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Polarity of microtubule assemblies during neuronal cell migration

(neural development/cerebellar granule cells/cell motility)

PASKO RAKIC*, ELIZABETH KNYIHAR-CSILLIK†, AND BERTALAN CSILLIK‡

Section of Neurobiology, Yale University School of Medicine, New Haven, CT 06511

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ABSTRACT **The active migration of neurons from their sites of origin to their final destinations requires the unidirectional translocation of the nuclei and somatic cytoplasm within the growing leading processes. To explore the cellular machinery underlying this translocation, we determined the polarity of microtubules situated within the leading and trailing processes of migrating cerebellar granule cells *in situ*. Our analysis reveals that the newly assembled positive ends of the microtubules in the leading process uniformly face the growing tip, while their disintegrating negative ends face the nucleus. In the trailing process, by contrast, microtubule arrays are of mixed polarity. We suggest that the dynamics of slow polymerization in combination with fast disintegration of oriented microtubules create "push" and "pull" forces that contribute to the piston-like saltatory displacement of the nucleus and cytoplasm within the membrane cylinder of the leading process of the migrating neuron.**

The cerebellar granule cell has fascinated developmental neurobiologists since the classical description by Ramon y Cajal (1) of its orderly and sequential migration across the molecular layer of the cerebellar cortex. Migrating neurons initially extend a cell extension called the leading process, which is followed by the translocation of the cell's nucleus within the extended membrane envelope (2–4). Although many aspects of neuronal cell migration have been elucidated in recent years (reviewed in refs. 5–8), the molecular machinery that translocates the cell nucleus itself remains unknown. It seems reasonable that a considerable mechanical force including a rearrangement of cytoskeletal scaffolding is an essential prerequisite for this translocation since the entire cell body is propelled across the developing molecular layer, an area that is densely packed with the cellular processes of earlier-generated neurons. Electron microscopic analyses have revealed parallel arrays of microtubules in both the leading and trailing processes of granule cells as they begin to move across the molecular layer (2). The large number of microtubules and their longitudinal deployment in the leading process have implicated them in the translocation of a cell's soma (9, 10), but beyond this there is little known about how microtubule proteins become assembled and how they function in migrating neurons. Therefore, knowledge about the polarity of the assemblies, sites of nucleation and posttranslational modifications of microtubule components in the cytoplasm of premigratory granule cells may provide new insights into the mechanism of the movement of cells during their migration.

Microtubules in mammalian neurons consist of α and β tubulin molecules polymerized in the form of a hollow tube (13). In general, new tubulin oligomeres are added to each microtubule at one pole, the positive end, and are deleted at the other pole, the negative end (14). The recently developed "hook assay" technique has permitted determination of the polarity of polymerization (direction of growth) of these microtubular assembly in individual cultured cells (11, 12). In this assay, a living cell is exposed to exogenous microtubule protein in the presence of a membrane destabilizer and energy source. This results in sheets of oligomeres attaching to the side of pre-existing microtubules in an arched manner that appear as a hook on high power electron micrographs. When one views an individual microtubule from the perspective of the distal end of a process facing toward the cell body, the clockwise and counterclockwise orientation of the hooks signifies, respectively, the positive and negative ends of that polymer (11, 13). Although this method was used to determine the polarity of microtubules in neurites of dissociated cultured neurons as well as in the axons of the peripheral nervous system (15–19), it has not been applied previously to the study of intact developing central nervous tissue. In the present study, we have used a modified hook method to examine the organization and polarity of microtubules in the leading and trailing processes of migrating granule cells of the developing cerebellar cortex in an effort to elucidate what underlies the translocation of cell nucleus within the cytoplasm of the migrating neurons.

MATERIALS AND METHODS

For the purpose of the present study, we modified the original hook assay method of Black and Baas (15) for use in intact, developing brain tissue. Briefly, unfixed blocks of cerebellar cortex were incubated with exogenous microtubule protein and cut transverse to the direction of the migratory pathway (Fig. 1*A*). The samples were then postfixed and processed for electron microscopic analysis. Specifically, blocks of the cerebellar cortex from 8- to 10-day-old rats were cut parallel to the pial surface at 4°C by free hand-held razor blade to expose the profiles of the leading and trailing processes in the transverse plane. The blocks were immersed for 2 min at 37°C in 0.5 M Pipes buffer containing 0.06% polyethylene glycol hexadecyl ether Brij 58 as a membrane destabilizer, an energy source (0.5 mM guanosine triphosphate), a chelating agent (0.01 mM EDTA), and exogenous microtubule protein, 5 mg/ml of the incubation solution. This was followed by incubation at 37°C in a similar microtubule assembly buffer without polyethylene glycol hexadecyl ether, for 30 min. Following these incubations, the first two freehand cut sections were fixed in cacodylate-buffered (pH = 7.2) 2% glutaraldehyde, treated with 0.15 mg/ml tannic acid to increase contrast, postfixed in 1% osmic acid for 30 min, dehydrated in graded alcohol, and embedded in an Epon/Araldite mixture. Semithin sections were obtained and stained with toluidine blue to control orientation of the tissue blocks. Serial thin sections were collected on slot grids by immersion (i.e., from below) to preserve their relative position and orientation, stained with

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*To whom reprint requests should be addressed.
†On leave of absence from: Department of Neurology, Albert Szent-Gyorgyi Medical University, Szeged, Hungary.
‡Fogarty Senior Scientist, on leave of absence from: Bay Zoltan Institute for Biotechnology, Szeged, Hungary.

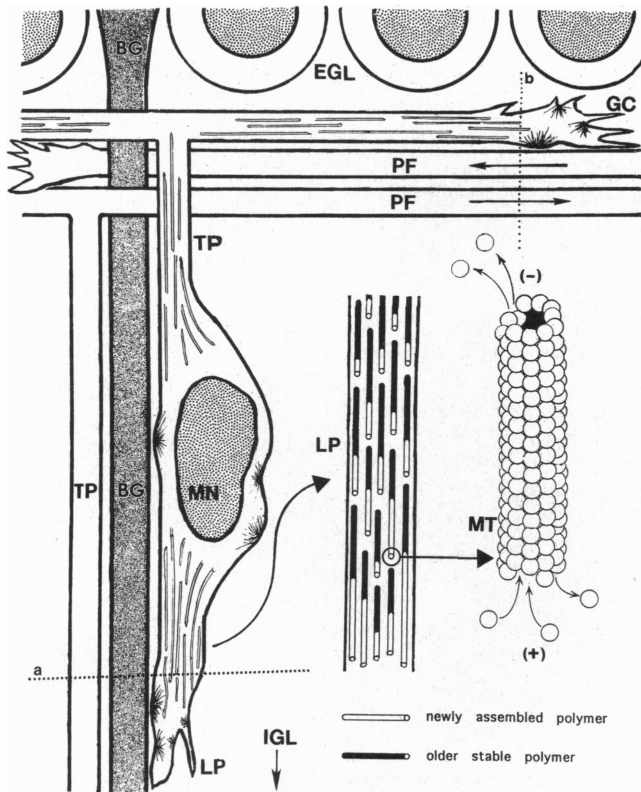

FIG. 1. A model of the distribution and polarity of microtubules (MT) within the processes of a migrating cerebellar granule cell (MN) as it moves from the external granular layer (EGL) to the internal granular layer (IGL). Microtubules become abundant in the postmitotic granule cell as it leaves the EGL, with its leading process (LP) and cell body aligned along the Bergmann glial fiber (BG). The migrating cell leaves behind a trailing process (TP) and the T-shaped axon with two growth cones (GC) that eventually form parallel fibers (PF). In the present study the polarity of microtubules was analyzed in the plane parallel to the cerebellar surface (dotted line "a"), which transect the leading and trailing processes at right angle, while the polarity of microtubules in the parallel fibers was examined in the sagittal plane (dotted line "b"). Newly assembled polymers (white cylinders) where tubulin molecules are added at the positive (+) end, and a stable polymers (black cylinders) with a negative (−) end where the microtubules (MT) become disassembled were recorded in each process. As documented in Fig. 2 and 3, microtubules in the leading process have their positive (+) ends containing newly synthesized polymer, uniformly oriented toward the growing tip, while the trailing processes (Fig. 4) contained microtubules of mixed orientation.

uranyl acetate and lead citrate, and then examined on a JEOL JEM 1010 electron microscope at ×30,000 to ×100,000.

To correctly assess the polarity of the individual microtubule within the identified cell process a proper orientation of each block and sequence of preincubated sections, both prior to and after fixation, was carefully monitored. We have examined the orientation of microtubules in the four principal cytoplasmic compartments of immature granule cell (7, 20): the leading processes, trailing process, growth cone, and parallel fibers. The trailing and leading processes could be identified by their orientation, size, shape and relationship to adjacent cellular elements in transverse sections cut parallel to pial surface (Fig. 1*A*), as well as by the distribution and electron density of cytoplasmic organelles (Figs. 2–4). Polarity of the microtubules in the parallel fibers and growth cones was examined in the sagittal sections, cut perpendicular to their orientation (Fig. 1*B*). After identification of these processes at low power, the orientation of the hooks attached to the individual microtubules was scored on the electron micrographs at final magnification of ×50,000 to ×25,000.

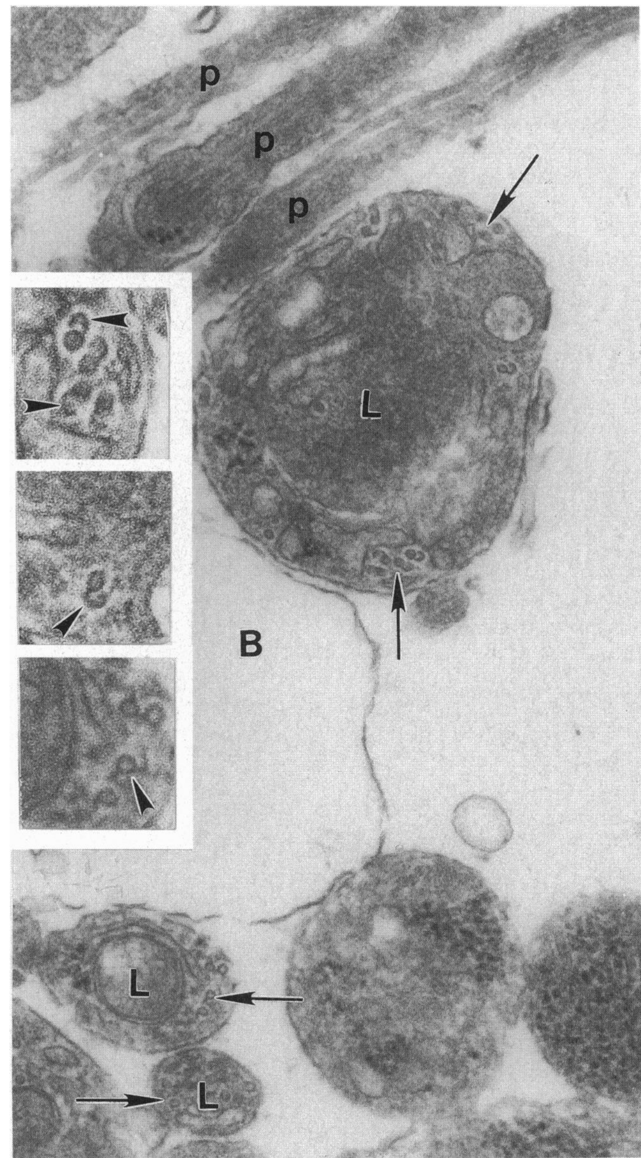

FIG. 2. Electron micrograph of transversely cut leading processes (L) of a migrating granule cell in a postnatal day 8 rat cerebellum, incubated with exogenous microtubule protein. The three leading processes (L) are characterized by a dark cytoplasm, compartments of microtubules and numerous ribosomes attached to the vacuous Bergmann glia process (B). Monomers of the exogenous tubuline protofilament sheets on the walls of the microtubules appear as hooks whose orientation signifies the direction of the assembly of the filament. This section, cut across the molecular layer, is viewed from the perspective of the cell body looking toward the internal granular cell layer. Therefore, the hooks directed counterclockwise signify that the growing, positive (+) end is oriented toward the internal granular layer, i.e., in the direction of cell movement. In the three leading processes illustrated, all hooks on the microtubules, proceeding in compartments (arrows), are oriented counterclockwise and therefore the (+) ends were directed toward the growing tip. Note the parallel fibers (p) that are cut longitudinally, and the highly hydrated cytoplasm of the transversely cut shaft of Bergmann glial cell (B) that is associated with the leading processes (L). (×75,000.) (*Insets*) Higher-power electron micrographs of three microtubule compartments. Note the uniform counterclockwise orientation of the hooks (arrowheads). (×187,500.)

RESULTS

Although treating unfixed, immature tissue with detergent, as required by this method, has deleterious effects on the preservation and integrity of cellular ultrastructure, it was possible

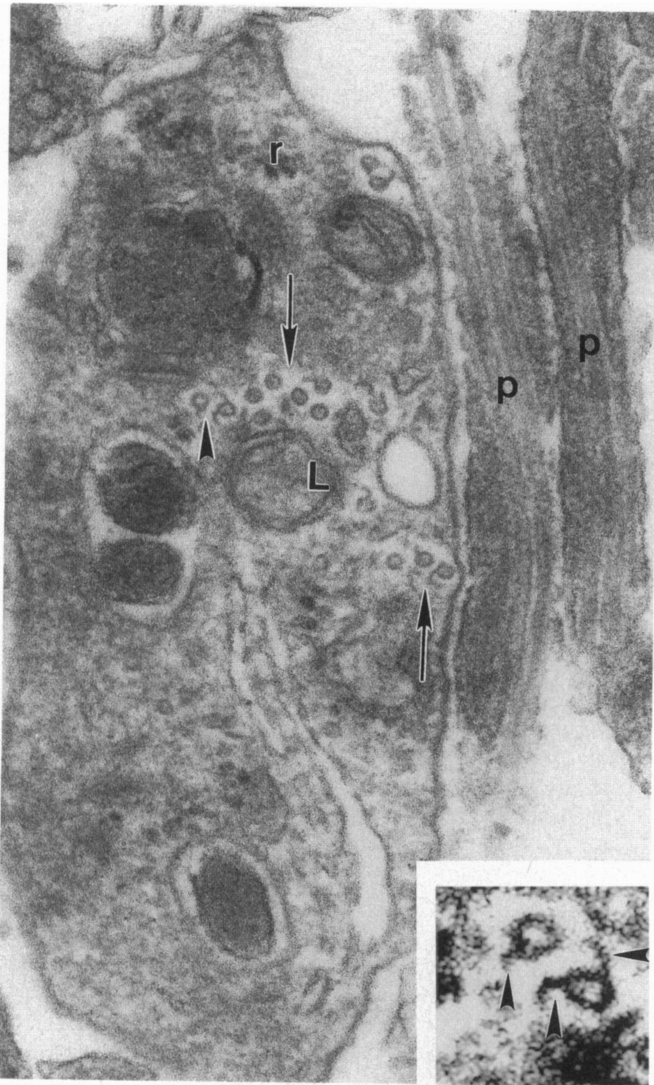

FIG. 3. Cross section of a leading process (L) from a cerebellum of 9-day-old rat, characterized by a dark cytoplasm, compartments of microtubules (arrows) and numerous ribosomes (r). Note the uniform counterclockwise orientation of the hooks, indicating that their positive (+) ends are oriented toward the internal granular layer. The orientation of hooks is more explicit when examined at higher magnification (*Inset*). p: longitudinal sections of parallel fibers. (×125,000; computer-enhanced and enlarged inset: ×437,500)

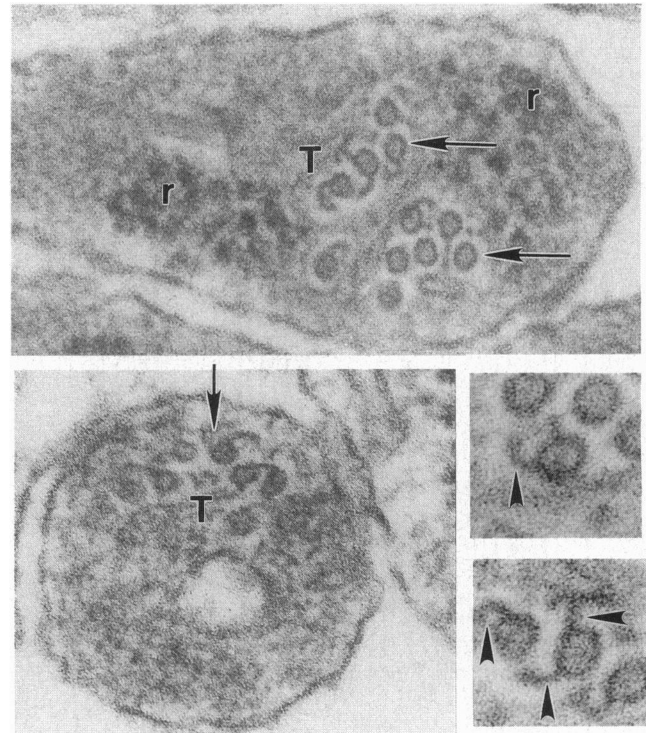

FIG. 4. Cross sections of two trailing processes (T) in the cerebellum of a postnatal day 9 rat. Microtubules are arranged in compartments (arrows); r: ribosomes. Note that the hooks attached to the microtubules are of mixed orientation, indicating mixed polarity of microtubules, i.e., within the same process one can find both positive (+) and negative (−) ends of microtubules oriented toward the internal granular layer. (×187,500; computer-enhanced and enlarged insets: ×437,500)

to recognize profiles of the leading and trailing processes of cerebellar granular cells as well as the Bergmann glial and parallel fibers. The more voluminous leading process has a typical, relatively dense cytoplasm containing organelles such as mitochondria, Golgi apparatus, endoplasmic reticulum, and free and attached ribosomes in addition to the arrays of microtubules (2). Trailing processes are of smaller diameter and generally contain more electron-lucent cytoplasm than the leading processes, but the cytoplasmic matrix was also relatively well preserved by this procedure. In contrast, treatment with membrane destabilizer caused the cytoplasm of Bergmann glial fibers to be highly hydrated and vacuous compared with untreated tissue. Parallel fibers and their growth cones are easily recognizable by their shape, orientation, and ultrastructural characteristics. Most importantly for this study, we were able to identify unambiguously the clockwise and counterclockwise orientation of the hooks attached to microtubules in all granule cell processes.

Leading processes were in close topographical relation to transversely cut Bergmann glial fibers and longitudinally oriented parallel fibers (Figs. 2 and 3). Within the leading processes, transected microtubules appear as hollow tubes, ≈28–30 nm in diameter, usually arranged in groups compartmentalized by other cytoplasmic organelles including numerous ribosomes. Material that was incubated with exogenous tubulin contained microtubule profiles that were decorated with distinctly oriented hooks. In less than 15% of microtubules the hooks were either absent or their orientation could not be determined. When sections cut through the molecular layer, parallel to the pia, are viewed from the perspective of the cell body looking toward the internal granular layer, a hook oriented counterclockwise signifies that the growing, positive (+) end of the microtubule is oriented toward the internal granular layer, i.e., in the direction of cell movement. The number of microtubules encountered in a cross section of a given leading process may vary between five and eight, although occasionally we have observed as many as 13. The orientation of the hooks attached to the microtubules was consistently unidirectional. Overall, more than 95% of the 89 identified microtubules in 14 leading processes had their hooks directed counterclockwise. In two cases, in which the leading process contained more then seven hooked microtubules, all assemblies were oriented counterclockwise. Therefore, we conclude that the positive ends of the microtubules in the leading process are overwhelmingly directed toward the direction of cell migration, i.e., to the internal granular layer.

In contrast to this uniform orientation of microtubules in the leading process, the trailing processes contained approximately equal numbers of hooks directed clockwise and counterclockwise (Fig. 4). This indicates that the positive and negative poles of microtubules in this granule cell compartment were oriented randomly in relation to the direction of nuclear displacement. We therefore conclude that microtu-

bules in the leading process have their growing, positive ends facing the direction of cell migration, whereas in the trailing process they are of mixed orientation.

In ultrathin sections cut in the sagittal plane, microtubules decorated with hooks were found in both parallel fibers as well as in their axonal growth cones (Fig. 5). Although in any individual parallel fiber, the orientation of hooks was identical; in some, all the hooks were arranged clockwise, while in others they were all oriented anti-clockwise. This diversity may reflect granule cell geometry in the molecular layer and the orientation of growth of their parallel fibers (Fig. 1). Thus, for any given axon in a section it is not possible to ascertain whether it grows toward or away from the observer (see Fig. 1) and therefore, some axons in a given section were viewed from the direction of the point of bifurcation, while others were viewed from the vantage point of the axonal growth cone. Likewise, one cannot determine whether an axon was cut close to its origin at the bifurcation, where one can expect mixed orientation, or in the proximity of the growth cone where extension occurs and one would expect uniformly polarized orientation. In the few instances in which growth cones were attached to the parallel fibers on the same or adjacent sections, the hooks attached to microtubules exhibited a consistent and uniform counterclockwise orientation (Fig. 5) suggesting that (+) pole were oriented in the direction of the axonal elongation.

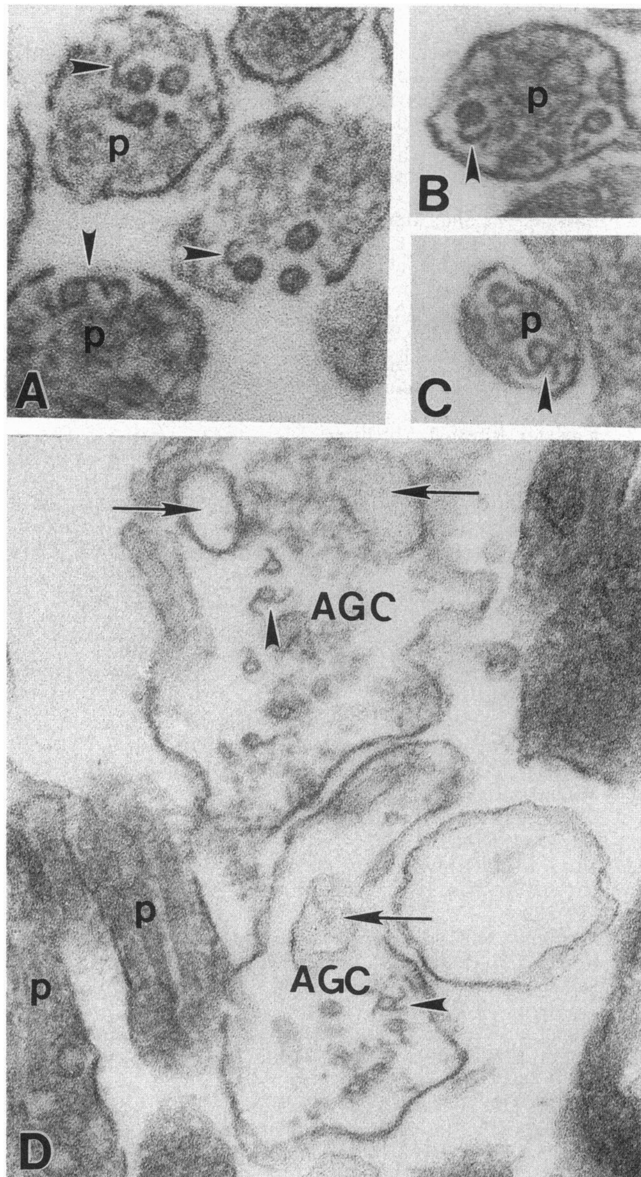

FIG. 5. (*A–C*) Cross sections of several parallel fibers (p) observed in a sagittal section of the cerebellar cortex in a 9 day old rat. Note the uniform polarity of the microtubules in each parallel fiber (identical orientation of hooks, arrowheads). The direction of hooks orientation varies from one parallel fiber to another, depending upon the vantage point (looking from the site of axonal bifurcation or from the perspective of growth cone). (×187,500.) (*D*) Axonal growth cones (AGC) belonging to parallel fibers in the cerebellum of a 8 day old rat. The plane of section is parallel to the cerebellar cortical surface. The growth cones are located amongst longitudinal sections of parallel fibers (p). Note growth cone vesicles (arrows) and the counterclockwise arrangement of the hooks upon microtubules (arrowheads), indicating their uniform polarity. (×124,000.)

DISCUSSION

Application of the modified "hook assay" method to intact tissue of the developing cerebellar cortex revealed a characteristic polarity of microtubule assemblies within distinct cytoplasmic compartments of migrating granule cells. This finding suggests a tangible molecular substrate that may be involved in control of both the directionality as well as the rate of nuclear displacement during neuronal migration. The orientation of microtubules in the two cytoplasmic compartments of the spindle shaped migrating granule cell suggests different dynamics of assembly and disassembly. In the leading process, positive ends of microtubules were uniformly oriented toward the growing tip of the process, while their negative, disintegrated ends were facing the nucleus. We propose that the extension of the leading process may, at least in part, be created by the "push" force of the array of polymers built up in the direction of leading process extension. In contrast, the nucleus and surrounding cytoplasm may be translocated within the membrane envelope because of the orchestrated dissociation of microtubules at their negative ends in front of the nucleus. Such a synchronized disintegration may create the cytoplasmic space devoid of stable cytoskeletal scaffolding, as well as "pull" forces essential for this displacement. These pull forces are expected to be stronger in the leading process, where the polarity of the microtubule array is uniform, than in the trailing process, where their orientation is mixed. A rapid, coordinated depolymerization of microtubule sheets alternating with a phase of relative stability at their (−) ends as observed during locomotion of nonneuronal cells (21–24) may underlie the alternation of movement and stationary periods observed during nuclear displacement in migrating neurons.

Our suggestion that the somatic translocation of the granule cell may, at least in part, depend on the dynamics of polymerization and depolymerization of microtubule protein in the cytoplasm of the leading and trailing processes is consistent with several observations. First, the rate for the linear growth of the microtubule (25, 26) is well within the range of the neuronal cell migration rate of 10–15 μm/hr (6, 7). Second, our model is consistent with the finding that the slow extension of the leading process of migrating neurons precedes the phase of more rapid nuclear displacement (3, 4, 6, 27). Third, it is in accord with the observed rapid nuclear movement alternating with a transient stationary periods (4, 27). The extension of the leading process may be related to slow, association phase (so-called rescues), while the rapid nuclear movement may be related to fast dissociation phase (catastrophes) of tubulin subunits. Finally, our hypothesis is in harmony with the finding that the disruption of microtubule structure results in the collapse of the migrating cell body and cessation of nuclear translocation (10).

The proposed role of microtubules in nuclear displacement during neuronal migration does not exclude the synergistic action of actin-like contractile proteins which may also participate in this event (9, 10). However, microtubule motors, like kinesin, which are involved in the intracellular transport of

macromolecules and small organelles (28, 29) via allosteric mechanisms (30), are probably inadequate to account for the translocation of the large cell's nucleus. Therefore, the uncertainty about the binding of kinesin and ncd (31) to plus (+) or minus (−) ends of microtubules (32, 33) should not have direct bearing on our model. However, the unidirectional stream of the cytoplasmic organelles carried by these motor molecules downstream, from the soma toward the leading tip, may add to the multitude of pull forces participating in this process (10).

The present results have also implications for understanding the emergence of polarity in the initially round postmitotic cells of the external granular layer and their morphogenetic transformation into T-shaped granule cells; first forming bipolar shape oriented parallel to pia, followed by an elaboration of the third process directed toward the internal granular layer to which the nucleus eventually descends (1, 2). Signals that induce this morphogenetic transformation are unknown, but repolarization of microtubules seems to be essential prerequisite (9). Caged by the cytoskeletal network, the nucleus could not move without rearrangement of microtubules. Although the leading process of a migrating granule cell does not give rise to an axon, we found that its microtubules are oriented uniformly with respect to the orientation of their (+) end, just as it has been observed in axons in tissue culture and in the intact peripheral nervous system (15–19). In contrast, the trailing process, which eventually transforms into the ascending portion of the granule cell axon, contains microtubules of mixed orientation, similar to the arrangement that has been observed in dendritic shafts. Thus, the orientation of microtubules in cytoplasmic compartments of immature granule cell does not predict their prospective transformation into axons or dendrites of mature neurons. Rather it reflects the direction of granule cell migration across the developing molecular layer.

The present findings also offer new insight into the possible intra- and extracellular mechanisms involved in the control of normal and abnormal rates of neuronal migration. The rate of assembling the microtubule polymer may depend on the concentration of cytosolic Ca^{2+}, which is essential for tubulin polymerization (34). Ca^{2+} influx in migrating granule cells, delivered through specific voltage and ligand gated channels (3, 8, 35), may provide signals for polymerization and depolarization of microtubules (27). In addition, it has been suggested that the integrity and orientation of cytoskeletal proteins may be related to membrane bound cell adhesion molecules (36) and to neuron–glial junctional complexes in migrating neurons (37, 38). Therefore, interruption of the molecular messages in the chain of signals controlling assembly and disassembly of the cytoskeleton in migrating neurons may explain diverse causes of abnormal neuronal placements observed in genetic and acquired brain malformations (39–42).

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1. Ramon y Cajal, S. (1890) *Int. Monatsschr. Anat. Physiol. (Leipzig)* **7,** 12–32.
2. Rakic, P. (1971) *J. Comp. Neurol.* **141,** 283–312.
3. Komuro, H. & Rakic, P. (1993) *Science* **260,** 95–97.
4. Komuro, H. & Rakic, P. (1995) *J. Neurosci.* **15,** 1110–1120.
5. Schachner, M., Fraissner, A., Fisher, G., Keilhauer, G., Kruse, J., Kunemund, V., Lindner, J. & Wernicke, H. (1985) in *The Cell in Contact*, eds. Edelman, G. M., Gall, W. E. & Thiery, J. P. (Wiley, New York), pp. 257–276.
6. Hatten, M. E. & Mason, C. (1990) *Experientia* **46,** 907–916.
7. Rakic, P., Cameron, R. & Komuro, H. (1994) *Curr. Opin. Neurobiol.* **4,** 63–69.
8. Rakic, P. & Komuro, H. (1995) *J. Neurobiol.* **26,** 299–315.
9. Rakic, P. (1985) in *The Cell in Contact,* eds. Edelman, G. M., Gall, W. E. & Thiery, J. P. (Wiley, New York), pp. 67–91.
10. Rivas, R. J. & Hatten, M. E. (1995) *J. Neurosci.* **15,** 981–989.
11. Heidemann, S. R. (1991) *Methods Enzymol.* **196,** 469–477.
12. Baas, P. W., Deitch, J. S., Black, M. M. & Banker, G. A. (1988) *Proc Natl. Acad. Sci. USA* **85,** 8335–8339.
13. Amos, L. & Banker, T. S. (1979) *Nature (London)* **279,** 607–612.
14. McIntosh, J. R. (1984) *J. Cell Biol.* **98,** 525–533.
15. Black, M. M. & Baas, P. W. (1989) *Trends Neurosci.* **12,** 211–214.
16. Tropp, K. S., Meade, L. B. & LaVail, J. H. (1994) *J. Neurosci.* **14,** 318–325.
17. Kidd, G. J., Andrews, S. B. & Trapp, B. D. (1994) *J. Neurocytol.* **23,** 801–810.
18. Baas, P. W., Slaughter, T., Brown, A. & Black, M. M. (1991) *J. Neurosci. Res.* **30,** 134–153.
19. Ahmad, F. J., Pienkowski, T. P. & Baas. P. W. (1993) *J. Neurosci.* **13,** 856–866.
20. Garcia-Segura, L. M. & Rakic, P. (1985) *Dev. Brain Res.* **23,** 145–149.
21. Saxton, W. M., Stemple, D. L., Leslie, R. J., Salmon, E. D., Zavortnik, M. & McIntosh, J. R. (1984) *J. Cell Biol.* **99,** 2175–2186.
22. Kirshner, M. W. & Mitchison, T. J. (1986) *Cell* **45,** 515–527.
23. Mitchison, T. J. & Kirschner, M. W. (1984) *Nature (London)* **312,** 237–242.
24. Cassimeris, L. Preyer, N. K. & Salamon, E. D. (1988) *J. Cell Biol.* **107,** 2223–2231.
25. Brown, A., Slaughter, T. & Black, M. M. (1992) *J. Cell Biol.* **119,** 867–882.
26. Reinsch, S. S., Mitcheson, T. J. & Kirschner, M. (1991) *J. Cell Biol.* **115,** 365–379.
27. Komuro, H. & Rakic, P. (1996) *Neuron*, in press.
28. Lafont, F., Burkhardt, J. K. & Simons, K. (1994) *Nature (London)* **372,** 801–803.
29. Gilbert, S. P., Webb, M. R., Brune, M. & Johnson, K. A. (1995) *Nature (London)* **373,** 671–676.
30. Hoenger, A., Sablin, E. P., Vale, R. D., Fletterick, R. J. & Milligan, R. A. (1995) *Nature (London)* **376,** 271–274.
31. Sablin, E. P. & Fletterick, R. J. (1995) *Proteins Struct. Funct. Genet.* **21,** 68–69.
32. Song, Y.-H. & Mandelkow, E. (1995) *J. Cell Biol.* **128,** 81–94.
33. Hirose, K., Fan, J. & Amos, L. A. (1995) *J. Mol. Biol.* **251,** 329–333.
34. Schulman, H. (1991) *J. Biol. Chem.* **266,** 346–354.
35. Komuro, H. & Rakic, P. (1992) *Science* **257,** 806–809.
36. Doherty, P. & Walsh, F. S. (1992) *Curr. Opin. Neurobiol.* **2,** 595–601.
37. Cameron, R. S. & Rakic, P. (1994) *J. Neurosci.* **14,** 3139–3155.
38. Anton, E. S., Cameron, R. & Rakic, P. (1996) *J. Neurosci.* **16,** 2283–2293.
39. Bart, P. (1987) *Can. J. Neurol. Sci.* **14,** 1–16.
40. Rakic, P. (1988) *Prog. Brain Res.* **73,** 15–37.
41. Rorke, L. B. (1994) *J. Neuropathol. Exp. Neurol.* **53,** 105–117.
42. Reiner, O., Albrecht, V., Gordon, M., Chianese, K. A., Wong, C., Gal-Gerber, O., Sapir, T., Siracusa, L. D., Burchberg, A. M., Caskey, C. T. & Eichele, G. (1995) *J. Neurosci.* **15,** 3730–3738.