Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 3269–3273, May 1981
Neurobiology

Polarity of axoplasmic microtubules in the olfactory nerve of the frog

(axoplasm/ultrastructure/detergent extraction/tubulin assembly/protofilament ribbons)

PAUL R. BURTON AND JEFFREY L. PAIGE

Department of Physiology and Cell Biology, University of Kansas, Lawrence, Kansas 66045

Communicated by Charles D. Michener, February 12, 1981

ABSTRACT **Pieces of olfactory nerve of the bullfrog were extracted in a tubulin assembly buffer medium containing detergents. With incubation at 37°C in such medium containing soluble tubulin, ribbons of protofilaments are formed on the surfaces of microtubules, with the ribbons curving in a clockwise or counterclockwise direction. The direction of hooking reflects the polarity of the microtubule. In nerve pieces oriented such that cross sections could be viewed toward the perikarya of the axons, over 90% of the ribbons on microtubules showed a clockwise orientation. When observers were looking toward the axonal terminals, most ribbons on microtubules showed a counterclockwise direction. In single axons in which ribbons appeared on all the contained microtubules, the ribbons showed a single directionality. The evidence suggests that microtubules in axons have a single polarity, probably reflecting their assembly from the perikarya outward through the axoplasm. If bidirectional transport is assumed in these axons, it is not reflected by the polarity of their microtubules, which may mean that the directionality of transport is provided by components other than microtubules.**

Bidirectional translocation of molecules and formed elements has been well demonstrated in axons, even though the mechanism of such transport remains to be elucidated. There has been much interest in the possibility that microtubules might provide, at the least, guideways and attachment sites along which axoplasmic transport occurs (1–5). Within the past few years, new efforts have been made to better define the chemistry and structure of the axoplasmic cytoskeleton in seeking clues to the mechanism underlying axoplasmic transport (6–8). The most prominent cytoskeletal components of both invertebrate and vertebrate axons are microtubules, which interconnect with each other and other filamentous elements of the axoplasmic latticework. As noted by Ellisman and Porter (7), the polarity of linear axoplasmic constituents is important to consider in framing hypotheses relating to the mechanisms of bidirectional axoplasmic transport. In this regard, the polarity of axonal microtubules is unknown, although Chalfie and Thomsom (9) noted differences in the appearance of distal and proximal ends (with reference to the cell body) of short microtubules in nematode axons and suggested that these differences may reflect the polarity of the microtubules.

Recently, a method for determining polarity of mitotic microtubules was described (10, 11). This method provides for extraction of soluble cell components in a buffered detergent medium formulated to preserve microtubules; the medium contains dimethyl sulfoxide, which at appropriate concentrations favors the assembly of soluble tubulin into ribbon element (12). With addition of tubulin (0.5–2.0 mg/ml) to this medium and incubation at 37°C, the tubulin assembles onto the preexisting microtubules as laterally attached ribbons consisting of protofilaments. When seen in cross section, the attached ribbons curve in a clockwise or counterclockwise direction, depending on the polarity of the microtubule with reference to its point of origin. Using the above method, or modifications thereof, we demonstrate that microtubules of axons of frog olfactory nerve all have the same polarity. The olfactory nerve is highly suited for this kind of study, because it mainly consists of small axons containing less than five microtubules. Also, the overall polarity of the nerve itself is readily ascertained, with the sensory axons having their cell bodies in the nasal epithelium and their terminations in the olfactory lobe of the brain.

METHODS AND MATERIALS

Bovine brain tubulin used in this study was obtained as described elsewhere (12, 13). In some cases, microtubule-associated proteins (MAPs) were removed from twice-polymerized tubulin by phosphocellulose chromatography (14), although tubulin with and without MAPs was used under conditions described below. Tubulin obtained by phosphocellulose chromatography, or PC-tubulin, was concentrated to about 13 mg/ml before use. Olfactory nerves were obtained from adult bullfrogs (*Rana catesbeiana*) after pithing of the spinal cord; the top of the skull was removed and the brain chamber was opened from the ventral aspect. By cutting bone in an anterior direction, access was gained to the olfactory lobes and olfactory nerves, which were quickly removed, placed in chilled amphibian nerve saline, and cut into appropriate pieces. These pieces were then immediately placed in the extraction-assembly medium described below.

In order to permeabilize pieces of nerve and provide access for free tubulin, an extraction–assembly buffer medium (hereafter designated EXAB) was used that was similar to the "lysis buffer" described by Heidemann *et al.* (11). The EXAB contained 0.5 M 1,4-piperazinediethanesulfonic acid (Pipes, pH 6.9), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 2.5% (vol/vol) dimethyl sulfoxide, 1% Triton X-100, 0.2% sodium deoxycholate, and 0.2% sodium dodecyl sulfate. In EXAB without tubulin, preexisting axonal microtubules are maintained in pieces of nerve, even though the axolemma and axoplasmic matrix are solubilized in a time-dependent manner. When PC-tubulin (0.5–2.0 mg/ml) and 0.5 mM GTP are added to EXAB containing pieces of nerve, the tubulin assembles onto the surfaces of axonal microtubules as laterally projecting ribbon elements. In the surrounding medium, the tubulin showed retarded spontaneous assembly of its own into ribbon elements, as noted by Heide-

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Abbreviations: MAP, microtubule-associated protein; EXAB, extraction–assembly buffer medium; PC-tubulin, tubulin obtained by phosphocellulose chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

mann *et al.* (11), who used tubulin that had been depleted of MAP-containing oligomers by prolonged centrifugation at high *g* forces. Microtubules with attached ribbon structures appear in cross section as circular elements with an attached ribbon curving in a clockwise or counterclockwise direction, the direction reflecting the polarity of the microtubule to which the ribbon is attached.

In some experiments, pieces of nerve were initially extracted at room temperature with two changes (3–5 min each) of EXAB without tubulin or GTP. The samples were then placed in EXAB plus tubulin for 5 min at 0–5°C to allow diffusion of tubulin into the permeabilized axons (preexisting microtubules are not depolymerized with such brief exposure to low temperature), GTP was added, and the samples were incubated at 37°C for 10–30 min. In other experiments, the nerve pieces were placed in EXAB without preextraction, tubulin and GTP were added, and samples were then incubated for 10–40 min at 37°C. To provide control samples, pieces of nerve were routinely extracted and incubated at 37°C without tubulin; these samples were often from the same nerve and they were incubated for the same length of time alongside their experimental counterparts. Although both PC-tubulin and MAP-containing tubulin were used in various experiments, similar results were obtained in terms of the formation and direction of ribbons on axonal microtubules.

In most experiments, a section of nerve including the anterior portion of the olfactory lobe was transected as shown in Fig. 2. In this way, the orientation of two pieces of nerve could be retained during processing, with one piece having a recognizable portion of the olfactory lobe at its end and the other piece split lengthwise at its end distal to the brain. An end of one piece was thus complementary to an end of the other, as further described below.

After incubation, pieces of nerve were fixed for 1–12 hr in 3% (vol/vol) glutaraldehyde in 0.1 M Pipes buffer (pH 6.9) containing 0.014%, 2%, or 4% tannic acid. The tannic acid concentration was varied depending on the degree to which microtubule or ribbon protofilaments were to be resolved. Fixation was at room temperature unless samples were to be left in fixative overnight, when they were placed under refrigeration. After initial fixation, nerve pieces were rinsed in 0.1 M sodium cacodylate buffer (pH 7.2), then secondarily fixed for 1 hr at room temperature in 1% osmium tetroxide buffered with cacodylate. Thereafter, samples were dehydrated in an acetone series and embedded in Araldite resin. Sections were cut with a diamond knife, captured on uncoated 400-mesh grids, and stained with methanolic–ethanolic uranyl acetate and lead citrate (15 min each), as described by Kim *et al.* (15).

RESULTS

Frog olfactory nerves are made up of thousands of unusually small axons arranged in bundles, with the axons ranging in diameter between 0.1 and 3.0 μm. The major formed elements composing the axoplasm are longitudinally oriented microtubules, neurofilaments, tubules of smooth endoplasmic reticulum, mitochondria, and a wispy filamentous material interconnecting the above structures within a latticework bridged to the inner surface of the axolemma (Fig. 1). Over 75% of the axons contain only two or three microtubules, and no more than five microtubules were ever seen in an axon. The number of neurofilaments is highly variable, ranging between 0 and 20.

In considering the polarity of microtubules in olfactory axons, it was important to bear in mind that the perikarya from which these sensory axons originate lie in the olfactory epithelium (O.E. in Fig. 2) lining the nasal cavity of the head, which opens to the outside by means of a nostril or external naris. The axons

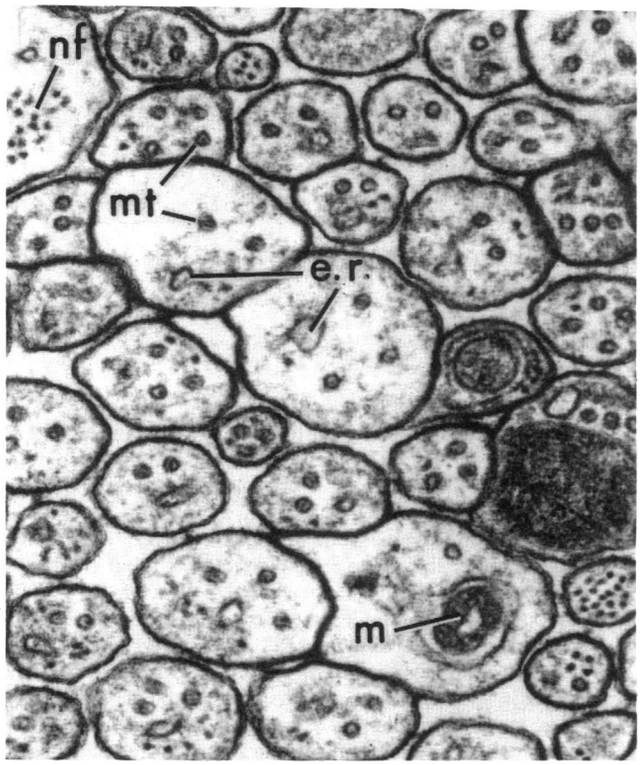

FIG. 1. Cross section of a portion of a normal untreated frog olfactory nerve, showing the small axons that compose the nerve. Only two or three microtubules (mt) are usually seen in these axons, along with a variable number of neurofilaments (nf). The other prominent formed elements seen are mitochondria (m) and cross-sectional profiles of tubules of smooth endoplasmic reticulum (e.r.). Variable amounts of a wispy matrix material compose the axoplasmic matrix. (×108,000.)

carry information from the olfactory epithelium to the olfactory lobe of the brain (O.L. in Fig. 2), where they terminate and synapse with other neurons. Fig. 2 shows an overview of the nerve pathway, with one of the two nerves transected to show the ends of one axon containing a microtubule with a ribbon of protofilaments attached to its surface. The microtubule with its attached ribbon would appear in cross section as shown in the diagram, and in views of the complementary surfaces of the transected axon the microtubule–ribbon complex would appear either as the number 6 or its mirror image, depending on the surface examined. In this study, pieces of nerve were often transected and processed in such a manner that complementary surfaces could be identified and sectioned. Thus, sections from one surface would represent a view toward the brain (axon terminals) and sections from the other surface would provide a view toward the nares (perikarya).

Fig. 3 shows cross sections of axons of extracted nerve pieces incubated with tubulin at 37°C, and the two sets of figures are complementary portions of the same olfactory nerve, with Fig. 3 *a–c* matching Fig. 3 *a′–c′*, and Fig. 3 *d* and *e* matching Fig. 3 *d′* and *e′*. The axons in Fig. 3 *a–e* are from the surface of a nerve piece providing a view toward the perikarya. Ribbons on microtubules are clearly evident, and all of them are oriented in a clockwise direction. In Fig. 3*a*, six axons are seen (arrows) in which all the microtubules have clockwise ribbons, and several axons are seen that contain microtubules without ribbons. Higher magnifications of individual axons are seen in Fig. 3 *b–e*. Discontinuities in the axolemma and the open areas in the axoplasm are evidence of detergent extraction; with prolonged extraction, boundaries of individual axons may be completely

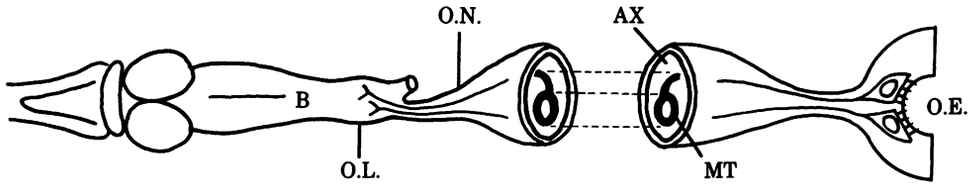

FIG. 2. Diagram of the olfactory nerve tract of the frog; the brain (B) is at the left and the olfactory epithelium (O.E.) is at the extreme right. Olfactory epithelium lines the cavities of the nose, which open to the outside through the paired nostrils (external nares) of the frog. The perikarya of the olfactory neurons are located in the olfactory epithelium and send axons, via the olfactory nerve (O.N.), to the olfactory lobe (O.L.) of the brain. Only one of the two olfactory nerves is shown, and this one is diagrammatically transected and enlarged to show the two opposing surfaces of one axon (AX). The two cut surfaces are complementary surfaces, and a microtubule with an attached ribbon structure is seen in cross section. When this microtubule is viewed looking at the cut surface to the right (i.e., toward the olfactory epithelium), its ribbon curves in a clockwise manner; however, when the cut surface to the left (i.e., toward the brain) is viewed, the ribbon on the microtubule curves in a counterclockwise manner. In this study, pieces of nerve were oriented to provide these kinds of views of axonal microtubules.

lost. Axons shown in Fig. 3 *d* and *e* were fixed in the presence of 2% tannic acid to better delineate microtubule wall protofilaments, and the ribbon-bearing microtubules shown have 13 protofilaments, which is the normal number for these microtubules. The axons shown in Fig. 3 *a'–e'* are from the surface of nerve pieces providing a view toward the brain of the frog and, as would be expected, in this orientation the axonal microtubules show ribbons directed in a counterclockwise manner. In numerous instances a ribbon can be seen attached to another ribbon (arrows), and the directionality assumed by the former is usually that of the latter. The subunit structure of the microtubule-ribbon complex is shown in Fig. 3*e'*, in which three ribbons, consisting of three protofilaments each, are attached to the surface of a microtubule made up of 13 protofilaments. The points of attachment or contact between the basal (or proximal) protofilament of a ribbon and the microtubule wall is usually at the point of junction between two wall protofilaments of the microtubule (arrows, Fig. 3*e'*); in other words, the basal protofilament of a ribbon makes contact with two wall protofilaments, and in fact the basal protofilament of a ribbon often appears to be wedged into the junction between the two wall protofilaments.

The micrographic evidence provided in Fig. 3 indicates that ribbons appearing on microtubules of olfactory axons are oriented in the same direction, and that all the microtubules in many individual axons display ribbons having a single directionality. To obtain quantitative information, counts were made of axonal microtubules showing ribbons directed clockwise or counterclockwise in a number of complementary samples. Two observers made numerous counts in a standardized manner, and the data are presented in Table 1. With sections cut such that axons were viewed as if looking toward the brain, between 83% and 98% of the ribbons were oriented in a counterclockwise manner, depending on the sample, while up to 98% of the ribbons were directed clockwise when sections were cut such that the view was toward the nares. These data support the observations that ribbons seen on microtubules of olfactory axons tend to have a uniform directionality.

Considerable variation was observed in the degree of extraction shown by axons in pieces of nerve; in some cases, membrane boundaries of axons were completely missing and only randomly distributed microtubules were seen, while in other cases the axolemma and axoplasm appeared to be largely intact. Also, not all the microtubules in the experimental samples showed ribbons. In some regions, over 50% of the microtubules displayed ribbons on their surfaces, while in other regions many fewer ribbons were seen. Counts of about 350 microtubules in each of two different samples indicated that about 20% of the microtubules showed ribbons. One other observation that should be noted is that ribbons were often seen on microtubules in pieces of nerve incubated *without* exogenous tubulin in the medium (i.e., in the control samples); further, these ribbons tended to be oriented in the same direction. The significance of this is unclear at the moment.

DISCUSSION

The studies of Heidemann and McIntosh (10) and Heidemann *et al.* (11) leave little doubt that the curvature of ribbons formed

Table 1. Directionality of ribbons attached to axonal microtubules in sections from complementary surfaces of transected olfactory nerve

Sample	Kind of tubulin	Observer	Directionality of ribbons on microtubules			
			Brain side		Nares side	
			Clockwise	Counterclockwise	Clockwise	Counterclockwise
A	×2	X	4 (2.3%)	174 (97.7%)	212 (97.2%)	6 (2.8%)
B	×2	X	17 (9.5%)	179 (90.5%)	198 (86.4%)	27 (13.6%)
B	×2	Y	10 (4.6%)	218 (95.4%)	400 (97.8%)	9 (2.2%)
C	PC	X	13 (17.0%)	74 (83.0%)	Insufficient extraction	
C	PC	Y	22 (9.7%)	225 (90.3%)	Insufficient extraction; a few clockwise ribbons were seen	

During preparation, two pieces of olfactory nerve were oriented to allow the observer to examine sections from complementary surfaces such that by examining the end of one nerve piece face-on, the view would be toward the nares (olfactory epithelium); the end of the complementary nerve piece would provide a view toward the brain (see Fig. 2). To obtain regions to be counted, a grid was scanned from the top downward, and the first section completely spanning a grid opening was selected. The section was scanned back and forth, from the top downward, at a magnification of ×20,000. Only distinct cross sections of microtubules bearing ribbons were counted, and counting was usually continued until at least 200 ribbon-bearing microtubules were tallied. Regarding the kind of tubulin used, ×2 tubulin is twice-polymerized tubulin containing MAPs, and PC-tubulin is tubulin freed of MAPs by phosphocellulose chromatography.

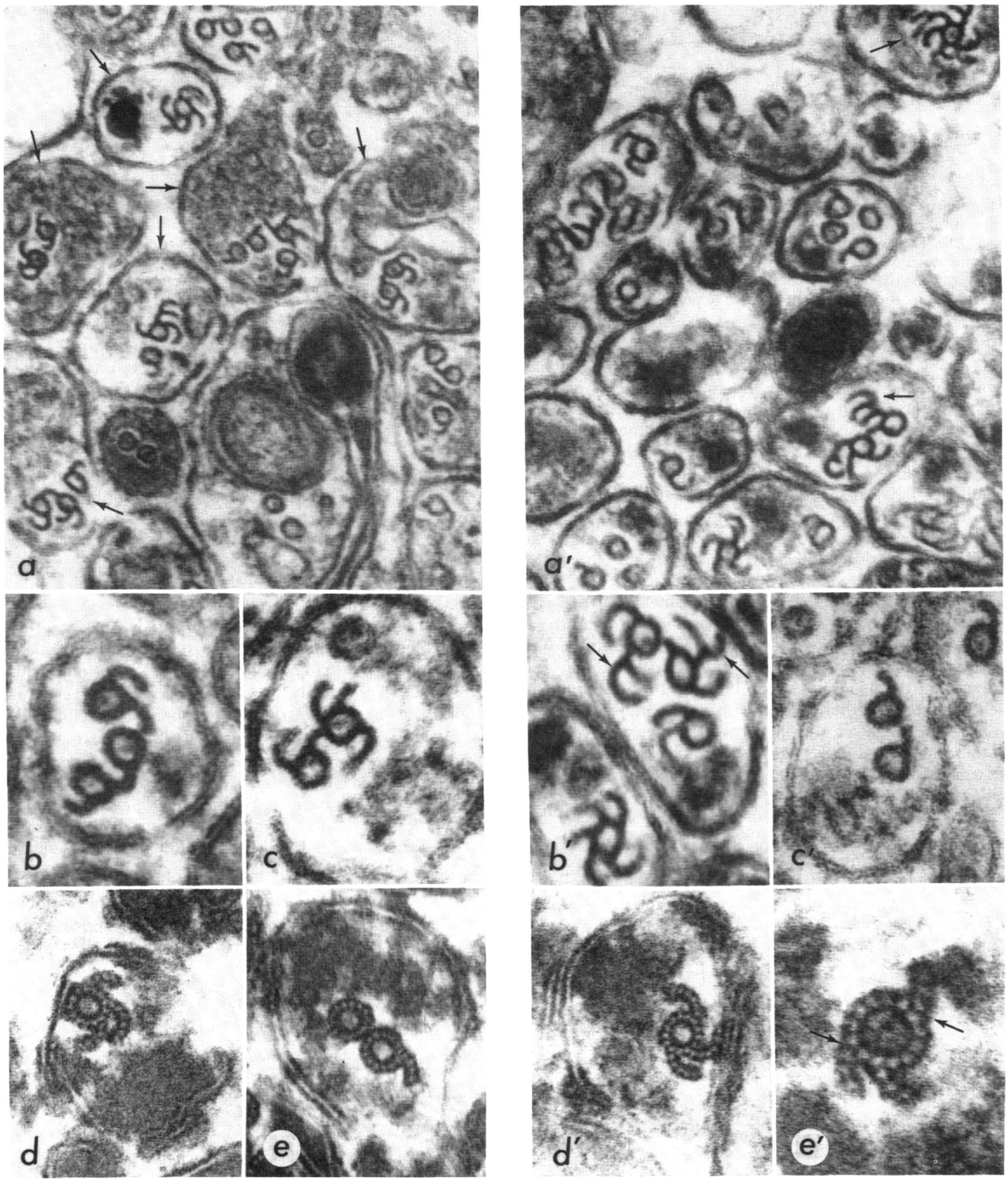

FIG. 3. Cross sections of experimental axons. (*Left*) The axons are viewed as if looking toward the olfactory epithelium; note that all the ribbons seen are oriented in a clockwise direction. *a–c* are from the end of nerve piece complementary to that shown in *a'–c'*. In *a*, the arrows indicate axons in which all the microtubules bear clockwise ribbons. Two axons are seen at higher magnification in *b* and *c*; note the empty-appearing regions and the discontinuities in the axolemma, which are effects of detergent treatment. *a–c* are of material fixed with 0.014% tannic acid in the fixative, whereas *d* and *e* show axons fixed with 2% tannic acid to better delineate wall protofilaments. The microtubules shown with ribbons attached have 13 wall protofilaments. The axons in *d* and *e* are from the end of a nerve complementary to that represented by axons seen in *d'* and *e'*. (*a*, ×165,000; *b–e*, ×265,000.) (*Right*) The axons are viewed as if looking toward the brain; note that all the ribbons seen are oriented in a counterclockwise direction. The arrows in *a'* and *b'* indicate ribbons that are attached to other ribbons. *b'–e'* show axons at high magnification, and at the lower left in *b'* an axon shows a microtubule with five attached ribbons. The axons shown in *a'–c'* were fixed as described for *a–c*, while the axons in *d'* and *e'* were fixed in the presence of 2% tannic acid. The microtubules shown in *d'* and *e'* have 13 wall protofilaments, and in *e'* a microtubule is shown with three ribbons, of three protofilaments each, attached to its surface. The arrows in *e'* indicate the points of junction between the basal protofilaments of the ribbons and the protofilaments of the microtubule wall. (*a'*, ×165,000; *b'–d'*, ×265,000; *e'*, ×511,000.)

on preexisting microtubules can reveal the intrinsic polarity of the microtubules. Their evidence indicates that ribbons formed on microtubules are directed in a clockwise manner when viewed toward their "minus" or slow-growing ends (10), which are normally associated with their nucleation sites. In the present study, the evidence indicates that microtubules of axons of frog olfactory nerves have a single polarity. If the perikaryon is the point of their origin, ribbons on these microtubules should be clockwise when viewed end-on looking in the direction of the olfactory epithelium, where the perikarya are located. This is indeed the case, which confirms and extends the observations of Heidemann and McIntosh (10). Further, evidence is presented indicating that axonal microtubules in these axons grow with their "plus" or fast-growing ends (10) distal to the perikarya from which they originated.

Numerous theories have been advanced to explain bidirectional axoplasmic transport, which proceeds at two rates, and several of these theories implicate microtubules in such translocations (refs. 1–4; see ref. 16 for review). Assuming that bidirectional transport occurs in axons of frog olfactory nerve, it could be theorized that microtubules of opposite polarity could provide for such directionality. This notion is not supported by the evidence, which indicates that microtubules of these axons have a single polarity. This may mean that the directionality of axoplasmic transport is provided by components other than microtubules.

It would have been satisfying if *all* the microtubules observed in treated nerve pieces displayed ribbons oriented in the same direction. It can be pointed out that if 20% of the microtubules show ribbons, and if 95% of the ribbons are oriented in the same direction, then two sets of microtubules failed to behave in a predictable manner in terms of ribbon addition. Why didn't ribbons form on all the microtubules? How can one account for the observation that 5% of the microtubules bear ribbons facing in the opposite direction? In the first place, it is likely that the degree of extraction and the limited diffusion of tubulin were factors serving to limit conditions providing for formation of ribbons on all the microtubules in all the axons. The nerve pieces used were of considerable size (the diameter of these nerves is about 1–1.5 mm, and pieces were sometimes 2–3 mm long when a portion of the olfactory lobe was left intact to provide orientation). As to the 5% of the microtubules with misdirected ribbons, a likely explanation is that some of these microtubules were newly formed during incubation and elongated in a direction opposite to the direction of elongation of their preexisting neighbors. As a result, their polarity would be opposite that of the preexisting microtubules. In this regard, we have preliminary evidence that this may be the case, for axons were sometimes seen with *more* than five microtubules, which represents the maximal number seen in normal, untreated axons (in one case, 14 microtubules were seen in an experimental axon).

In summary, the evidence presented is taken as strongly supporting the view that microtubules of frog olfactory axons have a single polarity, particularly in view of the convincing frequency with which *all* the microtubules in individual axons showed ribbons having the same polarity.

Note Added in Proof. After this article was accepted for publication, Euteneuer and McIntosh (17) reported that all microtubules in the axopodia of the heliozoan *Actinosphaerium* have the same polarity, as do all microtubules in the processes of angelfish melanophores. Because bidirectional transport is known to occur in these cytoplasmic extensions, the authors suggested that "... cellular systems for motility, and even those capable of bidirectional transport, can be constructed from microtubules of a single polarity." Because axons are also cytoplasmic extensions, the present work complements and supports the above suggestion.

We are indebted to our colleagues Drs. Brower Burchill and Richard Himes and to Dr. John Hansen and Ms. Cheryl Craft (University of Texas Health Science Center, San Antonio), for critically reading the manuscript. We are also indebted to Dr. Peter Cooke (Department of Physiology, University of Connecticut Health Science Center, Farmington) for reminding us that axons of frog olfactory nerve might be useful model systems for studies of cytoskeletal elements. This research was supported by U.S. Public Health Service Grant GM 24992.

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