

Distribution of the microtubule-related protein ninein in developing neurons

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Abstract

Ninein associates with the centrosome in many cell types, where it recaptures minus-ends of microtubules after their release. In more complex and polarized cells, ninein has also been observed at noncentrosomal locales, where its function is not as well understood. We have found that cultured neurons contain both centrosomal and noncentrosomal ninein, and that the noncentrosomal ninein, typically observed as small particles, is both abundant and widespread. Noncentrosomal ninein is also dispersed throughout the cytoplasm of non-neuronal cells present within the cultures, but is particularly rich in the cytoplasm of neurons, where it may compete with centrosomal ninein to impede the recapture of microtubules by the centrosome after their release. Interestingly, noncentrosomal ninein is concentrated in regions of both neurons and non-neuronal cells undergoing retraction, such as in the trailing processes that retract during neuronal migration. These results suggest that noncentrosomal ninein may contribute to the configuration of the microtubule array underlying alterations in cellular morphology, and that such a contribution is likely to be particularly important for neuronal cells.

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1. Introduction

Simple nonpolarized cells contain a radial microtubule array that emanates from the centrosome, to which the minus-ends of the microtubules are attached. Recent studies suggest that microtubules are released from the centrosome after their nucleation, but then their minus-ends are “recaptured” by a protein called ninein that is present within the centrosome (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Piel et al., 2001; Abal et al., 2002; Chen et al., 2003). In polarized epithelial cells, a portion of the ninein is relocated to the apical surface, where it affiliates with microtubules that have been transported after

their release from the centrosome (Mogensen et al., 2000; Piel et al., 2001). Thus, it appears that ninein is a likely participant in configuring microtubules into distinct patterns of organization in polarized as well as nonpolarized cells. At present, the prevailing view is that ninein affiliates with minus-ends of microtubules during their transit, and then associates with structures in the apical membrane to which the minus-ends of microtubules become anchored. Direct evidence for this model has yet to be established, and little is known about ninein across cell types. For example, nothing is known about ninein in neurons, which are even more dramatically polarized than epithelial cells. In neurons, virtually all of the microtubules are released from the centrosome and then deployed to various compartments and locations in the neuron where they are organized into distinct subcellular patterns of organization (Ahmad and Baas, 1995; Baas, 1996).

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Many types of neurons migrate during their development, utilizing a leading process which provides a direction for migration and possibly tugs the neuron along as well as a trailing process which usually retracts as the neuron progresses forward (Rakic, 1990; Hatten 1999; Lambert de Rouvroit and Goffinet, 2001). Microtubules form a cage around the nucleus of the migrating neuron, and this cage somehow affiliates with the microtubules in the leading process, such that the nucleus is propelled forward during migration (Rivas and Hatten, 1995; Rakic et al., 1996). In conjunction, the microtubules within the trailing process move inward as the process shortens and is re-absorbed into the cell body. Recent work has indicted key roles for microtubule-related proteins such as doublecortin, cytoplasmic dynein, and Lis1 in this process (Feng and Walsh, 2001), but very little work has been done on the potential involvement of proteins involved in the release and capture of microtubules. Here, we have begun a series of analyses on ninein in neurons by examining its distribution in migrating rat granule neurons in culture.

2. Methods

2.1. Cell culture

Reaggregate cultures of cerebellar cells, which consist mostly of granule neurons, were prepared as described with minor modifications (Gao et al., 1992; Hatten et al., 1998). Briefly, two cerebella were dissected from euthanized rat pups on postnatal day 3–5. (All animal methods followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee.) Cerebella were treated with trypsin and dissociated by titration in narrow bore pipettes. To form reaggregates, cells were then resuspended in 10 ml serum-containing medium (MEM supplemented with 10% horse serum (Roche Applied Science, Indianapolis) 10% fetal calf serum (HyClone, Logan, Utah), 1 mg/ml glucose, 2 mM L-glutamine (Roche) and penicillin/streptomycin (200 units/ml each; Roche), placed in a loosely capped, 15 ml centrifuge tube, and incubated for 18–24 h at 37 °C, 5% CO₂. Reaggregates were plated in serum-containing medium into coverslip-bottomed wells (Baird et al., 1994) coated with 25 µg/ml laminin (Roche). After reaggregates adhered to the laminin-coated coverslips, serum-containing medium was replaced with serum-free medium consisting of MEM containing N₂ supplement (Roche), 0.1% glucose, 2 mM L-glutamine (Roche) and penicillin/streptomycin (200 units/ml each; Roche). Cells immediately began migrating out of the reaggregates and were imaged live or fixed and immunostained within 1–2 days after plating.

2.2. Anti-ninein antibody

The peptide (“Pep3”) used to produce the ninein antibody was a 275 amino-acid sequence tagged to GST (corresponding to 952–1227 of the mouse sequence). This was expressed in *E. coli* (BL21) and purified using a glutathione sepharose binding column. The total protein content of the elution was checked and showed two major bands corresponding to Pep3–GST and GST. The ninein antibody was generated by injecting rabbits with the purified Pep3–GST peptide (Harlan Labs, UK). Serum was tested by immunolabeling; pre-immunization serum gave no specific labeling and final bleed serum gave centrosomal labeling. The centrosomal labeling could be blocked by pre-incubating with Pep3–GST peptide. Affinity purified antibody was produced by passing serum through a GST bound column and then passing flow-through over a Pep3–GST column. Antibodies were eluted from the second column. Affinity purified antibodies were again tested by immunolabeling and found to specifically label the centrosome and could be blocked with Pep3–GST peptide. Dot blots of Pep3–GST and UE-1 lysates (inner ear epithelial cell line) were carried out. The affinity purified antibody recognized Pep3–GST but not GST alone. Western blot (using UE1 lysates) of N3 labeled a band at approximately 240 kDa.

2.3. Immunostaining

Primary cerebellar neuronal cultures were fixed using 0.2% glutaraldehyde for 15 min at 20 °C, and were either co-extracted or post-extracted using 0.2% Triton-100. After fixing, cells were washed with PBS three times for 5 min and then incubated in 10 mg/ml sodium borohydride in PBS two times for 15 min. For post-extraction, the 0.2% Triton-100 was dissolved in the sodium borohydride solution prior to exposure to the fixed cells. Following treatment with 10 mg/ml sodium borohydride, all fixed cultures were washed in PBS three times for 5 min. Cultures were then blocked for 1 h at 20 °C using filter sterilized 10% NGS and 10 mg/ml BSA in PBS (blocking medium). Cultures were rinsed once briefly with PBS followed immediately by exposure to rabbit polyclonal ninein antibody (1:250) for 1 h at 37 °C. The primary antibody was then removed and blocking medium was applied for 30 min. This was followed by application of the secondary antibody solution which consisted of goat anti-rabbit antibody conjugated to Cy3 (Sigma, 1:200) together with a mouse FITC conjugated β-tubulin antibody (Sigma, 1:100) for 1 h. Cells were then rinsed extensively in PBS, and mounted in a medium that reduces photobleaching. Image acquisition was performed using an Axiovert 200 M inverted microscope equipped with a 100x/1.3NA Plan Apo oil immersion objective (Carl Zeiss,

Germany), and ninein and tubulin staining was visualized using appropriate wavelength filter cubes (Chroma Corp.) for Cy3 and FITC fluorescence, respectively.

2.4. Imaging of GFP–ninein

After dissociation as described under “Cell Culture”, cells were resuspended in Nucleofector solution (Amaxa Biosystems, Gaithersburg, Maryland), and then transfected with GFP–ninein cDNA (10 μ g per 100 μ l suspension) using an Amaxa electroporator nucleofection device according to manufacturer’s instructions and recommended settings. Cells were then allowed to reaggregate, and reaggregates plated as described under “Cell Culture”. After plating and adhesion of the reaggregates, serum-free medium was replaced with identical medium, except lacking sodium bicarbonate. Images were obtained using the same Axiovert 200M microscope and objective lens described above, equipped with heated stage and objective lens, a high resolution cooled CCD camera (Orca ER, Hamamatsu, Japan) and a computer running Axiovision software (Carl Zeiss).

3. Results

We took two approaches for visualizing the distribution of ninein in neuronal cultures. First, we immu-

nostained fixed cultures with a polyclonal antibody to ninein, while simultaneously immunostaining them to visualize microtubules. Second, we expressed a GFP–ninein construct (Abal et al., 2002) and observed the distribution of the fluorescent protein in living cells. The first approach generally revealed a more widespread ninein distribution than the second. Given the specificity of the anti-ninein antibody, we therefore assume that immunolabeling better represents the total ninein pool. Visualization using GFP–ninein provided information on dynamic changes in ninein distribution over time. In general, the GFP–ninein distribution was simpler than that revealed by the immunostaining, perhaps because it reflects the portion of the endogenous ninein that most rapidly exchanges with the newly synthesized pool. Expression was generally moderate in all of the cells in our study; we saw no evidence that ninein was over-expressed to the point that it altered the normal organization of microtubules (as in Abal et al., 2002).

3.1. Ninein distribution in non-neuronal cells

Cerebellar cultures consist predominantly of granule neurons, but also contain a small number of non-neuronal cells that include both glia and fibroblasts. As previously reported for fibroblasts and non-neuronal cell lines, immunostained ninein was abundant at the centrosome of non-neuronal cerebellar cells (identified

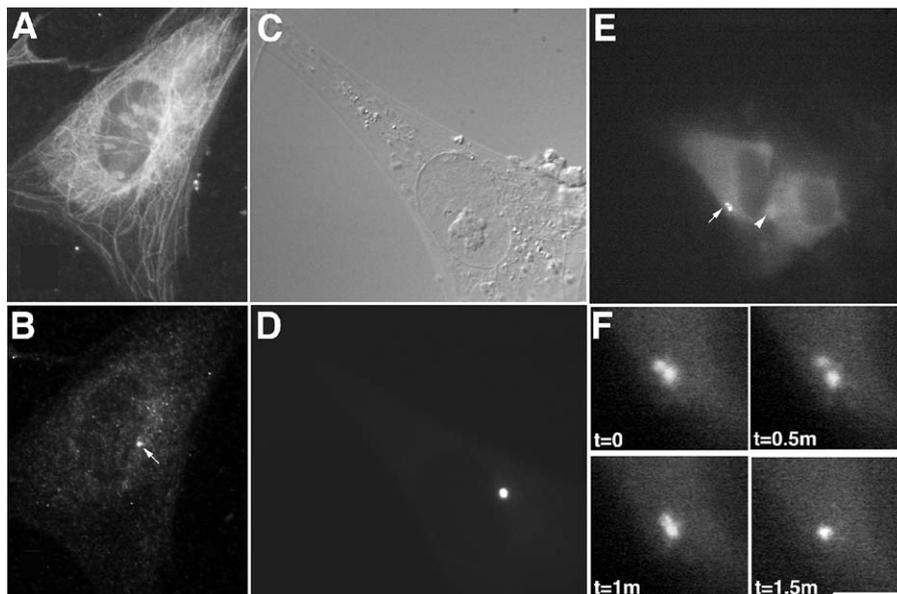


Fig. 1. Ninein in non-neuronal cells derived from cerebellum. (A) Cell immunolabeled for β -tubulin. (B) Same cell immunolabeled for ninein. Ninein fluorescence is highest at a single location (arrow), presumably the centrosome, but low levels of punctate fluorescence are present throughout most of the cell. Note apparent location of centrosome based on microtubules in A corresponds to brightest ninein fluorescence in B. (C) DIC image of a single cell. (D) Same cell as C showing fluorescence from ninein–GFP. A single region near the nucleus is brightly fluorescent, presumably the centrosome. (E) Two cells expressing GFP–ninein. In one cell, the centrosome is clearly visible (arrow), in the second cell, the centrosome is out of the plane of focus (arrowhead). (E) Time-lapse images at higher magnification of apparent centrosome in E. What appear to be two centrosomes move with respect to one another. Scale bar = 30 μ m (A–E); 7.5 μ m F.

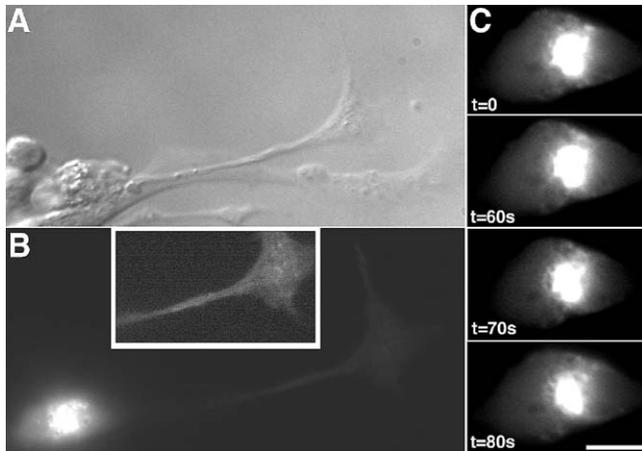


Fig. 2. Ninein in a migrating neuron. (A) DIC image of a cerebellar granule neuron with a cell body that had translocated in prior time-lapse images. Note leading process extending to upper right from cell body at lower left; cell body appears to be translocating on a process of another cell. (B) GFP-ninein fluorescence, same cell as in A. Note bright fluorescence from region of centrosome and surrounding areas; leading process has faint GFP-ninein fluorescence, better seen with increased contrast (inset). (C) Time-lapse images at higher magnification of cell body in B. Note that the distribution of pericentrosomal ninein changes from image to image. Scale bar = 16 μ m (A,B); 10 μ m (C).

by its convergence with the point from which splayed microtubules emanated), and also appeared as a scattering of noncentrosomal particles (Fig. 1A/B). In transfected non-neuronal cells, GFP-ninein was most abundant at the centrosome. It was often quite bright

at the centrosome with little or no noncentrosomal particles visible (Fig. 1C/D), suggesting that centrosomal ninein may be particularly dynamic compared to noncentrosomal ninein with regard to binding newly synthesized ninein protein. Sometimes a pair of apparent centrioles containing GFP-ninein could be discerned, with centrioles rapidly moving short distances with respect to each other during time-lapse imaging (Fig. 1E/F). Noncentrosomal ninein sometimes appeared as a diffuse haze in these cells. Discrete particles more akin to the immunostaining were also detected in some cells, particularly in retracting regions (see Fig. 4).

3.2. Ninein distribution in neurons

In comparison to non-neuronal cells, expression of the GFP-ninein was generally higher, with a distribution that varied considerably from cell to cell. In the majority of expressing neurons where GFP-ninein was detected at the centrosome, the amount of GFP fluorescence at the centrosome was high, and the region occupied by centrosomal and pericentrosomal ninein was large in comparison to non-neuronal cells. This type of distribution was seen in migrating cerebellar granule cells which contained abundant labeling in the vicinity of what appeared to be the centrosome, and additional extracentrosomal ninein, more distant from the centrosome, but still in the cell body (Fig. 2A/B; supplemental movie #1 online). Cells were considered migrating if their cell bodies translocated a distance at

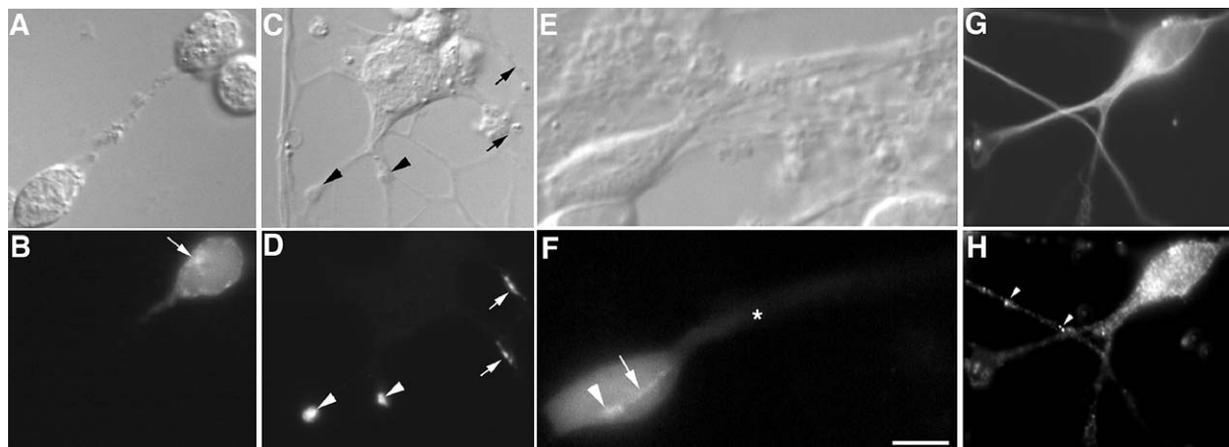


Fig. 3. Ninein in neurons with processes. (A) DIC image of a neuron (upper right) that recently extended a process onto another process from a second neuron (lower left). (B) GFP-ninein fluorescence, same cell as A. Note that regions of higher GFP-ninein are scattered around the nucleus, distinct from the apparent centrosome (arrow). (C) DIC image of a neuron with several processes. Two processes have bulbous endings (arrowheads), while two are more tapered (arrows). (D) GFP-ninein fluorescence, same cell as C. Note that GFP-ninein is only detected in processes, not in the cell body. Also note how GFP-ninein conforms to the shape of the processes; bulbous in bulbous process endings (arrowheads) and more extended in tapered processes (arrows). (E) DIC image of a neuron with a process. (F) GFP-ninein fluorescence, same cell as E. Apparent centrosome contains GFP-ninein (arrowhead) along with a filamentous structure (arrow) directed from the centrosome toward the process. Diffuse GFP-ninein is present throughout the cell body and at lower levels within the process (asterisk). (G) Cerebellar cell immunolabeled for β -tubulin. (H) Same cell as in G, immunolabeled for ninein. Note abundant ninein throughout cell body. Ninein particles (arrowheads) are also present in processes, but in lesser amounts. Scale bar = 16 μ m (A–D); 10 μ m (E, F); 3.5 μ m (G, H).

least that equal to their diameter during time-lapse observations. Both the pericentrosomal and non-centrosomal ninein in migrating neurons showed a great deal of movement, as seen in images from a time-lapse series (Fig. 2C). GFP–ninein was also present in neuronal processes including the leading process of migrating neurons, but at very low levels (Fig. 2B). Noncentrosomal ninein was found in neurons in a number of stages of development in addition to migration. Fig. 3B is an image of noncentrosomal ninein in a neuron that had already extended a process. Time-lapse observations of this cell revealed that non-centrosomal GFP–ninein also showed movement (data not shown). Noncentrosomal GFP–ninein particles moved in a directed fashion around the nucleus, and therefore most or all of these movements cannot be attributed to Brownian motion.

Also in contrast to non-neuronal cells, some transfected neurons lacked GFP–ninein at their centrosomes. Fig. 3D shows a neuron in which concentrations of GFP–ninein are only visible in processes, while the cell body is devoid of detectable fluorescence. Neurons in which processes had recently stopped growing often contained concentrations of ninein in their processes, approximately 5–10% of neurons. Another aspect of the transfected neurons was the occasional appearance of ninein in the form of filaments, perhaps reflecting the ninein protein associating along the length of a microtubule. Fig. 3F shows an example of this phenomenon. This filamentous distri-

bution of GFP–ninein persisted over an observation period of several minutes. Other examples of filamentously distributed GFP–ninein were more transient, with filaments appearing and then disappearing within seconds or minutes. Even within the filamentous formations, however, including the one shown in Fig. 3F, there was evidence of GFP–ninein movement; time-lapse images revealed movement of brighter substructures within the filaments (data not shown). Immunostaining for endogenous neuronal ninein showed abundant quantities of noncentrosomal ninein, far more than observed in non-neuronal cells. The levels were generally so high that we could usually not discern a clear centrosome amid the noncentrosomal particles, which often appeared as larger clumps (Fig. 3H).

3.3. *Ninein is concentrated in regions of neurons and non-neuronal cells undergoing retraction*

A particularly intriguing aspect of noncentrosomal ninein was its association with cellular regions undergoing retraction, as illustrated in Fig. 4, and online in supplemental movie #2. Fig. 4A–C are from a time-lapse series showing retraction of one portion of a non-neuronal cell. Note the relatively high levels of noncentrosomal ninein particles within and proximal to the region being retracted. The association between retraction and ninein was also seen in neurons, both during retraction of neurites (Fig. 4D/E) and in the

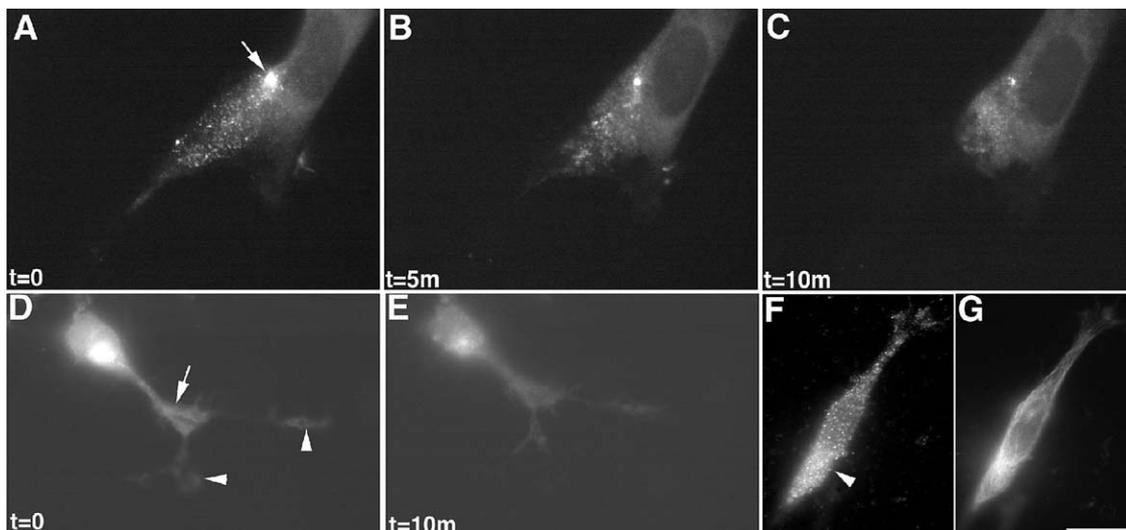


Fig. 4. Ninein associated with retraction. (A–C) GFP–ninein fluorescence in a non-neuronal cell, images from a time-lapse series. GFP–ninein is present at high levels at the apparent centrosome (arrow). Particles of GFP–ninein are present away from the centrosome in one region of the cell. In panels B and C, the region containing high levels of ninein retracted. (D and E) GFP–ninein in a spontaneously retracting neuronal processes. Process partially retracts between panels D and E (arrowheads). GFP–ninein is present within the more proximal process (arrow) from which the retracting processes branch (arrowheads). The cell body also contains GFP–ninein. (F) Cultured cerebellar cell with morphology characteristic of a migrating cell, immunostained for ninein. Ninein-containing particles are scattered throughout the cell, but are concentrated within the apparent trailing process (arrowhead). (G) Same cell as F immunostained for β -tubulin, which is also abundant in the trailing process. Scale bar = 30 μ m (A–E); 8 μ m (F, G).

trailing processes of migrating neurons, which are retracted during migration (Fig. 4F/G). Unlike non-neuronal cells, where ninein could often be detected only in and near the regions of retraction, in neurons, ninein was elevated within, and proximal to, the process or cellular region to be retracted, but lower levels of ninein were also present in many other regions.

4. Discussion

Traditional views of the microtubule cytoskeleton have derived mainly from simple fibroblastic cells that display a radial array of microtubules emanating from the centrosome during mitosis, and a bipolar spindle during mitosis. In recent years, a great deal of information has emerged on more sophisticated and polarized cell types such as neurons and certain kinds of epithelial cells, which do not display radial microtubule arrays, and in the case of neurons, are terminally post-mitotic. While neurons certainly express microtubule-related proteins that are fairly specific to neurons, we have proposed that key features of the neuronal microtubule arrays are established and organized by essentially the same toolbox of proteins that organize microtubules in mitotic cells (Baas, 1999; Baas and Buster, 2004). For example, microtubules are nucleated from the centrosome of the neuron by gamma-tubulin, released by katanin, and are then subjected to essentially the same motor proteins that generate spindle movements. In the neuron, these motor proteins drive the microtubules into axons and dendrites (Sharp et al., 1997; Ahmad et al., 1998). The protein called NuMA, which localizes to spindle poles during mitosis and is driven into the nucleus during interphase, appears in neurons in the form of cytoplasmic particles that may have a role in organizing microtubules, particularly in dendrites (Ferhat et al., 1998). In the present study, we have documented that neurons are also rich in non-centrosomal particles of ninein, the distribution of which suggests a potential role in reconfiguring microtubules during changes in cellular morphology.

Centrosomal, ninein is proposed to contribute to recapturing microtubule minus-ends. In most non-neuronal cells, ninein is concentrated at the centrosome, but is also present in scattered noncentrosomal particles, where its function is not well understood. Neurons contain far more of the particles, such that a well-defined centrosome is not apparent from ninein immunostaining. However, GFP-ninein is detected at the centrosome, suggesting that the neuronal centrosome does contain ninein, probably the most dynamic pool. Neurons are different from most non-neuronal cells in that it appears that microtubules are rarely recaptured by the centrosome after their release (Yu et al., 1993; Ahmad and Baas, 1995). We suspect that this is due to

competition between the ninein present at the centrosome and the comparatively larger pool of extra-centrosomal ninein that would tend to diminish the recapture of microtubules by the centrosomal pool. This inhibition of recapture may be crucial for the formation of the cage of microtubules around the nucleus that characterizes migratory neurons, as well as for the capacity of individual microtubules to vacate the cell body as they transit into developing processes. In non-neuronal cells, we see the greatest abundance of non-centrosomal ninein particles in regions of the cell that are undergoing retraction, and this theme is also observed in neurons, where we see ninein concentrated in processes undergoing retraction during neuronal migration. The abundance of ninein particles during retraction may reflect the importance of microtubules and associated ninein during movements of polarized cells, and the rearrangements of microtubules that accompany retraction, as demonstrated during retraction of neuronal processes (He et al., 2002). The concentration of ninein in retracting processes, however, is likely to reflect a specific role for ninein, rather than ninein being abundant simply because microtubules are prominent in the retracting region. This view is supported by observations of many regions of neurons where microtubules were abundant, but did not contain high levels of ninein.

In images and some frames of our movies, we have captured moments when the ninein particles seem more filamentous in nature, presumably reflecting transient binding or movement along the microtubules. In other images, we see a complete lack of any GFP-ninein at the centrosome and an accumulation in distal regions of processes, which is reminiscent of the accumulation of ninein at the apical surface of epithelial cells (Mogensen et al., 2000). Given the differing distribution of ninein in neurons in different stages of development and the fact that ninein appears stable in some neurons and is associated with moving particles in others suggests that ninein may be involved in more than one function in neurons, or at least that its function is regulated developmentally.

In neurons, there is no apparent reason why minus-ends of microtubules would be “captured” by any structure far from the centrosome. In fact, it appears to be the plus-ends that are generally directed outward, particularly in distal regions such as growth cones (Baas and Buster, 2004). Therefore, it is unclear, based on current views on ninein, exactly what ninein might be doing to facilitate microtubule organization at the tips of processes or within retracting processes. The fact that ninein concentrates within cells and regions of cells undergoing morphological changes suggests that it may be part of a cascade of events, possibly including motor proteins and other factors such as NuMA, that assist in the rapid reorganization of microtubules. We

are enthusiastic about future studies on ninein that will seek to directly ascertain its functions in developing and migratory neurons.

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