Changes in Microtubule Polarity Orientation during The Development of Hippocampal Neurons in Culture

Peter W. Baas,* Mark M. Black,* and Gary A. Banker‡

* Department of Anatomy, Temple University School of Medicine, Philadelphia, Pennsylvania 19140; ‡ Department of Anatomy, Cell Biology, and Neurobiology, Albany Medical College, Albany, New York 12208

Abstract. Microtubules in the dendrites of cultured hippocampal neurons are of nonuniform polarity orientation. About half of the microtubules have their plus ends oriented distal to the cell body, and the other half have their minus ends distal; in contrast, microtubules in the axon are of uniform polarity orientation, all having their plus ends distal (Baas, P. W., J. S. Deitch, M. M. Black, and G. A. Banker. 1988. *Proc. Natl. Acad. Sci. USA.* 85:8335–8339). Here we describe the developmental changes that give rise to the distinct microtubule patterns of axons and dendrites. Cultured hippocampal neurons initially extend several short processes, any one of which can apparently become the axon (Dotti, C. G., and G. A. Banker. 1987. *Nature [Lond.].* 330:477–479). A few days after the axon has begun its rapid growth, the remaining processes differentiate into dendrites (Dotti, C. G., C. A. Sullivan, and G. A. Banker. 1988. *J. Neurosci.* 8:1454–1468). The polarity orientation of the microtubules in all of the initial processes is uniform, with plus ends distal to the cell body, even though most of these processes will become dendrites. This uniform microtubule polarity orientation is maintained in the axon at all stages of its growth. The polarity orientation of the microtubules in the other processes remains uniform until they begin to grow and acquire the morphological characteristics of dendrites. It is during this period that microtubules with minus ends distal to the cell body first appear in these processes. The proportion of minus end–distal microtubules gradually increases until, by 7 d in culture, about equal numbers of dendritic microtubules are oriented in each direction. Thus, the establishment of regional differences in microtubule polarity orientation occurs after the initial polarization of the neuron and is temporally correlated with the differentiation of the dendrites.

N ERVE cells are highly polarized. Typical neurons give rise to two distinct classes of neurites, axons and dendrites, that differ in form and function (for review see Lasek, 1988). To elucidate the cellular mechanisms that underlie the generation and maintenance of neuronal polarity, we have focused on studies of cultured neurons derived from the hippocampal region of the cerebral cortex. Like their counterparts in situ, the axons and dendrites that develop in embryonic hippocampal cultures differ from one another in morphology, observed at both the light and electron microscopic levels, and in the macromolecular composition of their cytoskeletal and membranous constituents (for review see Banker and Waxman, 1988). In addition, as in situ, axons are presynaptic and dendrites are postsynaptic (Bartlett and Banker, 1984b).

It has recently been discovered that the axons and dendrites of cultured hippocampal neurons also differ with respect to the polarity orientation of their microtubules (Baas et al., 1988). Microtubules are intrinsically polar structures, with the "plus" end of the microtubule favored for assembly over the "minus" end (Binder et al., 1975; Bergen and Borisy, 1980). Microtubule polarity orientation in the axons of cul-

tured hippocampal neurons, as in all other types of axons examined (Heidemann et al., 1981; Burton and Paige, 1981; Filliatreau and DiGiamberdino, 1981; Baas et al., 1987a; Viancour and Forman, 1987; Burton, 1988), is uniform, with the plus ends of the microtubules distal to the cell body. In sharp contrast, microtubule polarity orientation in the dendrites is nonuniform, with roughly equal numbers of microtubules having each orientation. Burton (1988) has independently reported similar findings for the dendrites of frog mitral cells, suggesting that nonuniform microtubule polarity orientation may be a common feature of dendrites. We have argued that the distinct microtubule patterns of axons and dendrites result in the routing of different complements of cytoplasmic organelles into each type of neurite, thus contributing directly to the establishment of neuronal polarity (Baas et al., 1988; Black and Baas, 1989).

Our goal in the present study was to determine when the regional differences in microtubule organization arise during the establishment of neuronal polarity. In culture, hippocampal neurons acquire their polarized form by a stereotyped sequence of developmental events (Dotti et al., 1988). After initially extending lamellipodia (developmental stage 1), the

cells elaborate several short, "minor" processes that are roughly equal in length and cannot be identified as axonal or dendritic (stage 2). After a period of 6–24 h, the cell undergoes its initial polarization; one of the minor processes begins to grow rapidly, becoming many times longer than the others within just a few hours (stage 3). This process becomes the cell's axon. A few days later, the remaining minor processes also begin to grow but at a significantly slower rate than the axon. These processes gradually acquire dendritic characteristics and become the cell's dendrites (stage 4). By 14 d in culture, the age at which we previously analyzed microtubule polarity orientation, the axons and dendrites of neighboring neurons have developed extensive synaptic connections (stage 5).

Our present results show that all of the neurites extended by an individual neuron initially contain microtubules of uniform polarity orientation with plus ends distal to the cell body. Microtubules with minus ends distal first appear in developing dendrites after the differentiation of the axon, early in stage 4 of development. These observations indicate that regional differences in microtubule polarity orientation do not contribute to the initial polarization of the neuron but may be important for the differentiation of the dendrites.

Materials and Methods

Cell Culture

Cultures of hippocampal neurons were prepared as previously described (Bartlett and Banker, 1984a). Briefly, hippocampi were dissected from the brains of 18-d rat fetuses, treated with 0.25% trypsin for 15 min at 37°C, and dissociated by trituration. The dissociated cells were plated onto polylysine-treated glass coverslips in MEM (Gibco Laboratories, Grand Island, NY) containing 10% horse serum. Before polylysine treatment, coverslips were treated with dichlorodimethylsilane (1% in benzene) to facilitate subsequent separation of the fixed, embedded cells from the coverslip (see below). After 3–4 h at 36°C, when the cells had attached, the coverslips were transferred, cell side down, into plates containing a confluent monolayer of astroglial cells (Booher and Sensenbrenner, 1972). Cultures were maintained in MEM supplemented with 0.1% ovalbumin, 0.01 mg/ml pyruvate (Selak et al., 1985), and the N₂ mixture of Bottenstein and Sato (1979). Microtubule polarity orientation was analyzed after 1, 2, 4, 5, 7, and 14 d in culture. From cultures of these ages, neurons were selected that exhibited morphological characteristics typical of cultured hippocampal neurons at developmental stages 2–5 according to the criteria of Dotti et al. (1988).

Microtubule Polarity Analyses

Microtubule polarity orientation was determined using the "hook" method (Heidemann and Euteneuer, 1982) as modified for cultured neurons (Baas et al., 1987a, 1988). This method involves lysing the neurons in a special microtubule assembly buffer in the presence of exogenous brain microtubule proteins. The exogenous tubulin adds onto existing microtubules in the form of lateral protofilament sheets that appear as hooked appendages when viewed in cross section. A clockwise hook indicates that the plus end of the microtubule is facing the observer, while a counterclockwise hook indicates that the minus end of the microtubule is facing the observer.

For these analyses, cultures were rinsed briefly with PBS and then treated for 2 min at 37°C with 0.06% Brij 58 in a microtubule assembly buffer (0.5 M Pipes, 0.1 mM EGTA, 0.01 mM EDTA, 0.1 mM MgCl₂, 2.5% DMSO, 0.5 mM GTP) containing 1.2 mg/ml microtubule protein. This mixture was then removed and replaced with the same mixture without the Brij 58 for 30 min at 37°C. Limiting detergent treatment to 2 min was necessary to maintain sufficient membrane to distinguish the borders of the neurites. Cultures were then fixed by the addition of an equal quantity of 4% glutaraldehyde in 0.1 M cacodylate, rinsed in 0.1 M cacodylate, treated for 5 min with 0.15 mg/ml tannic acid, rinsed again for 5 min in 0.1 M cacodylate, postfixed for 5 min with 1% OsO₄, dehydrated in ethanol series, and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA).

After polymerization of the resin overnight at 60°C, the coverslips were removed from the resin by alternate exposures to boiling water and liquid nitrogen. Cells were chosen by phase-contrast microscopy, photographed, circled with a diamond marker objective, and sectioned perpendicular to the long axes of the neurites. Sections were picked up on 75 mesh formvar-coated grids, stained with uranyl acetate and lead citrate, and viewed electron microscopically. To preserve the correct orientation of the hooks, particular care was taken to insure that neither sections nor grids were inverted during handling. Because consecutive sections showed identical hooking patterns, the hooks from one representative section were scored for each neurite (or neurite region) analyzed (Baas et al., 1988). The hooking data were interpreted and analyzed as previously described (Heidemann and Euteneuer, 1982).

Results

Microtubule Polarity Orientation in Neurons with Well-developed Dendrites

Our previous study on microtubule polarity orientation in cultured hippocampal neurons focused on neurons maintained in culture for 14 d (stage 5), when dendrites and axons are well developed (Baas et al., 1988). In that study, we determined that microtubule polarity orientation in the axon was uniform, plus ends distal to the cell body, regardless of where in the axon we sampled. In sharp contrast, microtubule polarity orientation in the dendrite differed at the two sites we sampled: the midregion of the dendrite contained roughly equal numbers of microtubules of each orientation, while the most distal regions contained only microtubules with plus ends distal to the cell body. Before considering developmental changes in microtubule polarity orientation, we first undertook to elucidate the variation in microtubule polarity orientation that occurs along the length of individual dendrites.

For these experiments, we sampled five regions along each of three 14-d dendrites. Because of the complexity of the neuron cultures at this stage, it was difficult to determine with confidence the precise point where individual dendrites terminated. Therefore, all sampling points were measured with reference to the cell body. Fig. 1 a shows a phase–contrast micrograph of one of the dendrites examined. The approximate length of the dendrite was 240 μm, and microtubule polarity orientation was sampled at 15, 110, 150, 180, and 220 μm from the cell body. At both 15 (proximal dendrite; Fig. 1 b) and 110 μm (midregion; Fig. 1 c) from the cell body, roughly equal numbers of hooks were clockwise and counterclockwise as viewed from the growth cone looking toward the cell body. At 150 (Fig. 1 d) and 180 μm (not shown), 64 and 71% of the hooks, respectively, were clockwise. At 220 μm from the cell body, 94% of the hooks were clockwise (Fig. 1 e). Similar results were obtained from the two other dendrites examined in this manner (Table I). These results indicate that roughly equal numbers of microtubules of each orientation are present throughout the proximal half of the dendrite, while, more distally, the proportion of microtubules with plus ends distal to the cell body gradually increases until microtubule polarity orientation reaches uniformity in the most distal 10–20% of the length of the dendrite.

Based on these observations, we sampled neurites for our developmental studies at or just proximal to the midregion. Our intention was to avoid the most distal regions of the neurites and, thus, maximize the probability of detecting non-
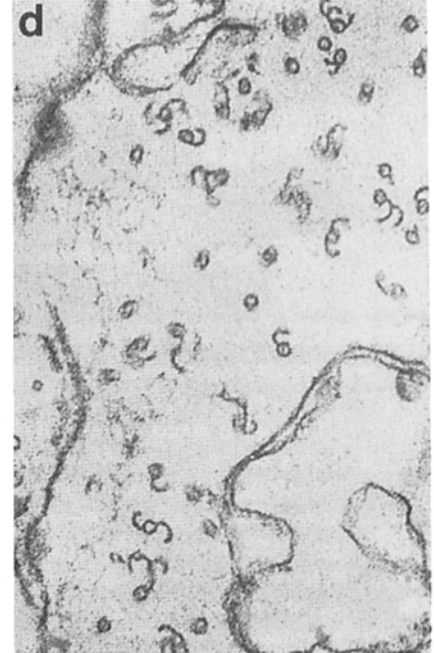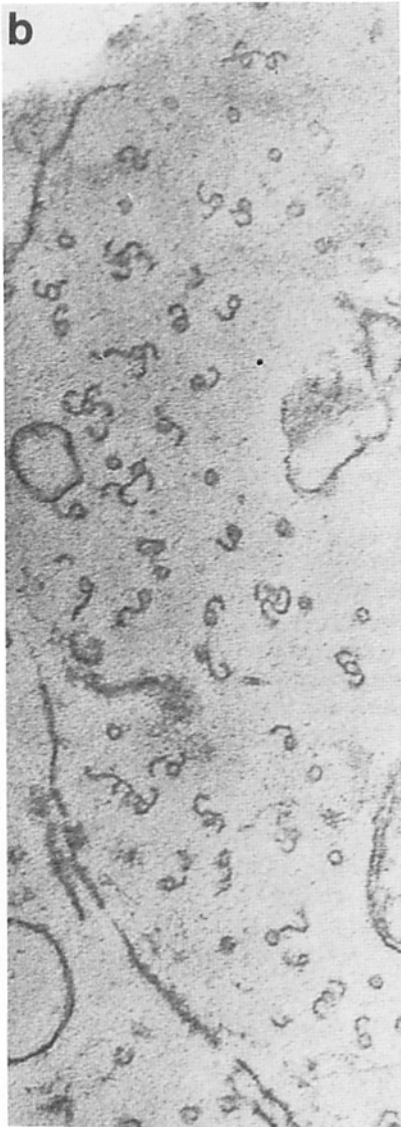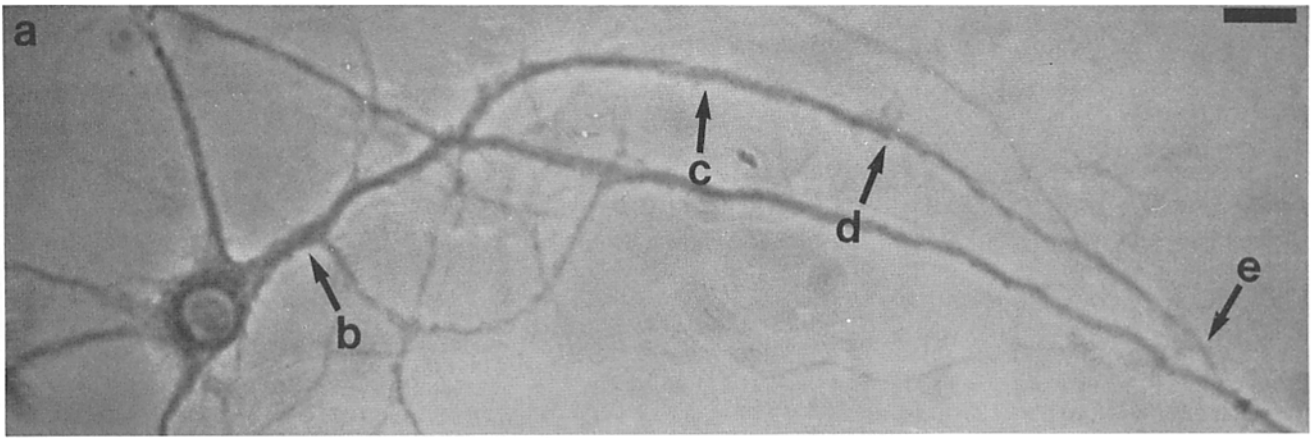
Figure 1. Microtubule polarity orientation in a dendrite from a stage 5 (14-d) cultured hippocampal neuron examined at various distances from the cell body. (*a*) Phase–contrast micrograph showing the dendrite that was sampled at distances (*b*) 15, (*c*) 110, (*d*) 150, and (*e*) 220 μm from the cell body (*arrows*). (*b–e*) Electron micrographs of representative regions of cross sections of the dendrite at each sample distance. At 15, 110, 150, and 220 μm, the hooks were 51, 51, 64, and 94% clockwise, respectively (hooks viewed from the dendrite tip looking toward the cell body). At 180 μm, the hooks were 71% clockwise (not shown in figure). These data indicate that roughly equal numbers of microtubules of each orientation are present in the proximal half of the dendrite. More distally, the proportion of microtubules with plus ends distal to the cell body gradually increases, until microtubule polarity orientation reaches uniformity in the most distal 10–20% of the dendrite. Bar: (*a*) 15 μm; (*b–e*) 0.15 μm.

Table I. Distance Studies on Microtubule Polarity Orientation in Dendrites of Stage 5 Neurons (14 d in culture)

Dendrite	Total length of dendrite	Approximate distance from cell body	CW	CCW	AMB	UHK	CW	HK
	μm	μm	n	n	n	n	%	%
Dendrite 1	~240	15	50	49	10	50	51	69
		110	47	46	10	55	51	65
		150	30	17	11	39	64	60
		180	22	9	13	58	71	43
		220	15	1	2	6	94	75
Dendrite 2	~200	15	37	35	7	15	51	84
		100	33	30	12	15	52	83
		125	14	9	4	3	61	90
		150	12	4	4	6	75	77
		185	11	1	4	2	92	89
Dendrite 3	~160	15	22	20	20	10	52	86
		80	22	19	20	13	54	82
		100	11	7	2	5	61	80
		120	8	4	3	3	67	83
		150	5	0	3	2	100	80

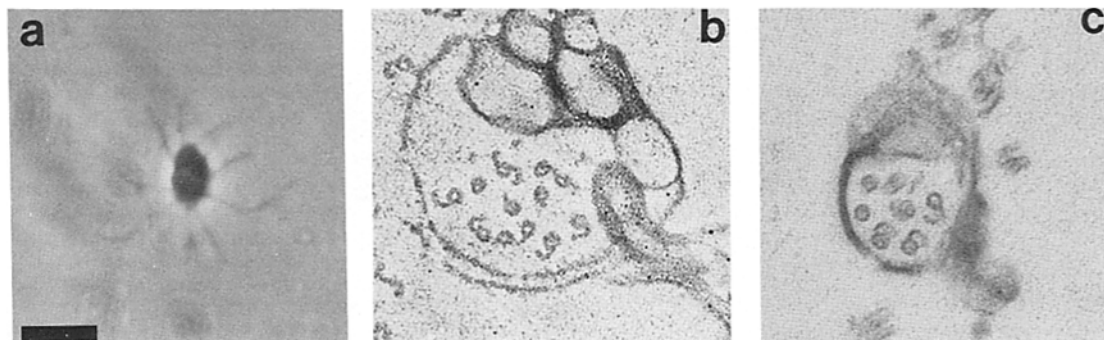CW, microtubules with clockwise hooks as viewed from the tip of the neurite looking toward the cell body; CCW, microtubules with counterclockwise hooks as viewed from the tip of the neurite looking toward the cell body; AMB, microtubules with ambiguous hooks; UHK, microtubules with no hooks; HK, microtubules with hooks.

uniform microtubule polarity orientation in any neurite where it might exist.

Microtubule Polarity Orientation in Stage 2 Neurons

We next examined neurons at stage 2, when neurites first appear. Fig. 2 a shows a typical stage 2 neuron with its several apparently identical minor processes. Because of the short length of these neurites coupled with the necessity of sectioning them perpendicular to their long axes to assess the direction of hooking, we could usually examine only one minor process per cell. In six such instances, the hooks on the microtubules were predominantly clockwise as viewed from the tip of the neurite looking toward the cell body. These results, summarized in Table II, indicate that microtubule polarity orientation in these minor processes was uniform with the plus ends of the microtubules distal to the cell body, which is the same organization observed in axons (Baas et al., 1988).

Do all of the minor processes growing from an individual neuron contain microtubules of uniform polarity orientation or, by chance, might each of the six processes we sampled have been destined to become axons? Since typically only one of the five to six minor processes becomes an axon, the chance of the latter possibility is extremely small ($p < 0.0001$). Nevertheless, to further examine this issue, we located two neurons that each had two minor processes growing colinear to one another, allowing both to be cut in cross section without having to retrim the block. Fig. 2 a is a phase-contrast micrograph of one of these neurons. As shown in Fig. 2, b and c, both minor processes contained microtubule hooks that were predominantly clockwise as viewed from the tips of the neurites. The percentage (mean ± SD) of clockwise hooks for all ten of the minor processes examined was 94 ± 6%. These observations indicate that all of the minor processes extended by a stage 2 neuron, not just the one that will become the axon, contain microtubules of uniform polarity

Figure 2. Microtubule polarity orientation in stage 2 neurons. (a) Phase-contrast micrograph of a stage 2 neuron, treated for microtubule polarity determination, showing its several minor processes. (b and c) Electron micrographs of cross sections from two minor processes extended by a single stage 2 neuron. The microtubule hooks were predominantly clockwise as viewed from the tip of the neurite looking toward the cell body, indicating that microtubule polarity orientation in these neurites is uniform, with the plus ends of the microtubules distal to the cell body. Bar: (a) 15 μm; (b and c) 0.15 μm.

Table II. Summary of Data on Microtubule Polarity Orientation during the Development of Cultured Hippocampal Neurons

| Stage	Time in culture	Type of neurite*	CW‡	CCW‡	AMB‡	UHK‡	HK§	CW§	Microtubule polarity orientation
	d		*n*	*n*	*n*	*n*	%	%	
1	<1	None							
2	1	Minor processes	73	5	7	15	85 ± 17	94 ± 6	Uniform, with plus ends distal to cell body
3	2	Minor processes	79	3	10	27	86 ± 17	97 ± 6	Uniform, with plus ends distal to cell body
3	2	Axons	62	4	7	12	88 ± 11	94 ± 9	Uniform, with plus ends distal to cell body
Early 4	4–5	Dendrites	197	81	44	267	64 ± 20	74 ± 20	Transition from uniform, with plus ends distal to cell body, to nonuniform
Early 4	4–5	Axons	54	1	9	32	68 ± 10	99 ± 3	Uniform, with plus ends distal to cell body
Late 4	7	Dendrites	200	154	115	276	66 ± 16	57 ± 4	Nonuniform
Late 4	7	Axons	55	1	2	21	76 ± 18	99 ± 4	Uniform, with plus ends distal to cell body

CW, microtubules with clockwise hooks as viewed from the tip of the neurite looking toward the cell body; *CCW*, microtubules with counterclockwise hooks as viewed from the tip of the neurite looking toward the cell body; *AMB*, microtubules with ambiguous hooks; *UHK*, microtubules with no hooks; *HK*, microtubules with hooks.
* Ten neurites of each type were analyzed.
‡ Indicates the sum of all ten neurites.
§ Mean ± SD for all ten neurites.

orientation, plus ends distal to the cell body. This conclusion is fully supported by data showing that the minor processes that remain after the differentiation of the axon also contain microtubules of uniform polarity orientation (see below).
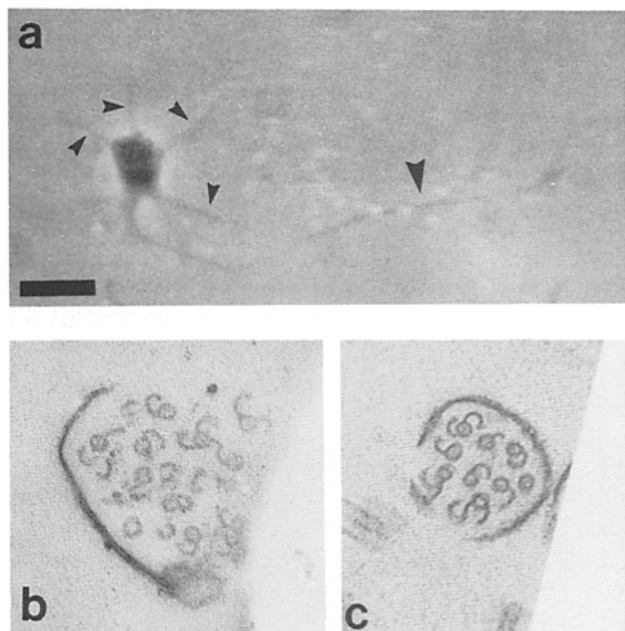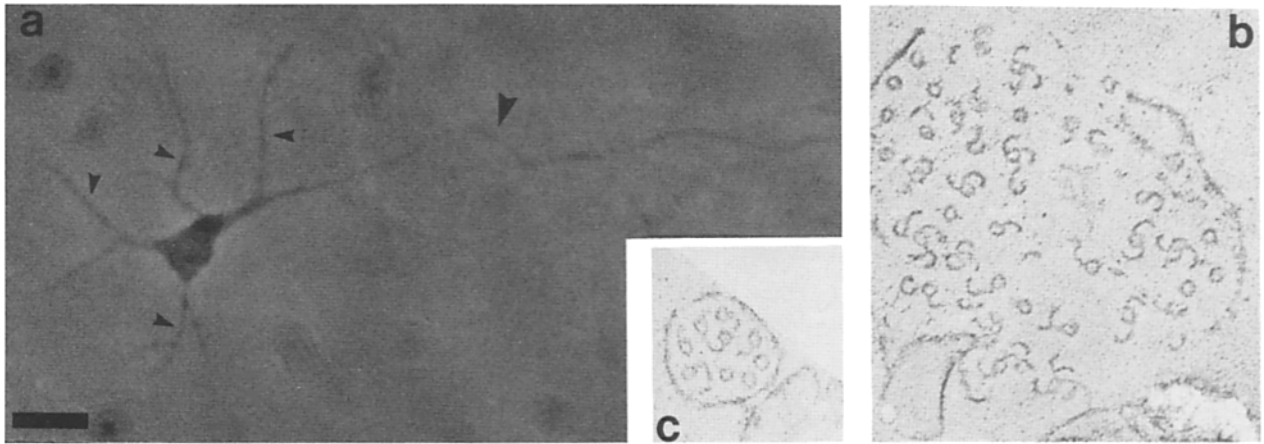

Figure 3. Microtubule polarity orientation in stage 3 neurons. (*a*) Phase–contrast micrograph of a stage 3 neuron, treated for microtubule polarity determination, showing an early axon (*large arrowhead*) and the several minor processes (*small arrowheads*). (*b* and *c*) Electron micrographs of cross sections through a minor process and the axon from a stage 3 neuron. The microtubule hooks in both neurites were predominantly clockwise as viewed from the tip of the neurite looking toward the cell body, indicating that microtubule polarity orientation in both neurites is uniform, with the plus ends of the microtubules distal to the cell body. Bar: (*a*) 15 μm; (*b* and *c*) 0.15 μm.

Microtubule Polarity Orientation in Stage 3 Neurons

We next examined microtubule polarity orientation in neurons at stage 3, when one of the minor processes had become the axon. Fig. 3 *a* is a phase–contrast micrograph of a typical stage 3 neuron, showing its one long axon and several remaining minor processes. The greater length of the early axon compared with that of the minor processes permitted us to more reliably reorient the embedded sample when necessary and hence to analyze microtubule polarity in both the axon and one minor process from each neuron. The microtubule hooking patterns of a minor process and the axon from the same stage 3 neuron are shown in Fig. 3, *b* and *c*, respectively. The hooks in this minor process and in all nine of the other stage 3 minor processes examined were predominantly clockwise (97 ± 5%) as viewed from the tips of the neurites. This was also the case in this axon and all nine of the other stage 3 axons examined (94 ± 5%). These data, summarized in Table II, indicate that both the minor processes and the axon of stage 3 neurons contain microtubules of uniform polarity orientation, plus ends distal to the cell body. It is important to note that the stage 3 minor processes will eventually become dendrites and contain microtubules of nonuniform polarity orientation. Thus, the differentiation of the axon, which represents the initial polarization of the neuron, occurs before the establishment of nonuniform microtubule polarity orientation in the processes that will become dendrites.

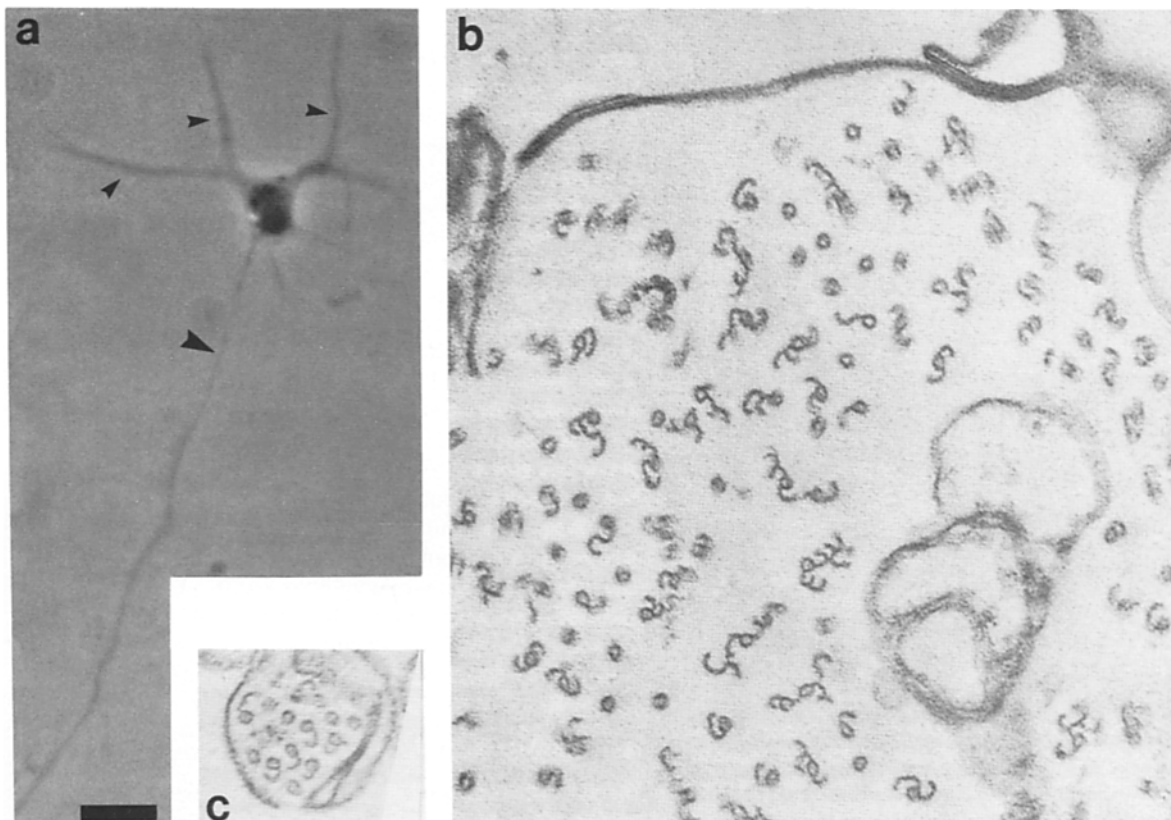## Microtubule Polarity Orientation in Stage 4 Neurons

We next examined microtubule polarity orientation in neurons at stage 4, when the remaining minor processes begin to acquire the characteristics of dendrites. We analyzed neurons after 4–5 d in culture, early in stage 4, as well as after 7 d in culture, late in stage 4. These data are summarized in Table II. Early in stage 4, after the developing dendrites had begun to elongate and to thicken proximally (Fig. 4 *a* and Fig. 5 *a*; see also Dotti et al., 1988), we first observed den-
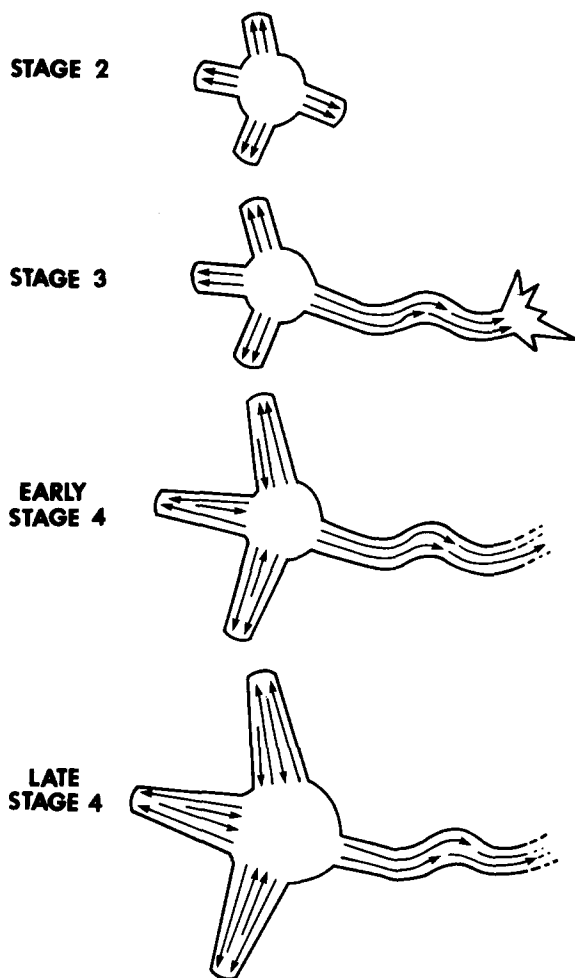
Figure 4. Microtubule polarity orientation in an early stage 4 neuron. (*a*) Phase micrograph of the neuron showing the axon (*large arrowhead*) and the nascent dendrites (*small arrowheads*). The nascent dendrites had thickened somewhat and lengthened beyond the typical dimensions of minor processes but did not yet display a clear proximodistal taper. (*b* and *c*) Electron micrographs of cross sections through a nascent dendrite and the axon, respectively. Both show predominantly clockwise microtubule hooks as viewed from the neurite tip, indicating uniform microtubule polarity orientation, plus ends distal to the cell body. Bar: (*a*) 15 μm; (*b* and *c*) 0.15 μm.

dritic microtubules with minus ends distal to the cell body. There was, however, pronounced variation among cells at this stage. Three developing dendrites contained predominantly clockwise hooks (94, 100, and 100%, respectively)

like the minor processes at stage 3 (Fig. 4 *b*). Three others contained roughly equal numbers of clockwise (52, 50, and 50%, respectively) and counterclockwise hooks, as in mature dendrites (Fig. 5 *b*). Four others contained between 71

Figure 5. Microtubule polarity orientation in an early stage 4 neuron. (*a*) Phase–contrast micrograph of a stage 4 neuron, treated for microtubule polarity determination, showing the axon (*large arrowhead*) and the dendrites (*small arrowheads*). The dendrites had lengthened, thickened proximally, and had begun to show a proximodistal taper. (*b* and *c*) Electron micrographs of cross sections through a dendrite and the axon, respectively. In the dendrite, roughly equal numbers of microtubule hooks were clockwise and counterclockwise as viewed from the tip of the dendrite, indicating nonuniform microtubule polarity orientation with roughly equal numbers of microtubules of each orientation. In the axon, the hooks were predominantly clockwise, indicating uniform microtubule polarity orientation, plus ends distal to the cell body. Bar: (*a*) 15 μm; (*b* and *c*) 0.15 μm.

STAGE 2

STAGE 3

EARLY STAGE 4

LATE STAGE 4

Figure 6. Diagram showing the changes in microtubule polarity orientation that occur during the development of hippocampal neurons in culture. The arrows represent microtubules, with the head of each arrow indicating the plus end of the microtubule. At stage 2, all of the neurites are minor processes. At stage 3, the neurite at the right of the diagram has become the axon. At early and late stage 4, the nonaxonal neurites have become dendrites and the axon has continued to lengthen (indicated diagramatically by the fading of the distal region of the axon).

4 dendrites was not due primarily to variation in sampling sites.

Another possibility is that the variation we observed is a reflection of differences among stage 4 cells in their state of development. To explore this possibility, we attempted to determine whether the microtubule hooking pattern of individual dendrites correlated with their morphology. We found that the neurites that contained microtubules of uniform polarity orientation had grown beyond the length of a typical minor process but had thickened only slightly in their proximal regions (Fig. 4 *a*). That is, they did not display the clear proximodistal taper that characterizes more mature dendrites. In contrast, those dendrites that contained microtubules of nonuniform polarity orientation had lengthened, thickened substantially in their proximal and middle regions, and clearly tapered with distance from the cell body (Fig. 5 *a*). These observations are consistent with the view that the acquisition of nonuniform microtubule polarity orientation in developing dendrites occurs coordinately with their acquisition of the morphological characteristics of dendrites.

It is worth noting that two of the early stage 4 dendrites originated from the same neuron but differed in their microtubule hooking patterns; one contained 94% clockwise hooks, while the other contained 77% clockwise hooks. This observation indicates that it is possible for two different dendrites from the same neuron to be at slightly different points in the transition from uniform to nonuniform microtubule polarity orientation.

Finally, we examined microtubule polarity orientation in neurons after 7 d in culture, late in stage 4 (Table II). In all ten 7-d dendrites we examined, similar numbers of microtubule hooks were clockwise and counterclockwise (57 ± 4% clockwise), indicating nonuniform microtubule polarity orientation with roughly equal numbers of microtubules having each orientation. As expected, in all ten axons examined from 7-d neurons, hooks were predominantly clockwise (99 ± 4%), indicating uniform microtubule polarity orientation with plus ends distal to the cell body. These observations are essentially identical to the findings for 14-d neurons (stage 5) as previously described (above and Baas et al., 1988). Thus, the distinct microtubule patterns that distinguish axons and dendrites appear to be fully established by 7 d in culture.

and 78% clockwise hooks. As expected, the microtubule hooks in all ten of the axons were predominantly clockwise (99 ± 3%), indicating uniform microtubule polarity orientation with plus ends distal to the cell body (Fig. 4 *c* and Fig. 5 *c*).

What accounts for the variation in the microtubule hooking patterns in early stage 4 dendrites? One possibility is that the variation we observed resulted from minor differences in the location of sampling sites along different dendrites. For example, the dendrites that appeared to contain predominantly plus end–distal microtubules might have contained microtubules of opposite orientation nearer to the cell body. To explore this possibility, we examined microtubule hooking patterns in several of the same early stage 4 dendrites at ∼15 µm from the cell body. In each case, the microtubule hooking pattern in the proximal region was similar to that observed in the midregion (data not shown), indicating that the variation in microtubule hooking patterns among early stage

Discussion

We previously reported that axons and dendrites of cultured hippocampal neurons differ with respect to the polarity orientation of their microtubules: in axons, all of the microtubules are oriented with plus ends distal to the cell body, while, in dendrites, roughly equal numbers of microtubules are oriented in each direction (Baas et al., 1988). In the present study, we have investigated the sequence of events by which this difference arises during the establishment of neuronal polarity. Our observations are summarized schematically in Fig. 6. Hippocampal neurons initially extend several apparently identical minor processes that ultimately give rise to the axon and the dendrites. Microtubule polarity orientation in each of the minor processes is uniform, plus ends distal to the cell body, regardless of whether the minor process ultimately becomes an axon or a dendrite. Thus, the polarity orientation of microtubules in the initial processes does not presage their ultimate fate. Uniform microtubule polarity

orientation persists in all of the processes during stage 3 of development, when one of the processes begins to acquire the distinct growth properties and molecular composition of the axon (Dotti et al., 1988; Goslin, K., D. J. Schreyer, J. H. P. Skene, and G. Banker, manuscript submitted for publication). Thus, during the initial polarization of the neuron, the polarity orientation of microtubules in all of the processes is identical: uniform, with plus ends distal to the cell body. Microtubules with minus ends distal to the cell body first appear in the nonaxonal processes during stage 4, when these processes begin to acquire the characteristics of dendrites.

At stage 5 of development, when the dendrites have acquired most of their mature properties but are nonetheless still growing, the ratio of microtubules of each orientation varies along the length of the dendrite. Throughout the proximal half of the dendrite, roughly equal numbers of microtubules are of each orientation. Beyond this point, the proportion of plus end–distal microtubules gradually increases until microtubule polarity orientation reaches uniformity in the most distal region of the dendrite. Thus, at all stages of development, the plus ends of microtubules extend into the advancing growth cones of both axons and dendrites. Several other observations point to a special role for plus ends of microtubules in growth cones. For example, amputated segments of the axon form new growth cones only at the end containing plus ends of microtubules (Baas et al., 1987a). In addition, there appear to be significant interactions between the plus ends of microtubules in growth cones and other growth cone constituents, including actin (Forscher and Smith, 1988) and 13H9 antigen (Goslin et al., 1989). Depolymerization of microtubules extending into growth cones impairs growth cone advance and process elongation (Yamada et al., 1970). All of these observations support the view that the presence of plus ends of microtubules in growth cones contributes directly to aspects of growth cone organization involved in the elongation of axons and dendrites.

Microtubule Patterns and the Generation of Neuronal Polarity

A fundamental question with respect to neuronal polarity concerns how the neuron generates two morphologically and functionally distinct types of neurites. Are processes determined at the time they arise or are they initially capable of becoming either axons or dendrites? In the case of neurons in culture, the present results indicate that the fate of the minor processes cannot be predicted on the basis of the polarity orientation of their microtubules, which is initially uniform in all of the processes extended by an individual neuron. In addition, previous studies have shown that minor processes are indistinguishable from one another in their morphology (Dotti et al., 1988), ultrastructure (Deitch, J., and G. Banker, unpublished observations), and complement of markers—such as MAP-2 (Dotti et al., 1987) and GAP-43 (Goslin, K., D. J. Schreyer, J. H. P. Skene, and G. Banker, manuscript submitted for publication)—which distinguish axons and dendrites of more mature neurons. In addition, experimental studies indicate that when the axon of a stage 3 neuron is amputated any one of its remaining minor processes, which would otherwise become dendrites, can differentiate into a new axon (Dotti and Banker, 1987; Goslin and Banker, 1989). Collectively, these observations suggest that the initial pro-

cesses extended by a cultured hippocampal neuron are not predetermined but rather are capable of becoming either axons or dendrites.

The present results provide insight into how differences in microtubule polarity orientation may contribute to the establishment and maintenance of neuronal polarity. The first overt sign of neuronal polarization occurs at stage 3 of development, when one of the minor processes differentiates into the axon. At a cellular level, this reflects the generation of asymmetries in the structure and metabolism of the neuron. Because microtubule polarity orientation is identical in all of the processes of stage 3 neurons, generation of the asymmetries that differentiate the axon from the minor processes cannot depend on regional differences in microtubule polarity orientation. For example, GAP-43, a neuron-specific membrane-associated phosphoprotein, selectively accumulates in the growth cone of the developing axon during the transition between stages 2 and 3 (Goslin, K., D. J. Schreyer, J. H. P. Skene, and G. Banker, manuscript submitted for publication). Likewise, the integral membrane protein synaptophysin, a component of synaptic vesicles, is preferentially distributed to the axonal domain of stage 3 neurons (Fletcher, T., P. De Camilli, G. Banker, unpublished observations). These membrane constituents are synthesized and assembled into vesicles in the cell body and conveyed into the axon by fast anterograde axonal transport. This transport presumably involves the translocation of membrane vesicles along microtubules specifically toward their plus ends (for reviews see Vale, 1987; Lasek, 1988). The selective transport of vesicles containing GAP-43 or synaptophysin into the axon during stage 3 cannot, however, be determined solely by their preferred direction of transport along microtubules because the microtubules in the axon and the minor processes have the same polarity orientation. Thus, other mechanisms, unrelated to microtubule polarity orientation, must exist in neurons to account for the segregation of such constituents during the initial phases of axonal outgrowth.

In contrast to axonal differentiation, the acquisition of certain dendritic characteristics appears to be temporally correlated with a change in microtubule polarity orientation, namely the appearance of microtubules with minus ends distal to the cell body. One example in this regard concerns the acquisition of proximodistal taper, a hallmark of dendrites. The nascent dendrites of hippocampal neurons begin to elongate very early in stage 4 and then somewhat later begin to exhibit a proximodistal taper. Our data indicate that minus end–distal microtubules appear in the developing dendrites at essentially the same time as they begin to acquire this taper (see Results).

Dotti and collaborators have recently presented evidence indicating that posttranslational glycosylation of proteins occurs in dendrites but not axons (Dotti, C., J. Van Woert, O. Steward, and G. Banker, unpublished observations; Steward et al., 1988). They reported that this property of dendrites arises early in stage 4, coincidently with the appearance of minus end–distal microtubules, and presumably reflects the selective transport of Golgi-like elements into dendrites but not axons. We have proposed that the selective routing of Golgi elements in neurons may result in part from their preferential transport toward the minus ends of microtubules (Baas et al., 1988; Black and Baas, 1989; see also Vale, 1987). During stages 2 and 3, when all of the neurites contain

exclusively plus end–distal microtubules, incorporation of sugars into glycoproteins occurs only in the nerve cell body. It is early in stage 4, when minus end–distal microtubules first appear, that dendrites acquire their unique capacity for protein glycosylation. Thus, the change in the presumed distribution of Golgi-like elements during neuronal development, like the acquisition of proximodistal taper, closely parallels the acquisition of nonuniform microtubule polarity orientation in the developing dendrites.

Finally, some aspects of neuronal polarity, such as the presence of ribosomes in dendrites but not axons, may be influenced by microtubule polarity orientation as well as by other factors. An active transport mechanism moves RNA selectively from the cell body into the dendrites (Davis et al., 1987), and we have raised the possibility that ribosomes, like Golgi elements, may achieve their selective distribution by a preferential transport toward the minus ends of microtubules (Baas et al., 1988; Black and Baas, 1989). However, during stage 3 of development, ribosomes are present in minor processes and in the most proximal portion of the axon (Deitch, J., and G. Banker, unpublished observations), both of which contain exclusively plus end–distal microtubules. Thus, ribosomes can apparently enter a short distance into all types of processes regardless of the polarity orientation of their microtubules. This might occur by Brownian motion given that ribosomes are sufficiently small that they can diffuse through cytoplasm (Luby-Phelps et al., 1986; Baas et al., 1987b). In contrast, an active transport directed specifically toward the minus ends of microtubules would enable ribosomes to reach far into the dendritic tree of mature neurons and would also prevent their active transport into the axon.

Mechanisms for Organizing Microtubules in the Neuron

Implicit in the above discussion is that the appearance of minus end–distal microtubules specifically in developing dendrites contributes to their acquisition of structural and molecular characteristics unique to dendrites. If this is correct, then the generation of minus end–distal microtubules plays a fundamental role in the development and maintenance of neuronal polarity. Unfortunately, the mechanisms that organize microtubules in the neuron are poorly understood. Ultrastructural analyses have failed to reveal any organizing structures for neuritic microtubules in the cell body of the neuron (Sharp et al., 1982; Lyser, 1968; Okabe and Hirokawa, 1988), and microtubule polarity analyses have failed to reveal organized microtubule arrays in the cell body comparable with those found in either the axon or the dendrites (Baas et al., 1988; Burton, 1988). Thus, mechanisms other than continuity with structural templates in the cell body must exist to organize the microtubule patterns of the axon and the dendrites.

Our observations suggest that there are at least two mechanisms involved in the organization of neuronal microtubules and that these mechanisms arise at distinct stages of neuronal development. The first begins at the level of the cell body and involves the coordinate formation of initial processes and the generation of plus end–distal microtubules in these processes. This presumably reflects interactions between the cortical region of the cell body from which growth cones form

and the underlying microtubules required to support the organization and advance of the growth cones. These interactions apparently have a stereospecificity with regard to the polarity of the microtubules; only plus ends of microtubules are directed into the advancing growth cones. The second mechanism comes into play after the axon has arisen and involves the generation of a second population of microtubules, with minus ends distal, specifically in the developing dendrites. We have proposed that this second population of dendritic microtubules is nucleated locally, within the dendrite itself, and that the capacity for local microtubule nucleation is not shared by the axon (Baas et al., 1988). Consistent with this view, experimental evidence suggests that all microtubule assembly in the axon may be limited to the elongation of preexisting microtubules (Baas and Heidemann, 1986), thus preserving the uniform polarity orientation of axonal microtubules. In this view, the distinct microtubule patterns of the axon and the dendrite result in part from regional differences in microtubule-nucleating capacity in the neuron. Experiments are in progress to test this hypothesis by identifying microtubule nucleation sites in the neuron.

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