Biochemical and Immunological Analyses of Cytoskeletal Domains of Neurons

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Abstract. We have used cultured sympathetic neurons to identify microtubule proteins (tubulin and microtubule-associated proteins [MAPs]) and neurofilament (NF) proteins in pure preparations of axons and also to examine the distribution of these proteins between axons and cell bodies + dendrites. Pieces of sympathetic ganglia containing thousands of neurons were plated onto culture dishes and allowed to extend neurites. Dendrites remained confined to the ganglionic explant or cell body mass (CBM), while axons extended away from the CBM for several millimeters. Axons were separated from cell bodies and dendrites by dissecting the CBM away from cultures, and the resulting axonal and CBM preparations were analyzed using biochemical, immunoblotting, and immunoprecipitation methods. Cultures were used after 17 d in vitro, when 40–60% of total protein was in the axons.

The 68,000-mol-wt NF subunit is present in both axons and CBM in roughly equal amounts. The 145,000- and 200,000-mol-wt NF subunits each consist of several variants which differ in phosphorylation state; poorly and nonphosphorylated species are present only in the CBM, whereas more heavily phosphorylated forms are present in axons and, to a lesser extent, the CBM. One 145,000-mol-wt NF variant was axon specific.

Tubulin is roughly equally distributed between CBM and axon-like neurites of explant cultures. MAP-1a, MAP-1b, MAP-3, and the 60,000-mol-wt MAP are also present in the CBM and axon-like neurites and show distribution patterns similar to that of tubulin. In contrast, MAP-2 was detected only in the CBM, while tau and the 210,000-mol-wt MAP were greatly enriched in axons compared to the CBM. In immunostaining analyses, MAP-2 localized to cell bodies and dendrite-like neurites, but not to axon-like neurites, whereas antibodies to tubulin and MAP-1b localized to all regions of the neurons.

The regional differences in composition of the neuronal cytoskeleton presumably generate corresponding differences in its structure, which may, in turn, contribute to the morphological differences between axons and dendrites.

NEURONS have very elaborate morphologies that are characterized by axonal and dendritic neurites. These highly anisotropic processes can be distinguished from each other on the basis of several morphological criteria (3, 51). For example, axons have lengths usually measured in mm to cm or more and maintain a relatively uniform diameter along their length, whereas dendrites are usually much shorter than axons, having lengths ≪1 mm, and taper as they extend away from the cell body. Axons and dendrites also generate very distinctive types of branching patterns (28). The morphological differences between axons and dendrites contribute to their unique physiological properties. Many of the morphological parameters that distinguish axons from dendrites can be generated by neurons grown in tissue culture (2, 3), suggesting that these features are endogenously determined. We have been studying the cellular basis for the differences between axonal and dendritic shape, with the long-term goal of defining the nature of endogenous determinants of neuronal morphology.

The regional differences in external shapes of individual neurons can be attributed to underlying differences in the cytoskeletons that provide their structural framework (9, 23, 62). For example, in many neurons, neurofilaments are abundant in axons but often sparse or absent in dendrites (51, 62). Microtubules in axons are generally organized into small tightly packed clusters that are interspersed within the neurofilament array (56), while microtubules are uniformly distributed in dendrites (62). Immunolocalization studies have revealed that the microtubule-associated protein (MAP)[1] designated MAP-2, is a component of dendritic microtubules, but not of axonal microtubules (20–22, 30), while the MAP designated tau is enriched in axons relative to the rest of the neuron (reference 5; see also Results). To obtain a more

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[1] *Abbreviations used in this paper:* CBM, cell body mass; MAP, microtubule-associated proteins; NFP, neurofilament proteins; NF68, NF145, and NF200, protein subunits of neurofilaments with apparent molecular weights of 68,000, 145,000, and 200,000; 1-D, one-dimensional SDS; 2-D, two-dimensional isoelectric focusing × SDS.

comprehensive understanding of the regional differentiation of the neuronal cytoskeleton, we have developed explant cultures of sympathetic neurons (24) for direct biochemical and immunochemical analyses of morphologically distinct neuronal domains.

Sympathetic neurons elaborate axon-like and dendrite-like neurites in culture (36, 37, 61). The axons attain lengths of many millimeters and maintain a relatively uniform diameter along their length, while the dendrites are relatively short, tapering processes with lengths of ~ 50 μm. In explant cultures, dendrites remain confined to the ganglionic explant or cell body mass (CBM), while axons extend beyond the CBM for many millimeters (see Fig. 1). Pure axons can be separated from cell bodies + dendrites by microsurgically removing the CBM from cultures with a scalpel blade (see Fig. 2), and the resulting preparations can then be assayed for cytoskeletal components. The present studies have focused primarily on the proteins of microtubules (tubulin and MAPs) and neuro-
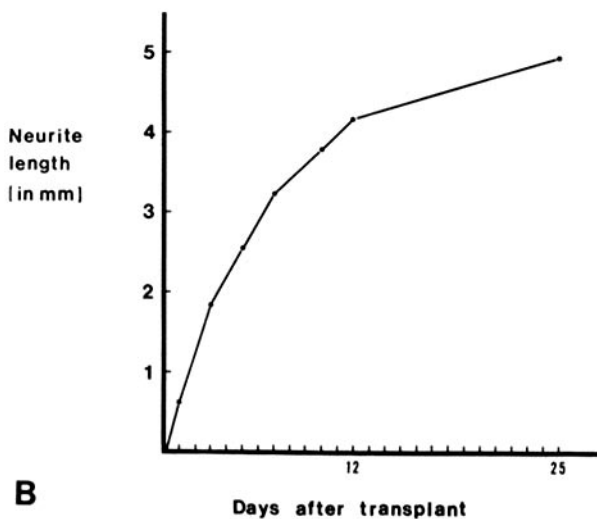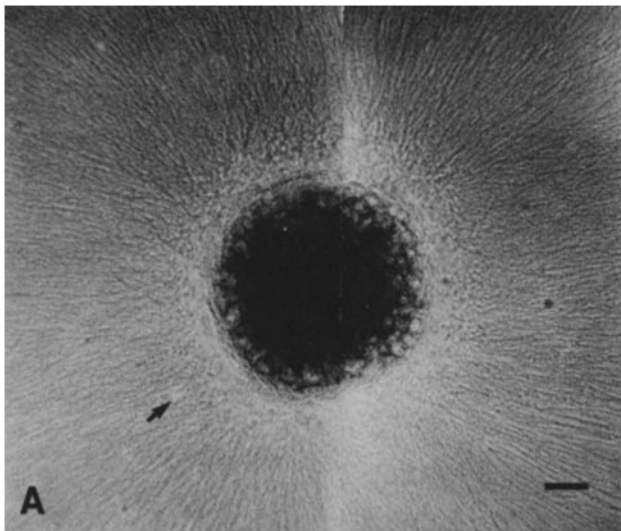

Figure 1. (A) Phase-contrast micrograph of an explant culture of sympathetic neurons. The arrow identifies the only nonneuronal cell detectable in the culture. Bar, 100 μm. (B) The lengths of the neurite outgrowth from the edge of the CBM are graphed as a function of time after transplantation. Each point is the average of at least four determinations.
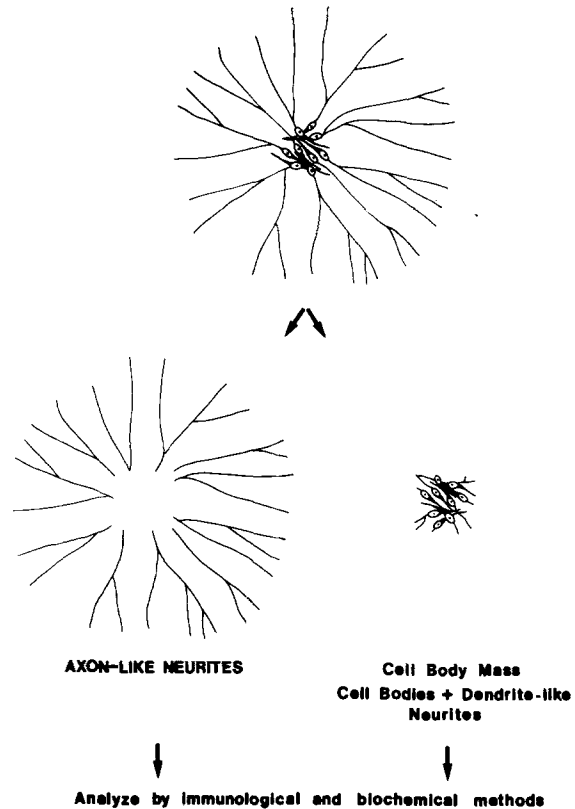

AXON-LIKE NEURITES

Cell Body Mass
Cell Bodies + Dendrite-like Neurites

Analyze by immunological and biochemical methods

Figure 2. Diagramatic illustration of how explant cultures are used to study the distribution of cytoskeletal proteins in cultured sympathetic neurons. Explant cultures consist of a centrally situated CBM, containing cell bodies and dendrites, from which extends the axons. Axons are separated from cell bodies and dendrites by cutting the CBM away from the cultures, and the resulting preparations are then assayed for cytoskeletal proteins.

filament proteins (NFPs) because the patterns of microtubule and neurofilament organization are the most striking structural differences between the axonal and dendritic cytoskeletons and are thus likely to contribute to the morphological differences between these neurites. Preliminary accounts of this work have been published previously (15, 48, 50).

Materials and Methods

Cell Culture

Dissociated sympathetic neurons prepared as described previously (49) were plated onto glass coverslips coated with polylysine or polylysine + collagen or laminin (Bethesda Research Laboratories, Gaithersburg, MD). To facilitate neurite growth on polylysine, cells were cultured in medium conditioned by mixed cultures of neurons, fibroblasts, and Schwann cells.

The procedures for setting up explant cultures were modified from Estridge and Bunge (24). Superior cervical ganglia from neonatal rat pups were stripped of their connective tissue sheaths and cut into 3–4 pieces of ~0.5 mm in diameter. Six to seven pieces were plated onto 35-mm collagen-coated culture dishes in N2 medium (43) supplemented with 1% human placental serum, 0.2 μg/ml triiodotyrosine, and 10 μM each of fluorodeoxyuridine and uridine (medium 1). On the day after plating, the culture medium was replaced with medium 2 (medium 1 with 10 μM cytosione arabinoside and without serum). Cultures were then fed with medium 3 (medium 2 without cytosine arabinoside) 2–5 d later.

The ganglionic explant or cell body mass (CBM) begins extending neurites within 12 h of plating, and after several days, the elongating neurites form a halo around the CBM. The neurite halos were often contaminated with neuron cell bodies which detached from the CBM during the first day in culture and also with nonneuronal cells. To obtain explant cultures in which neurite halos

were free of contaminating cells, CBMs were dissected out of cultures 5 d after the initial plating and replated on fresh dishes in medium 1. Beginning on the day after re-plating, cultures were fed on alternate days with fresh medium 3. Cultures were maintained for 12 d prior to use. These procedures reproducibly (≥90%) yielded explant cultures with extensive neurite halos that were free of detectable neuron cell bodies and nonneuronal cells (Fig. 1 A). Only such cultures were used in the experiments reported here.

Metabolic Labeling

Neurite proteins are synthesized in the neuron cell bodies and then transported into the neurites (16, 38). To ensure uniform labeling of the cell bodies and their neurites, cultures were labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) continuously for 5 to 12 d prior to use. During the labeling period, cultures were fed every third day with medium 3 containing 13% of the normal level of methionine plus 5–20 µCi of [^{35}S]methionine per ml. On nonfeeding days, cultures were supplemented with 5–20 µCi of [^{35}S]methionine in 100 µl of complete medium. In some experiments, cultures were labeled with 1 mCi/ml of [^{32}P]PO$_4$ for 60–90 min in phosphate-free Dulbecco's modified Eagle's medium supplemented with nerve growth factor, and then rinsed with Tris-buffered saline prior to analysis.

Identification of MAPs and Neurofilament Proteins (NFPs) in Neurites and CBM

The first step in these analyses involved dissecting the CBM away from the neurites under a dissecting microscope. The resulting CBM and neurite preparations were then assayed for MAPs and NFPs by immunoblotting, immunoprecipitation, taxol co-assembly, and/or two-dimensional isoelectric focusing × SDS (2-D) gel electrophoresis. The basic design of the experiments is illustrated schematically in Fig. 2.

Immunoprecipitation and immunoblotting procedures were as described previously (49). In most immunoblotting experiments, CBM from several cultures were pooled as were the corresponding neurite preparations. Pooled samples were then divided into several aliquots which were assayed for microtubule proteins or NFPs by immunoblotting. The following antibodies were used in the present study: monoclonal antibodies against brain MAP-2 (AP14) (6) and tau (5), monoclonal antibodies against α- and β-tubulin (Amersham Corp.), monoclonal antibodies against phosphorylated and nonphosphorylated forms of the 145,000- and 200,000-mol-wt NFPs (Sternberger-Meyer Immunocytochemicals, Jarrettsville, MD), monoclonal antibodies against brain MAP-1a (12) and MAP-1b (13) (provided by Dr. Richard Vallee), a monoclonal antibody against brain MAP-3 (31) (provided by Dr. Andrew Matus), and monoclonal antibodies against the 68,000- and 145,000- (recognizing all phosphorylated variants) mol-wt NFPs (provided by Dr. Virginia Lee).

CBM and neurites were also assayed for MAPs by co-assembly with brain microtubule proteins in the presence of taxol as described previously (49), but with the following modifications. CBM and neurites were lysed in cold (0–4°C) reassembly buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, 1 mM MgSO$_4$, 1 mM GTP, and protease inhibitors [see below]) containing 0.1% Triton X-100. The lysed samples were centrifuged in a Beckman Type 65 rotor (Beckman Instruments Inc., Palo Alto, CA) at 50,000 rpm (180,000 g) for 90 min at 4°C. The supernates from the samples were mixed with a high speed supernate from brain (prepared in the above described buffer, except without Triton X-100), adjusted to 20 µM taxol, incubated at 33°C for 30 min, and the resulting microtubules were harvested by centrifugation. MAPs were extracted from the taxol-microtubules by incubation with reassembly buffer containing 20 µM taxol, 0.75 M NaCl, and 10 mM 2-mercaptoethanol, incubated in a boiling water bath for 5 min, and then centrifuged at 150,000 g for 15 min to obtain heat-soluble material, which contained thermostable MAPs.

Immunofluorescence Staining of Dissociated Cultures

For most experiments, cultures were fixed by immersion in either cold methanol or cold acetone for 6–10 min and then rinsed with phosphate-buffered saline (PBS). Rinsed coverslips were incubated with 10% normal goat serum + 1% bovine serum albumin (BSA) (blocking solution) for 10–15 min and then incubated with primary antibody for 45–60 min at 35°C. After extensive rinsing with PBS, the cover slips were then incubated with blocking solution for 10–15 min followed by rhodamine-labeled goat anti-mouse IgG (affinity purified, Cappel Laboratories, Cochranville, PA) in blocking solution for 45–60 min at 35°C. The cover slips were then rinsed with PBS, mounted on glass slides, and viewed with a Zeiss inverted microscope using epifluorescence optics.

In some experiments, cultures were fixed for 10 min in a buffer containing 80 mM Pipes, pH 6.9, 1 mM MgSO$_4$, 10 mM EGTA, 0.3% glutaraldehyde, and 0.1% of the nonionic detergent Empigen BB. The coverslips were rinsed

with PBS containing 10 mg/ml Na borohydride, and then processed for MAP-2 and tubulin localization as described above.

Polyacrylamide Gel Electrophoresis and Peptide Mapping

One-dimensional SDS (1-D), and 2-D gel electrophoresis and one-dimensional peptide mapping were as described previously (7), with the following modifications. The electrode buffer for 1-D gels consisted of 0.394 M glycine, 0.05 M Tris, and 0.1% SDS. Also, samples for 1-D gels were dissolved in BUST (5% 2-mercaptoethanol, 8 M Urea, 2% SDS, 0.1 M Tris, pH 6.8) by incubation at room temperature. BUST as well as buffers used in taxol assembly assays also contained a cocktail of protease inhibitors which included 10 µg/ml each of leupeptin, chymostatin, antipain, 0.2–1 trypsin inhibitory units/ml of approtinin, and 0.1 mg/ml of n-tosyl-L-arginine methyl ester. Labeled proteins were visualized by fluorography (14).

Results

Explant Cultures: General Features

Typical explant cultures (Fig. 1 A) consist of a centrally situated CBM of ~0.5 mm in diameter that contains thousands of neurons; from this CBM a dense halo of neurites extends. The neurite halo has few or no nonneuronal cells as determined by light (Fig. 1 A) and electron (7, 24) microscopy. The CBM also has few nonneuronal cells as determined by trypsinizing CBM, plating out the resulting cell suspension, and then scoring for neuronal and nonneuronal cells 6 h after plating; 91% (n = 2) of the cells in the CBM were neurons.

Fig. 1 B shows the extent of neurite outgrowth from explant cultures expressed as a function of time after transplantation. Neurite length increased almost linearly at a rate of 0.35 mm/d for ~12 d, after which the rate declined (see also reference 1). In the experiments reported here, explant cultures were used at 12 d after transplantation. At this time, 40–60% (n = 3) of the total protein in the culture was present in the neurites. Thus, the distribution of individual proteins between the CBM and axon-like neurites can be evaluated without appreciable bias resulting from an uneven distribution of total protein between CBM and axonal preparations.

Dendrite-like Neurites Remain Confined to the CBM

Dendrites of cultured sympathetic neurons are relatively short (36, 37, 61), and are expected to remain confined to the CBM. To test this prediction, we determined the distribution of MAP-2, a cell body and dendrite-specific marker (20–22, 30), in explant culture. CBM and neurite halos from cultures labeled for 5 d were assayed for MAP-2 by immunoprecipitation. Fig. 3 shows 1-D gel profiles of total labeled material in CBM and neurites and also the resulting immunoprecipitates. Total protein (as methanol-precipitable radioactivity) and many individual proteins, including tubulin, were roughly equally distributed between CBM and neurites (Fig. 3). In contrast, the vast majority of labeled MAP-2 was present in the CBM, with only trace amounts detectable in neurites. Comparable results were also obtained with two other antibodies to brain MAP-2 (not shown). Quantification showed that 93–98% (n = 4) of the immunoprecipitated methionine-labeled MAP-2 was present in the CBM. These results demonstrate that cell bodies and dendrites remained confined to the CBM, and, that by removing the CBM from explant cultures, cell bodies and dendrites can be effectively separated from axons (Fig. 2).
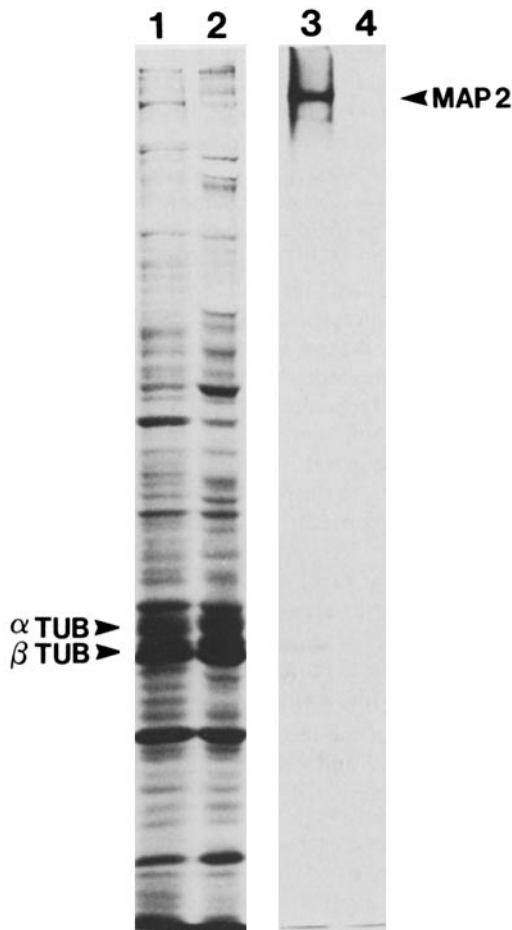
Figure 3. MAP-2 distribution in explant cultures. CBM and axons from cultures labeled for 5 d were dissolved in 1% SDS and then assayed for MAP-2 by immunoprecipitation. Lanes 1 and 2 show profiles of total labeled proteins in CBM and axons, respectively. Lanes 3 and 4 show immunoprecipitates from CBM and axons, respectively. α- and β-Tubulin (αTub and βTub, respectively) and MAP-2 are indicated. Note that the tubulins were relatively evenly distributed between CBM and axons, whereas MAP-2 was only detected in the CBM.

NFPs of CBM and Axon-like Neurites

In the present studies, axons and CBM were assayed for NFPs by immunoblotting and 2-D gels. Fig. 4 shows typical immunoblots obtained with antibodies against protein subunits of neurofilaments with apparent molecular weights of 68,000, 145,000, and 200,000, designated NF68, NF145, and NF200, respectively. NF68, which is the principal core protein of neurofilaments (41), is present in both the CBM and axon-like neurites in roughly equal amounts (Fig. 4A). NF145 and NF200 were also present in CBM and axons, but in antigenically distinct forms (Fig. 4, b and c). NF145 and NF200 each consist of several variants that differ in phosphorylation state and that can be distinguished from each other with appropriate antibodies and on 2-D gels (8, 34, 59). Antibodies against poorly or nonphosphorylated forms of NF145 and NF200 localize exclusively to the CBM of explant cultures, while antibodies to more heavily phosphorylated forms of these proteins localize to axons, and, to a lesser extent, the CBM (Fig. 4, b and c).
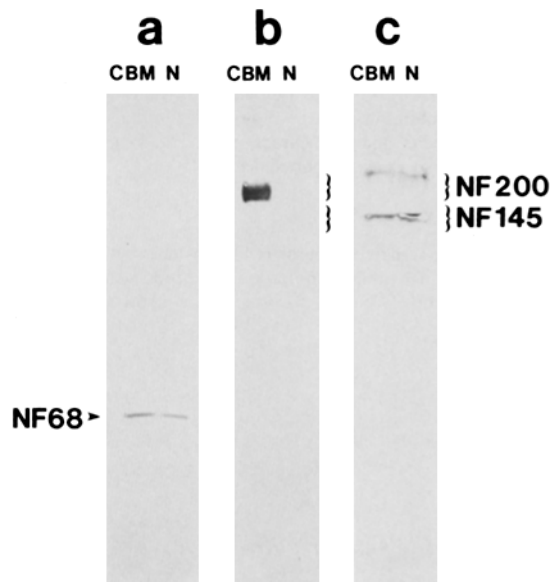

Figure 4. Distribution of NFPs in explant cultures. Immunoblots of CBM and axons obtained with monoclonal antibodies to NF68 (a), non- or poorly phosphorylated NF145 + NF200 (b), and heavily phosphorylated NF145 + NF200 (c). In b, trace amounts of the nonphosphorylated NF145 were detectable although they did not reproduce in the photograph.

The distribution of NF145 was analyzed in greater detail. This subunit can be resolved by 2-D gel electrophoresis into five variants that differ in apparent molecular weight and, to a lesser extent isoelectric point. One variant is nonphosphorylated (8), while the remaining four are phosphorylated. (While all four phosphorylated variants of the NF145 can be resolved in our 2-D gels, the two slowest migrating species often run together, generating an asymetrically shaped spot [see for example Fig. 5b].) Nonphosphorylated NF145 is not apparent in Fig. 5, although from other results (Figs. 4 and 6), it is only detectable in the CBM. The pattern of phosphorylated variants in the CBM is quite distinct from that in the axons (Fig. 5, compare panels a and c with b and d). In particular, one variant that is consistently detected in axons (Fig. 5d, arrowhead) has not been observed in the CBM (Fig. 5c).

Immunofluorescence Localization of NFP

Immunostaining analyses of NFP distribution in sympathetic neurons grown in dissociated cultures (Fig. 6) confirmed the results obtained with explant cultures. Antibody to NF68 stained all neurons in the cultures, localizing to the cell bodies and all of their neurites. Antibodies to non- and poorly phosphorylated NF145 + NF200 intensely stained all cell bodies and, to a lesser degree, proximal neurites; distal neurites remained unstained. In contrast, antibody to heavily phosphorylated NF145 + NF200 localized primarily to neurites, proximal as well as distal, with cell bodies staining relatively weakly.

Microtubule Proteins of CBM and Axon-like Neurites

Fig. 7 shows immunoblots of CBM and axons obtained with monoclonal antibodies against α- and β-tubulin and a variety of MAPs. α- and β-Tubulin are distributed evenly between the CBM and axons (see also Fig. 3). The α- and β-tubulins
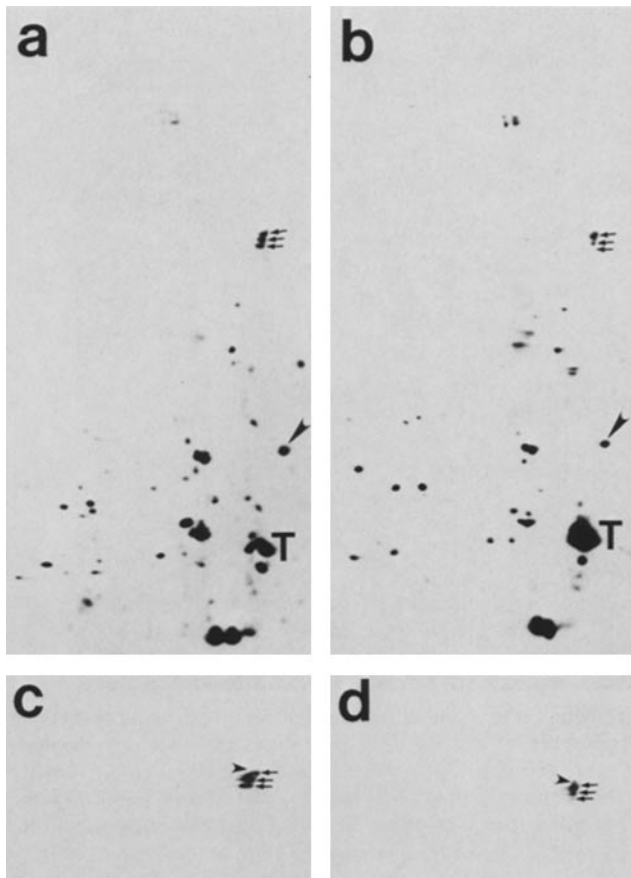
Figure 5. 2-D gel analyses of the NF68 and NF145 in CBM and axons. (*a* and *b*) CBM and axons from a culture labeled for 12 d were extracted with 0.1% Triton X-100 as described in the Materials and Methods section (see section on taxol co-assembly), and the resulting insoluble fractions, which contained the NFP (8), were analyzed on 2-D gels. Fluorographs of the labeled insoluble proteins in CBM and axons are shown in *a* and *b*, respectively. The NF68 and NF145 are indicated by arrowheads and arrows, respectively. *T*, tubulin. The identification of NFP is based on our earlier work (8). Note that NF68 in the CBM is indistinguishable from that in axons. In contrast, the subunit composition of NF145 in the CBM differs from that in axons. (*c* and *d*) CBM and axons from an explant culture were dissolved in sample buffer and analyzed by 2-D immunoblotting using an antibody against phosphorylated NF145. The resulting blots of CBM and axons are shown in *c* and *d*, respectively. The arrows identify NF145 species in CBM and axons, while the arrowhead indicates an NF145 variant that was only detected in axons.

are each composed of several isoforms (25). Two α-tubulins and two β-tubulins can be clearly resolved by 2-D electrophoresis (7, 10), and at this level of resolution, the tubulins of axons are indistinguishable from those of the CBM (data not shown).

MAP-1a, MAP-1b, and MAP-3 are all present in axons as well as the CBM, and each exhibits a pattern of distribution that is similar to that of tubulin. In contrast, MAP-2 is localized almost entirely to the CBM, while tau is greatly enriched in axons compared to the CBM. Comparable results for MAP-2 (Fig. 3) and tau were also obtained by immunoprecipitation analyses. The immunoprecipitation experiments on tau distribution used cultures labeled with [^{35}S]methionine (not shown) or [^{32}P]PO$_4$ (Fig. 8). Results obtained with both

labeling methods showed that tau was enriched in axons compared to the CBM. Note that the material in the CBM that is recognized by antibody to MAP-1a and MAP-1b differs from the corresponding material in axons. These differences are not trivial artifacts of separating CBM from axons because MAP-1a and MAP-1b from whole cultures solubilized directly in SDS + protease inhibitor–containing sample buffer resembled the sum of that seen in CBM and axons (not shown).

The distribution of some MAPs in explant cultures can also be evaluated by preparing taxol microtubules from CBM and axon preparations (see Materials and Methods). One advantage of this approach is that it is not dependent on the availability of anti-MAP antibodies. Taxol-microtubules were prepared from labeled extracts of CBM and axons that were mixed with unlabeled brain microtubule proteins, and the labeled co-assembling neuronal proteins were identified. Many proteins were present in the resulting preparations (Fig. 9, lanes 1 and 2), making identification of known MAPs difficult. To facilitate identification of MAPs, we selected for heat-stable proteins in the taxol-microtubule preparations. Several MAPs of sympathetic neurons are heat stable, including MAP-2, tau, and the 210,000-mol-wt MAP (49). As heat stability is a relatively unusual property for proteins, selecting for heat stable MAPs provides a simple way to enrich for the above mentioned MAPs. As seen in Fig. 9, lanes 3 and 4, these analyses confirmed that MAP-2 was only localized to the CBM, while tau was greatly enriched in axons compared to the CBM, and also demonstrated for the first time that the 210,000-mol-wt MAP was enriched in axons. Quantitative information on the distribution of MAP-2, tau, and the 210,000-mol-wt MAP in explant cultures was obtained by scanning fluorographs of heat-stable MAPs in CBM and axon preparations (Table I). The results fully confirmed the impressions obtained by visual inspections of the fluorographs and also the immunoblots. All of the detectable MAP-2 was present in the CBM, while 85% or more of tau and the 210,000-mol-wt MAP were present in the axons. In comparable cultures, ~60% of the tubulin (Table I) was present in the axons.

Finally, CBM and axons were assayed for the 60,000-mol-wt MAP described previously (9). The 60,000-mol-wt MAP consists of five variants that are generated by differential phosphorylation and are readily resolved in 2-D gels. All five 60,000-mol-wt MAP species are present in the CBM, but only four are present in axons (Fig. 10).

Immunofluorescence Localization of Tubulin, MAP-2, and MAP-1b

Tubulin antibodies localized to all neurons in the cultures, intensely staining cell bodies and all neurites (Fig. 11, *A*), and antibodies to MAP-1b produced similar staining patterns (Fig. 11, *E* and *F*). Antibodies to MAP-2 stained all neuron cell bodies in the cultures, but many neurites remained unstained (Fig. 11, *B*, *C* and *D*). MAP-2 staining in neurites did not extend very far away from the cell body (1.6 ± 0.2 [x ± SEM; n = 21] cell body diameters, or ~ 50 μm). These observations were obtained with two very different fixation procedures. In favorable preparations (Fig. 11, *C* and *D*), it could be seen that individual neurons gave rise to both MAP-2–positive and MAP-2–negative neurites. MAP-2–negative neurites resembled axons in that they were long, thin, nontapering, and
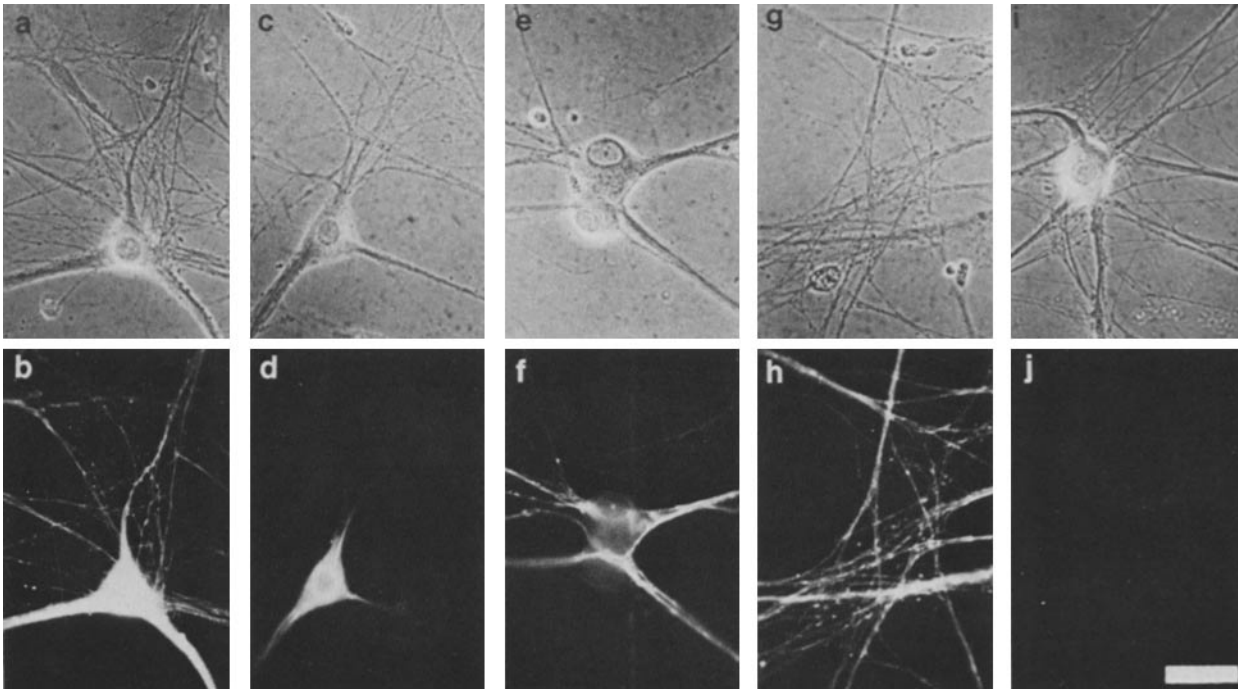
Figure 6. Immunostaining analyses of NFPs in dissociated cultures of sympathetic neurons. 7-d-old cultures grown on polylysine + collagen were fixed by immersion in cold methanol and then stained with antibodies against the various NFP. The figure shows phase and corresponding fluorescent images of neurons stained with antibodies to NF68 (*a* and *b*), non- or poorly phosphorylated NF145 + NF200 (*c* and *d*), heavily phosphorylated NF145 + NF200 (*e–h*), and no first antibody (*i* and *j*). In these images, the cell bodies are usually in a different focal plane than the neurites, and most neurites are in fascicles. Also, only some of the neurites in any given field orginate from the cell body in that field. In *e* and *f,* the cell bodies are in focus, while many of the neurites are not. *g* and *h* show a field of neurites without cell bodies. Comparable results were also obtained with cultures fixed with 3.5% paraformaldehyde in PBS followed by permeabilization with 0.1% Triton X-100 in PBS, or by immersion in cold acetone. Bar, 40 μm.

frequently had varicosities, while MAP-2–positive neurites resembled dendrites in that they were relatively broad at their base and tapered as they extended away from the cell body. Fibroblasts and Schwann cells in the cultures stained very weakly, if at all, with MAP-1b and MAP-2 antibodies (not shown).

Comparison of phase and fluorescent images of MAP-2–stained neurons suggested that MAP-2–positive neurites extended beyond the region stained by the antibody (Fig. 11, compare panels *C* and *D*). In interpreting these observations, it is relevant that axons of cultured neurons commonly arise from dendrites (3, 61), and neurites of cultured sympathetic neurons have a strong tendency to fasciculate (61). Thus, the MAP-2–negative neurites that extend from MAP-2–positive neurites may represent axons arising from dendrites and/or axons that have fasciculated with dendrites.

Discussion

Explant Cultures Provide Preparations of Pure Axons and Enriched Fractions of Cell Bodies + Dendrites

Cultured sympathetic neurons produce neurites that can be distinguished as axons or dendrites on the basis of morphological and physiological criteria (36, 37, 61; and see Fig. 11). The enormous difference between the lengths of axon-like and dendrite-like neurites (several millimeters vs ≤ 50 μm) has provided the basis for using explant cultures to physically separate axons from cell bodies and dendrites. The purity of the axon preparation has been established morphologically

(Fig. 1 and references 7 and 24) and biochemically, by showing that markers specific for cell bodies and dendrites are not detectable in axonal halos (Figs. 3 and 4). The CBM is less pure than axon preparations in that proximal axonal segments are present along with cell bodies and dendrites. In spite of this, we were able to clearly detect two MAPs (Figs. 7–9) and several other proteins (Fig. 3) that were substantially enriched in axons compared to the CBM, indicating that the amount of axonal material in the CBM is quite small relative to the axonal halo. Thus, explant cultures represent a novel system with which direct biochemical procedures can be used to identify cytoskeletal components in pure axonal preparations, and to evaluate the distribution of these components between axons and cell bodies + dendrites.

Regional Differentiation of the Neuronal Cytoskeleton

The composition of the axonal cytoskeleton differs in several respects from that of cell bodies + dendrites (see Figs. 3–11). MAP-2 is present in cell bodies and dendrites but not in axons (Figs. 3, 7, 9, and 11), while tau and the 210,000-mol-wt MAP are greatly enriched in axons compared to the rest of the neuron (Figs. 7–9). The presence of axon-enriched MAPs in the CBM is expected because these proteins are synthesized in the neuron cell bodies, which are localized entirely to the CBM, and the CBM contains proximal portions of axons. Whether these axon-enriched MAPs are also minor components of the cell body and/or dendritic cytoskeletons is not known. In this regard, immunohistochemical analyses suggest that tau is not present in dendrites (5).
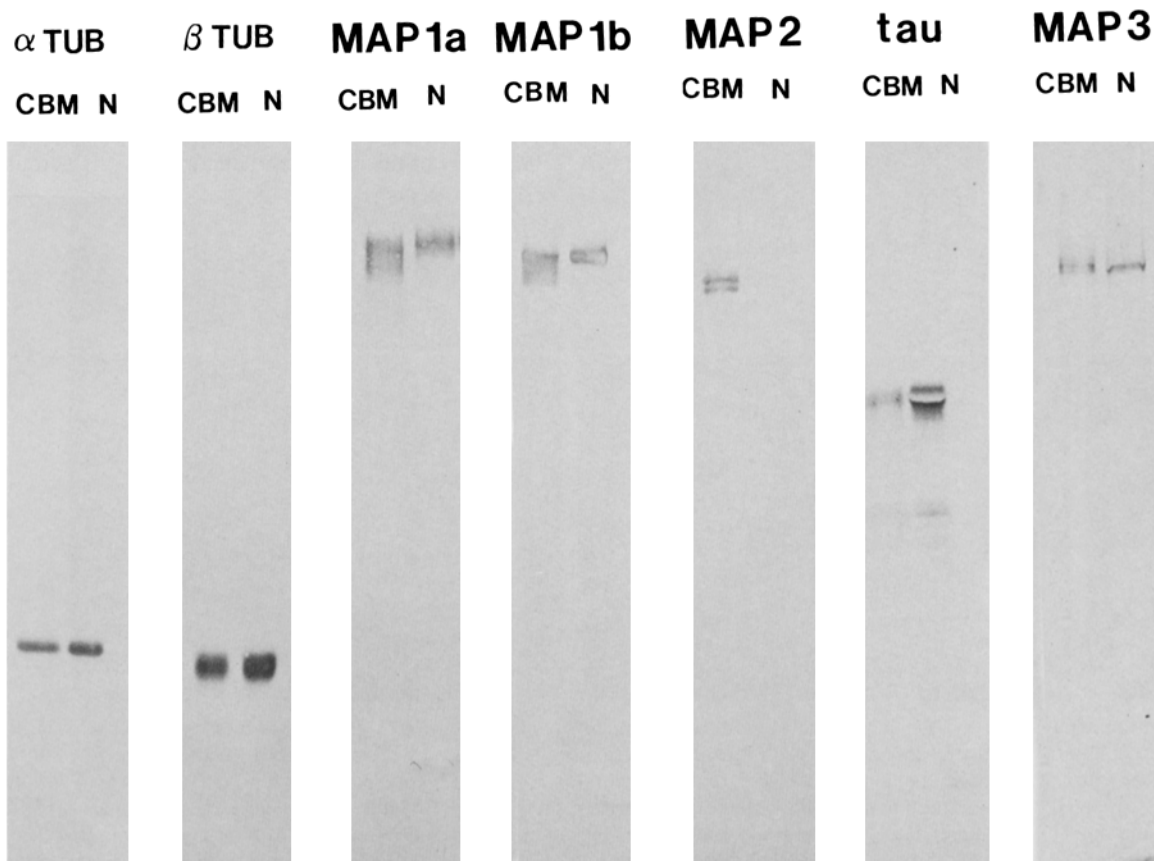
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Figure 7. The distribution of tubulin and several MAPs in explant cultures. CBM and axons (*N*) were probed for microtubule proteins by immunoblotting. The samples were analyzed on 8% gels (MAP-3), or gels consisting of gradients of 5–12.5% (α-tubulin [α-*TUB*], β-tubulin [β-*TUB*], and tau) or 4–8% (MAP-1a, MAP-1b, and MAP-2). One experiment was performed with antibody to MAP-3, while at least four experiments were performed with all other antibodies.

The cytoskeleton of axons differs in other ways from that of the cell bodies + dendrites. For example, nonphosphorylated and many of the phosphorylated variants of NF145 and NF200 compose neurofilaments in cell bodies and proximal (≤50 μm) neurites (see also reference 8), but only the more heavily phosphorylated forms are present in axons (Figs. 4–6), including one NF145 variant (in Fig. 5*d*) that was only detected in axons. Comparable observations have also been made with other neuronal systems (4, 23, 29, 59). Some MAPs also consist of variant forms which are differentially distributed between CBM and axons. For example, the 60,000-mol-wt MAP consists of five variants (9); all five are present in the CBM, but only four are detectable in the axons (Fig. 10). Also, MAP-1a and MAP-1b in the CBM differ from their counterparts in axons with respect to mobility in 1-D gels (Fig. 7), raising the possibility that MAP-1a and MAP-1b species in axons differs from those in cell bodies and dendrites.

One issue of considerable interest is whether tubulin shows regional variation in isoform composition. Brain tubulin consists of a variety isoforms, as many as 10 α-tubulins and 10 β-tubulins (25), and many of these are present in sympathetic neurons (26). This variation reflects the expression of multiple tubulin genes as well as posttranslational modifications (15). Two α-tubulins and two β-tubulins were identified in axons and the CBM, and at the level of resolution studied, axonal tubulins were indistinguishable from CBM tubulins. We are presently using high resolution isoelectric focusing gels (25)

to more completely compare the tubulins of axons and cell bodies + dendrites.

The differential distribution of variants of a given cytoskeletal protein between axons and CBM may reflect posttranslational modifications that are preferentially expressed in one domain of the neuron compared to others. The axon-specific variant of the NF145 is probably generated by such a mechanism because axons lack the capacity for protein synthesis (11). It is also possible that cytoskeletal proteins are modified in the cell body, with only some of the resulting variants undergoing transport into the axons.

In the present discussion, we have compared the composition of the axonal cytoskeleton with that of the cell bodies + dendrites. It is important to bear in mind that the cell body and dendrite are distinct domains of the neuron, and that the cytoskeleton of the cell body and dendrite may differ from each other as well as from that of the axon.

Consequences of Regional Differences in Composition of the Neuronal Cytoskeleton

These and other results (4, 5, 11, 12, 17, 20–23, 29–31, 59) indicate that neuronal cytoskeletal proteins can be classed as dendrite enriched, axon enriched, cell body enriched, or present in all regions of the neuron. Cytoskeletal proteins that show selective partitioning are probably specialized for either the cell body, axonal, or dendritic cytoskeletons, while those
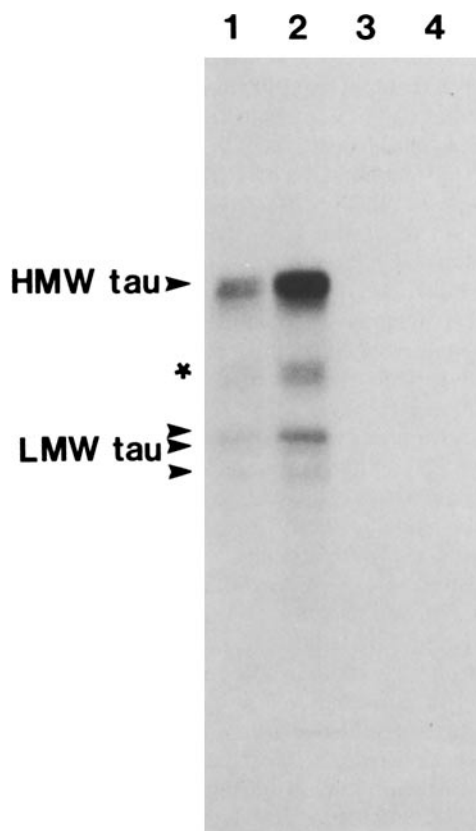
258

Figure 8. Distribution of phosphorylated tau in explant cultures. CBM and axons from explant cultures labeled with [^{32}P]PO$_4$ were dissolved in 1% SDS and then assayed for tau by immunoprecipitation. Lanes *1* and *2* show immunoprecipitates from CBM and axons, respectively. Tau species of high (100,000–110,000) and low (55,000–68,000) molecular weight (49) (*HMW tau* and *LMW tau*, respectively) are indicated. Lanes *3* and *4* are control (without first antibody) precipitates from the CBM and axons, respectively. * identifies a band of immunoreactive material that is a fragment of the higher molecular weight tau (49).

etons are synthesized primarily in the neuron cell body. Thus, segregation of these proteins occurs initially within the cell body. The nature of the segregation process is unknown, although it may be coupled to the assembly of the axonal and dendritic cytoskeletons (8, 39, 47). Implicit in this possibility is that the assembly of the axonal and dendritic cytoskeletons
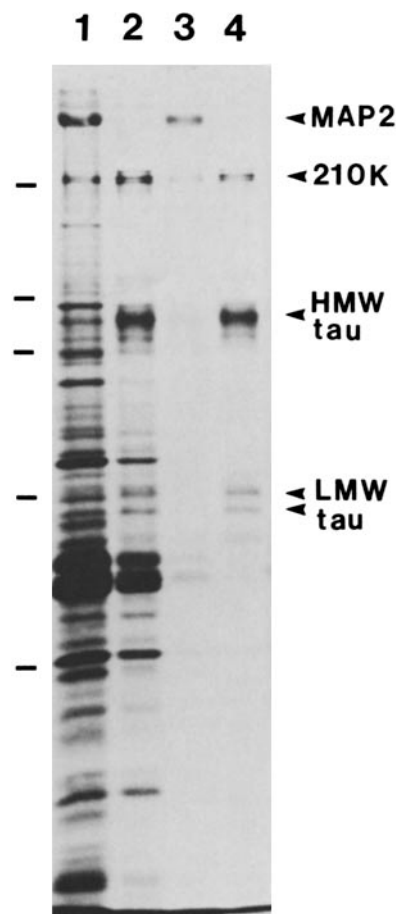

Figure 9. Identification of MAPs in CBM and axons by co-assembly with brain microtubule proteins in the presence of taxol. Soluble extracts from CBM and axons of cultures labeled for 12 d were assayed for MAPs by co-assembly with unlabeled brain microtubule protein in the presence of taxol (see Materials and Methods). MAPs were released from the taxol-microtubules by salt extraction and then incubated in a boiling water bath to prepare heat-stable MAPs. Lanes *1* and *2*, salt extract from taxol-microtubules of CBM and axons, respectively. Lanes *3* and *4*, heat-stable MAPs of CBM and axons, respectively. MAP-2, the 210,000-mol-wt MAP (*210K*) and high and low molecular weight taus (*HMW tau* and *LMW tau*) are indicated. The positions of molecular weight standards are indicated on the left: these are, from top to bottom, 200,000, 116,000, 94,000, 68,000, and 43,000. Control experiments in which soluble and insoluble extracts were analyzed by 2-D gels indicated that most of the labeled tubulin in the CBM was solubilized, but a majority of the labeled tubulin in the neurites was not solubilized. The basis for this difference in tubulin solubility in CBM and axons is being studied. Other experiments in which soluble and insoluble extracts were probed for MAP-1a, MAP-1b, and tau by immunoblotting established that the vast majority of tau in the CBM and axon-like neurites was solubilized (not shown). However, most of the MAP-1a and MAP-1b in both CBM and axons remained insoluble and thus was not available for co-assembly onto microtubules.

present throughout the neuron probably have more generalized functions with respect to these cytoskeletons.

It is likely that the regional differences in composition of the neuronal cytoskeleton generate corresponding differences in its structure. For example, the spacing between microtubules in axons differs dramatically from that in dendrites (reference 62 and see introduction). MAPs influence the spacing between microtubules (19, 35, 58, 60) and also cross-link microtubules with other cytoskeletal structures in vitro (27, 35, 40, 44, 45, 55). Moreover, the various MAPs appear to differ with respect to these parameters. For example, MAP-2, but not tau, is able to cross-link microtubules with actin filaments (52) and neurofilaments (54). MAP-2 and tau also differ in the extent to which they extend away from the microtubule wall (63). These considerations suggest that the differences in the complement of MAPs in axons and dendrites represent the molecular basis for the differences in microtubule organization seen in these neurites.

Differentiation of the Neuronal Cytoskeleton and Neuronal Form

The proteins comprising the axonal and dendritic cytoskel-

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occurs at distinct locations in the neuron cell body, and that the composition of the axonal and dendritic cytoskeletons is specified at these locations. Enzymes involved in the post-translational processing of cytoskeletal components may also become associated with the axonal or dendritic cytoskeletons at these assembly sites. Regardless of the specific mechanism(s), this segregation process represents a key event in the differentiation of the axonal and dendritic cytoskeletons.

Table I. Distribution of MAPs between CBM and Axons

MAP	Exp.	% in CBM	% in axons
MAP-2	1	100	0
	2	100	0
210,000-mol-wt MAP	1	11	89
	2	16	84
Tau	1	7	93
	2	17	83
Tubulin*	1	41	59
	2	41	59

These results are based on analyses of MAP distribution beween CBM and axons as determined with the taxol co-assembly assay (see Materials and Methods). 1-D gel profiles of the heat-stable fractions of taxol-microtubules from CBM and axons were scanned with a laser densitometer. The area under the peaks corresponding to the MAPs was quantified using a digitizer, and the distribution of the various MAPs between CBM and axons was calculated as follows:

$$\frac{\text{area of MAP peak in CBM or axons}}{\text{area of MAP peak in CBM + axons}} \times 100.$$

The results from two separate experiments (*Exp.*) are shown. The cultures were labeled for 11 d (*Exp. 2*) or 12 d (*Exp. 1*). The results of experiment 1 were derived from lanes 3 and 4 of the fluorograph depicted in Fig. 9.

* The results for tubulin were obtained by dissolving CBM and axonal preparations from explant cultures labeled for 12 d in sample buffer, analyzing the resulting material on 2-D gels, excising the tubulin spots from the gels, and quantifying their [^{35}S]methionine content as described previously (8).

Individual neurons consist of morphologically distinct domains such as the axon and dendrites (2, 51, 62). The generation of several of the morphological features that distinguish axons from dendrites has been attributed to factors that are intrinsic to the neuron (2, 3). It has been suggested that the differences in the morphology of axons and dendrites reflect underlying differences in their cytoskeletons. If this is correct, then the segregation of axonal from dendritic cytoskeletal proteins may be a manifestation of endogenous determinants of neuronal morphology.

Although aspects of axonal and dendritic morphology are apparently endogenously specified, these neurites exhibit considerable plasticity in their external shapes in response to a variety of environmental stimuli (53). This morphological flexibility is indicative of a dynamic quality to the cytoskeletons that provide the architectural framework of these neurites. Cytoskeletal dynamics is defined by the equilibria that govern the assembly and disassembly reactions of the various cytoskeletal proteins. Posttranslational modification of cytoskeletal proteins influences these reactions in vitro (32, 33, 42, 44, 45, 52, 54, 57), and may thus constitute a mechanism for modulating them in vivo. Subtle alterations of cytoskeletal composition occur locally within axons, and possibly in dendrites, as a result of posttranslational modifications (18, 46). For example, we have identified a variant of the NF145 that is apparently generated by posttranslational processing of other NF145 variants within the axon (Fig. 5). We have also obtained direct evidence for phosphorylation of tau (Fig. 8), NF145, NF68, the 60,000-mol-wt MAP, and tubulin in axons (Black, M. M., and P. Keyser, unpublished observations). These as well as other posttranslational modifications may contribute to the dynamic qualities of the neuritic cytoskeletons that are essential for the expression of morphological plasticity.
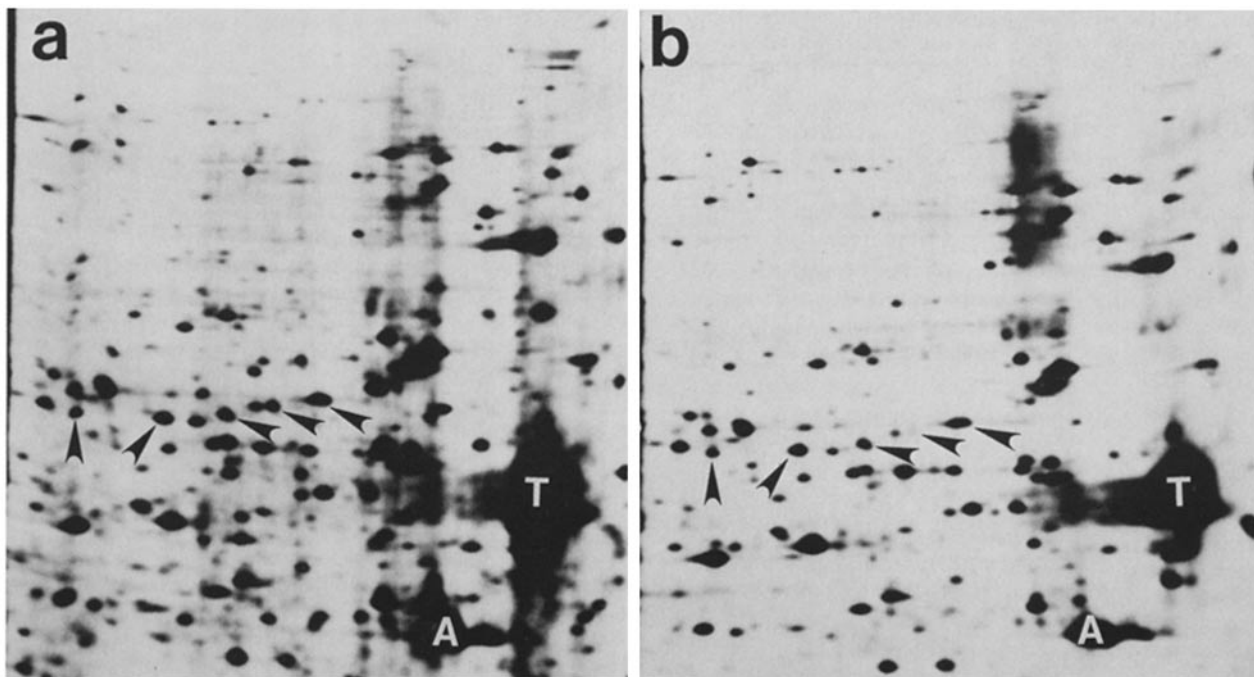

Figure 10. 2-D gel profiles of total proteins in CBM and axons. CBM and axons from a culture labeled for 5 d were dissolved in sample buffer and analyzed directly on 2-D gels. Fluorographic exposures of the CBM (*a*) and axons (*b*) are shown. The 60,000-mol-wt MAPs (arrowheads), tubulin (*T*), and actin (*A*) are indicated. The spots identified as the 60,000-mol-wt MAPs were confirmed as such in peptide mapping experiments (not shown) in which the proteins in 2-D gels of SDS extracts were compared to the bonafide MAPs obtained by a selective extraction procedure (9). These data are representative of more than six experiments.
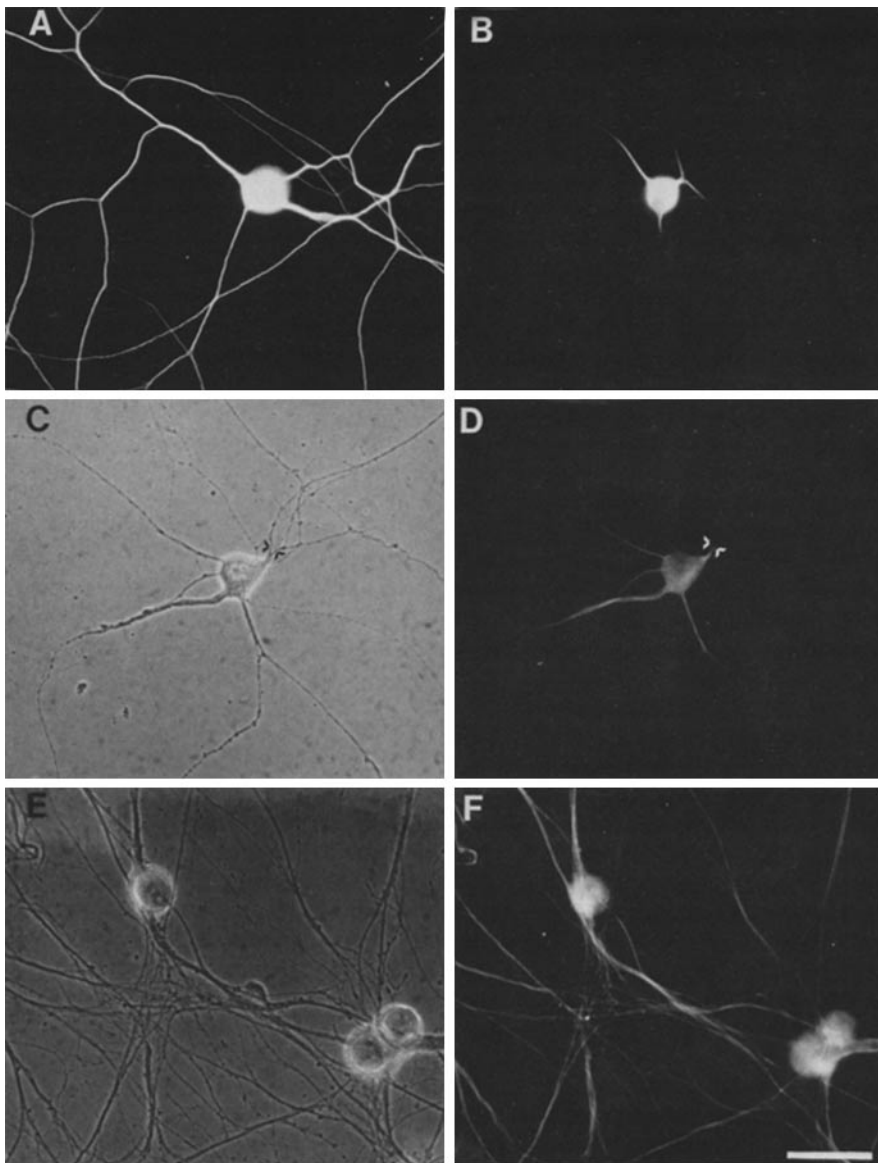
Figure 11. Immunostaining of cultured sympathetic neurons with antibodies to MAP-1b, MAP-2, and tubulin. (*A* and *B*) 1-d-old cultures were extracted and fixed with glutaraldehyde as described in the Materials and Methods section and then stained with antibodies to tubulin (*A*) or MAP-2 (*B*). The phase-contrast images of the cells were very faint and did not photograph well. However, the fields depicted in *a* and *b* had comparable neurite densities. Note that the neuron cell bodies stain with both antibodies. Also most and probably all neurites stain with antibody to tubulin. In contrast, only a few neurites stained with antibodies to MAP-2, and these were generally short relative to most neurites in the cultures. (*C* and *D*) Phase-contrast (*c*) and immunofluorescent (*d*) images of a 2-d-old culture stained with antibody to MAP-2. While some of the neurites elaborated by the neuron depicted in *c* and *d* stained with the antibody, others did not (>). (*e* and *f*) Phase-contrast (*e*) and immunofluorescent (*f*) images of a 7-d-old culture stained with antibody to MAP-1b. All cell bodies and most neurites stain with this antibody. Bar, 40 μm.

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