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The nuclear/mitotic apparatus protein NuMA is a component of the somatodendritic microtubule arrays of the neuron

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Summary

Neurons are terminally post-mitotic cells that utilize their microtubule arrays for the growth and maintenance of axons and dendrites rather than for the formation of mitotic spindles. Recent studies from our laboratory suggest that the mechanisms that organize the axonal and dendritic microtubule arrays may be variations on the same mechanisms that organize the mitotic spindle in dividing cells. In particular, we have identified molecular motor proteins that serve analogous functions in the establishment of these seemingly very different microtubule arrays. In the present study, we have sought to determine whether a non-motor protein termed *NuMA* is also a component of both systems. NuMA is a ~230 kDa structural protein that is present exclusively in the nucleus during interphase. During mitosis, NuMA forms aggregates that interact with microtubules and certain motor proteins. As a result of these interactions, NuMA is thought to draw together the minus-ends of microtubules, thereby helping to organize them into a bipolar spindle. In contrast to mitotic cells, post-mitotic neurons display NuMA both in the nucleus and in the cytoplasm. NuMA appears as multiple small particles within the somatodendritic compartment of the neuron, where its levels increase during early dendritic differentiation. A partial but not complete colocalization with minus-ends of microtubules is suggested by the distribution of the particles during development and during drug treatments that alter the microtubule array. These observations provide an initial set of clues regarding a potentially important function of NuMA in the organization of microtubules within the somatodendritic compartment of the neuron.

Introduction

A hallmark feature of the neuron is that each of its compartments is characterized by a distinct pattern of microtubule organization. Within the axon, the microtubules are uniformly oriented with their plus-ends distal to the cell body (Heidemann *et al.*, 1981). Within the dendrites, the microtubules are nonuniformly oriented with regard to their polarity (Baas *et al.*, 1988, 1991; Burton, 1988). Within the cell body, the microtubules splay in many directions but coalesce into bundles in the axonal and dendritic hillock regions (Yu *et al.*, 1994). Because these microtubule arrays are very specialized, most efforts to understand the mechanisms by which they are established have focused on neuron-specific proteins such as tau and MAP2. However, recent studies from our laboratory strongly suggest that the neuronal microtubule arrays may be organized by variations of the same mechanisms and even the same molecules that organize microtubules within the mitotic spindle of dividing cells.

The best evidence for this idea derives from studies on molecular motor proteins, which are known to generate forces on microtubules necessary for the formation and functioning of the mitotic spindle. We have shown that at least three distinct motor proteins that are components of the mitotic spindle are also components of the microtubule arrays of post-mitotic neurons. The motor known as Eg5 is associated with the microtubules of duplicating centrosomes in prophase (Blangy *et al.*, 1995) and is also concentrated on the microtubules within the growing tips of axons and dendrites (Ferhat *et al.*, 1998a). The motor known as CHO1/MKLP1 is associated with the nonuniformly oriented microtubules within the spindle midzone (Sellitto & Kuriyama, 1988) and is also associated with the

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nonuniformly oriented microtubules of the dendrite (Yu *et al.,* 1997; Sharp *et al.,* 1997). Cytoplasmic dynein is important for establishing the fusiform shape of the mitotic spindle (Heald *et al.,* 1996) and is also essential for conveying microtubules from the centrosome into developing axons (Ahmad *et al.,* 1998).

In the present study, we have sought to determine whether a non-motor protein that is essential for mitotic spindle formation might also be a component of the neuronal microtubule arrays. This protein, termed *NuMA,* is present during interphase in most cell types exclusively within the nucleus, where it forms a structural matrix (Luderus *et al.,* 1994). During mitosis, NuMA concentrates at each pole of the mitotic spindle, presumably as a result of its interaction with motor proteins that convey it toward the minus-ends of microtubules (Gaglio *et al.,* 1996; Merdes *et al.,* 1996). NuMA also self-aggregates and binds to microtubules (Compton & Cleveland, 1994; Merdes *et al.,* 1996; Merdes & Cleveland, 1997). As a result of these various interactions, NuMA is thought to draw together and tightly focus the minus-ends of microtubules during spindle formation (Maekawa *et al.,* 1991; Gaglio *et al.,* 1995, 1996; Merdes *et al.,* 1996). Identification of NuMA as a component of the neuronal microtubule arrays is important for two reasons. First, given its role during mitosis, it seems reasonable that NuMA could help organize microtubules within the neuron. Second, an association of NuMA with neuronal microtubules would provide yet another link between the manner by which microtubule organization is regulated in the mitotic spindle and post-mitotic neurons.

Materials and methods

CELL CULTURES

For studies on cultured neurons, we obtained the neuronal tissue from rats, because cultures of rat hippocampal and sympathetic neurons are well characterized, and also because there is sufficient crossreactivity of cultured rat neurons with the human auto-antibody (SP-H) specific to NuMA (Maekawa *et al.,* 1991; Maekawa & Kuriyama, 1993). Cultures of embryonic rat hippocampal neurons were prepared as previously described (Goslin & Banker, 1991; Sharp *et al.,* 1995; Ferhat *et al.,* 1998b). Briefly, hippocampi were dissected from 18day rat embryos, treated with trypsin for 15 min at 37°C, and triturated with fire-polished Pasteur pipettes. The cells were plated at a density of 1000 cells/cm^2 onto glass coverslips coated with 1 mg/ml poly-L-lysine in Minimum Essential Medium (MEM, Gibco-BRL, Grand Island, NY) containing 10% horse serum. After 2–4 h, the coverslips plated with neurons were co-cultured into plastic tissue-culture dishes containing a monolayer of astroglial cells. The astroglial cells had been grown in medium containing MEM and 10% fetal bovine serum. One day prior to co-culture, the medium was changed to a fresh medium containing MEM, the N2 supplements described by Bottenstein (see Goslin & Banker, 1991), 0.1% ovalbumin, and 0.01 mg/ml sodium pyruvate.

Cultures of sympathetic neurons from the superior cervical ganglia were prepared from newborn rat pups. After dissection, the ganglia were treated with 0.25% collagenase for 1 h followed by 0.25% trypsin for 45 min, and then triturated with fire-polished Pasteur pipettes into a single cell dispersion as previously described (Baas & Ahmad, 1993). Before plating the cells, the glass coverslips were coated for 3 h with 1 mg/ml poly-D-lysine, rinsed extensively, and then treated with 10 μg/ml laminin for 4 h as described by Higgins *et al.* (1991). Cells were then plated in Leibovitz' L15 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 100 μg/ml nerve growth factor for 24 h. For long-term culture, the medium was replaced the next morning by N2 medium (see Baas & Ahmad, 1993) supplemented with 5% fetal bovine serum and 100 ng/ml nerve growth factor. Cytosine arabinoside was added at 10 μM to reduce the proliferation of nonneuronal cells.

Cultures of human HeLa cells and mouse neuroblastoma cells (N2a) were maintained as previously described (Yu *et al.,* 1997; Ferhat *et al.,* 1998a).

WESTERN BLOT ANALYSES

For Western blot analyses, cultures were washed three times with phosphate buffered saline (PBS 1X), scraped and homogenized at 4°C in 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% NP40, 5 mM EDTA with 1 mM PMSF and 10 μg/ml each of aprotinin and leupeptin. Samples were centrifuged at 15,000 g for 20 min at 4°C. Finally, protein concentrations of culture extracts were determined by the DC protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein samples were boiled for 10 min, and the same amounts were loaded into each well and resolved on 4% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA). Blots were blocked with 5% nonfat dried milk and 0.2% Tween 20 in PBS 1X (PBS-milk) for 3 h at room temperature and incubated overnight at 4°C with the NuMA antibody described above at 1/5000 in PBS-milk. The membranes were washed six times for 15 min with a solution containing PBS 1X and 0.1% Tween 20, incubated with horseradish peroxidase goat anti-human Ig at 1/5000 in PBS-milk for 1 hr at room temperature, washed, and immunodetected using the enhanced chemiluminescence system (ECL, Amersham Corp., Arlington Heights, IL).

IMMUNOFLUORESCENCE MICROSCOPY

For immunofluorescence analyses, the cultures were fixed for 6 min in cold methanol (−20°C), rehydrated three times for 5 min each in PBS 1X, and incubated for 30 min in blocking solution containing 5% normal goat serum in PBS 1X. The cells were then exposed overnight at 4°C to both a mouse monoclonal antibody that specifically recognizes β-tubulin (used at 1/5000, Amersham Corp.) and to the human autoantibody (SP-H) specific to NuMA (used at 1/600). The cells were washed extensively in PBS 1X and incubated with a goat anti-mouse antibody conjugated with fluorescein (FITC) and a goat anti-human antibody conjugated with Texas Red.

Fluorescent second antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). After washes in PBS 1X, cells were mounted in a medium that reduces photobleaching and were then viewed with either a confocal microscope (LSM 410, Carl Zeiss Incorporated, Thornwood, NY) or a microscope interfaced with a cooled CCD camera (Photometrics, Tuczon, AZ). The confocal microscope permitted us to select the optical section on which the NuMA particles were most abundant, while the cooled CCD camera permitted the acquisition of higher-resolution images.

PHARMACOLOGICAL STUDIES

In one set of experiments, hippocampal neurons that had been cultured for 7 days were incubated for 30 min or 2 h at 37°C in culture media supplemented with 10 μg/ml of cytochalasin B (Sigma Chemical Co., St. Louis, MO). In another set of experiments, hippocampal neurons cultured for 7 days were exposed to 10 μg/ml of nocodazole (Aldrich Chemical Co., Milwaukee, WI) for 30 min, 1 h, 2h. Cultured N2a cells were also incubated in the presence of 10 μg/ml taxol (gift from the National Cancer Institute) for 4 h at 37°C. In some cases, cytochalasin B was added to a final concentration of 10 μg/ml during the last 15 min, 30 min, or 1 h of the 4 h taxol treatment. After incubation with these drugs, cells were prepared for immunofluorescence microscopy as described above.

MICROTUBULE POLARITY ANALYSES

In order to determine the polarity orientation of microtubules within the processes extended by N2a cells treated with pharmacologic agents, we used the standard "hooking" protocol. In this procedure, the cells are lysed in the presence of exogenous brain tubulin in a special microtubule assembly buffer that promotes the formation of lateral protofilament sheets on the existing microtubules. When viewed in cross section with the electron microscope, these sheets appear as hooked appendages on the microtubules. The curvature of the "hooks" reveals the orientation of the microtubule. A clockwise hook as viewed from the distal end of the process indicates that the microtubule is oriented with its plus-end distal to the cell body, and a counterclockwise hook indicates the opposite. The procedure was carried out and the data were interpreted as previously described (Sharp *et al.*, 1995, 1996; Yu *et al.*, 1997).

Results

IDENTIFICATION AND REGULATION OF NUMA PROTEIN IN DEVELOPING NEURONS

In a first set of studies, we wished to determine whether NuMA is expressed in terminally post-mitotic neurons at various stages of their development. For these studies, we analyzed protein obtained from cultures of rat sympathetic neurons. HeLa cells were used as a positive control, and we also analyzed N2a cells because they have neuronal characteristics and yet are mitotic. Western blot analyses were performed using a human autoimmune serum that contains an autoantibody (SP-H) specific to the NuMA antigen (Maekawa *et al.*, 1991; Maekawa and Kuriyama, 1993). In all cases, 50 μg of total protein were loaded per lane. The results of these analyses are shown in Figure 1. HeLa cells showed a single major band at about 230 kDa. Similar results were obtained with the SP-H antibody on N2a cells that had not been treated with an agent that causes them to differentiate. The 230 kDa protein was also expressed in N2a cultures that had been induced to differentiate by the addition of either retinoic acid (RA)

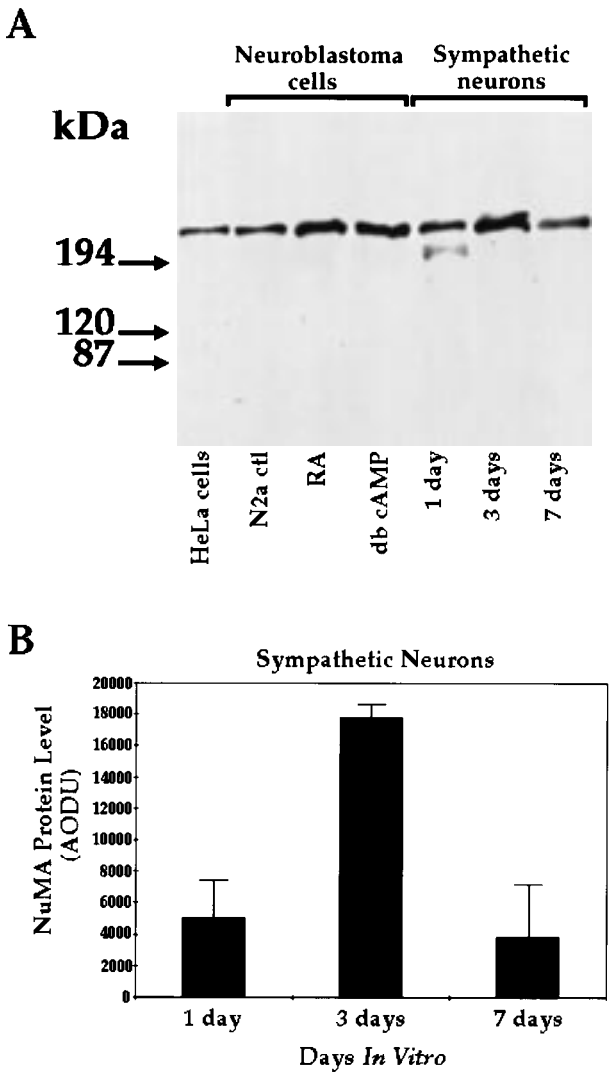

Fig. 1. Western blot analyses. (A) Shown here are Western blot analyses using the SP-H antibody to detect the NuMA antigen in HeLa cells, N2a cells, and primary cultures of rat sympathetic neurons. In all cases, 50 μg of total protein was loaded per lane. HeLa cells showed a major band at about 230 kDa, as did the undifferentiated N2a cells, the differentiated N2a cells, and the sympathetic neurons. (B) Shown here are quantitative analyses of NuMA levels in cultured sympathetic neurons derived from the Western blots. The sympathetic neurons showed higher expression levels at 3 days than at 1 or 7 days.

or dibutyryl cyclic AMP (db cAMP) (Fig. 1A). The quantitative analyses were performed on unsaturated films, where the resulting signals were still in the linear range. In differentiated N2a cells, the expression levels were substantially upregulated compared with the undifferentiated N2a cells (data not shown). The sympathetic neurons showed a comparable 230 kDa band at 1, 3, and 7 days in cultures (Fig. 1A). At 1 day, there was an additional band of slightly lower molecular weight that might be the result of either protein degradation (Maekawa *et al.*, 1991) or alternative splicing (Tang *et al.*, 1993) (Fig. 1A). At 3 days, the expression of the 230 kDa band was substantially higher than at 1 or 7 days (Fig. 1B). No bands were observed in control studies, in which the primary antibody was deleted. These results indicate that NuMA is expressed in post-mitotic neurons and that its expression is developmentally regulated.

COMPARISON OF NUMA DISTRIBUTION DURING THE CELL CYCLE AND DURING NEURONAL DEVELOPMENT

Unless otherwise noted, all images were acquired with the confocal microscope. Optical sections were roughly 50 μm in thickness and were selected in the region of the neuron that contained the highest intensity of NuMA immunoreactivity. Although the distribution of NuMA during the cell cycle has been previously reported (Maekawa *et al.*, 1991; Compton *et al.*, 1991, 1992; Yang *et al.*, 1992; Maekawa and Kuriyama, 1993), it was important for us to reexamine this issue in finer detail in order to draw direct comparisons with NuMA distribution during neuronal development. For these studies, we used HeLa cells because the stages of the cell cycle could be assessed by DIC microscopy in these relatively flat cells on the basis of nuclear morphology (Fig. 2, left-hand panels). Figure 2 shows also micrographs of HeLa cells immunostained with the SP-H antibody (right-hand panels). In interphase cells (Fig. 2A), NuMA is found throughout the nucleus, except in the nucleoli, and the staining is relatively evenly distributed (Fig. 2A′). Although nuclear staining is quite intense, the cytoplasm shows only faint background staining similar to cells stained with the secondary anti-

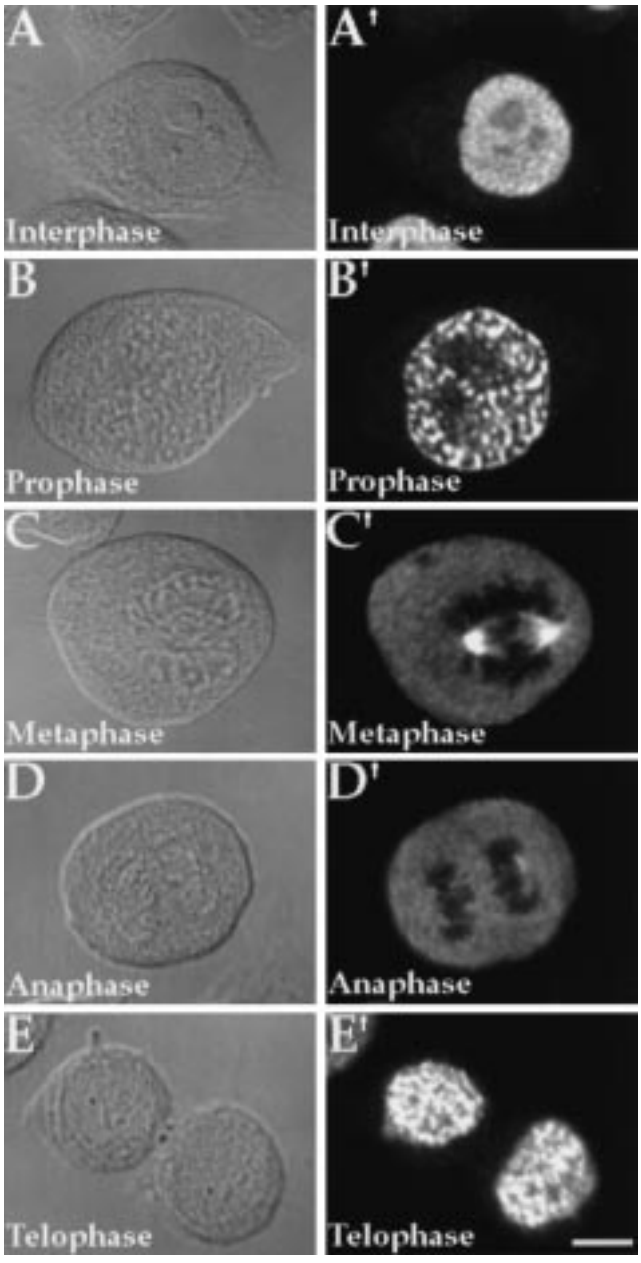

Fig. 2. NuMA distribution in HeLa cells during the cell cycle. Shown here are HeLa cells in various stages of the cell cycle, which can be assessed by DIC microscopy on the basis of their nuclear morphology (left-hand panels). The right-hand panels are images of the same cells immunostained for NuMA with the SP-H antibody. In interphase cells (A), NuMA is found throughout the nucleus, except in the nucleoli, and the staining is relatively evenly distributed (A′). Nuclear staining is intense, but the cytoplasm is not labelled. At prophase (B), the NuMA antigen is still confined within the nucleus, but its distribution is condensed into nonuniform patches (B′). As in interphase, the intensity of the NuMA signal is high in the prophase nucleus, with no staining observed in the cytoplasm. At metaphase (C), the NuMA antigen becomes concentrated in the centrosomal region of each half spindle (C′). The antigen can also be observed along the microtubules of the spindle but is more concentrated on the regions of the microtubules near the centrosome. In addition, uniform diffuse staining is present in the cytoplasm. At anaphase (D), a small amount of NuMA is concentrated at the pericentrosomal region of each half spindle, and the rest of the staining is uniformly distributed, as observed at metaphase (D′). As the cells progress from anaphase into telophase (E), the NuMA antigen no longer associates with the centrosomal region of the spindle but rather forms dense aggregates within the newly formed nuclei of the future daughter cells (E′). At this stage, there is virtually no diffuse cytoplasmic staining such as that observed at metaphase and anaphase. Bar: 40 μm.

body alone. At prophase (Fig. 2B), the NuMA antigen is still confined within the nucleus, but its distribution is condensed into nonuniform patches (Fig. 2B′) compared to interphase. As in interphase, the intensity of the NuMA signal is high in the prophase nucleus, with no staining above background observed in the cytoplasm. At metaphase (Fig. 2C), the NuMA antigen becomes highly concentrated in the region of each half spindle corresponding to the duplicated centrosomes (Fig. 2C′). The antigen is also present along the microtubules of the spindle and is more concentrated on the regions of the microtubules near the duplicated centrosomes as compared to the regions near the midzone. In addition, we observed uniform diffuse staining in the cytoplasm compared to interphase and prophase. At anaphase (Fig. 2D), a very small amount of the NuMA antigen can be observed concentrated at the pericentrosomal region of each half spindle, and the rest of the staining is uniformly diffused in the cytoplasm (Fig. 2D′), as observed at metaphase. Finally, as the cells progress from anaphase into telophase (Fig. 2E), the NuMA antigen no longer associates with the centrosomal region of the spindle but rather forms dense aggregates within the newly formed nuclei of the future daughter cells (Fig. 2E′). At this stage, there is virtually no diffuse cytoplasmic staining such as that observed at metaphase and anaphase.

Having completed these analyses on NuMA distribution during the cell cycle, we next wished to determine NuMA distribution in developing neurons. For these analyses, we used two well-characterized culture systems of terminally post-mitotic neurons, one from the peripheral and one from the central nervous system. Sympathetic and hippocampal neurons were obtained from newborn rat pups and from rat fetuses at times when most of them had completed their terminal mitotic division (Higgins *et al.*, 1991; Goslin & Banker, 1991). The Western blot analyses show that NuMA is present within cultured sympathetic neurons and that its expression is developmentally regulated. The NuMA antigen as revealed by the SP-H antibody was found in the nucleus at all the time periods examined (Fig. 3). Figures 3A and 3B show the cellular morphology of cultured sympathetic neurons at 4 h and 1 day in culture, respectively, as revealed by β-tubulin immunostaining. At 4 h of culture (Fig. 3A′), there was dim and rather diffuse immunofluorescence staining for NuMA within the cytoplasm of the cell body of all neurons but not within the processes. At 1 day (Fig. 3B′), the NuMA antigen started to become more visible in the cell body (see arrows) of most of the neurons. At 3 days (Fig. 3C), all the neurons displayed high NuMA immunoreactivity in the cell body, while at 7 (Fig. 3D) and 14 (Fig. 3E) days, the neurons displayed a significant diminution in NuMA immunoreactivity compared to 3 days. Even though cytoplasmic NuMA levels were downregulated at 7 days, the protein was still clearly found in the dendritic compartment. With regard to the appearance of the cytoplasmic NuMA staining, 1 day cultures showed several small, relatively discrete particles. The particles became significantly brighter and more numerous at 3 days and then dimmer and less numerous at 7 and 14 days.

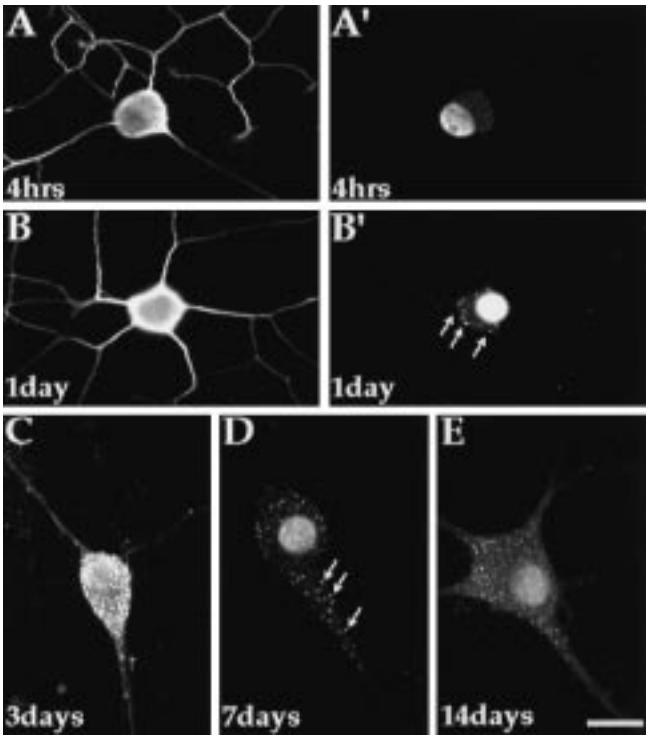

Fig. 3. NuMA distribution in developing sympathetic neurons. The NuMA antigen as revealed by the SP-H antibody was found in the nucleus at all the time periods examined. (A, B) These panels show the cellular morphology of cultured sympathetic neurons at 4 h and 1 day in culture, respectively, as revealed by β-tubulin immunostaining. At 4 h of culture (A′), there was only dim and diffuse NuMA staining in the cytoplasm of the cell body. At 1 day (B′), the NuMA antigen became more visible in the cell body (see arrows) of most of the neurons. At 3 days (C), all the neurons displayed high NuMA immunoreactivity in the cell body and early dendrites, while at 7 (D) and 14 (E) days, the neurons displayed a significant diminution in NuMA immunoreactivity compared to 3 days. Even though cytoplasmic NuMA levels were downregulated at 7 days, the protein was still clearly found in the dendritic compartment (see arrows in D). With regard to the appearance of the cytoplasmic NuMA staining, 1 day cultures showed several small, relatively discrete particles. The particles became significantly brighter and more numerous at 3 days and then dimmer and less numerous at 7 and 14 days. At least 300 cells were analyzed for each time point. Bar: 20 μm.

Hippocampal neurons are particularly useful for developmental analyses because they differentiate axons and dendrites in a well-characterized sequence of stages (Dotti *et al.*, 1988). The cells initially extend lamellipodia (stage 1) that coalesce into immature proc-

esses within a few hours after plating (stage 2). One of these immature processes becomes the axon by 1.5 days in culture (stage 3), after which those remaining differentiate into dendrites by 4 days in culture (stage 4). Figures 4A, B, C, D, E, and F show hippocampal neurons at various stages of development immunostained for β-tubulin to reveal their morphology. At all stages studied, the NuMA antigen was observed in the nucleus. At stages 1 and 2 (Fig. 4A) of development, cultured hippocampal neurons showed only very dim and diffuse NuMA immunoreactivity within the cell body (Fig. 4A′). At stage 3 (Fig. 4B), the protein is still localized within the nucleus but also starts to become more visible in the cell body (Fig. 4B′). The staining in the cell body appears as a few discrete particles (Fig. 4B′). At early stage 4 (Fig. 4C and 4D), the number of particles increased significantly compared to stage 3, and the particles were distributed within the cell body and the dendrites (Fig. 4C′ and 4D′). At late stage 4 (Fig. 4E), in most cases, the number of particles was still high, but the particles were more restricted to the cell body (Fig. 4E′). At stage 5 (Fig. 4F), the number of particles was significantly diminished in the somatodendritic compartment, and most of the immunoreactivity appeared more diffuse (Fig. 4F′).

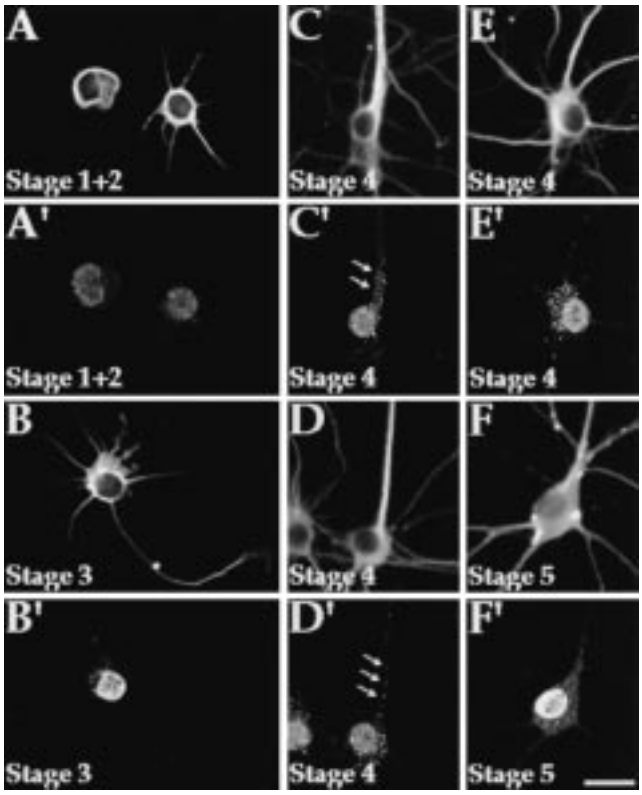

Fig. 4. NuMA distribution in developing hippocampal neurons. Shown here are pairs of immunofluorescence images of cultured hippocampal neurons labelled for β-tubulin (A, B, C, D, E, F) and NuMA (A′, B′, C′, D′, E′, F′). At all stages studied, the NuMA antigen was observed in the nucleus. At stages 1 and 2 (A) of development, the neurons showed only dim and diffuse NuMA immunoreactivity within the cell body (A′). At stage 3 (B), the protein is still localized within the nucleus but also starts to become visible in the cell body (B′). The staining in the cell body appears as a few discrete particles (B′). At early stage 4 (C, D), in all the cells, the number of particles increased significantly compared to stage 3, and the particles were distributed within the cell body and dendrites (C′, D′; see arrows). At late stage 4 (E), in most cases, the number of particles was still high, but the particles were more restricted to the cell body (E′). At stage 5 (F), the number of particles was significantly diminished in the somatodendritic compartment, and the staining became more diffuse in appearance (F′). At least 300 cells were analyzed for each developmental stage. Bar: 20 μm.

INTEGRITY OF CYTOPLASMIC NUMA PARTICLES DEPENDS ON NORMAL MICROTUBULE LEVELS AND NORMAL MICROTUBULE ORGANIZATION

In the mitotic spindle, the distribution of NuMA is dependent upon the microtubules that emanate from the centrosome (Price & Pettijohn, 1986; Kallajoki *et al.*, 1992; Tousson *et al.*, 1991). Specifically, NuMA is thought to associate with the microtubules and translocate via motor proteins toward their minus-ends (Gaglio *et al.*, 1996; Merdes *et al.*, 1996). To ascertain whether the NuMA particles observed in neurons are associated with microtubules, we investigated whether there is any alteration in the appearance of the particles during drug treatments that alter the microtubule arrays of the neuron. For these studies, we incubated the cells for 1 h in nocodazole, a drug known to depolymerize microtubules. As a negative control, we first treated the neurons for 2 h with cytochalasin B, a drug that is known to disrupt actin filaments but not microtubules. In one set of experiments, the disruption by cytochalasin B of the hippocampal neuron's actin filaments was assessed and confirmed using phalloidin-conjugated FITC (data not shown). Shown in Figure 5 are images of cultured hippocampal neurons at stage 4 double-stained for β-tubulin and NuMA. After treatments with cytochalasin B or nocodazole, the morphology of the neurons is not significantly altered. Tubulin staining was similar to controls after treatment with cytochalasin B (Fig. 5A) but was reduced after nocodazole treatment (Fig. 5B). The distribution of NuMA particles was unchanged in the cells treated with the anti-actin drug (Figs. 5A′). However, there was a marked diminution in the brightness of the particles in the neurons treated with nocodazole, and the staining appeared more diffuse (Fig. 5B′). After 1 h of recovery from nocodazole treatment, the tubulin staining was increased compared to the nocodazole-treated cells, indicating the reassembly of microtubules (data not shown). Under these conditions, the NuMA particles became brighter and more numerous (not shown). In most cases, the brightness of the NuMA particles was indistinguishable from that of controls, but in some

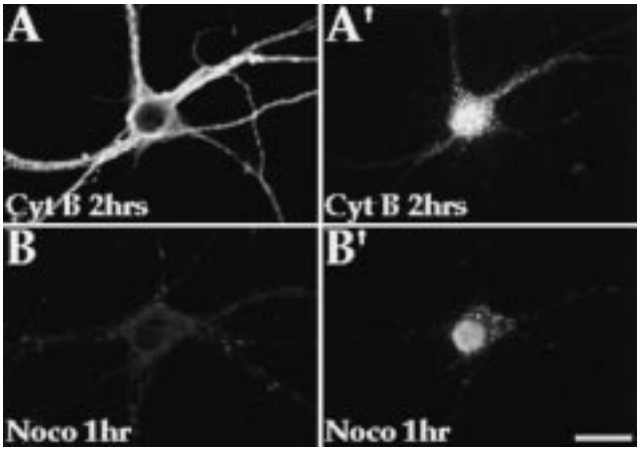

Fig. 5. NuMA distribution in neurons treated with drugs that disrupt actin filaments or microtubules. Shown in this figure are images of cultured hippocampal neurons (at stage 4) double-stained for β-tubulin and NuMA. After treatments with cytochalasin B or nocodazole, the morphology of the neurons was not significantly altered. Tubulin staining is similar to controls after cytochalasin treatment (A) but is substantially diminished after nocodazole treatment (B). The distribution of NuMA particles is indistinguishable from controls in neurons treated with cytochalasin B (A'). However, there is a marked diminution in the number of bright particles in neurons treated with nocodazole, and the staining appears more diffuse (B'). Bar: 20 μm.

cases the particles were actually somewhat brighter than in controls. Collectively, these results indicate that the integrity of the NuMA particles depends on the presence of an intact microtubule array but not an intact array of actin filaments.

DOES NUMA CO-LOCALIZE WITH MINUS-ENDS OF MICROTUBULES IN NEURONAL CELLS?

The pharmacologic studies on cultured hippocampal neurons described above indicate that NuMA is associated with the somatodendritic microtubule arrays. It has been shown that NuMA concentrates at spindle poles, suggesting that NuMA is transported along microtubules toward their minus-ends during mitosis (Gaglio *et al.*, 1996; Merdes *et al.*, 1996). The fact that NuMA particles are present within the cell body and dendrites but not the axon of neuronal cells is consistent with the possibility that NuMA is also transported toward minus-ends of microtubules in the neuron. Attempts to investigate this possibility within primary neurons proved problematic. For example, we tried to perform immunoelectron microscopy, but these attempts were not successful because the NuMA particles were lost during the fairly strong extraction procedures necessary to introduce the colloidal-gold-conjugated antibodies. In addition, the treatments with drugs that affect the cytoskeleton, such as nocodazole, cytochalasin B, or taxol (data not shown), did not produce morphological changes or microtubule rearrangements that would permit us to better elucidate the minus-ends of microtubules. Fortunately, however, we found that N2a cells, which have neuronal characteristics and yet are still mitotic, undergo alterations in response to certain growth factors and pharmacologic agents that provide some information on the relationship of NuMA to the minus-ends of microtubules.

Figure 6 shows N2a cells in various stages of the cell cycle, double-labelled to reveal microtubules and NuMA. At metaphase (Fig. 6A and A') and anaphase (Fig. 6B and B'), the NuMA staining pattern was similar to that described for HeLa cells (Maekawa *et al.*, 1991; Compton *et al.*, 1991, 1992; Yang *et al.*, 1992; see Fig. 2). At telophase (Fig. 6C), the NuMA staining pattern (Fig. 6C') differed somewhat from that described for HeLa cells. In addition to the reaggregation of NuMA into the newly formed nuclei of the future daughter cells, a few NuMA particles were clearly present in the cytoplasm of the N2a cells. As the N2a cells progress from telophase into interphase (Fig. 6D), smaller and more numerous NuMA particles appeared throughout the cytoplasm (Fig. 6D'). Notably, when the cells are rendered post-mitotic by db cAMP or retinoic acid (Yu *et al.*, 1997), NuMA particles appeared within the developing dendrite-like processes but not the axon-like processes (data not shown). Thus, N2a cells distribute NuMA in similar fashion to other dividing cells during mitosis and in similar fashion to primary neurons during interphase.

Fig. 7 shows N2a cells treated with taxol or a combination of taxol and cytochalasin B, double-labelled to reveal microtubules and NuMA. In mitotic N2a cells, the application of taxol for 4 h induced the assembly of numerous small microtubule asters (Fig. 7A). This result was similar to that observed with other mitotic cells (Maekawa *et al.*, 1991; Verde *et al.*, 1991; Kallajoki *et al.*, 1992). As previously shown, the central region of the asters was strongly stained by the SP-H antibody (Fig. 7A'). When the N2a cells were in interphase and treated with taxol for 4 h, the microtubules were bundled and rearranged into peripheral rings under the plasma membrane, and there was a loss of centrosomal microtubules (Fig. 7B). Under these conditions, the NuMA particles appeared throughout the cytoplasm, but it was impossible to determine whether they co-localized with the minus-ends of microtubules within the peripheral microtubules rings (Fig. 7B'). In the cells bearing short spontaneous processes, microtubule bundles appeared within the processes (Fig. 7C), and the NuMA particles appeared within the more proximal region of the bundle (Fig. 7C'). The addition of cytochalasin B alone produced no detectable effects on morphology (not shown). The addition of cytochalasin B to taxol-treated mitotic N2a cells did not affect the microtubule asters (Fig. 7D) or the NuMA antigen distribution (Fig. 7D'). However, when taxol-treated interpha-

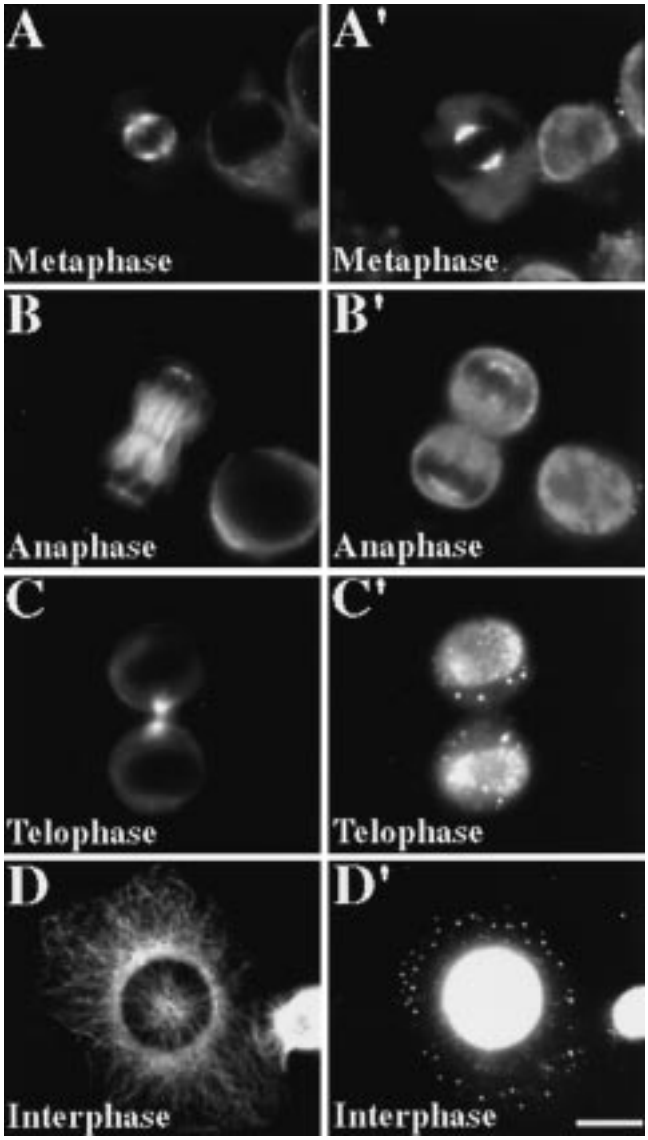

Fig. 6. NuMA distribution in N2a cells during the cell cycle. N2a cells in various stages of the cell cycle have been double-labelled to reveal microtubules (left-hand panels) and NuMA (right-hand panels). At metaphase (A, A′) and anaphase (B, B′), the NuMA staining patterns were similar to those described for HeLa cells. At telophase, the NuMA staining pattern differed from that reported in HeLa cells (C, C′). In addition to the reaggregation of NuMA into the newly formed nuclei of the future daughter cells, a few NuMA particles were present in the cytoplasm of the N2a cells. As the N2a cells progress from telophase into interphase, smaller and more numerous NuMA particles appeared throughout the cytoplasm (D, D′). Bar: 15 μm.

sic N2a cells were additionally exposed to cytochalasin B, the cells that did not bear spontaneous processes underwent a striking change in shape, leading to the formation of short processes that contained bundles of microtubules. At 15 or 30 min in cytochalasin B, the processes were relatively thin (see Fig. 7E and F), while at 1 h, the processes were notably thicker (not shown). In almost all cases, the NuMA particles remained within the cell body (see Fig. 7E′ and 7F′). In some cases (see arrows in Fig. 7E, E′, F, and F′), the particles appeared to co-localize with one end of a microtubule bundle, but it was impossible to make this correlation in all cases. In all such cases in which the microtubule bundle extended into a process, the particles appeared in the vicinity of the proximal rather than the distal end of the microtubule bundle. To determine the polarity orientation of the microtubules within the bundles, we used the standard "hooking" protocol (see Materials and Methods above). As shown in Figure 8, the hooks on the microtubules were predominantly clockwise as viewed from the distal tips of the processes (85 ± 1.7%; $n = 4$), indicating that the microtubules are uniformly or nearly uniformly oriented with their plus-ends distal to the cell body. Taken together, these observations on N2a cells indicate that the distribution of NuMA particles corresponds roughly but not perfectly with the distribution of minus-ends of microtubules.

Discussion

NuMA has been shown to be present in the nuclei of a variety of proliferating and terminally differentiated cell types, where it is thought to play a structural role (see for example, Kallajoki *et al.*, 1992; Tang *et al.*, 1993; Compton & Cleveland, 1994). In addition, NuMA has been shown to play an essential role in the organization of the mitotic spindle (Compton & Cleveland, 1994; Merdes & Cleveland, 1997). During mitosis, NuMA becomes exposed to microtubules when the nuclear envelope disintegrates. At this point, NuMA self-aggregates, associates with microtubules, and interacts with minus-end-directed motor proteins (Gaglio *et al.*, 1995, 1996; Merdes *et al.*, 1996). As a result of these associations, NuMA is thought to move toward the minus-ends of microtubules, thereby drawing them together at each of the two spindle poles. As the cell completes division and reenters interphase, all the detectable NuMA once again becomes localized within the nucleus.

In the present study, we have documented that NuMA is present in the cytoplasm of terminally post-mitotic neurons. This is not the first observation of this kind in that NuMA has been detected in the cytoplasm of frog sperm and human granulocytes (Merdes & Cleveland, 1998). The cytoplasmic NuMA in these cells does not reflect protein degradation and therefore probably reflects a physiological role for NuMA in the cytoplasm (Merdes & Cleveland, 1998). Similarly, in the case of the neuron, there are also strong reasons to believe that the presence of NuMA in the cytoplasm does not reflect its degradation. For example, NuMA has a very specific distribution in the neuron, as well as a consistent pattern by which it appears during development.

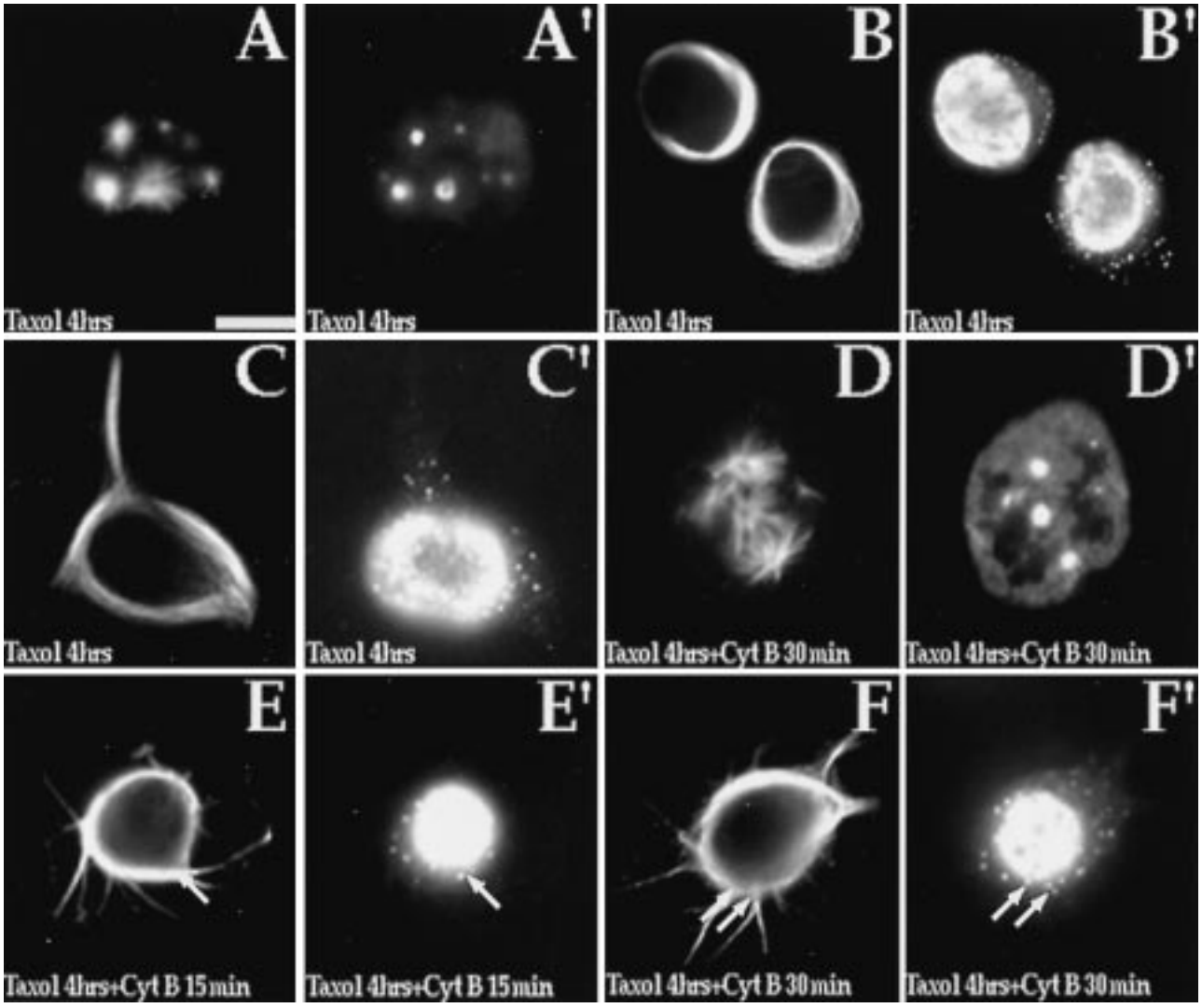

Fig. 7. NuMA distribution in N2a cells treated with taxol or a combination of taxol and cytochalasin B. Shown here are N2a cells double-labelled to reveal microtubules (left-hand micrograph of each pair) and NuMA (right-hand micrograph of each pair). In mitotic N2a cells, the application of taxol for 4 h induced the assembly of numerous small microtubule asters (A). NuMA is enriched in the central region of the asters (A'). When the N2a cells were in interphase and treated with taxol for 4 h, the microtubules became bundled and rearranged into peripheral rings under the plasma membrane, and there was a loss of centrosomal microtubules (B). Under these conditions, the NuMA particles appeared within the cytoplasm, but it was impossible to ascertain whether they co-localized with the minus-ends of microtubules (B'). In the cells bearing short spontaneously processes, microtubule bundles appeared within the processes (C), and the NuMA particles appeared within the more proximal region of the bundle (C'). The addition of cytochalasin B to taxol-treated mitotic N2a cells did not affect the microtubule asters (D) or the NuMA antigen distribution (D'). However, when taxol-treated interphasic N2a cells were additionally exposed to cytochalasin B, the cells that did not bear spontaneous processes underwent a striking change in shape, leading to the formation of short processes that contained bundles of microtubules. At 15 or 30 min in cytochalasin B, the processes were relatively thin (E, and F). In almost all cases, the NuMA particles remained within the cell body, and in some cases, the particles appeared in the general vicinity of the proximal end of a microtubule bundle extending into a process (see arrows in E, E', F, and F'). No NuMA particles were observed at the distal tips of the processes where the plus-ends of microtubules concentrate (see panels E' and F'). Bar: 15 μm.

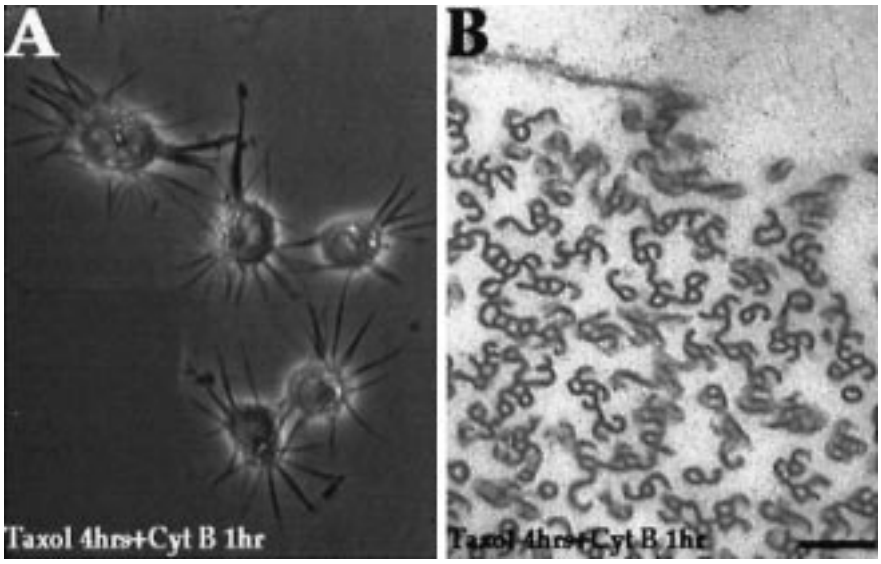

Fig. 8. Microtubule polarity analyses on processes extended by N2a cells in the presence of taxol and cytochalasin B. (A) shows a DIC image of N2a cells treated with taxol and cytochalasin B as described in the text (see Results). The cells have extended numerous short processes. Panel (B) shows an electron micrograph of a cross section through one of these processes after the cells had been prepared for microtubule polarity visualization by the standard "hooking" protocol. The hooks are predominantly clockwise as viewed from the distal tips of the processes, indicating that the microtubules are uniformly or nearly uniformly oriented with their plus-ends distal to the cell body. Bar: (A), 10 μm; (B), 85 nm.

In addition, Western blots reveal a single sharp band similar to that observed in mitotic cells rather than the multiple indistinct lower-molecular-weight bands observed in muscle cells, in which NuMA is known to be degraded (Merdes & Cleveland, 1998).

The NuMA molecule contains a nuclear recognition site in its C-terminal region (Tang *et al.*, 1994). The presence of NuMA within the cytoplasm of the neuron suggests that this site is either masked by factors in the neuronal cytoplasm, removed by selective proteolysis, or not expressed due to alternative splicing. Regarding the last possibility, there is direct evidence that NuMA can undergo splicing events in other cell types (Tang *et al.*, 1993). At present, our data do not confirm or refute any of these possibilities. We detected only one band on our Western blots (except at one day in culture, at which time we detected two), but the possibility remains that the number of amino acids deleted from the cytoplasmic form is too small to produce a difference in electrophoretic migration.

In the neuron, the levels of NuMA in the nucleus are consistent throughout development, but the levels in the cytoplasm vary with different development stages. NuMA is relatively low in the cytoplasm during early axonal development but is significantly higher during early dendritic development. After the dendrites have formed, the levels of cytoplasmic NuMA are diminished. These results suggest that cytoplasmic NuMA may have an important function during early neuronal development but not after the neuron has completed its differentiation.

The cytoplasmic NuMA appears in the form of multiple small particles that are present in the cell body and the dendrites but not in the axon. The particles are dispersed when microtubules are pharmacologically depolymerized, indicating that the integrity of the particles is dependent upon their association with microtubules. The particles are also dispersed after the dendrites have fully formed, which probably suggests an alteration in the interaction of the NuMA protein with microtubules. The somatodendritic distribution of the particles suggests that they may be actively transported toward minus-ends of microtubules. This is because dendrites contain nonuniformly oriented microtubules, whereas axons contain uniformly oriented microtubules. Therefore, cytoplasmic constituents that are transported toward minus-ends of microtubules will be transported into dendrites but not axons (Black & Baas, 1989). Further support for the conclusion that NuMA moves toward minus-ends of microtubules derives from studies on N2a cells, in which alterations in the distribution of minus-ends of microtubules roughly correlate with changes in the distribution of the NuMA particles. Taxol treatment induces the formation of either asters or a small number of short microtubule bundles that focus the minus-ends of microtubules, and also results in a redistribution of the NuMA particles to the vicinity of the minus-ends of microtubules. These results indicate that NuMA is a component of the somatodendritic microtubule arrays of the neuron and suggest that it probably interacts with minus-end-directed microtubule motors. Cytoplasmic dynein is a

good candidate for such a motor, given that NuMA has been shown to interact with cytoplasmic dynein in mitotic cells (Gaglio *et al.*, 1996; Merdes & Cleveland, 1996).

All these observations indicate that cytoplasmic NuMA is tightly regulated within the neuron and hence strongly suggest that it has a functional role. As noted above, the putative function of NuMA in mitotic cells is to draw together the minus-ends of microtubules at the spindle poles (Compton & Cleveland, 1994; Merdes and Cleveland, 1997), and it seems reasonable that the cytoplasmic NuMA might play a similar role in the neuron. However, neurons do not form microtubule spindles or asters, even when they are treated with taxol. One possibility is that the association of NuMA with microtubules may be weaker in the neuronal cytoplasm compared to mitotic cells. Indeed, we have found the cytoplasmic NuMA is almost completely lost during triton-extraction of neurons, whereas a substantial portion of the mitotic NuMA remains associated with microtubules under similar conditions (Maekawa *et al.*, 1991). The weaker association in the neuron may be the result of differences in NuMA phosphorylation, which is known to regulate the binding of NuMA with microtubules (Compton *et al.*, 1995; Saredi *et al.*, 1997), or may be the result of proteins such as MAP2 that are present in the neuron but not mitotic cells. In any event, the movement of the NuMA particles towards minus-ends of microtubules probably helps to align and orient the microtubules, but the association with the microtubules and the forces generated upon them are not sufficiently strong to form asters nor to result in a precise co-localization of the particles with minus-ends of microtubules.

How might these interactions influence microtubule organization in the neuron? The organization of microtubules within the neuronal cell body is not well characterized, but two observations suggest that there is a tendency for microtubules to be oriented with their plus-ends outward. First, the localization of the Golgi apparatus to the center of a cell is known to result from the transport of Golgi elements toward minus-ends of microtubules, and the Golgi apparatus is centrally situated within the neuron (Ahmad *et al.*, 1998). Second, tyrosinated tubulin is more enriched toward the plus-ends of microtubules, and immunostaining for tyrosinated tubulin extends closer to the periphery of the cell body than staining for detyrosinated tubulin (Baas *et al.*, 1991). Perhaps the transport of NuMA towards minus-ends of microtubules helps to orient these ends towards cell center. In this regard, the microtubule array of the entire cell body might be likened to an imperfect aster. However, it is important to note that the same tendency for microtubules to be oriented with plus-ends outward can be observed in younger neurons in which the NuMA particles are not yet apparent. Thus if NuMA plays a role in orienting microtubules in the cell body, one must conclude that such a role is important for meeting new challenges that arise during the time when dendrites start to develop. For example, when dendrites start to develop, the motor protein termed *CHO1/MKLP1* begins to transport microtubules from the cell body into these processes with their minus-ends leading (Sharp *et al.*, 1997). Perhaps NuMA is important for maintaining a plus-end-distal orientation of those microtubules that remain in the cell body.

It is possible that the principal role of the NuMA particles is more profound and relates directly to the targeting of microtubules during dendritic differentiation. During axonal development, microtubules are preferentially transported into the axon (Yu *et al.*, 1994); and then, during dendritic development, they are preferentially transported into the dendrites (Sharp *et al.*, 1995). Experimental analyses suggest that the transport of microtubules into the axon is suppressed during early dendritic development, as is the transport of plus-end-distal microtubules into the dendrite (Sharp *et al.*, 1995). Other recent studies indicate that cytoplasmic dynein is the motor protein that transports plus-end-distal microtubules from the neuronal cell body (Ahmad *et al.*, 1998). Given all this, it would appear that dynein-driven microtubule transport is suppressed over the window of time during which CHO1/MKLP1-driven microtubule transport is targeted to dendrites. It is compelling to speculate that the transport of the NuMA particles during this window of time might monopolize the cytoplasmic dynein that would otherwise have been used for plus-end-distal microtubule transport. Such a scenario, which obviously needs rigorous testing, is attractive because it is consistent with the available data and provides a satisfactory answer to an important question regarding the establishment of neuronal polarity.

In conclusion, the present studies have documented that NuMA, a protein previously thought only to associate with microtubules only during mitosis, is a component of the somatodendritic microtubule arrays of terminally post-mitotic neurons. While the precise function of this protein remains unclear, it seems reasonable that cytoplasmic NuMA may be important for organizing microtubules during dendritic development. These observations complement our previous work showing that at least three molecular motor proteins known to organize microtubules within the mitotic spindle are also important components of the post-mitotic microtubule arrays of the neuron. Thus, it would appear that the mechanisms that organize microtubules during mitosis and within the post-mitotic neuron are variations on the same theme, and that many of the same molecules are utilized in both cases.

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