

Brain Dynein (MAP1C) Localizes on Both Anterogradely and Retrogradely Transported Membranous Organelles In Vivo

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Abstract. Brain dynein is a microtubule-activated ATPase considered to be a candidate to function as a molecular motor to transport membranous organelles retrogradely in the axon. To determine whether brain dynein really binds to retrogradely transported organelles in vivo and how it is transported to the nerve terminals, we studied the localization of brain dynein in axons after the ligation of peripheral nerves by light and electron microscopic immunocytochemistry using affinity-purified anti-brain dynein antibodies. Different classes of organelles preferentially accumulated at the

regions proximal and distal to the ligated part. Interestingly, brain dynein accumulated both at the regions proximal and distal to the ligation sites and localized not only on retrogradely transported membranous organelles but also on anterogradely transported ones. This is the first evidence to show that brain dynein associates with retrogradely transported organelles in vivo and that brain dynein is transported to the nerve terminal by fast flow. This also suggests that there may be some mechanism that activates brain dynein only for retrograde transport.

THE axons of nerve cells have served as a good model system for studying the mechanism of organelle transport. There is bidirectional movement of numerous classes of membranous organelles in the axon (Nakai, 1956; Lubinska and Niemierko, 1971; Tytell et al., 1981; Allen et al., 1982), and structural studies after the blockage of transport have revealed that different classes of organelles are preferentially transported by respective types of flow (Smith, 1980; Tsukita and Ishikawa, 1980). Several theories have been proposed to explain the underlying mechanisms (for review, see Grafstein and Forman, 1980; Schliwa, 1984). Electron microscopic studies of the neuronal cytoskeleton in vivo have suggested that microtubules (MTs)¹ and cross-bridges between MTs and membranous organelles form the structural basis for this class of motility (Smith, 1971; Hirokawa, 1982; Miller and Lasek, 1985; Hirokawa and Yorifuji, 1986; Hirokawa et al., 1989a). In fact, observations of organelle movements in the isolated axoplasm by video-enhanced light microscopy have demonstrated that bidirectional organelle transport occurs along MTs (Brady et al., 1985; Schnapp et al., 1985; Vale et al., 1985a). Recently, candidates for the motor molecules of these transports, namely kinesin and brain dynein (MAP1C), have been identified (Brady, 1985; Vale et al., 1985b; Paschal and Vallee, 1987; Paschal et al., 1987; Schnapp and Reese, 1989). Brain dynein-like molecules were identified in *Caenorhabditis elegans* (Lye et al., 1987), *Reticulomyxa* (Euteneuer et al., 1988, 1989), and squid giant axons (Gilbert and Sloboda, 1986, 1989; Pratt, 1986) as well. Because MTs on cover glasses coated with brain dynein move from minus to plus

ends, brain dynein is thought to function as a motor of retrograde transport (Paschal and Vallee, 1987). However, it has not been resolved whether brain dynein really associates with retrogradely transported membranous organelles and is a motor for retrograde transport in vivo. A related question is whether the retrograde motor is transported to the nerve terminals by fast or slow transport, and whether brain dynein attaches only to retrogradely transported organelles or if the motor binds to both anterogradely and retrogradely transported ones. If the latter were the case, some mechanism that activates brain dynein only for the retrograde transport would have to exist.

To address these issues, we studied the localization of brain dynein in the axons after ligation of peripheral nerves by light and electron microscopic immunocytochemistry using affinity-purified anti-brain dynein antibodies. Our study clearly demonstrated that brain dynein accumulates both anterogradely and retrogradely at regions both proximal and distal to the ligation and that it binds to both retrogradely and anterogradely transported membranous organelles. This suggests that brain dynein is really a motor for retrograde transport in vivo, that it is transported to the nerve terminal by fast flow, and that there may possibly exist some mechanism that activates brain dynein only for the retrograde transport.

Materials and Methods

Purification of Brain Dynein and Antibody Production

Brain dynein (MAP1C) was purified by the method of Paschal et al. (1987) with slight modifications as described previously (Yoshida et al., 1990).

1. *Abbreviation used in this paper:* MTs, microtubules.

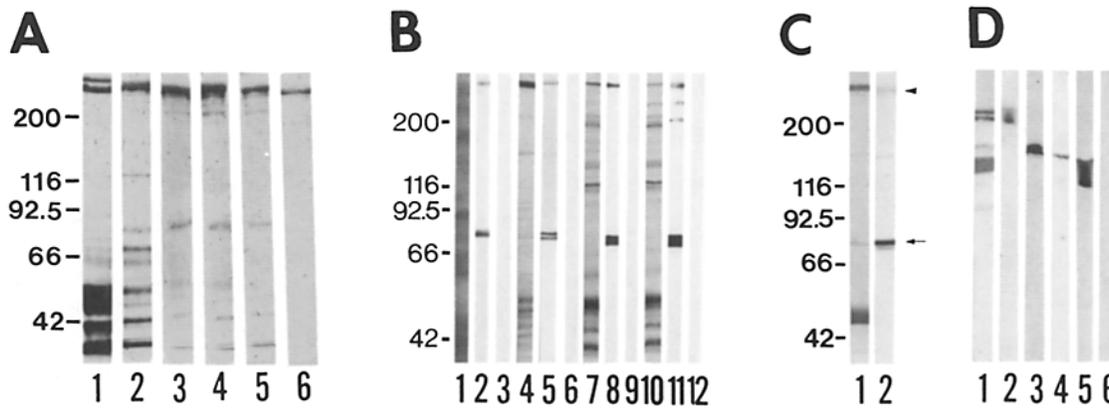


Figure 1. (A) SDS PAGE of purification steps of bovine brain dynein (MAPIC). Lane 1, microtubule proteins polymerized with taxol. Lane 2, supernatant after ATP extraction. Lane 3, fraction obtained after sucrose density gradient centrifugation. Lane 4, fraction obtained after DEAE-Sepharose column chromatography. Lane 5, fraction obtained after second sucrose density gradient centrifugation. Lane 6, purified MAP2 from bovine brains is electrophoresed for comparison. Molecular mass markers are indicated in kilodaltons. (B) Immunoblot analysis of crude extracts of mouse peripheral nerves (lanes 1-3), cytoplasmic dynein purified from rat livers (lanes 4-6), and fractions containing mouse brain dyneins (lanes 7-9), and photocleaved mouse brain dynein fractions (lanes 10-12). The samples were separated on 7.5% polyacrylamide gels. One part of the gel was stained with Coomassie brilliant blue (lanes 4, 7, and 10). The rest of it was translated to nitrocellulose, strips of which were stained with India ink (lane 1), anti MAPIC antibody (lanes 2, 5, 8, and 11) or nonimmune rabbit IgG (lanes 3, 6, 9, and 12). The affinity-purified anti-MAPIC antibodies stain a high-molecular weight band and low-molecular weight bands that correspond to the heavy and light chains of brain dynein, respectively, in the blots of crude extracts of mouse peripheral nerves (lane 2), rat liver cytoplasmic dynein (lane 5), mouse brain dynein fractions (lane 8), and photocleaved mouse brain dynein fractions (lane 11). Both heavy and light chains are stained, but the light chains are stained more intensely. Although mouse brain dynein fractions still contain other contaminated polypeptides, these antibodies recognize only the heavy chain and light chains. After UV photocleavage, the heavy chain split into smaller fragments (lane 10) which are also stained by this antibody (lane 11). (C) Immunoblot analysis of purified liver MAPIC bound to microtubules. The pellet of rat liver MAPIC bound to microtubules polymerized from PC tubulin in the presence of taxol was separated on 7.5% polyacrylamide gels. One part of the gel was stained with Coomassie brilliant blue (lane 1). The rest of it was translated to nitrocellulose, strips of which were stained with anti MAPIC antibody (lane 2). Anti MAPIC antibody stained the heavy chain (arrowhead), light chains (arrow), and additional 150-kD intermediate chain. (D) Immunoblot analyses of crude microtubule proteins prepared from mouse brain on a 4% polyacrylamide gel. Lane 1, Amido black staining of microtubule proteins transferred to nitrocellulose paper. Lane 2, staining with an anti-MAP1A mAb. Lane 3, staining with an anti-MAP1B mAb. Lane 4, staining with affinity-purified anti-MAPIC antibodies. Staining of only the heavy chain is shown. Lane 5, staining with an anti-MAP2 mAb. Lane 6, staining with nonimmune rabbit IgG. High molecular weight parts are shown. Affinity-purified anti-brain dynein (MAPIC) antibody reacts only with brain dynein.

Microtubules were prepared from bovine brain white matter and brain stem using taxol. After GTP extraction, MAPIC was extracted in 10 mM ATP solution from the MTs and purified by sucrose density gradient centrifugation and DEAE-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) chromatography. The antigen to be used as a ligand for affinity purification was further purified by additional sucrose density gradient centrifugation. Anti-MAPIC antibody was raised against rabbits by immunization with the purified MAPIC subsequently affinity purified by a method described previously (Yoshida et al., 1985), and adsorbed with Sepharose beads conjugated with a sufficient amount of purified MAP2. MAP2 was purified from microtubule proteins by the methods of Kim et al. (1979) or Hirokawa et al. (1988). MAPIC was also purified from rat livers by a modified procedure (Bloom and Brasher, 1989; Collins and Vallee, 1989) based on the method developed by Paschal et al. (1987). Anti-MAPIC serum was applied to a protein A-Sepharose column, and the collected IgGs were incubated with Cyanogen bromide-activated Sepharose-4B conjugated with bovine brain dynein or rat liver MAPIC. After washing the unbound proteins, the bound antibodies were eluted and then incubated with cyanogen bromide-activated Sepharose-4B conjugated with porcine brain MAP2 to completely adsorb the reactivity to MAP2.

Immunoblotting Procedure

Sciatic nerves and brains were dissected from adult mice and homogenized separately in extraction buffer (0.1 M Pipes, 1 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM DTT, pH 6.6) and then centrifuged to yield the crude sciatic nerve and brain extracts. Taxol was added to the crude brain extracts to a final concentration of 20 μM (Vallee, 1982), and MT proteins were pelleted by centrifugation and rehomogenized in the extraction buffer. Cytoplasmic dyneins were purified from rat livers and

mouse brains by a modification of the methods of Paschal et al. (1987), Bloom and Brasher (1989), and Collins and Vallee (1989). Briefly, dissected tissues were immediately homogenized at 4°C in PEM buffer (100 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 0.1 mM DTT, 1 μg/ml leupeptin, 0.1 mM PMSF, 10 μg/ml tosyl arginyl methyl ester, 1 μg/ml pepstatin A [pH 6.8]) and centrifuged at 100,000 g for 20 min at 4°C. The supernatant was recovered and centrifuged for another 60 min. This supernatant was supplemented with 1 U/ml hexokinase, 50 mM glucose, 500 μg/ml phosphocellulose column-purified tubulin (PC tubulin), and 5 μM taxol. After incubation at 37°C for 5 min, the microtubules were sedimented at 4°C, washed by resuspension and centrifugation in PEM buffer alone, and then in PEM buffer containing 5 mM Mg GTP. Dyneins were extracted from microtubules using 10 mM Mg ATP and 400 mM KCl, and further fractionated by sucrose density gradient centrifugation.

Fractions containing brain dyneins were photocleaved by irradiation at 365 nm in the presence of 100 μM vanadate and 50 μM ATP for 30 min (Lee-Eiford et al., 1986). Crude sciatic nerve extracts, brain MT proteins, cytoplasmic dyneins purified from rat livers, fractions containing brain dyneins, and photocleaved brain dynein-containing fractions were respectively separated on polyacrylamide gels as described by Laemmli (1970). Liver cytoplasmic dyneins were bound to microtubules polymerized from PC tubulin in the presence of taxol. After centrifugation at 30°C for 30 min at 10,000 g, the resulting pellets were also separated by SDS gel electrophoresis. One part of the gel was stained with Coomassie brilliant blue and the rest was electrophoretically blotted to nitrocellulose as described by Towbin et al. (1979) and stained with India ink, anti-brain dynein antibody, or nonimmune rabbit IgG. Nitrocellulose sheets were incubated with 5% skim milk PBS before incubation with either affinity-purified anti-MAPIC antibodies, monoclonal antibodies against MAP1A (Shiomura and Hirokawa, 1987), MAP1B, MAP2 (Sato-Yoshitake et al., 1989), or by nonimmune rab-

bit IgG. For immunoblots against crude sciatic nerve extracts, primary antibodies were detected by incubation with 10 nm gold-conjugated goat anti-rabbit antibodies (Janssen Pharmaceutica, Beerse, Belgium) and visualized by silver enhancement as described by Jones and Moermans (1988). For immunoblots against brain MT proteins, liver cytoplasmic dyneins, brain dynein fractions, and liver cytoplasmic dyneins bound to microtubules nitrocellulose sheets were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies before the subsequent color development with 4-chloronaphtol.

Ligation of Mouse Peripheral Nerves

Saphenous nerves of female albino mice were ligated very tightly under anesthesia and kept within small cardboard boxes where movement was severely restricted. After 6–10 h, the mice were transcardially perfused with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer. Small pieces of nerves both proximal and distal to the ligated portions were processed for conventional ultrathin-section EM immunofluorescence microscopy, and cryoultrathin-section immunocytochemistry.

Immunofluorescence Microscopy

The nerves were sectioned on a cryostat (4–5- μm sections) and stained with affinity-purified anti-brain dynein antibodies ($\sim 30 \mu\text{g/ml}$), anti-tubulin antibodies ($\sim 10 \mu\text{g/ml}$) (Polysciences, Inc., Warrington, PA) or normal rabbit IgG ($\sim 30 \mu\text{g/ml}$) and rhodamine labeled anti-rabbit IgG (Cappel Laboratories, Malvern, PA). Sections were preincubated for 1 h with 5% skim milk in PBS and stained as described previously (Hirokawa et al., 1984, 1985). Nonligated nerves were also stained similarly.

Immunocytochemistry of Brain Dynein Using Ultrathin Cryosections

Procedures for immunolabeling of ultrathin cryosections were performed as described previously (Tokuyasu, 1980; Hirokawa et al., 1989b) with some modifications. After cryoprotection in graded series of sucrose PBS, the nerves were embedded in 2% gelatin and frozen with liquid Freon 22. We used affinity purified anti-brain dynein IgG ($\sim 30 \mu\text{g/ml}$) in PBS or normal rabbit IgG ($\sim 30 \mu\text{g/ml}$) in PBS as the first antibodies, and 10 nm gold-labeled anti-rabbit IgG in PBS as the second antibodies. The antibodies were centrifuged at 5,000 rpm for 15 min at 4°C before being used for removal of the aggregates.

EM and Measurement

The ultrathin sections were observed with a JEOL 2000 EX electron microscope at 100 kV. For measurement, the negatives were printed at a magnification of 200,000. To determine the localization of gold particles in the axoplasm quantitatively, the numbers of gold particles were counted in four regions from six proximal nerve sections with a total area of 23–49 μm^2 for each region and in four regions from six distal nerve sections with a total area of 20–45 μm^2 for each region at the axoplasm relatively removed from the ligation. The four different types of areas in the sections examined were: (a) regions where membranous organelles had accumulated; (b) cytoskeleton-enriched areas; (c) myelin sheath; and (d) extracellular space. In each of these regions, the average number of gold particles per unit area was determined. Furthermore, the distance between gold particles and membranes of membrane organelles was measured with a magnifying glass in areas where membranous organelles were relatively sparsely localized. 10 micrographs from 3 samples for each portion (proximal and distal) were used for measurement.

Results

Brain Dynein Accumulates at Axoplasms Both Proximal and Distal to the Ligation

Peripheral nerves (saphenous nerves) of female albino mice were ligated under anesthesia. 6–10 h later, the mice were fixed and the nerves were dissected out. The proximal and distal portions of the ligated nerves were processed for immunofluorescent microscopy, electron microscopy and electron

microscopic immunocytochemistry by cryoultramicrotomy. Brain dynein was purified from bovine brains as described previously (Paschal et al., 1987; Yoshida et al., 1990) (Fig. 1 A). Polyclonal antibodies against bovine brain dynein were raised against rabbits. The antiserum was purified by affinity columns using purified cytoplasmic dynein and absorbed with purified MAP2. In the crude extracts of mouse peripheral nerves, this affinity-purified IgG specifically recognized a high-molecular mass band and low-molecular mass bands corresponding to the heavy chain and light chains of brain dyneins, respectively (Fig. 1 B, lanes 1, 2, and 3). Whereas it recognized both heavy and light chains of brain dynein, the reaction was more intense with the light chains (Fig. 1, B and C). It stained the same bands in mouse brain MT proteins (data not shown). The specificity of this antibody was confirmed by immunoblotting data, indicating that it reacted with cytoplasmic dyneins purified from rat liver (Fig. 1 B, lanes 4, 5, and 6) and mouse brain dynein (Fig. 1 B, lanes 7, 8, and 9), and also that it recognized brain dynein heavy chains after UV light-induced cleavage in the presence of vanadate and ATP (Fig. 1 B, lanes 10, 11, and 12). As shown in Fig. 1 D, it did not react with MAP1A, MAP1B, and MAP2. This antibody also faintly stained the 150-kD band of liver cytoplasmic dynein, which is possibly an intermediate chain (Fig. 1 C) (Collins and Vallee, 1989). Fig. 1 C demonstrates liver MAPIC bound to tubulin in the presence of taxol (lane D). We can identify the heavy chain, intermediate 150-kD chain, and light chains. In this sample anti-MAPIC antibody clearly stained the heavy chain (arrowhead), light chains (arrows), and the 150-kD intermediate chain (lane 2). These immunoblotting data collectively indicated that this antibody specifically recognizes the heavy chain and light chains of brain dyneins.

Fig. 2, A, B, and C display the immunofluorescence micrographs of the proximal (Fig. 2, A and C) and distal portions (Fig. 2 B) of ligated nerves. Interestingly, anti-brain dynein antibodies strongly stained not only the distal portions but also the proximal portions. We also noticed that the staining in both the proximal and distal portions followed a gradational pattern, tending to be much brighter at the regions closer to the ligated portions, despite the ligated points having been somewhat damaged (Fig. 2, A and B). Furthermore, the diameter of the nerves tended to become wider at regions closer to the ligated parts (Fig. 2, A and B). As indicated in Fig. 2 C (higher magnification), the staining pattern tended to appear as dots at the more distant regions from the ligation. This was observed even more clearly when nonligated control peripheral nerves were stained with this antibody. Anti-brain dynein antibody tended to stain normal axons (nonligated) in the form of dots on the faint background, and much less intensely than in the ligated nerves (Fig. 2 E). These results suggest that brain dynein accumulates both anterogradely and retrogradely at the ligated portion, and certain parts become localized on the discontinuous small dot structures. In control sections we did not find any positive staining (Fig. 2 F).

Anti-tubulin antibodies stained axoplasms at both proximal and distal parts of the ligated nerves diffusely, and the above gradational patterns of staining were not observed. The intensity of staining with the anti-tubulin antibodies at the ligated regions was similar to that at the nonligated axoplasm (data not shown; see Fig. 8 in Hirokawa et al., 1985).

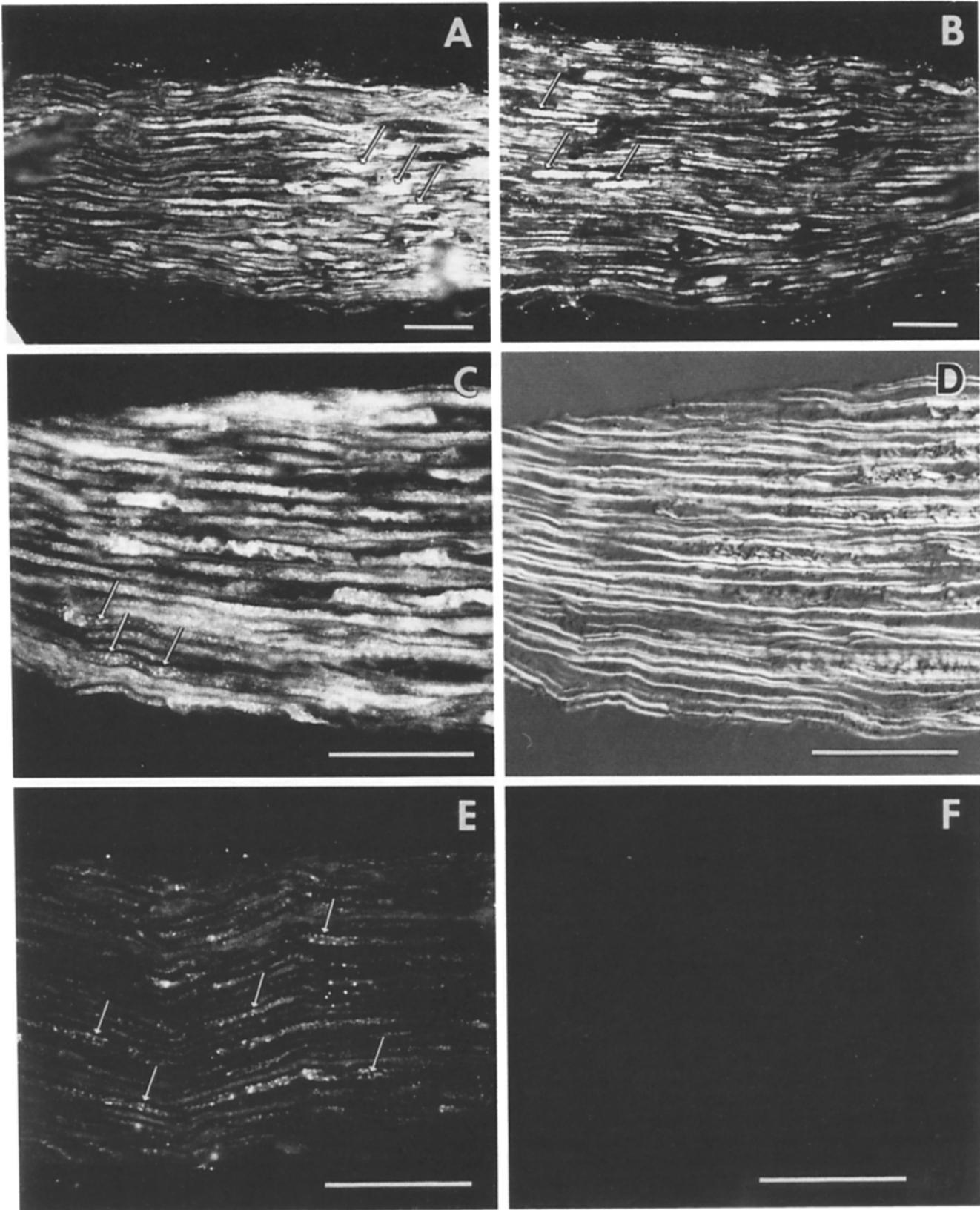


Figure 2. (A-F) Immunofluorescence micrographs of ligated (A, B, C, and F) and nonligated peripheral nerves (saphenous nerves) (E) stained with anti-brain dynein (MAPIC) antibodies. (A) Axons proximal to the ligated region (right). Ligated portion is between A and B. (B) Axons distal to the ligated region (left). Note that axons in A and B are wider at regions closer to the ligated parts, where staining is very bright. (arrows). (C) Higher magnification of axons proximal to the ligated region (right). Arrows indicate punctate staining. (D) Nomarski micrograph of the same region as C. (E) Nonligated axons stained with the same antibodies. Note the punctate staining pattern (arrows). (F) Axons proximal to the ligation stained with normal rabbit IgG as with the first antibody. Bars: (A and B) 50 μm ; (C, E, and F) 100 μm .

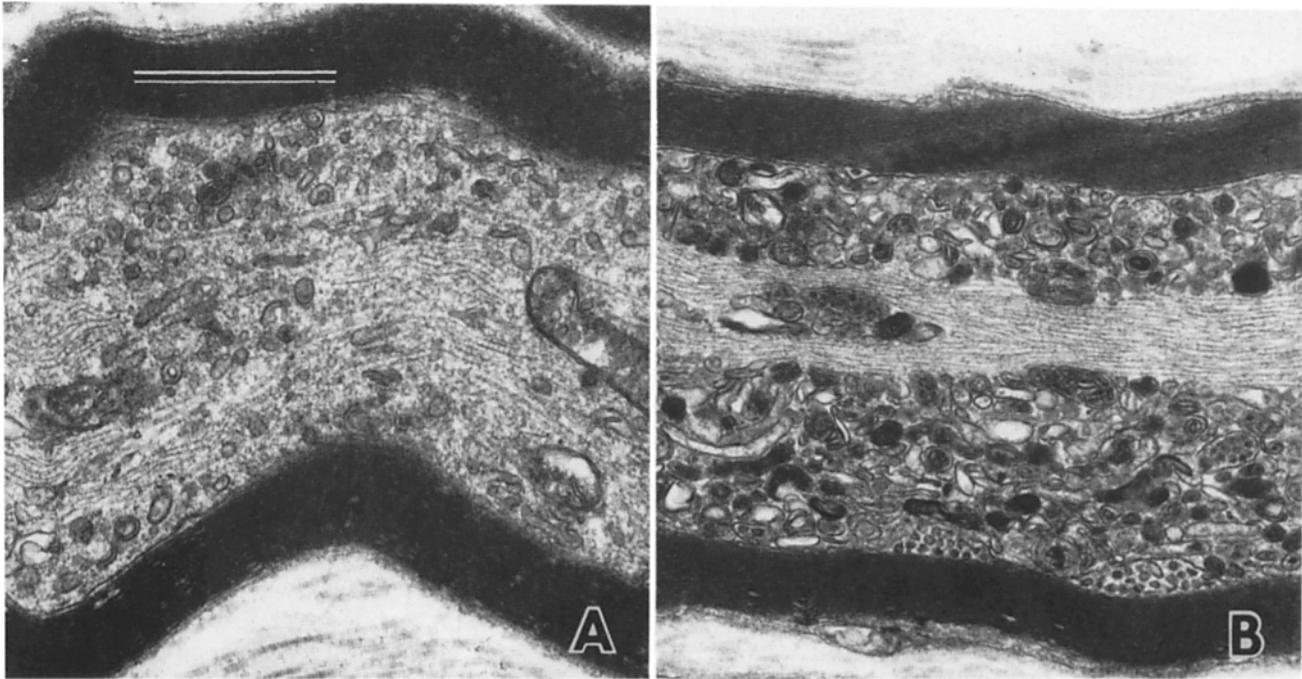


Figure 3. (A) An electron micrograph of axonal cytoplasm proximal to the ligated region. Tubulovesicular membranous organelles and mitochondria have accumulated. (B) An electron micrograph of axonal cytoplasm distal to the ligated region. Lysosomes, multivesicular bodies and mitochondria have accumulated. Bars: (A and B) 1 μ m.

Brain Dynein Associates with Both Anterogradely and Retrogradely Transported Membranous Organelles

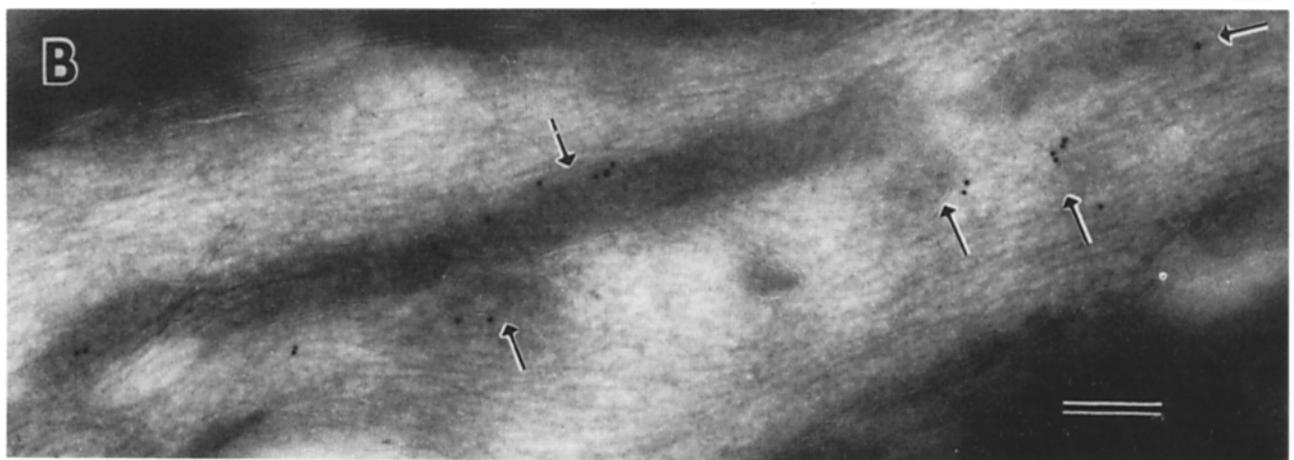
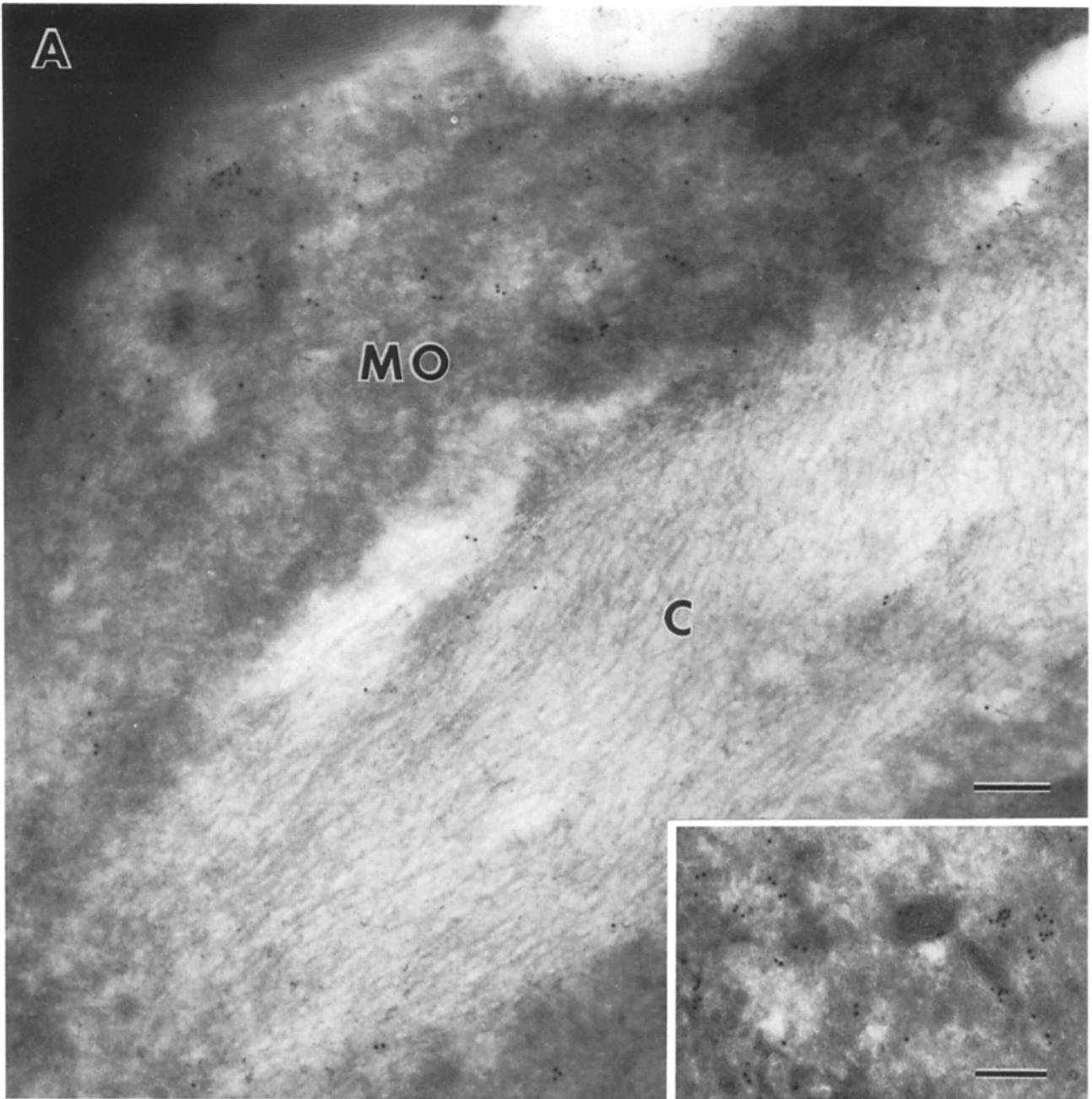
We further analyzed the localization of brain dynein at the electron microscopic level. We observed that different classes of organelles preferentially accumulated at the regions both proximal and distal to the ligated portion (Fig. 3). As previously reported (Smith, 1980; Tsukita and Ishikawa, 1980), tubulovesicular membranous structures and mitochondria increased in number in the axons proximal to the ligated part (Fig. 3 A), whereas membranous organelles such as lysosomes, multivesicular bodies and mitochondria accumulated at the regions distal to the ligated part (Fig. 3 B). We could identify three distinct regions in the axoplasm of the ligated nerves relatively distant from the ligation. They were the anterogradely transported membranous organelle-rich regions on the proximal side, the retrogradely transported membranous organelle-rich regions on the distal side, and cytoskeleton-enriched regions on both sides of the ligation. At the axoplasm very close to the ligation, only organelle-enriched regions were recognizable, while those removed from the ligation we could identify both membranous organelle-enriched regions and cytoskeleton-enriched region in the same area (Figs. 4 A and 5).

Antibody-labeling experiments using immunogold-labeled second antibodies indicated that gold particles tended to accumulate at the regions where membranous organelles accumulated and, further, that the gold labels were closely associated with the membranous organelles at the regions both proximal and distal to the ligated portion, although some gold particles were also observed at some distance from the membranous organelles (Figs. 4 and 5). Fig. 4 A shows an axoplasm proximal and a little bit away from the ligation, in an area where regions enriched in membrane-bound organ-

elles were readily distinguished from cytoskeletal domains. Proteins associated with membrane-bound organelles should be restricted to the membrane-enriched regions, whereas freely diffusible proteins would be expected to equilibrate between membrane-enriched regions and cytoskeleton-enriched regions. Gold particles were preferentially localized in the membrane-enriched regions and were very closely associated with the membranous organelles (Fig. 4 A). In an axoplasm away from ligation gold particles tended to be closely associated with the sparsely distributed membranous organelles (Fig. 4 B).

In the axoplasm proximal to the ligation, gold labels were associated with both tubulovesicular membranous organelles and mitochondria (Fig. 4). We calculated the relative density of gold particles for the membrane-enriched areas, cytoskeleton-enriched areas, the myelin sheath, and the extracellular space. For the calculations we chose areas away from the ligation in which we could identify both membranous organelle-enriched and cytoskeleton-enriched regions such as shown in Figs. 4 and 5 C. As Table I illustrates, gold particles were primarily localized in the membrane-enriched areas.

Fig. 5 displays axoplasm distal to and away from the ligation where membranous organelles accumulated between, or aside from, the cytoplasm filled with cytoskeletons. Gold labels tended to be localized mainly in the membrane-enriched area. At these regions distal to the ligation, we identified gold particles close to presumptive lysosomes, multivesicular bodies, and mitochondria (Fig. 5). We also calculated the relative density of gold particles for the membrane-enriched area, cytoskeleton-enriched area, the myelin sheath, and the extracellular space at the axoplasm distal to the ligation. Gold particles were primarily localized at the membrane-enriched areas (Table I). In control sections only a few, randomly dispersed gold particles were observed (Fig.



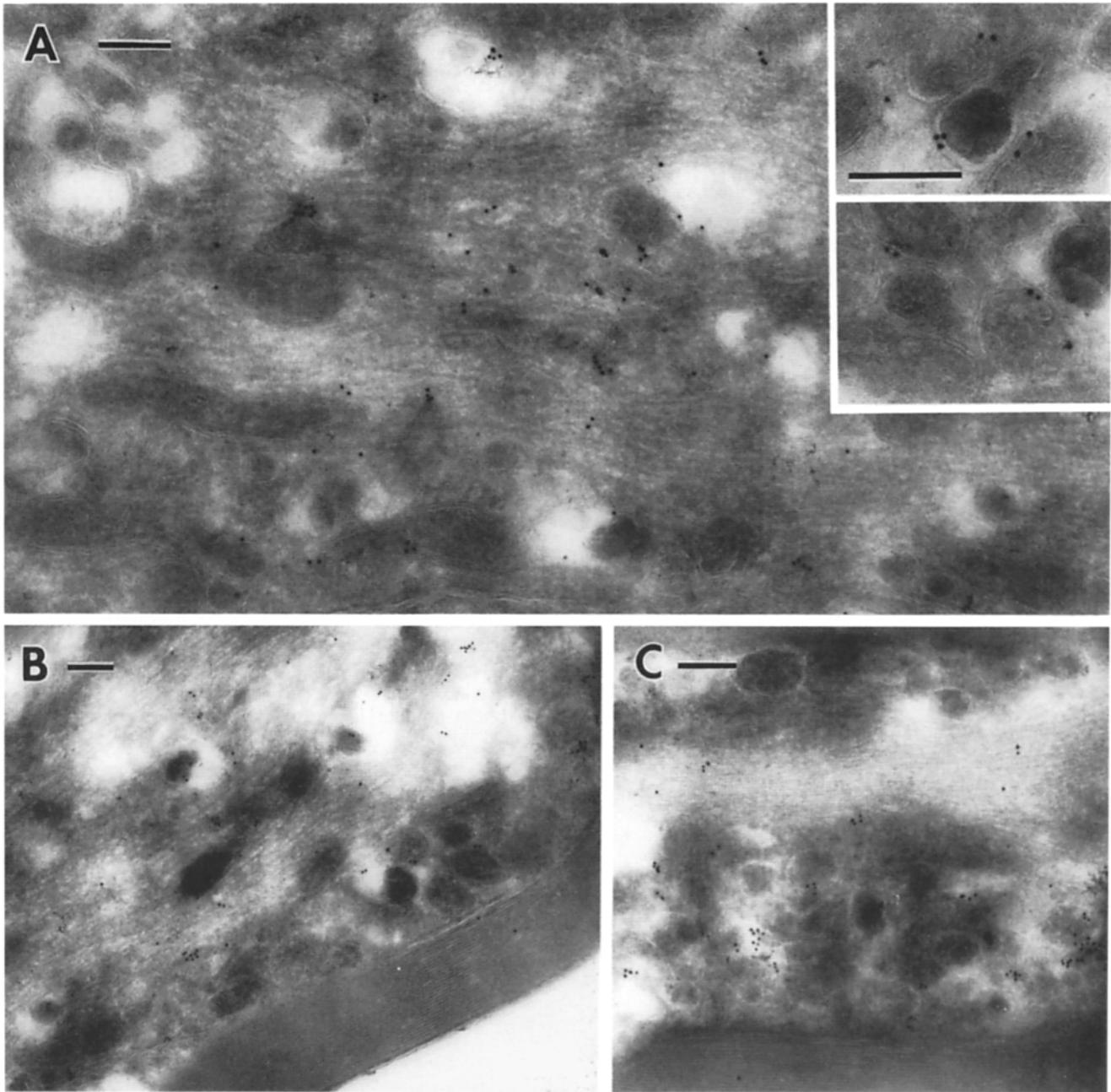


Figure 5. (A–C) Cryoultrathin sections of axoplasms distal to the ligation stained with anti-brain dynein antibodies and 10 nm gold-labeled second antibodies. These electron micrographs show axoplasms where membranous organelles have accumulated between, or aside from, cytoskeletons. Many gold particles are associated with membranous organelles such as multivesicular bodies, lysosomes and mitochondria. Insets in A show gold particles associated with lysosomes and multivesicular bodies. Bars: (A–C) 200 nm.

6). We noticed in both proximal and distal portions that gold particles were associated with the surface of membranous organelles in a sporadic manner rather than densely covering the entire surface of membranous organelles. This could re-

fect the number of brain dynein molecules binding to the membranous organelle.

We measured the relationship of the distance between the surface membranes of organelles and gold particles. For this

Figure 4. Cryoultrathin sections of axoplasms proximal to the ligation stained with anti-brain dynein antibodies and 10 nm gold-labeled second antibodies. (A) This particular section is relatively thick. The regions where membranous organelles (MO) (tubulovesicular membranous organelles and mitochondria) have accumulated are separated from the region where cytoskeletons (C) are enriched. Note gold particles are mostly located at the membranous organelles-enriched axoplasm. (Inset) Thinner section showing accumulation of gold particles in a membrane organelle enriched region. (B) An axoplasm away from the ligation. Membranous organelles are sparsely distributed and gold labels are closely associated with membranous organelles (arrows). Bars: (A and B) 200 nm.

Table I. Gold Particles per Unit Area in Sections of Nerve Proximal and Distal to the Ligation

Region	No. of gold particles/ μm^2	
	Proximal	Distal
Extracellular space	2.4	1.8
Myelin sheath	3.2	1.6
Cytoskeleton	3.8	2.8
Membrane-bounded organelles	17.3	19.3

measurement we chose areas where membrane organelles were relatively sparse, such as the areas shown in Figs. 4 B and 5. Fig. 7 indicates the results in a histogram. As can be seen, most of the gold particles (74% in the proximal portion, 70% in the distal portion) existed <60 nm from the surface of the membranous organelles. Together, these data strongly suggest that brain dynein binds to both anterogradely and retrogradely transported membranous organelles in vivo.

Discussion

In this study we identified three different domains in the axoplasms of ligated nerves. They are the anterogradely transported membranous organelle-enriched region on the proximal side, the retrogradely transported membranous-organelle enriched region on the distal side, and cytoskeleton-enriched regions on both sides of the ligation. At the axoplasms very close to the ligation, only organelle-enriched regions were recognizable, whereas at those removed from the ligation we could identify both membranous organelle-enriched regions and cytoskeleton-enriched region in the same area. Our measurements of the relative density of gold particles for the

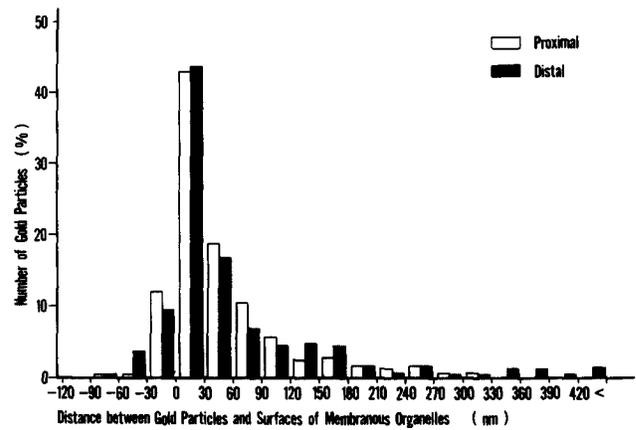


Figure 7. A histogram of the relationship of the distance between the surface membranes of organelles and gold particles. Minus and plus mean gold particles located inside and outside the membranous organelles, respectively. 453 and 471 gold particles were examined at proximal and distal regions, respectively.

membrane-enriched area, cytoskeleton-enriched area, myelin sheath, and extracellular space in the area away from the ligation indicated that a greater number of particles tended to localize in both the anterogradely transported membranous organelle-enriched and retrogradely transported membranous organelle-enriched regions.

Furthermore we measured the distance between the gold particles and surfaces of the membranous organelles. Because the lengths of IgG and brain dynein are ~ 20 and ~ 50 nm, respectively (Vallee et al., 1988), the maximum distances between gold particles and the surfaces of membrane could be $50 \text{ nm (brain dynein)} + 20 \text{ nm} \times 2 \text{ (IgG} \times 2) = 90 \text{ nm}$ at the outside of the surface membranes and 20 nm

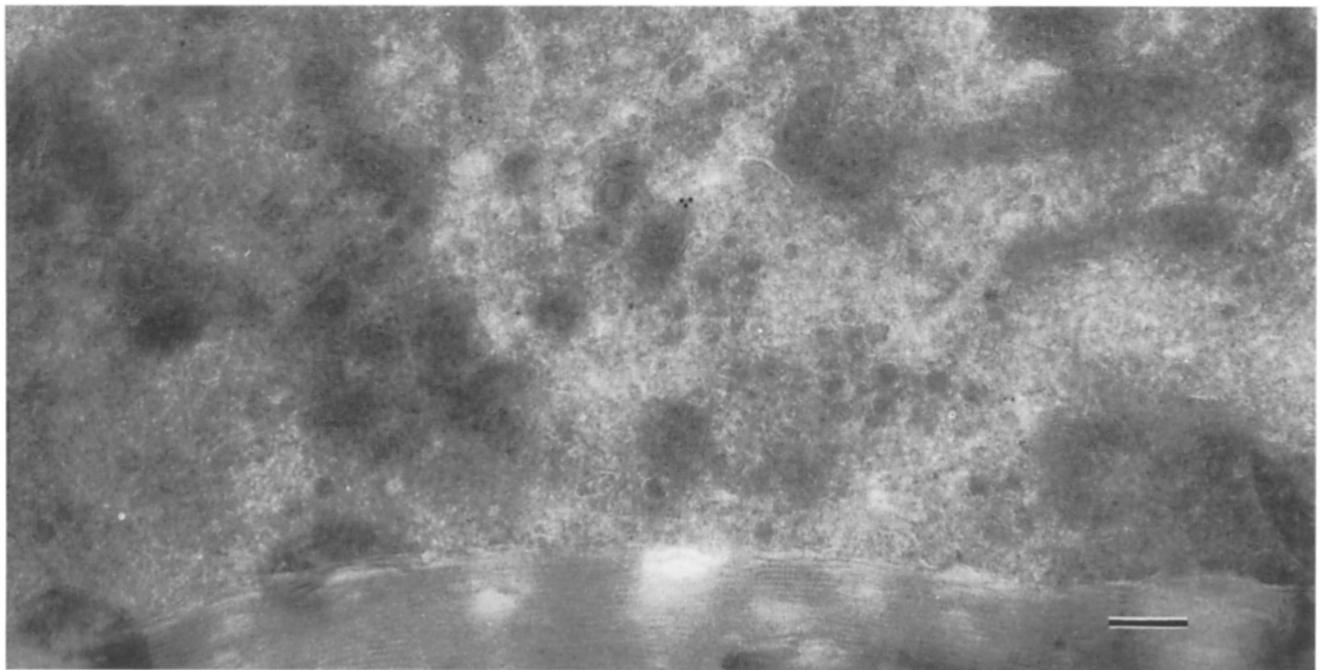


Figure 6. Axoplasm proximal to the ligation stained with normal rabbit IgG as with the first antibody. Bar, 200 nm.

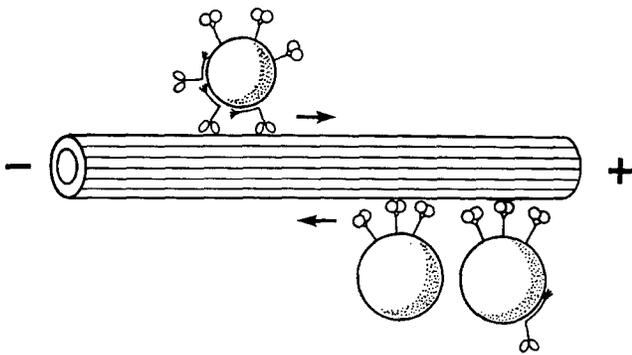


Figure 8. Schematic drawing of the conclusions obtained from our studies (this study, and Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K. K. Pfister, G. S. Bloom, and S. T. Brady, manuscript in preparation). Brain dynein molecules are associated with both anterogradely and retrogradely transported organelles. Although kinesin is associated with small percentages of retrogradely transported membranous organelles, it is primarily bound to anterogradely transported membranous organelles. Some mechanism is thought to activate brain dynein for the retrograde flow.

$\times 2$ (IgG $\times 2$) = 40 nm at the inside of the surface membranes. Our data indicated that $\sim 85\%$ of the gold particles were located within 90 nm outside and 40 nm inside of the organelle membranes. Together, these results mean that many brain dynein molecules are associated with both anterogradely and retrogradely transported organelles. This conclusion is supported by an in vitro study showing that a substantial amount of a high-molecular mass ATPase that comigrated with a flagellar dynein heavy chain associated with vesicles from squid giant axons (Pratt, 1986, 1989), as well as a recent study demonstrating that antibodies against a brain dynein-like high molecular weight protein in squid giant axons immunofluorescently stained membrane vesicle-like particles in the dissociated squid axoplasm (Gilbert and Sloboda, 1989). Our study, however, does not exclude the possibility that some brain dynein molecules exist in the cytoplasm freely or in association with other organelles such as cytoskeletal structures, and in fact rather suggests that although it is a small population, some brain dynein molecules are apparently located at some distance from the membranous organelles as well.

In this study we did not find any membranous organelles with their surfaces covered heavily with gold particles. Of course we should bear in mind the sensitivity of immunogold cytochemistry using cryoultrathin sections; our data also indicate that the number of brain dynein molecules associated with a single membranous organelle are not many. This is consistent with our previous findings that the cross-bridges between an MT and a membranous organelle in the axon are small in number (Hirokawa, 1982; Hirokawa et al., 1985; Hirokawa and Yorifuji, 1986; Hirokawa et al., 1989a).

Previous studies have shown that kinesin translocates polystyrene beads along MTs from minus ends to plus ends and moves MTs on cover glasses from plus ends to minus ends (Vale et al., 1985a,b; Porter et al., 1987; Saxton et al., 1988). On the other hand, brain dynein translocates microtubules on cover glasses from minus ends to plus ends (Paschal et al., 1987) and membranous organelles on MTs from plus ends to minus ends in vitro (Schroer et al., 1989; Schnapp and Reese, 1989). Most MTs in axons are aligned with their

plus ends toward the periphery (Heidemann et al., 1984). Therefore, kinesin and dynein appear to represent ideal candidates for anterograde and retrograde translocators, respectively.

There are three main possibilities concerning the mechanism of bidirectional transport of organelles. One is that organelles transported anterogradely bind only to the anterograde transporter, kinesin, and those transported retrogradely bind only to the retrograde translocator, brain dynein. The other possibilities are first, that both translocators are localized on all organelles transported by fast flow, and the respective translocators are activated for the specific flow (anterograde kinesin, retrograde dynein) or, second, that one translocator binds to specific organelles, whereas the other translocator is localized on all organelles and will be activated for the specific flow. However, the precise localization of these translocators in vivo was not known and the question as to whether these molecules are really associated with membranous organelles has not been answered.

Recent immunofluorescence and quantitative immunoblotting studies using anti-kinesin antibodies have suggested that in fibroblasts a certain percentage of kinesin molecules is bound to membranous organelles (Pfister et al., 1989; Hollenbeck, 1989). Very recently, using cryoultramicrotomy of ligated nerves, we found that kinesin is primarily associated with anterogradely transported membrane organelles (Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K. K. Pfister, G. S. Bloom, and S. T. Brady, manuscript in preparation). This study demonstrated for the first time that brain dynein is associated with membranous organelles transported both anterogradely and retrogradely in the axons. Schliwa's group identified a dynein-like ATPase in the giant ameba, *Reticulomyxa*. Because sucrose density fractions that contained this protein as a major ATPase caused bidirectional movements of beads along MTs this protein was considered to be a bidirectional motor (Koonce and Schliwa, 1985, 1986; Euteneuer et al., 1988, 1989). We found in this study that brain dynein associates with both anterogradely and retrogradely transported membranous organelles. From these results it can be supposed that brain dynein also could function as a bidirectional motor in the mammalian axons, like the high-molecular weight ATPase in the *Reticulomyxa*. However, because brain dynein moves membranous organelles or beads only from plus to minus ends along MTs in vitro (Schroer et al., 1989; Schnapp and Reese, 1989), and because most MTs in the axons are aligned with their plus ends toward the periphery (Heidemann et al., 1984), it is more likely that brain dynein is transported to the nerve terminal in an inactive form to be activated there to work as a retrograde motor by some unknown mechanism. This may be related to the additional cytosolic factors necessary for producing retrograde organelle motility in vitro as suggested recently by Schroer et al. (1989), or may be related to some other mechanisms such as phosphorylation.

Still to be resolved was how the retrograde motors (brain dynein) are transported to the nerve terminals, by fast or slow transport. Here we presented evidence to show that the retrograde motors are conveyed to the nerve terminals at least by fast flow because brain dyneins accumulated at the proximal portions of ligated nerves and mainly associated with the membranous organelles that were conveyed by fast transport, whereas slowly transported tubulin was not accu-

mulated in large amounts at the proximal portions during the period passed between the operation and killing. We studied the localization of kinesin in the same system and found it to bind primarily to anterogradely transported organelles, although kinesin was associated with certain percentages (13.5%) of retrogradely transported membranous organelles (Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K. K. Pfister, G. S. Bloom, and S. T. Brady, manuscript in preparation). Fig. 8 shows these conclusions in a schematic manner. Kinesin works as a motor for the anterograde transport which conveys mitochondria and tubulovesicular membranous organelles, possible precursors of synaptic vesicles, and detaches from the vesicles at the nerve terminal, possibly be mostly degraded, whereas some kinesin may associate with small percentage of retrogradely transported membranous organelles in an inactive form (Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K. K. Pfister, G. S. Bloom, and S. T. Brady, manuscript in preparation). On the other hand, brain dynein could be conveyed to the nerve terminals anterogradely as a membrane bound inactive form, detach from the membrane organelles at the nerve terminal, and reassociate with the retrogradely transported membranous organelles in an activated form. This conclusion agrees with the observation that some organelles switch their direction of movement rapidly (Breuer et al., 1975; Freed and Lebowitz, 1970; Forman et al., 1983, 1987; Hirokawa and Yorifuji, 1986). Probably this is mainly because the activation of inactive brain dynein would happen accidentally. The mechanism for the activation of brain dynein for only retrograde transport is a very important issue, indeed, and will need to be resolved in the near future.

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