

Rearrangement of Microtubule Polarity Orientation During Conversion of Dendrites to Axons in Cultured Pyramidal Neurons

Daisuke Takahashi,¹ Wenqian Yu,² Peter W. Baas,² Rika Kawai-Hirai,³ and Kensuke Hayashi^{1*}

¹*Life Science Institute, Sophia University, Tokyo, Japan*

²*Department of Neurobiology and Anatomy, Drexel University College of Medicine, Queen Lane, Philadelphia, United States of America*

³*Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma, Japan*

Axons and dendrites of neurons differ in the polarity orientation of their microtubules. Whereas the polarity orientation of microtubules in axons is uniform, with all plus ends distal, that in dendrites is nonuniform. The mechanisms responsible for establishment and maintenance of microtubule polarity orientation in neuronal processes remain unclear, however. We previously described a culture system in which dendrites of rat cortical neurons convert to axons. In the present study, we examined changes in microtubule polarity orientation in such dendrites. With the use of the hooking procedure and electron microscopy, we found that microtubule polarity orientation changed from nonuniform to uniform, with a plus end-distal arrangement, in dendrites that gave rise to axons during culture of neurons for 24 h. Microtubule polarity orientation remained nonuniform in dendrites that did not elongate. Axon regeneration at the dendritic tip thus triggered the disappearance of minus end-distal microtubules from dendrites. These minus end-distal microtubules also disappeared from dendrites during axon regeneration in the presence of inhibitors of actin polymerization, suggesting that actin-dependent transport of microtubules is not required for this process and implicating a previously unidentified mechanism in the establishment and maintenance of microtubule polarity orientation in neuronal processes. *Cell Motil. Cytoskeleton* 64: 347–359, 2007. © 2007 Wiley-Liss, Inc.

Key words: axon formation; axon regeneration; dendrite formation; cell polarity; hooking procedure

INTRODUCTION

Neurons are highly polarized cells, possessing two distinct types of process, dendrites and a single axon. These two types of process differ in morphology, protein content, and function [Craig and Banker, 1994]. They also differ in the polarity orientation of their microtubules [Baas et al., 1988; Burton, 1988]. Microtubule polarity orientation in axons is uniform, with the plus end of each microtubule being directed away from the cell body toward the growth cone. This arrangement of microtubules facilitates the continuous and rapid transport of material along the length of the axon. In contrast, microtubule polarity orientation in dendrites is nonuni-

Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology, Japan; Contract grant numbers: 16500193 and 16027207.

*Correspondence to: Kensuke Hayashi, Life Science Institute, Sophia University, 7-1 Kioicho, Chiyoda-ku, Tokyo 102-8554, Japan. E-mail: kensuk-h@sophia.ac.jp

Received 20 November 2006; Accepted 11 January 2007

Published online 6 March 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cm.20188

form, with about half of the microtubules having their plus ends distal and half their minus ends distal.

Various signaling molecules are implicated in the early events of neuronal polarization that determines the neurite fate, axons vs. dendrites [Arimura and Kaibuchi, 2005], but little is known of how these molecules affect microtubule polarity orientation in neuronal processes. Microtubule polarity orientation in such processes is an important determinant of their differentiation and maintenance because of the role of microtubules as guiding rails for membrane traffic and the delivery of dendrite- or axon-specific proteins to their appropriate locations [Burack et al., 2000; Setou et al., 2003]. It is thus important to understand the mechanisms that underlie the establishment and maintenance of microtubule polarity orientation in neuronal processes in order to understand those responsible for the establishment and maintenance of neuronal polarity itself.

The uniform polarity orientation of microtubules in axons is thought to be achieved in part through actin- and dynein-dependent microtubule transport [Ahmad et al., 1998] and as a result of the enhanced polymerization of microtubules at growth cones [Fukata et al., 2002]. These two mechanisms thus result in the addition of plus end-distal microtubules to the axon as it elongates. The nonuniform polarity orientation of microtubules in dendrites is established by transport of minus end-distal microtubules, a process likely mediated by kinesin-like motor proteins [Sharp et al., 1997], into these processes during their development [Baas et al., 1989]. The mechanisms responsible for the maintenance of microtubule polarity orientation in axons and dendrites, however, remain unclear.

An experimental system in which neuronal processes change their identity is ideal for studying the mechanisms by which microtubule polarity orientation is maintained in neuronal processes. We previously described a culture system in which dendrites convert to axons [Hayashi et al., 2002]. We isolated neurons that bore an apical dendrite from the neonatal rat cerebral cortex. When cultured, ~65% of these neurons regenerated an axon from the dendritic tip. We found that not only the regenerated axon but also the distal half of the original dendrite became positive for immunological markers of axons [Hayashi et al., 2002]. The regeneration of an axon from the dendritic tip required 5 h longer after plating than did the regeneration of axons from the cell body. This delay likely reflects a reorganization of the dendritic cytoskeleton required to support the efficient membrane transport needed for axonal elongation.

We have now examined changes in microtubule polarity orientation in dendrites during their conversion to axons. We found that the minus end-distal microtubules present in the original dendrites disappeared within

24 h of the onset of culture. This change also occurred in the presence of inhibitors of actin polymerization, indicating that it does not require microtubule movement along actin filaments. Our results thus suggest the existence of a previously unknown mechanism for the arrangement of microtubules in neuronal processes.

MATERIALS AND METHODS

Isolation and Culture of Pyramidal Neurons

The cerebral cortex of neonatal Wistar rats was cut into pieces and incubated for 30 min at 30°C in a buffered saline solution [15 mM HEPES (pH 7.0), 120 mM NaCl, 5 mM KCl, 0.7 mM KH₂PO₄, 25 mM glucose, 60 mM sucrose] supplemented with 0.15% trypsin (Difco, Detroit, MI) and 0.1% collagenase (Wako, Osaka, Japan). The tissue fragments were then gently dissociated with a pipette in the same buffered saline solution supplemented with pyruvate (0.1 mg/ml), glutathione (50 µg/ml), *N*-acetylcysteine (25 µg/ml), trisodium citrate (0.1 mg/ml), transferrin (50 µg/ml) (Wako), superoxide dismutase (10 U/ml) (Wako), DNase 1 (10 mg/ml) (Sigma, St. Louis, MO), soybean trypsin inhibitor (10 mg/ml) (Wako), 1 mM CaCl₂, and 1 mM MgCl₂. Dissociated cells were suspended in Neurobasal Medium (Invitrogen, Carlsbad, CA) supplemented with 0.5 mM glutamine and 2% B27 supplement (Invitrogen) and then plated at a density of 2 × 10⁴/ml on tissue culture dishes that had been treated with poly-D-lysine (Sigma) and 10% fetal bovine serum. After incubation for 1 h, when neurons had attached to the dish, we selected those that bore an apical dendrite with a length of ~100 µm, photographed them, and cultured them. For depletion of actin filaments, cytochalasin D or E (Sigma) or latrunculin A (Molecular Probes, Eugene, OR) was added to the culture medium at a final concentration of 0.5 µM at the beginning of culture.

Immunostaining of Neurons

For double staining of neurons for axonal and dendritic markers, neurons were fixed on ice for 30 min with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 min, and incubated for 1 h at room temperature with 5% normal donkey serum in PBS. They are then incubated for 1 h with rabbit polyclonal antibodies to microtubule-associated protein 2 (MAP2) (Chemicon, Temecula, CA) and either a mouse monoclonal antibody to phosphorylated neurofilaments (SMI-31; Covance, Berkeley, CA) or a mouse monoclonal antibody to dephosphorylated tau (Tau-1, Chemicon), washed with 0.1% Triton X-100 in PBS for 30 min, incubated for 1 h with fluorescein isothiocya-

nate-conjugated goat antibodies to mouse immunoglobulin G (Jackson Immuno Research, West Grove, PA) and rhodamine-conjugated goat antibodies to rabbit immunoglobulin G (Jackson Immuno Research), and washed again. For double staining of actin filaments and tubulin, neurons were fixed for 30 min at room temperature with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and exposed to 0.1% Triton X-100 in PBS for 15 min. They were then incubated for 1 h with rhodamine-conjugated phalloidin (Molecular Probes) and a fluorescein isothiocyanate-conjugated mouse monoclonal antibody to β -tubulin (clone TUB2.1, Sigma) before washing with 0.1% Triton X-100 in PBS for 30 min. Specimens were observed with a fluorescence microscope (Olympus IX-70).

Hooking Procedure and Electron Microscopy

The polarity orientation of microtubules was determined by the hooking protocol [Baas et al., 1987]. Neurons were exposed to porcine brain tubulin (1.2 mg/ml) in an assembly buffer [0.5 M PIPES (pH 6.9), 2.5% dimethyl sulfoxide, 0.1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM GTP, 1% saponin] for 3 min at 37°C and then to a fixative solution [0.1 M sodium cacodylate (pH 7.4), 0.2% tannic acid, 2% glutaraldehyde] for 15 min at room temperature. After washing twice with 0.1 M sodium cacodylate (pH 7.4), the neurons were treated with 1% osmium tetroxide for 15 min, dehydrated with a graded series of ethanol solutions (50, 70, and 100%), embedded in epoxy resin, and cured at 60°C for 24 h. Cross-sections of processes were prepared and analyzed with an electron microscope (EM 906; Carl Zeiss, Oberkochen, Germany). As seen by an observer facing the tip of the process, clockwise hooks indicate that microtubules are oriented with their plus ends distal relative to the cell body and counterclockwise hooks indicate those with their minus ends distal.

Time-Lapse Video Microscopy

Culture dishes containing neurons were secured in a chamber placed on an Olympus inverted phase-contrast microscope (IX50) equipped with a system for control of temperature and CO_2 concentration. Images were acquired at 6-min intervals with a Cool SNAP CCD digital camera (Roper Scientific, Trenton, NJ) controlled by IPLab software (Scanalytics, Fairfax, VA). The sequence of PICT images was converted into a Quick Time file. The procedure was described in detail in a previous study [Hayashi et al., 2003].

RESULTS

Conversion of Dendrites to Axons

We previously described a culture system in which dendrites convert to axons [Hayashi et al., 2002]. Neurons

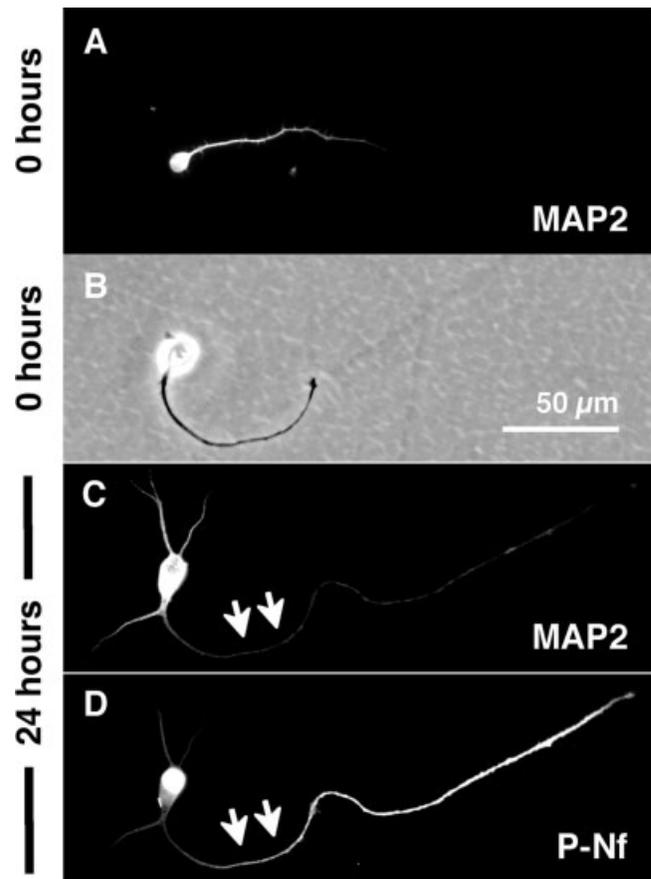


Fig. 1. Immunocytofluorescence analysis of process identity in cultured dendrite-bearing neurons isolated from neonatal rat cortex. (A) Freshly isolated neuron with a long process stained for the dendritic marker MAP2. (B) Phase-contrast micrograph of a dendrite-bearing neuron before culture. (C, D) The neuron shown in (B) was cultured for 24 h and then stained for both MAP2 (C) and an axonal marker (D), phosphorylated neurofilaments (P-Nf). Not only the regenerated process but also the distal half of the original dendrite (arrows) were negative for the dendritic marker and positive for the axonal marker.

were isolated by mild dissociation of neonatal rat cerebral cortex. We selected neurons bearing a thick process with a length of $\sim 100 \mu m$ (Figs. 1A and 1B). This process had several characteristics of apical dendrites, so we call it the original dendrite. Beside the original dendrite, these neurons sometimes possessed several pre-existing minor processes. When we cultured these neurons, additional minor processes were newly formed on the cell body. Small growth cones were found at the tip of the all processes including the original dendrite, pre-existing minor processes and newly formed minor processes. Only one of them suddenly enlarged within 24 h and elongated to form an axon. In our previous study, we analyzed the neurons after 3 days of culture and found that axons were often formed from the tip of the original dendrites and that the distal portion of the original dendrite became positive for axonal markers [Hayashi

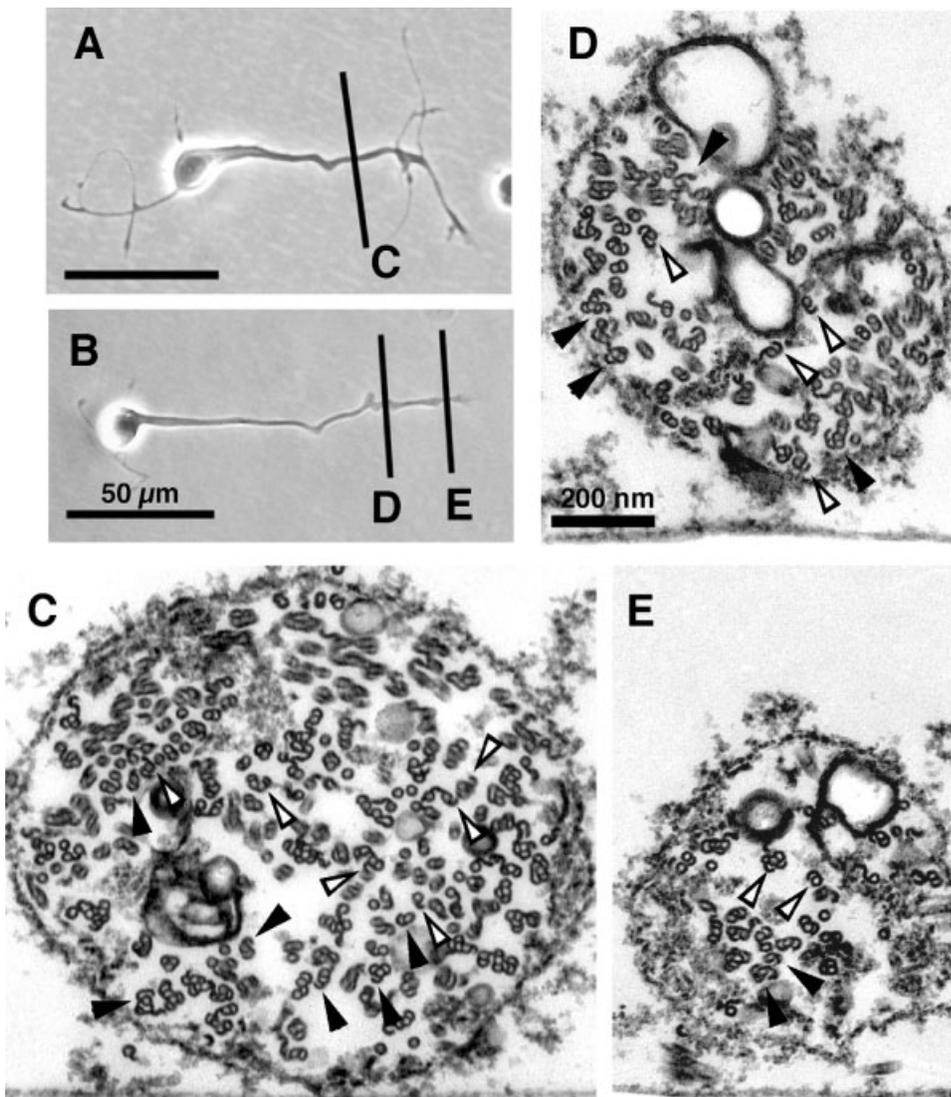


Fig. 2. Microtubule polarity orientation in the original dendrite of neurons before culture. (A, B) Phase-contrast images of dendrite-bearing neurons before culture. The vertical bars indicate the positions of the cross-sections shown in (C) through (E). (C) Section of the mid-region of the dendrite shown in (A). Arrowheads and open arrowheads indicate examples of plus end-distal and minus end-distal microtubules, respectively, identified on the basis of the orientation of the hooks. (D, E) Distal sections of the dendrite shown in (B), with microtubule polarity orientation indicated as in (C). Microtubule polarity orientation was nonuniform in all three sections.

et al., 2002]. In the present study, we examined the dendrites of these neurons after culture for 24 h. About 75% of neurons (44 of 59) exhibited axon regeneration from the tip of the original dendrite, a value similar to that (66%) apparent after culture of the neurons for 3 days in our previous study [Hayashi et al., 2002]. Axonal conversion of the distal region of the original dendrite was also evident in such cases by immunocytofluorescence analysis; the distal region of the dendrite thus became negative for MAP2 and positive for phosphorylated neurofilaments (Figs. 1C and 1D).

To investigate changes in microtubule polarity orientation in the original dendrites, we first analyzed the orientation at the onset of culture with the use of the hooking procedure (see Materials and Methods). Transverse sections of the mid-region of the original dendrite, $\sim 50 \mu\text{m}$ from the cell body, were examined (Fig. 2A). Less than half of microtubules had unambiguous hooks;

among these, 38%–48% of microtubules had counterclockwise hooks (minus end-distal microtubules), with the remainder manifesting clockwise hooks (plus end-distal microtubules) (Fig. 2C, Table 1). Examination of more distal regions of the dendrite, including those $\sim 25 \mu\text{m}$ from the tip (Figs. 2B and 2D) and immediately behind the tip (Figs. 2B and 2E), revealed that microtubule polarity orientation was also nonuniform in these regions (40 and 25% minus end-distal microtubules, respectively). This nonuniform polarity orientation of microtubules in the most distal region of dendrites is consistent with the previous report that two population of microtubules with opposite polarity were seen in both the proximal and distal region of dendrites (Burton, 1988), but is different from the report that microtubules in the distal portion of dendrites of cultured neurons were uniformly plus end-distal [Baas et al., 1988]. It is possible that the

TABLE 1. Summary of Microtubule Polarity Orientation in the Mid-region of Dendrites

Addition	Culture time (h)	Plus end-distal	Minus end-distal
Original dendrite that regenerated an axon			
None	0	23	14 (38%)
		16	15 (48%)
	11	12	9 (43%)
		19	3 (14%)
		10	9 (47%)
		7	4 (36%)
	24	16	3 (16%)
		16	1 (6%)
		10	0 (0%)
		26	1 (4%)
72	26	1 (4%)	
Original dendrite that did not elongate			
None	24	25	19 (43%)
	72	14	10 (42%)
		13	19 (59%)
Dendrite generated from a minor process			
None	24	30	24 (44%)
Original dendrite that regenerated an axon			
Cytochalasin D	24	55	4 (7%)
		13	0 (0%)
		12	0 (0%)
		11	1 (8%)
		31	3 (9%)
Cytochalasin E	24	31	3 (9%)

Data represent the number (percentage) of microtubules with the indicated polarity orientation in an individual process.

microtubule polarity pattern along the length of the dendrite depends on the particular developmental stage of the dendrite, such as whether or not it is growing.

We next analyzed microtubule polarity orientation in dendrites after culture of neurons for 24 h (Fig. 3). In neurons that regenerated an axon from the tip of the dendrite, 84%–100% of microtubules in the mid-region of the original dendrite were plus end-distal (Figs. 3A, 3B, and 3E; Table 1), indicating that microtubule polarity orientation in the original dendrite changed during 24 h of culture. Although the onset of axon regeneration from the dendritic tip occurs on average >13 h after plating [Hayashi et al., 2002], some neurons exhibited obvious axonal regeneration after culture for only 11 h. Examination of the mid-region of the original dendrites of such neurons revealed that microtubule polarity orientation was nonuniform; in one of the three neurons examined, however, the proportion of minus end-distal microtubules had already decreased to 14% (Figs. 4A–4C, Table 1). The change in microtubule polarity orientation thus appears to begin at an early stage of axon regeneration.

Microtubule polarity orientation remained nonuniform in the proximal region of the original dendrite after culture of neurons for 24 h (Figs. 3B and 3C; 27% minus end-distal microtubules in this example). Although it

was hard to identify the original dendrite among the complicated network of processes in old cultures, we could find one example of the original dendrite that elongated to form an axon after 3 days of culture. Almost all microtubules were plus end-distal in the most proximal region of the original dendrite (Figs. 4D–4F; 5% minus end-distal microtubules in this example) as well as in the mid-region (Figs. 4E and 4G; Table 1). These observations suggest that the change in microtubule polarity orientation propagated proximally between ~11 and 72 h of culture.

To exclude the possibility that our culture conditions were not sufficient for dendrites to maintain nonuniform polarity orientation of microtubules, we analyzed neurons that regenerated an axon from a pre-existing minor process rather than from the dendrite (Figs. 5A and 5B). In such instances, the dendrite did not elongate and retained a dendritic morphology. Microtubule polarity orientation in the mid-region of such dendrites was found to be nonuniform after culture of neurons for 24 h (Figs. 5A–5C, Table 1) or 3 days (Table 1), indicating that nonuniform polarity orientation of the dendrite is maintained under our culture conditions unless an axon is regenerated from the tip. In some neurons, a pre-existing minor process became longer and thicker but retained a tapering morphology and did not develop a growth cone at its tip, indicative of differentiation into a dendrite. The polarity orientation of microtubules in the mid-region of such dendrites was also nonuniform after culture for 24 h (Figs. 5D–5F, Table 1), indicating that a nonuniform polarity orientation can be established within this time period.

Axon Regeneration in the Presence of Actin Polymerization Inhibitors

While it is conceivable that the disappearance of minus end-distal microtubules from dendrites that gave rise to axons might be attributable to selective depolymerization of such microtubules, earlier studies suggest that minus-end distal microtubules can be cleared from dendrites by the selective movement of minus-end-distal microtubules back to the cell body [Yu et al., 2000]. This movement would presumably be based on dynein motors, and would be generated at least in part against the actin cytoskeleton [Dillman et al., 1996; Ahmad et al., 1998; Hasaka et al., 2004; He et al., 2005]. Therefore, we reasoned that a simple test of this model for our present experimental system would be to determine whether depletion of actin filaments was deleterious to the disappearance of minus-end-distal microtubules.

Actin filaments were detected along the length of the dendritic membrane and at growth cones in neurons that had regenerated an axon after culture for 24 h (Fig. 6A). Neurons cultured for 24 h in the presence of

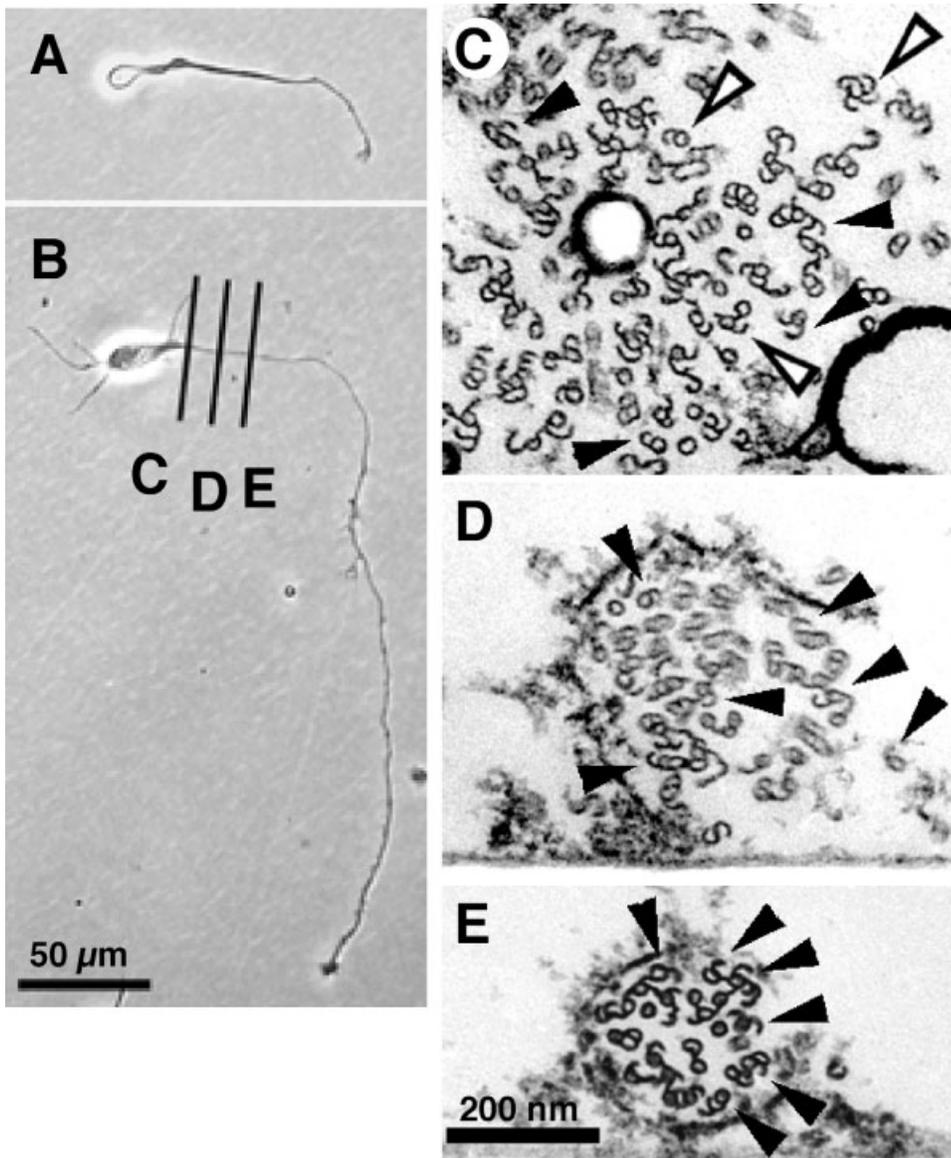


Fig. 3. Microtubule polarity orientation in the original dendrite after culture for 24 h of a neuron that regenerated an axon from the dendritic tip. (A) Phase-contrast image of a dendrite-bearing neuron before culture. (B) The neuron shown in (A) after culture for 24 h. The original dendrite elongated and regenerated an axon. (C–E) Cross-sections of the process of the neuron shown in (B) at the positions indicated by the vertical bars; the section in (E) corresponds to the mid-region of the original dendrite, whereas those in (D) and (C) represent more proximal regions. Arrowheads and open arrowheads indicate examples of plus end-distal and minus end-distal microtubules, respectively, identified on the basis of the orientation of the hooks. Most microtubules are plus end-distal in (D) and (E), whereas microtubule polarity orientation is nonuniform in (C).

inhibitors of actin polymerization (cytochalasin D, cytochalasin E, or latrunculin A) manifested only sparse punctate staining with phalloidin at these sites (Figs. 6B–6D). Although filopodial and lamellipodial structures were not evident, a new process appeared and elongated during culture of neurons in the presence of these inhibitors; in 94% of neurons (46 of 49), this new process appeared at the tip of the dendrite. Both these new processes and the distal region of the original dendrite were negative for the dendritic marker MAP2 and positive for axonal markers (phosphorylated neurofilaments and dephosphorylated tau) (Figs. 6E–6H).

Time-lapse analysis of the growth of these regenerating processes in the presence of cytochalasin D or cytochalasin E revealed that the original dendrite did not retract before elongation of the new process and that the

process elongated without formation of a growth cone (Fig. 7). Examination of microtubule polarity orientation in the mid-region of original dendrites that exhibited process elongation after culture for 24 h in the presence of cytochalasin D or cytochalasin E revealed that most (91%–100%) microtubules were plus end-distal (Fig. 8, Table 1), indicating that minus end-distal microtubules had disappeared even in the absence of actin filaments.

DISCUSSION

Two mechanisms have been proposed for the establishment of uniform polarity orientation of microtubules in axons. First, tubulin polymerization is enhanced at the plus ends of microtubules at growth cones. Such enhancement is thought to result from stabilization of

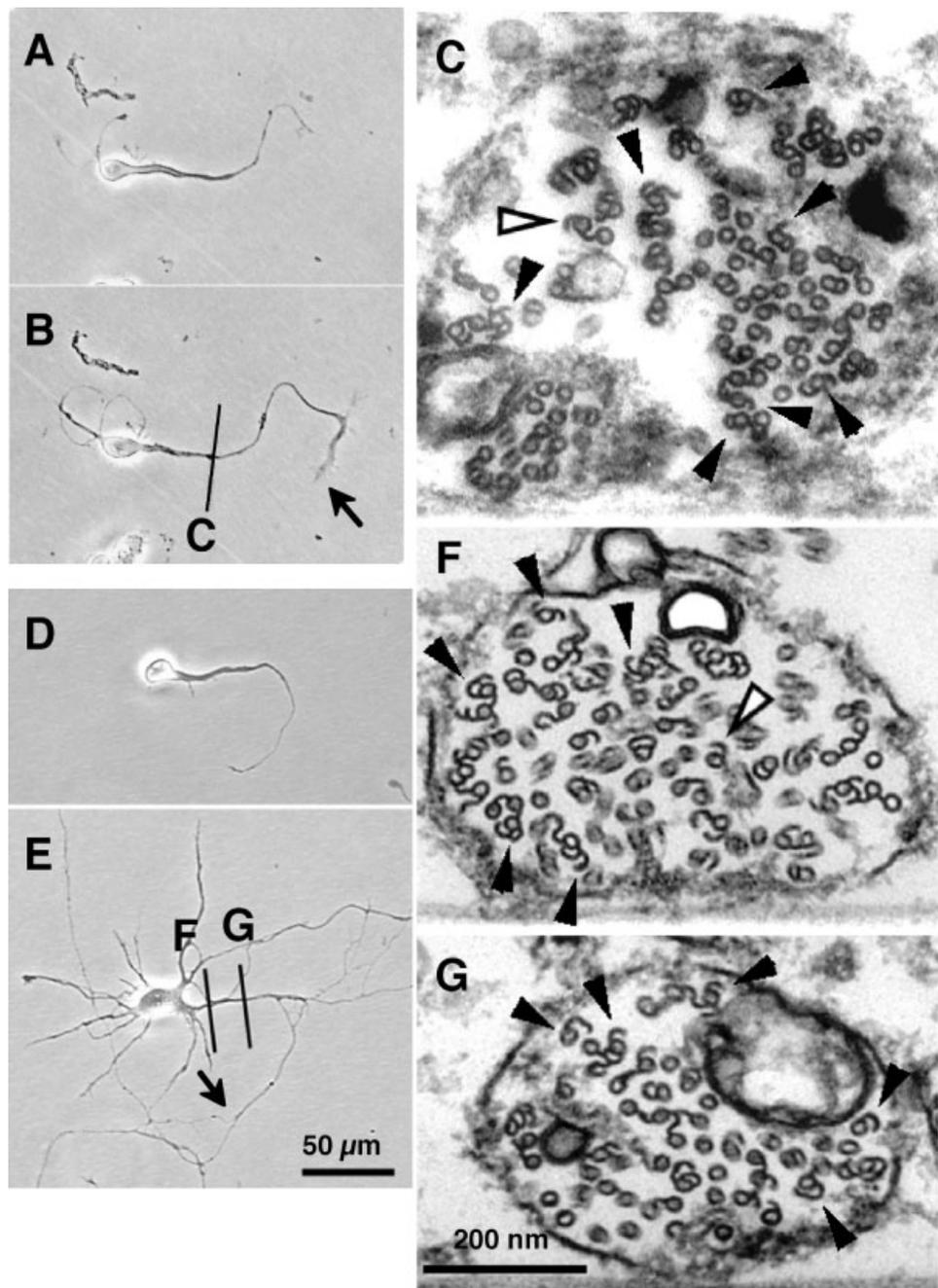


Fig. 4. Microtubule polarity orientation in the original dendrite after culture for 11 h or 3 days of neurons that regenerated an axon from the dendritic tip. (A–C) Phase-contrast images of a dendrite-bearing neuron before culture (A) and after culture for 11 h (B). The original dendrite elongated and developed a growth cone at the tip (arrow). A cross-section of the process at the position indicated in (B) is shown in (C). Arrowheads and open arrowheads indicate examples of plus end-distal and minus end-distal microtubules, respectively; most microtubules are plus end-distal. (D–G) Phase-contrast images of a dendrite-bearing neuron before culture (D) and after culture for 3 days (E). The original dendrite elongated (arrow) and regenerated an axon. Cross-sections of the process in the mid-region and a more proximal region of the original dendrite indicated in (E) are shown in (G) and (F), respectively. In both sections, most of the microtubules are plus end-distal.

the plus ends with plus end-tracking proteins such as the EB1-APC and CLIP-CLASP complexes [Akhmanova and Hoogenraad, 2005] and from promotion of microtubule assembly by CRMP2 [Fukata et al., 2002], which mediates tubulin transport toward the plus ends of microtubules [Kimura et al., 2005]. At the end of microtubule array, plus ends of plus end-distal microtubules, but not minus ends of minus end-microtubules, elongate to form an axon, which thus contains only plus end-distal microtubules. Second, plus end-distal microtubules are sup-

plied to axons by transportation [Ahmad et al., 1998]. Although most microtubules in the axon are stationary, a substantial number of them are not [Wang and Brown, 2002; Hasaka et al., 2004; He et al., 2005]. Actin- and dynein-dependent transport of microtubules is thought to be especially important for establishment of uniform polarity orientation of microtubules in axons because it is polarity specific. Microtubules are thought to slide, at least in part, on dynein-dynactin complexes immobilized on the actin matrix with their plus end leading, so that plus end-distal

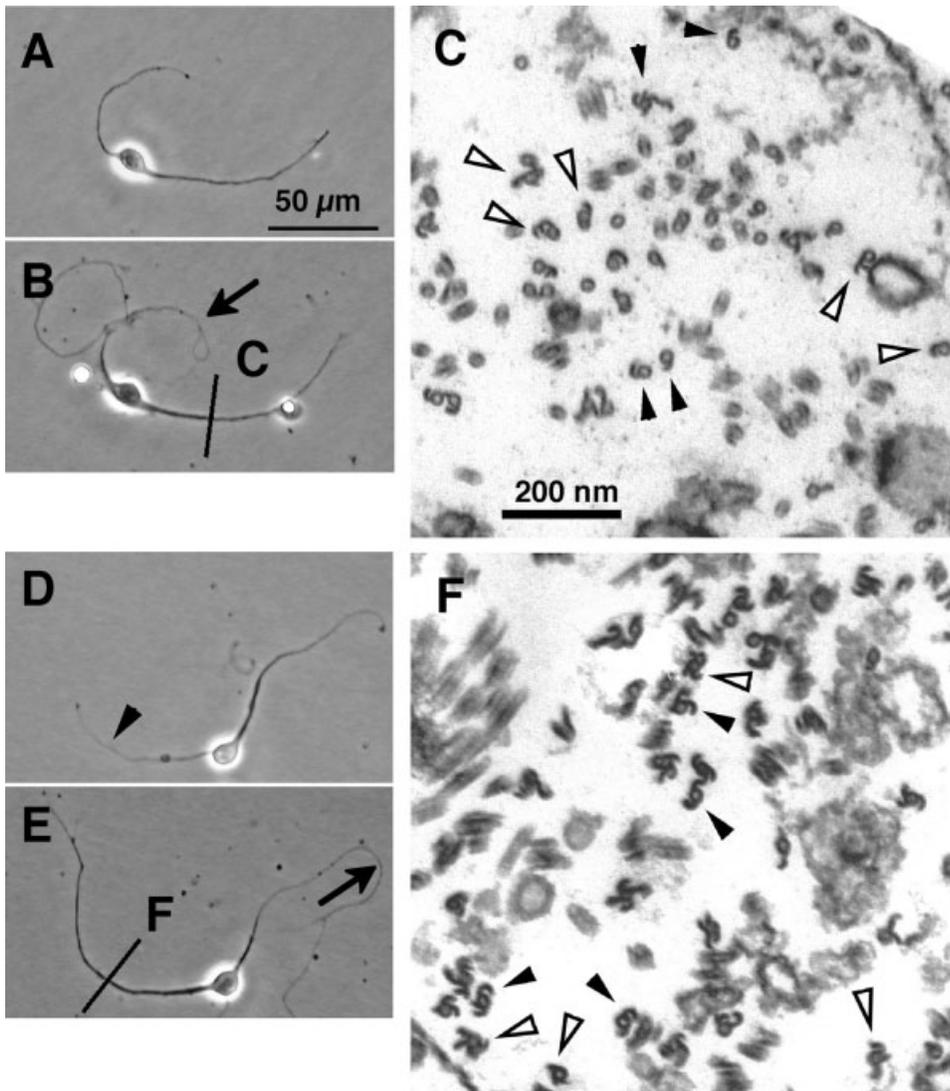


Fig. 5. Microtubule polarity orientation in dendrites of neurons cultured for 24 h. (A–C) Phase-contrast images of a dendrite-bearing neuron before culture (A) and after culture for 24 h (B). The original dendrite did not elongate but an axon regenerated from a pre-existing minor process (arrow). A cross-section of the dendrite at the position indicated in (B) is shown in (C). Arrowheads and open arrowheads indicate examples of plus end-distal and minus end-distal microtubules, respectively; the polarity orientation of microtubules was nonuniform. (D–F) Phase-contrast images of a dendrite-bearing neuron before culture (D) and after culture for 24 h (E). The original dendrite elongated and regenerated an axon (arrow), whereas a pre-existing minor process (arrowhead) matured into a dendrite. (F) Cross-section of the new dendrite at the position indicated in (E), revealing a nonuniform polarity orientation of microtubules.

microtubules move distally in axons. Impairment of this movement inhibits both outward progression of microtubules in cells and outgrowth of axons [Ahmad et al., 1998].

These two mechanisms, however, are not able to account for our present observations. Time-lapse analysis showed that the change in microtubule polarity orientation from nonuniform to uniform in original dendrites that give rise to axons does not result from retraction of the dendrite and regrowth of a new axon. Furthermore, the change in polarity orientation was observed in the presence of actin polymerization inhibitors. Although we cannot completely exclude the possibility that small amount of actin filaments below the sensitivity of phalloidin-staining remained, our data indicate that transport of microtubules on intact network of actin filaments is not required for the change in microtubule polarity orientation. Our results therefore suggest the existence of a previously unknown mechanism for removal of minus

end-distal microtubules and consequent establishment and maintenance of a uniform arrangement of microtubules in axons.

Role of Actin Filaments on Axon Regeneration

About 70% of neurons in our present and previous [Hayashi et al., 2002] studies exhibited axon regeneration from the tip of the original dendrite, although growth cone-like accumulations of actin filaments were observed not only at the tip of the dendrite but also at the tip of minor processes of the cell body soon after plating [Hayashi et al., 2002]. It is very interesting that the probability of axon regeneration from the original dendrite increased to 94% in the presence of actin polymerization inhibitors. This indicates that actin filaments at the tip of the original dendrites have an inhibitory effect on the elongation of dendrites, while they are the main structure of growth cones. A role for actin filaments in mainte-

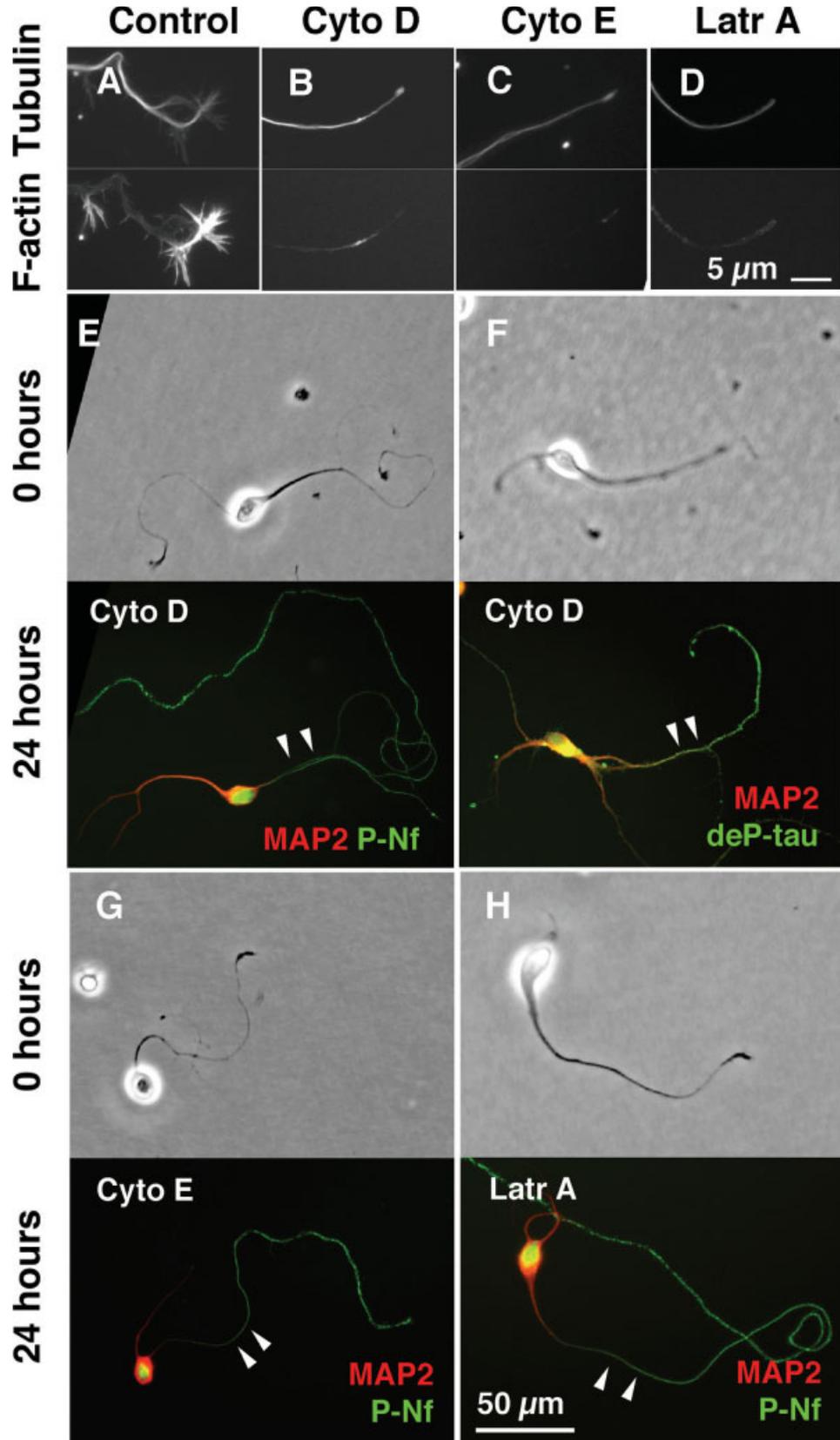
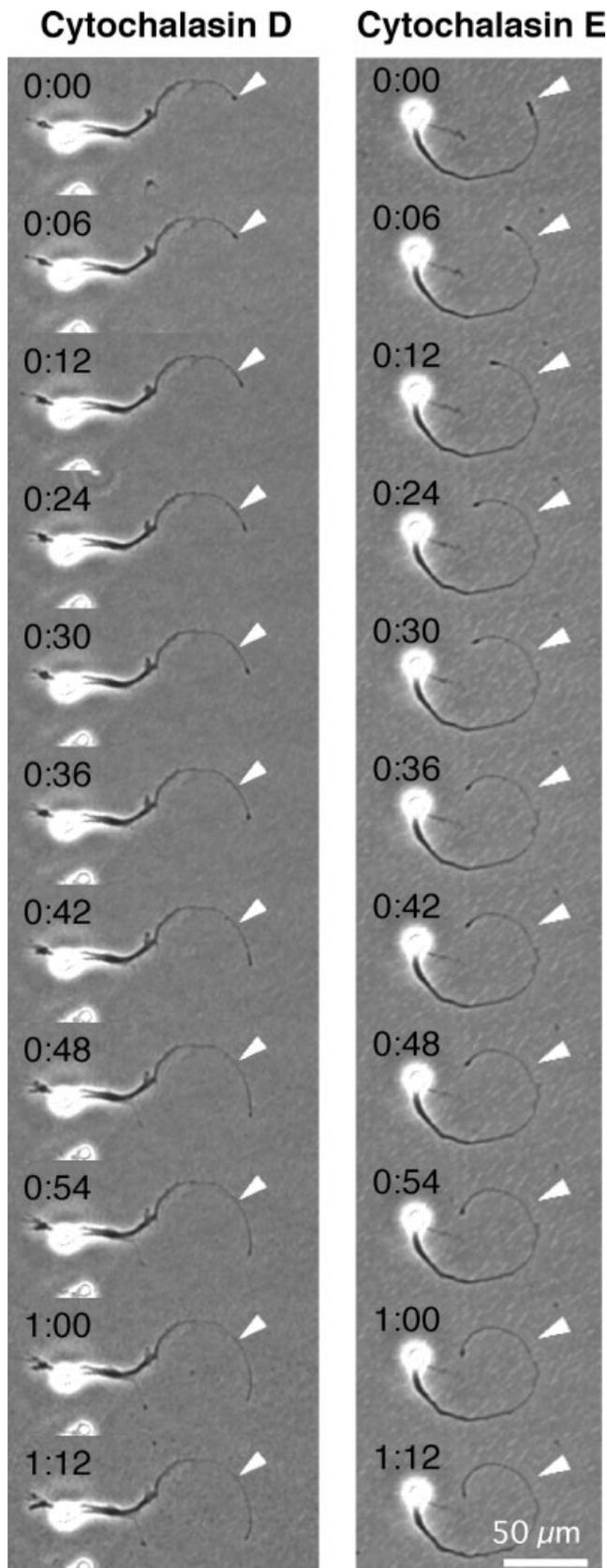


Fig. 6. Effects of actin polymerization inhibitors on axon regeneration in dendrite-bearing neurons. (A–D) The tips of processes regenerating from neurons cultured for 24 h in the absence (A) or presence of cytochalasin D (B), cytochalasin E (C), or latrunculin A (D), each at 0.5 μM, were stained with both antibodies to tubulin (upper panels) and rhodamine-conjugated phalloidin (lower panels). No growth cone is apparent at the tip of the elongating processes in the presence of actin polymerization inhibitors. (E–H) Phase-contrast images of dendrite-bearing neurons before culture (upper panels) and fluorescence images (lower panels) of the same neurons after culture for 24 h in the presence of cytochalasin D (E, F), cytochalasin E (G), or latrunculin A (H). Neurons were stained with both the dendritic marker MAP2 (red) and either phosphorylated neurofilaments (P-Nf) or dephosphorylated tau (deP-tau) as axonal markers (green). In each instance, the distal region of the original dendrite became dendritic marker negative and axonal marker positive (arrowheads).



nance of dendritic fate was also demonstrated by Bradke and Dotti [2000], who found that differentiated dendrites of cultured hippocampal neurons grew as axons after depletion of actin filaments.

Possible Biochemical Changes in the Original Dendrite

Before culture, dendrites in the present study manifested two dendritic characteristics: MAP2 localization and nonuniform polarity orientation of microtubules. After culture for 24 h, however, the dendrites showed an increase in phosphorylation of neurofilaments and dephosphorylation of tau, a decrease in MAP2 concentration, and the loss of minus end-distal microtubules. These changes occurred concurrently and only when an axon elongated from the dendritic tip. It is likely that generation of a growth cone at the tip of the dendrite triggers an intracellular signaling mechanism that results in these various changes in the original dendrite and their progression in a retrograde manner.

Glycogen synthase kinase-3 β (GSK-3 β) may participate in such a signaling pathway. Inactivation of GSK-3 β induces the formation of multiple axons and a constitutively active form of GSK-3 β inhibits axon formation in hippocampal neurons [Jiang et al., 2005; Yoshimura et al., 2005]. Furthermore, inhibition of GSK-3 β resulted in the conversion of preexisting dendrites to axons [Jiang et al., 2005]. It is thus possible that inactivation of GSK-3 β in the original dendrite underlies the regeneration of axons from the dendritic tip in our experimental system. It is unknown how signaling by GSK-3 β or other molecules might result in rearrangement of microtubules. The signaling for the changes observed in the present study apparently does not require actin filaments, however, because the increase in immunoreactivity for phosphorylated neurofilaments and dephosphorylated tau, the decrease in MAP2 immunoreactivity, and the loss of minus end-distal microtubules occurred in the absence of actin filaments.

Possible Mechanisms for the Change in Microtubule Polarity Orientation in the Original Dendrite

Based on earlier studies, our first thought was the disappearance of minus end-distal microtubules from the original dendrite results from movement of such microtubules into the cell body. Yu et al. (2000) showed that

Fig. 7. Time-lapse analysis of dendrite-bearing neurons cultured in the presence of cytochalasin D or cytochalasin E. Phase-contrast images were obtained every 6 min from the onset of culture. The tip of the original dendrite (arrowheads) did not retract before elongation, and there is no growth cone at the tip of the regenerating process. Time stamps are hours:minutes.

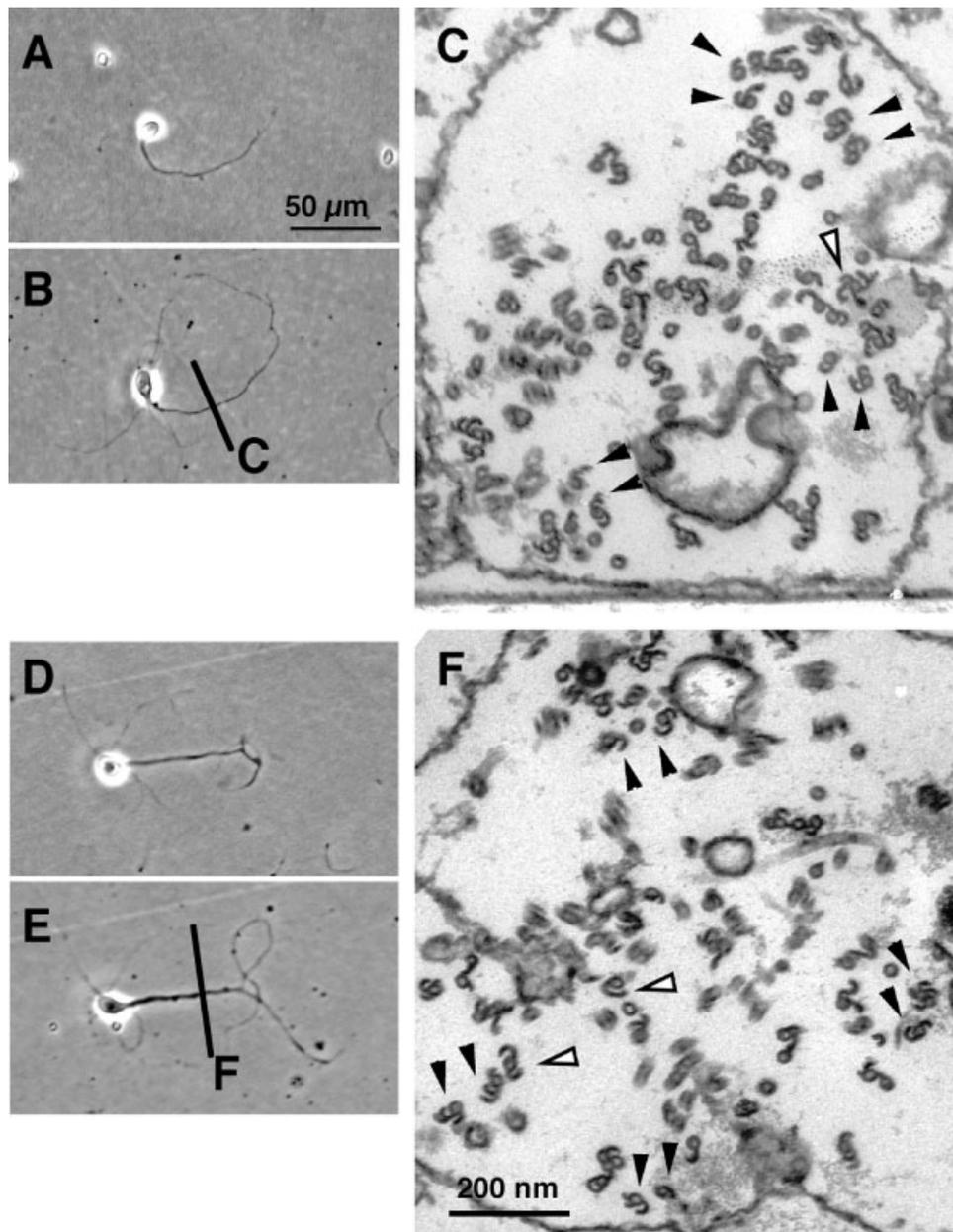


Fig. 8. Microtubule polarity orientation in dendrites of neurons cultured for 24 h in the presence of actin polymerization inhibitors. (A, D) Phase-contrast images of dendrite-bearing neurons before culture. (B, E) The neurons shown in (A) and (D), respectively, were cultured for 24 h in the presence of cytochalasin D or cytochalasin E, respectively. (C, F) Cross-sections of the mid-region of the original dendrites at the positions indicated in (B) and (E), respectively. In both instances, most microtubules are plus end-distal. Arrowheads and open arrowheads indicate examples of plus end-distal and minus end-distal microtubules, respectively.

depletion of the kinesin-like protein CHO1 (MKLP1) from cultured sympathetic neurons resulted in a loss of minus end-distal microtubules from dendrites. CHO1 is a motor protein that is enriched in, and is thought to transport minus end-distal microtubules into, dendrites. Depletion of this protein from neurons was also found to suppress dendrite development [Sharp et al., 1997], and its expression in nonneuronal cells induced the formation of dendrite-like processes with a nonuniform microtubule polarity orientation [Sharp et al., 1996]. It has thus been proposed that cytoplasmic dynein transports microtubules with their plus ends leading along the actin cytoskeleton into dendrites, whereas CHO1 transports microtubules anterogradely with

their minus ends leading [Yu et al., 2000; Baas and Bister, 2004]. Depletion of CHO1 results in loss of the force pushing minus end-distal microtubules distally, and such microtubules are pushed back, presumably by forces generated by cytoplasmic dynein.

Our observations, however, with regard to the role of actin filaments are not completely consistent with this model. Thus, we would conclude that, if the minus-end-distal microtubules are chased back to the cell body, their movement would have been due to dynein motors using a substrate other than actin filaments, or due to kinesin motors pushing against some other substrate. The substrate may be other microtubules, or it may be

other structures as internal membranous elements. It is likely that CHO1 is inhibited in the original dendrite during axon regeneration in our system, with the result that minus end-distal microtubules do not enter the dendrite. Loss-of-function experiments on dynein or CHO1 would reveal how these molecules contribute to the rearrangement of microtubules in neuronal processes. Although injection of antibodies or transfection of dominant-negative constructs are too harmful for dendrite-bearing neurons to survive, loss-of-function experiments are possible on dendrite-bearing neurons isolated from genetically modified animals in the future.

Live-cell imaging studies have recently shown that the rapid movement of microtubules in axons is restricted to only the very shortest microtubules [Hasaka et al., 2004; He et al., 2005], which leads us to believe that there may also be other mechanisms at play with regard to the clearing of minus end-distal microtubules from these dendrites. One possibility is that the microtubules move as a result of treadmilling. In this process, the plus end of microtubules grows while the minus end shortens, resulting in apparent movement of microtubules with their plus end leading. Treadmilling would thus result in the gradual transfer of the minus-end-distal microtubules back into the cell body (or their eventual disappearance before reaching the cell body). Related to this, it is conceivable that signaling triggered by the growth cone at the tip of the original dendrite in our experimental system may induce dissociation of minus end-capping proteins such as ninein [Dammermann et al., 2003] from microtubules and thereby contribute to their shortening or treadmilling.

Finally, it is possible that the conversion of a dendrite to an axon involves the upregulation of microtubule-severing proteins [Baas and Buster, 2004]; this would render the microtubules shorter and less stable, so that they can be cleared by treadmilling or motor-driven transport, or some combination of both. Further studies on the molecular regulation of the vast array of microtubule behaviors will likely reveal how neurite identity is established and maintained during neuronal morphogenesis.

CONCLUSIONS

With the use of the culture system in which dendrites convert to axons, we found that minus end-distal microtubules disappeared from dendrites during the conversion. Their disappearance cannot be explained by previously reported movements of microtubules in neuronal processes. Our results therefore suggest the existence of an unknown mechanism for removal of minus end-distal microtubules and consequent establishment and maintenance of a uniform arrangement of microtubules in axons.

ACKNOWLEDGMENTS

We thank Naoko Kamata (Life Science Institute, Sophia University) for excellent assistance with cell culture and immunostaining. We also thank Toru Higashinakagawa (Waseda University) for his help and support to this project.

REFERENCES

- Ahmad FJ, Echeverri CJ, Valee RB, Baas PW. 1998. Cytoplasmic dynein and dynactin are required for the transport of microtubules into the axon. *J Cell Biol* 140:391–401.
- Akhmanova A, Hoogenraad CC. 2005. Microtubule plus-end-tracking proteins: Mechanisms and functions. *Curr Opin Cell Biol* 17:47–54.
- Arimura N, Kaibuchi K. 2005. Key regulators in neuronal polarity. *Neuron* 48:881–884.
- Baas PW, Buster DW. 2004. Slow axonal transport and the genesis of neuronal morphology. *J Neurobiol* 58:3–17.
- Baas PW, White LA, Heidemann SR. 1987. Microtubule polarity reversal accompanies regrowth of amputated neurites. *J Cell Biol* 84:5272–5276.
- Baas PW, Deitch JS, Black MM, Banker GA. 1988. Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc Natl Acad Sci USA* 85:8335–8339.
- Baas PW, Black MM, Banker GA. 1989. Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. *J Cell Biol* 109:3085–3094.
- Bradke F, Dotti CG. 2000. Differentiated neurons retain the capacity to generate axons from dendrites. *Curr Biol* 10:1467–1470.
- Burack MA, Silverman MA, Banker G. 2000. The role of selective transport in neuronal protein sorting. *Neuron* 26:465–472.
- Burton PR. 1988. Dendrites of mitral cell neurons contain microtubules of opposite polarity. *Brain Res* 473:107–115.
- Craig AM, Banker G. 1994. Neuronal polarity. *Annu Rev Neurosci* 17:267–310.
- Dammermann A, Desai A, Oegema K. 2003. The minus end in sight. *Curr Biol* 13:614–624.
- Dillman JF, Dabney LP, Pfister KK. 1996. Cytoplasmic dynein is associated with slow axonal transport. *Proc Natl Acad Sci USA* 93:141–144.
- Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K. 2002. CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* 4:583–591.
- Goslin K, Banker G. 1989. Experimental observations on the development of polarity by hippocampal neurons in culture. *J Cell Biol* 108:1507–1516.
- Hasaka TP, Myers KA, Baas PW. 2004. Role of actin filaments in the axonal transport of microtubules. *J Neurosci* 24:11291–11301.
- Hayashi K, Kawai-Hirai R, Ishikawa K, Tanaka K. 2002. Reversal of neuronal polarity characterized by conversion of dendrites into axons in neonatal rat cortical neurons in vitro. *Neuroscience* 110:7–17.
- Hayashi K, Kawai-Hirai R, Harada A, Takata K. 2003. Inhibitory neurons from fetal rat cerebral cortex exert delayed axon formation and active migration in vitro. *J Cell Sci* 116:4419–4428.
- He Y, Francis F, Myers KA, Yu W, Black MM, Baas PW. 2005. Role of cytoplasmic dynein in the axonal transport of microtubules and neurofilaments. *J Cell Biol* 168:697–703.

- Jiang H, Guo W, Liang X, Rao Y. 2005. Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3 β and its upstream regulators. *Cell* 120:123–135.
- Kimura T, Arimura N, Fukata Y, Watanabe H, Iwamatsu A, Kaibuchi K. 2005. Tubulin and CRMP-2 complex is transported via kinesin-1. *J Neurochem* 93:1371–1382.
- Setou M, Hayasaka T, Yao I. 2003. Axonal transport versus dendritic transport. *J Neurobiol* 58:201–206.
- Sharp DJ, Kuriyama R, Baas PW. 1996. Expression of a kinesin-related motor protein in Sf9 cells induces them to extend dendrite-like processes with nonuniform microtubule polarity orientation. *J Neurosci* 16:4370–4375.
- Sharp DJ, Yu W, Ferhat L, Kuriyama R, Rueger DC, Baas PW. 1997. Identification of a motor protein essential for dendritic differentiation. *J Cell Biol* 138:833–843.
- Wang L, Brown A. 2002. Rapid movement of microtubules in axons. *Curr Biol* 12:1496–1501.
- Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K. 2005. GSK-3 β regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 120:137–149.
- Yu W, Cook C, Sauter C, Kuriyama R, Kaplan PL, Baas PW. 2000. Depletion of a microtubule-associated motor protein induces the loss of dendritic identity. *J Neurosci* 20:5782–5791.