estimate that the injected volume is <10% of the cell body volume (see Materials and Methods). We have injected biotin-tubulin at 2.8 mg/ml and estimated that the injection would result in an up to 14% increase of the total tubulin in the cell assuming that the total concentration of tubulin in the cell is 2 mg/ml (10). There is a possibility that this increase might cause spontaneous polymerization or additional assembly from the ends of MTs. However, Schulze and Kirsch-
Okabe and Hirokawa

Figure 7. Mean labeling densities per unit length of labeled segments vs. distance from the cell body. We have chosen several areas at different parts of the same neurite in one section and measured the mean labeling density per unit length of labeled segments. The cells were incubated for three different incubation times after injections and permeabilized. The error bars are ±1 SEM.

Figure 8. A two-dimensional reconstruction of a proximal neurite of the cell permeabilized 223 s after injection. Lines represent labeled MTs. Long segments and short segments coexisted in the same area. Arrowheads point to the MT ends that are continuous to the unlabeled segments. Bar, 1.0 μm.

Okabe and Hirokawa Microtubule Dynamics in Nerve Cells

659
**Figure 9.** Length distributions and growth rate of reconstructed labeled segments. (a) Histograms of the lengths of reconstructed labeled segments from cells permeabilized 111, 145, 223, and 509 s after injection. (b) Mean lengths of the reconstructed labeled segments in the proximal neurites vs. time of incubation after injection. The error bars are ±1 SEM. (c) The longest segment length in the proximal neurite vs. time of incubation after injection.

**Figure 10.** Stereo views of fully labeled and unlabeled MTs running in one direction in the neurites. The cell was permeabilized 614 s after injection. Three-dimensional organization of MTs and electron dense structures can be seen clearly. Bar, 0.1 μm.
Figure II. Stereo pair micrographs of thick-sectioned cells showing putative beginnings and endings of MTs in the neurites. The cell was permeabilized 614 s after injection. (a) An unlabeled MT appears to start within the section. The beginning of the MT is in the close vicinity of electron dense material (arrow). (b) A putative beginning of a fully labeled MT associated with electron dense material (arrow). (c) A fully labeled MT appears to terminate in the close vicinity of electron dense material (arrow). Bar, 0.1 \( \mu m \).
The Journal of Cell Biology, Volume 107, 1988 662

The movement of free tubulin subunits into the neurites is mainly due to the spreading of biotin-tubulin. Our model also suggests that neurite MTs are dynamic structures and assembly of subunits continues to occur within the neurites in situ. Several recent studies indicate that rapid reorganization of neurite MTs occurs after severe perturbation of the cytoskeleton (1, 8). Even without these perturbations, it is likely that the neurite cytoskeleton has some flexibility even after the termination of its growth. It is known that the axonal cytoskeleton itself is transported along the neurites as the slowest component of axonal transport (17) and should be separated at branch points of the axon into distinct bundles of the cytoskeleton. Disassembly and reassembly of the cytoskeleton would be necessary to separate a bundle of numerous MTs and neurofilaments entangling each other.

Tubulin Incorporation Sites in the Neurites

Our studies show the incorporation of labeled subunits at the distal ends of MTs in the neurites. It is known that MTs in
the neurites have uniform polarity orientation and their plus end is distal to the cell body (9). Thus the distal labeling of MTs in the neurites suggests the incorporation at the plus ends.

Several studies indicate that stable MT fragments serve as nucleating elements for MT assembly within the neurites (3, 8, 19). Whether new nucleation of MTs from any particular structures other than preexisting MTs occurs within the neurites is an important problem. We have frequently observed that MTs labeled along their length appear to have their beginnings in the close vicinity of electron dense structures. This may reflect new nucleation off some structures in the neurites, but there remains the possibility that the procedure of extraction before fixation affects the native spatial relationship between membranous organelles and MTs. Other mild extraction conditions should be used to confirm the possibility of new nucleation off some organelles in the neurites.

**MT Growth Rate in the Neurites**

Our studies suggest that mean elongation rate of MTs in the neurites would be <0.3 μm/min. Because serial reconstruction analysis was used to estimate the growth rate of MTs, it would be important to know whether our reconstruction procedure properly connected the corresponding segments of labeled MTs or not. To test whether long labeled segments were reconstructed by our procedure, we plotted the lengths of the longest labeled segment in each cell against time (Fig. 9 c). The longest segment length increases linearly up to 223 s and this suggests that long labeled MTs are reconstructed properly during first 200 s. Although the longest segment length at 509 s is not as much as the expected length estimated from the first three time-points, this would result from the limited length of the whole reconstructed region. The whole reconstructed region at 509 s is ~6.5 μm long along the neurite and labeled MTs longer than 5 μm would run out of the reconstructed region and would be omitted selectively from the measurement. Of course it is also possible that the real attenuation of MT growth or depolymerization of some MTs may occur during incubation and we can not discern either of these possibilities from our present data.

If long labeled segments are selectively excluded from the reconstruction, the estimated growth rate would be affected and become smaller than the real rate of elongation. We counted the number of omitted segments and it was always <10% of the total number of measured segments. The mean length would increase by up to 60% assuming that the lengths of all omitted segments are equal to that of the longest segment in the reconstructed region. This indicates that the estimated growth rate might increase up to 0.38 μm/min. However, the reported MT growth rate in fibroblasts, 3.6 μm/min (30), is 10-fold faster and this difference of the elongation rate would not be due to the artifact of reconstruction procedure. We therefore conclude that MT turnover in PC12 cells is quantitatively different from that in fibroblasts.

Fibroblasts are well-differentiated, unidifferentiated cells that change their shape and the organization of MTs through their course of cell cycle and their movement. In contrast, differentiated PC12 cells cease to divide and form long processes that hold their shape for several weeks (18), and it would not be surprising if MTs, in such a stable structure, exchange subunits more slowly. Although most MTs in fibroblasts are unstable, they contain stable MTs as a minor population (31). Since there is a small population of MTs in the neurites that elongate three- to fivefold faster than the mean elongation rate, it is likely that MT dynamics in differentiated neuron and fibroblast cells are not different qualitatively, but the population of stable MTs are different. If this differentiation of MTs reflect functional distinctions, fast elongating MTs in the neurites might play a role in motile activity such as the growing and branching of the neurites.

The results presented here suggest that neuronal MTs are not static polymers but dynamic structures that continue to elongate even within the neurites. A major goal of the study of the neuronal cytoskeleton is a full understanding of its molecular architecture and of the molecular dynamics of its formation and maintenance. Several recent studies using the quick-freeze, deep-etch technique combined with the immunocytochemical procedure (12, 16) have shown the localization of component proteins of the neuronal cytoskeleton on a molecular level and the information about the molecular architecture of the neuronal cytoskeleton has increased rapidly. In contrast, the study of the turnover of the cytoskeletal components in the neuron is just beginning and immunocytochemical visualization of chemically fused PC12 cells combined with the microinjection of haptenic cytoskeletal proteins is a promising model system for understanding the dynamics of the neuronal cytoskeleton in vivo.

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