Selective Stabilization of Tau in Axons and Microtubule-associated Protein 2C in Cell Bodies and Dendrites Contributes to Polarized Localization of Cytoskeletal Proteins in Mature Neurons

Nobutaka Hirokawa, Takeshi Funakoshi, Reiko Sato-Harada, and Yoshimitsu Kanai

Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, Hongo, Tokyo 113, Japan

Abstract. In mature neurons, tau is abundant in axons, whereas microtubule-associated protein 2 (MAP2) and MAP2C are specifically localized in dendrites. Known mechanisms involved in the compartmentalization of these cytoskeletal proteins include the differential localization of mRNA (MAP2 mRNA in dendrites, MAP2C mRNA in cell body, and Tau mRNA in proximal axon revealed by in situ hybridization) (Garner, C.C., R.P. Tucker, and A. Matus. 1988. *Nature (Lond.).* 336: 674–677; Litman, P., J. Barg, L. Rindzooski, and I. Ginzburg. 1993. *Neuron.* 10:627–638), suppressed transit of MAP2 into axons (revealed by cDNA transfection into neurons) (Kanai, Y., and N. Hirokawa. 1995. *Neuron.* 14:421–432), and differential turnover of MAP2 in axons vs dendrites (Okabe, S., and N. Hirokawa. 1989. *Proc. Natl. Acad. Sci. USA.* 86:4127–4131). To investigate whether differential turnover of MAPs contributes to localization of other major MAPs in general, we microinjected biotinylated tau, MAP2C, or MAP2 into mature spinal cord neurons in culture (~3 wk) and then analyzed their fates by antibiotin immunocytochemistry. Initially, each was detected in axons and dendrites, although tau persisted only in axons, whereas MAP2C and MAP2 were restricted to cell bodies and dendrites. Injected MAP2C and MAP2 bound to dendritic microtubules more firmly than to microtubules in axons, while injected tau bound to axonal microtubules more firmly than to microtubules in dendrites. Thus, beyond contributions from mRNA localization and selective axonal transport, compartmentalization of each of the three major MAPs occurs through local differential turnover.

D**IFFERENT** compartmentalization of the cytoskeletal elements in nerve cells is an important feature occurring in the highly polarized structural differentiation shown by dendrites and axons of these cells (Dotti and Banker, 1987; Cleveland, 1990; Hirokawa, 1994). Among the cytoskeletal proteins showing a polarized distribution, microtubule-associated protein 2 (MAP2)[1] is specifically localized in dendrites (Bernhardt and Matus, 1984; Caceres et al., 1984; Cumming et al., 1994; De Camilli et al., 1984), while tau is abundant in axons (Binder et al., 1985; Kosik and Finch, 1987; Peng et al., 1986; Brion et al., 1988; Mandell and Banker, 1995). Furthermore, MAP2C, an isoform derived from the MAP2 gene by alternative splicing, is specifically localized in both axons and dendrites in juvenile neurons (Tucker et al., 1988a; Meichsner

et al., 1993), whereas it is no longer present in axons in mature neurons (Tucker et al., 1988b). Recent molecular biological approaches have revealed that cDNAs of MAP2 (1828 amino acids), MAP2C (467 amino acids), and tau (432 amino acids) are composed of an NH_2-terminal projection domain having different lengths, with homologous microtubule-binding domains consisting of three to four repeats of 18 amino acids being located at the COOH-terminal region (reviewed by Goedert et al., 1991). Moreover, when MAP2 and tau are expressed in nonneuronal cells, these cells grow thin processes in which microtubules (MT) form bundles (Kanai et al., 1989; Lewis et al., 1989; Knops et al., 1991). In fact, MT bundles resembling those found in dendrites and axons are formed in the thin processes induced by expression of MAP2 and tau, respectively (Knops et al., 1991; Chen et al., 1992). Our recent gene targeting study of tau protein indicated its importance in the organization and stabilization of MTs in small caliber axons and also suggested that several MAPs may play a group role in affecting their functions (Harada et al., 1994). Hence, the polarized distribution of MAPs may have an important relationship with neuronal morphogen-

---

Address all correspondence to Nobutaka Hirokawa, Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 Japan. Tel: 81-3-3812-2111 ext. 3326. Fax: 81-3-5689-4856.

---

1. *Abbreviations used in this paper:* MAP, microtubule-associated protein; MT, microtubule; RT, room temperature.

esis. Concurrently, the clarification of the mechanisms of this polarized distribution of MAP2, MAP2C, and tau is of major importance, since they may not only be involved with neurons in general, but also with other cells that compartmentalize cytoskeletal proteins.

In terms of the mechanism for this compartmentalization of MAPs, evidence has been presented for three mechanisms (see Fig. 1). The first is differential localization of mRNA. In situ hybridization studies have indicated that MAP2 and tau mRNAs extend into dendrites and axons, respectively, while MAP2C mRNA is confined to cell bodies (Garner et al., 1988; Litman et al., 1993). These studies have proposed that differential localization of mRNA results in the final localization of MAPs.

The second mechanism demonstrated is differential turnover of MAP2 in the axon vs in the dendrites. This possibility arose from the microinjection of biotin-labeled MAP2 into cultured mature spinal cord neurons and subsequent analysis of the localization of injected MAP2 in the axon and dendrites (Okabe and Hirokawa, 1989). This study indicated that injected MAP2 is conveyed to both dendrites and the axon after 1 d, but MAP2 in the axon turned over quickly and disappeared ∼3–4 d after microinjection, while MAP2 is stabilized in dendrites ∼3–4 d after microinjection.

The third mechanism is the suppressed transit into axons. This was shown for MAP2 by transfection of a cDNA encoding it into cultured spinal cord neurons (Kanai and Hirokawa, 1995). Although tau and MAP2C were transported into the axon, MAP2 was not.

Although all three mechanisms are apparently used to localize MAP2, the relative contribution of each for two of the known MAPs, tau and MAP2C, is lacking. No earlier efforts have examined the potential that differential turnover of these in axons and dendrites may contribute to compartmentalization. In the case of MAP2C, mRNA is confined to the cell body (Garner et al., 1988) and despite its transport of the protein into the axon (Kanai and Hirokawa, 1995), MAP2C is compartmentalized in cell bodies and dendrites in matured neurons (Tucker et al., 1988b). This strongly suggests that compartmentalization of MAP2C requires differential stabilization in the cell body and dendrites.

To test this and to extend the analysis of differential stability to tau, we have microinjected biotin-labeled tau, biotin-MAP2C, and biotin-MAP2 into the mature spinal cord neurons, and subsequent analyses of the fates of injected MAPs at light microscopic and electron microscopic levels were performed.

Materials and Methods

Preparation of Biotin-labeled Proteins

Porcine brain MTs were prepared by temperature-dependent assembly and disassembly (Shelanski et al., 1973). MT proteins were conjugated with N-hydroxy-succinimidyl biotin (Kristofferson et al., 1986; Okabe and Hirokawa, 1989). Briefly, we heated 500 mg of MT proteins in 100 ml of assembly buffer (25% (vol/vol) glycerol, 100 mM Pipes, 1 mM MgCl$_2$, 1 mM EGTA, pH 6.8) containing 1 mM ATP and 10 mg/ml leupeptin at 37°C for 5 min and then added 100 mg of N-hydroxy-succinimidyl biotin. The biotin labeling was allowed to proceed for 15 min, after which the reaction was quenched by adding 1 g of potassium glutamate. The labeled MTs

were further purified by two assembly–disassembly cycles. Biotin-tau and -MAP2 were separated from MTs by a method described previously (Hirokawa et al., 1988a, b). MAP2C was purified from porcine brain according to Takeuchi et al. (1992). Purified MAP2C was incubated with tubulin purified from porcine brains by phosphocellulose column chromatography in one-third vol glycerol in PEM (0.1 M Pipes, 1 mM EGTA, 1 mM MgCl$_2$, pH 6.8) at 37°C for 30 min. The resultant MT proteins were labeled with N-hydroxy-succinimidyl biotin as described above. After the reaction was quenched by K-glutamate, the MT proteins were centrifuged at 38,000 rpm (L8-8M Ultracentrifuge; Beckman Instruments, Inc., Fullerton, CA) at 35°C for 30 min. The pellets were suspended in 1 M NaCl–PEM at 4°C for 40 min, which was then applied to a PD10 column (Pharmacia Biotech AB, Uppsala, Sweden) to remove tubulin. The flow-through was collected and centrifuged at 2°C for 10 min. After adding 2 mM DTT, the supernatant was boiled for 5 min, immediately cooled on ice for 30 min, and then brought to 50% saturation in ammonium sulfate by the addition of saturated ammonium sulfate. The sample was stirred for 30 min at 4°C and centrifuged at 12,000 g for 20 min at 4°C to precipitate biotin-labeled MAP2C. The precipitate was suspended in PEM buffer containing 0.75 M NaCl and then dialyzed against PEM.

Binding of Biotin-labeled Proteins to MT

Tau, biotin-tau, MAP2, biotin-MAP2, MAP2C, biotin-MAP2C, and tubulin in PEM buffer were centrifuged (TL 100; Beckman Instruments, Inc.) at 55,000 rpm for 30 min at 2°C. The supernatants were used for the experiments. All of these MAPs were mixed with tubulin (concentrations of MAPs and tubulin were 1.5 and 2 mg/ml, respectively) and incubated at 37°C for 30 min in the presence of 1 mM GTP. Then they were again centrifuged at 55,000 rpm for 30 min at 35°C. Supernatants and pellets were separated, and the pellets were suspended in PEM of equal volume to the supernatant. The same volume of supernatants and pellets was subsequently analyzed by SDS-PAGE.

Cell Culture and Microinjection

Spinal cord neurons of mouse embryos were cultured according to the method of Ranson et al. (1977). Mature neurons (∼3-wk culture) were used. PC12 cells were cultured and fused using polyethylene glycol as described previously (Okabe and Hirokawa, 1988). Microinjection was performed according to Graessmann et al. (1980). For microinjection, the stored sample was dialyzed and diluted with an injection buffer (50 mM potassium glutamate, 1 mM MgCl$_2$, pH 6.8) to 2–2.3 mg/ml. Cells were placed on a Diaphot inverted microscope (Nikon Inc., Garden City, NY) and then pressure injected using a micromanipulator (Narishige Scientific Laboratory, Tokyo, Japan). During the injection procedure, the cells were videotaped with a time-lapse videotape recorder to determine the exact incubation time after injection. The volume injected per cell was estimated by the method of Graessmann et al. (1980). Under a constant flow rate of the sample solution, we recorded the meniscus movement within the pipette and determined the sample solution flow rate. Using this flow rate and the mean injection time per cell, the volume injected per cell was estimated at <10^{-10} ml. Because the cell body of the smallest cell used was ∼30 mm in diameter and mostly hemispheric in shape, and because the height of the cell body was ∼5 mm, the calculated volume of this cell body was ∼2.4 × 10^{-9} ml. From these data, the injected volume was estimated at <4.2% of the cell body volume.

Immunofluorescence Localization and Immunoblotting of Native Tau, MAP2, and MAP2C in Spinal Cord Neurons in Culture

Cultured neurons (∼3-wk culture) were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PEM buffer for 30 min at room temperature (RT). After washing with PBS, they were permeabilized by immersion in 100% methanol at −20°C for 6 min. Next, they were washed with PBS and incubated with 5% skim milk in PBS containing 10% normal goat serum for 30 min at 35°C. As the first antibodies, we used 100-fold diluted affinity-purified anti–MAP2 rabbit IgG (Hirokawa et al., 1988b) and 50-fold diluted anti–tau mouse mAb Tau-1 (Binder et al., 1985). In some cases, anti–MAP2 mouse mAb (HM-2; Sigma Chemical Co., St. Louis, MO) was used as the first antibody. The cells were stained with these first antibodies for 1 h at 35°C. After thorough washing, they were stained for 1 h at 35°C with 100-fold diluted FITC-labeled anti–rabbit goat IgG or rhodamine-labeled anti–mouse goat IgG in 5% skim milk, and 10% nor-

mal goat serum in PBS for 1 h at 35°C. Control cells were incubated with normal rabbit IgG or normal mouse IgG as the first antibodies. After washing, cells were mounted and observed with a microscope (Axiophoto; Zeiss, Inc., Thornwood, NY). In the phosphatase treatment study, cells were incubated with 20 U/ml calf intestine alkaline phosphatase (TOYOBO, Tokyo, Japan) in alkali phosphatase buffer (50 mM Tris, pH 8.0) at 37°C for 2 h (Papasozomenos and Binder, 1987; Kanai et al., 1989). After washing with PBS, the cells were stained as described above.

For Western blotting, MT proteins were purified from cultured spinal cord cells (~3-wk culture) by the taxol-dependent procedure described by Vallee (1982). The cells were scraped from culture dishes after washing with PBS and then homogenized in 1.5 vol of PEM. The homogenates were centrifuged at 180,000 g for 90 min at 4°C, the supernatant was collected, and GTP and taxol were added (final 1 mM GTP, 20 mM taxol). This was followed by warming the supernatant at 37°C for 15 min to allow MT polymerization, after which they were centrifuged at 100,000 g for 40 min at 30°C. The resultant pellets (MT proteins) were separated by SDS-PAGE and transferred onto nitrocellulose sheets. The sheets were immunoblotted using anti-MAP2 mAb (HM2; Sigma Chemical Co.), which stains MAP2A, B, C, and anti-tau mAb (tau-1; Boehringer Mannheim Biochemicals, Indianapolis, IN), followed by ^{125}I goat anti–mouse IgG (ICN Pharmaceuticals, Inc., Irvine, CA).

Immunofluorescence Localization of Biotin-MAPs

Cells were fixed with 0.3% glutaraldehyde in a PEM buffer for 20 min, washed with PBS, treated with 1 mg/ml NaBH$_4$ in PBS for 30 min, and permeabilized with 1% Triton X-100 in PBS for 30 min at RT. The cells were sequentially incubated with 5% skim milk in PBS for 30 min at RT and then again with 100-times diluted rabbit antibiotin (Enzo Biochem., Inc., New York, NY) in 5% skim milk in PBS for 1 h at RT. After thorough washing, they were again sequentially incubated with 5% skim milk in PBS for 30 min at RT, followed by incubation with 100-times diluted rhodamine-conjugated goat anti–rabbit IgG (Cappel Laboratories, Cochranville, PA) in 5% skim milk in PBS for 1 h at RT. For some biotin-MAP2–injected cells, the cells were double stained with anti–biotin rabbit IgG and anti–MAP2 mouse mAbs (Sato et al., 1989) as first antibodies, and rhodamine-conjugated goat anti–rabbit IgG and FITC-conjugated goat anti–mouse IgG as second antibodies. In other cases, injected cells were permeabilized with a solution containing 30% glycerol, 80 mM Pipes, pH 6.8, 10 mM MgCl$_2$, 1 mM EGTA, 2.5 mM GTP, and 1% Triton X-100 for 2 min, and fixed with 5 mM ethylene glycol-bis-succinimidyl succinate (Gorbsky et al., 1987) in a PEM buffer plus 1 mM GTP and 10 mM taxol for 20 min at 37°C. The cells were washed several times with PBS and quenched with 100 mM glycine–PBS for 30 min. After blocking with 5% skim milk in PBS for 1 h, they were incubated with 100-times diluted anti–biotin rabbit IgG in 5% skim milk in PBS for 1 h. After washing with PBS they were again incubated with 5% skim milk in PBS for 30 min at 35°C, followed by staining with 100-times diluted rhodamine-conjugated rabbit anti–goat IgG in 5% skim milk in PBS for 1 h at RT. For PC12 cells microinjected with biotin-MAP2C, the cells were treated as described above and stained with a mixture of anti–biotin rabbit IgG and anti–tubulin mouse IgG as the first antibodies, and rhodamine-goat anti–rabbit IgG and fluorescein goat anti–mouse IgG being used as second antibodies. Cells were examined with a Zeiss Axiophoto fluorescence microscope. The fluorescence micrographs of spinal cord neurons were taken and printed under the same conditions.

Electron Microscopy Immunocytochemistry

Spinal cord neurons and PC12 cells were microinjected with biotin-MAP2, -MAP2C, or -tau and permeabilized as described above. The cells were subsequently fixed with 0.3% glutaraldehyde in PEM buffer plus 1 mM GTP and 10 mM taxol for 30 min at RT and then processed for EM using anti-biotin antibodies and colloidal gold (5 nm)–labeled second antibodies (Janssen Pharmaceutica, Beerse, Belgium) according to Okabe and Hirokawa (1989).

Immunoblotting and Quantitation of MAP2 in Peripheral Nerve Axons

MT protein was purified from mouse sciatic nerves by the taxol-dependent procedure described by Vallee (1982). Briefly, sciatic nerves from 30 mice were homogenized in 1.5 vol of PEM buffer (100 mM PMSF, 1 mg/ml leupeptin) and centrifuged at 180,000 g for 90 min at 4°C. The superna-

tant at this stage is referred to as cytosolic extract. Taxol was added to 20 mM and GTP to 1 mM, the solution was warmed to 37°C for 30 min, and an MT pellet was formed by centrifuging at 100,000 g for 40 min at 30°C. For the quantitation of MAP2 and tubulin, MT protein was separated by SDS-PAGE and then transferred onto nitrocellulose sheets. Purified tubulin and MAP2 from porcine brain were loaded onto the same sheets as the concentration standards. The sheets were immunoblotted using anti-MAP2 polyclonal antibody (Hirokawa et al., 1988b) followed by ^{125}I protein A (ICN Pharmaceuticals, Inc.) or anti-α-tubulin mAb (DMIA; Sigma Chemical Co.), followed by ^{125}I goat anti–mouse IgG (ICN Pharmaceuticals, Inc.). The quantitation of protein concentration was performed using an image analyzer (Bas 2000; Fuji Photo Film Co., Ltd., Tokyo, Japan). Immunoblotting of MAPs in sciatic nerve cytosolic extract was similarly performed. For the first antibodies, we used anti-MAP1A mAb (Shiomura and Hirokawa, 1987a), anti-MAP2 mAb (HM2; Sigma Chemical Co.) and anti-tau mAb (tau 1; Boehringer Mannheim Biochemicals).

Axon Measurements

To measure the length of the axon where the biotin-MAPs had been conveyed after 24 h, micrographs were printed at a magnification of 213, and the length of the fluorescent axon from the leading edge of detectable fluorescence showing higher fluorescence than the background staining of axons of uninjected neurons to the cell body was measured.

Results

Biotinylated MAPs Bound to MTs In Vitro and In Vivo

To examine whether biotinylated tau, MAP2, or MAP2C binds to tubulin, we incubated native tau, native MAP2, native MAP2C, biotin-tau, -MAP2, or -MAP2C with phosphocellulose column–purified tubulin in PEM buffer containing 1 mM GTP. After incubation at 37°C for 30 min, the samples were centrifuged at 107,000 g at 35°C for 30 min. Fig. 2 *A* shows SDS gel electrophoresis patterns of biotinylated tau (Fig. 2, lane *1*), biotinylated MAP2 (Fig. 2, lane *4*), and biotinylated MAP2C (Fig. 2, lane *7*); supernatants and pellets of native tau plus tubulin (Fig. 2, lane *2*), biotinylated tau plus tubulin (Fig. 2, lane *3*), supernatants and pellets of native MAP2 plus tubulin (Fig. 2, lane *5*), and biotinylated MAP2 plus tubulin (Fig. 2, lane *6*); and supernatants and pellets of native MAP2C plus tubulin (Fig. 2, lane *8*) and biotinylated MAP2C plus tubulin (Fig. 2, lane *9*). Note that biotin-tau, -MAP2, and -MAP2C (Fig. 2, lanes *3*, *6*, and *9*) effectively bound to tubulin. When we microinjected biotin-tau, -MAP2, or -MAP2C into PC12 cells to determine if these proteins bound to MTs in vivo, it was found that they (Fig. 2, *B*, *C*, and *D*, respectively) bound to MTs that were permeabilized before fixation. These data indicate that biotin-tau, -MAP2, and -MAP2C are capable of effectively binding to tubulin both in vitro and in vivo.

Cultured Spinal Cord Neurons Develop Polarized Distribution of Tau, MAP2, and MAP2C

First we confirmed whether the cultured mature spinal cord neurons (~3 wk) compartmentalize native tau mainly to axons and MAP2 and MAP2C to dendrites. Double staining with anti-tau antibodies and anti-MAP2 antibodies indicates that anti-tau antibodies (Tau-1) mainly stained axons, whereas anti-MAP2 antibodies specifically stained dendrites. In addition, these antibodies can only stain neurons. Because tau-1 does not recognize tau proteins phosphorylated at tau-1 site, and because it was suggested that tau proteins phosphorylated at tau-1 site exist in certain
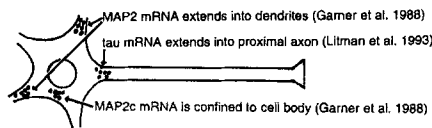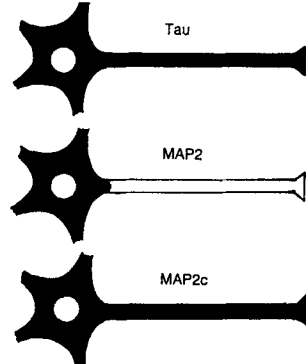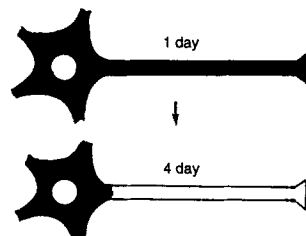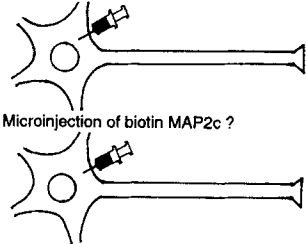
Figure 1. Possible mechanisms for the compartmentalization of MAP2, MAP2C, and tau based on previous studies and subjects of the present study.

amounts in dendrites and in cell bodies in vivo (Papasozomenos and Binder, 1987), we stained the spinal cord neurons with tau-1 antibodies after treatment with alkaline phosphatase (Papasozomenos and Binder, 1987). Tau-1 stained many axons and some dendrites and cell bodies. In terms of the dendritic staining, double staining with anti-MAP2 antibodies revealed that tau-1 staining tended to be restricted to cell bodies and some proximal dendrites, while many dendrites were not stained. Thus, our data demonstrate that mature spinal cord neurons localize tau abundantly in axons, whereas cell bodies and some proximal dendrites localize tau phosphorylated at tau-1 site in small amounts. Concerning the localization of MAP2 and MAP2C, it should be realized that antibodies that specifically stain MAP2C are not available. However, because anti-MAP2 antibodies that recognize MAP2A, MAP2B, and MAP2C (HM-2; Sigma Chemical Co.) stain only cell bodies and dendrites, MAP2C is not localized immunofluorescently in the axon of spinal cord neurons at this stage. Immunoblotting of MT proteins from spinal cord–cultured
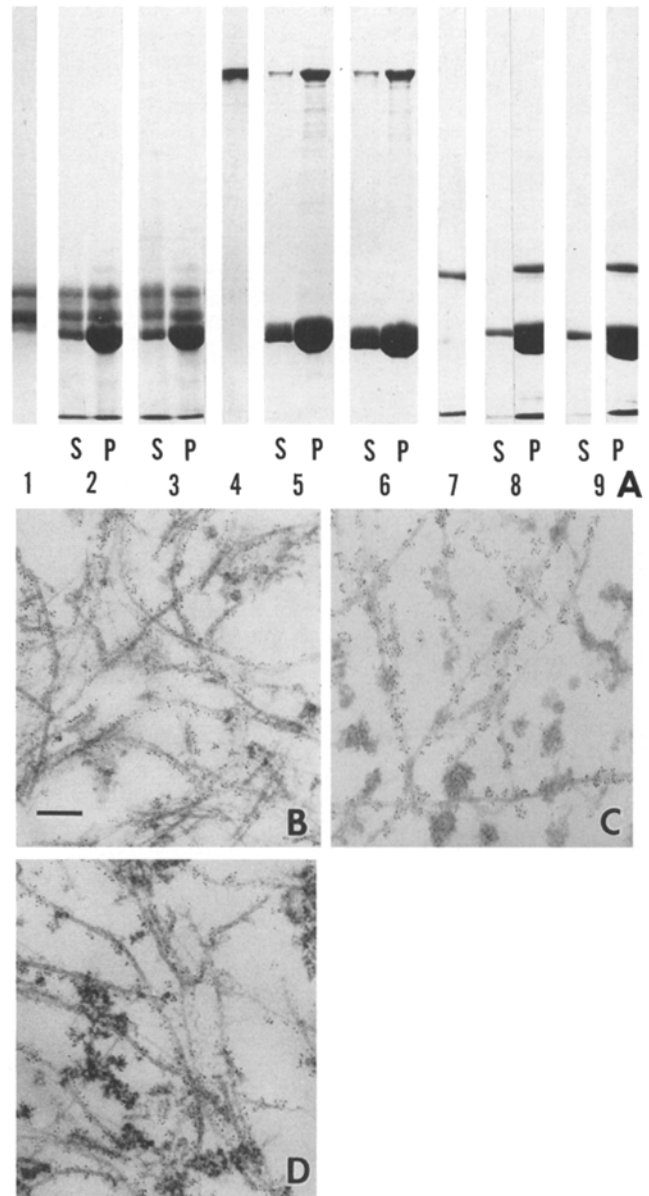
Figure 2. (A) SDS-PAGE analysis of biotinylated tau, MAP2, and MAP2C stained with Coomassie brilliant blue. (Lane 1) Biotinylated tau; (lane 2) supernatant (S) and pellet (P) of tau plus tubulin; (lane 3) supernatant (S) and pellet (P) of biotinylated tau plus tubulin; (lane 4) biotinylated MAP2; (lane 5) supernatant (S) and pellet (P) of MAP2 plus tubulin; (lane 6) supernatant (S) and pellet (P) of biotinylated MAP2 plus tubulin; (lane 7) biotinylated MAP2C; (lane 8) supernatant (S) and pellet (P) of MAP2C plus tubulin; (lane 9) supernatant (S) and pellet (P) of biotinylated MAP2C plus tubulin. Note that biotinylated tau, MAP2, and MAP2C efficiently bind to tubulin. Electron micrographs (B–D) of PC12 cells that were permeabilized, fixed, and stained with anti-biotin antibodies and colloidal gold–labeled second antibodies after microinjection of biotin-tau (B), -MAP2 (C), or -MAP2C (D). Note that injected tau, MAP2, and MAP2C efficiently bind to microtubules. B–D are the same magnification. Bar, 200 nm.

cells showed the existence of tau, MAP2, and MAP2C (data not shown). These data, in conjunction with immunofluorescence microscopy results, confirm the evidence that mature spinal cord neurons localize MAP2C in cell bodies and in dendrites.

Microinjection of Biotin-labeled MAPs into Mature Spinal Cord Neurons in Culture and Analysis of Their Fates and MT Binding

Equal amounts of biotinylated tau, MAP2, and MAP2C were separately microinjected into primary-cultured mature spinal cord neurons, with the microinjected cells then being fixed after specific time periods and processed for immunofluorescent microscopy using anti-biotin first antibodies and rhodamine-labeled second antibodies. 24 h after the injection, tau, MAP2, and MAP2C were found in both axons and dendrites in 100% of the cells examined (Tau, 95 cells; MAP2, 92 cells; MAP2C, 107 cells). Tau, MAP2, and MAP2C in the axon moved progressively, traveling 2.30 ± 0.85 mm ($n = 22$), 1.58 ± 0.49 mm ($n = 30$), and 1.7 ± 0.42 mm ($n = 10$), respectively, away from cell bodies after 24 h. Since biotin-MAP2 was detected with anti-biotin antibodies in axons that normally are not

stained with anti-MAP2 antibodies, the question arises whether anti-biotin staining really reflects the localization of labeled MAP2. To clarify this, we double stained the microinjected neurons with anti-biotin antibodies and anti-MAP2 antibodies. MAP2 was detected in the axons of a microinjected neuron.

Next, we examined the affinity of injected tau, MAP2, and MAP2C for microtubules in axons and dendrites. To do this, 4 h after the injection, the cells were permeabilized with 1% Triton X-100 in MT-stabilized buffer, and then fixed and processed for anti-biotin staining. This method enabled us to extract soluble proteins and observe only tau, MAP2, and MAP2C that bind to the cytoskeleton. Interestingly, although tau was found in both axons and dendrites (Fig. 3 A), MAP2 tended to be confined to dendrites and cell bodies (Fig. 3 B). MAP2C also tended to be localized in dendrites and cell bodies (Fig. 3 C). This pattern of localization of MAP2 and MAP2C did not change at 24 h after injection. However, in the case of tau, the staining in dendrites and cell bodies became weak, while persisting in the axons (Fig. 4). As shown, all three are intensely bound to the neuronal cytoskeleton. At the EM level, we found that the gold particles were scattered in the cytoplasm of axons and dendrites in nonpermeabilized cells (Fig. 5 A).
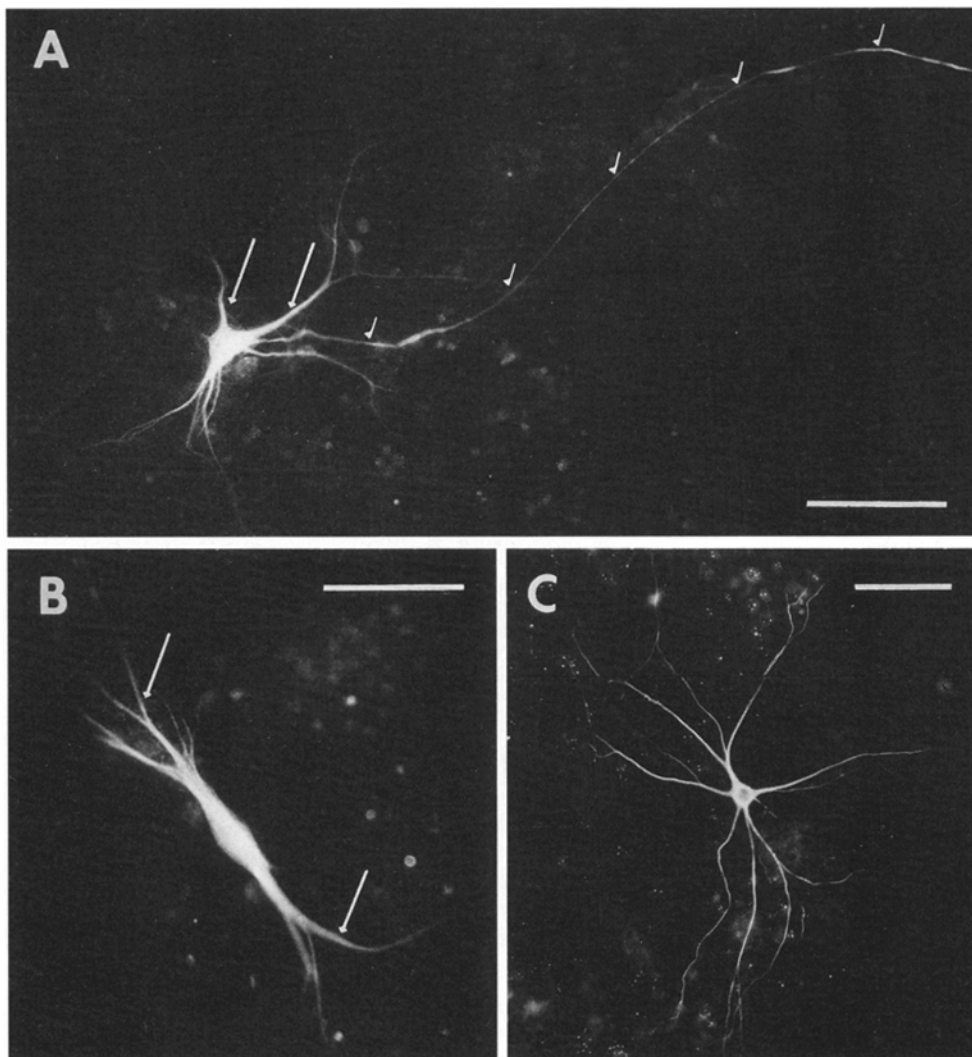

Figure 3. Immunofluorescence localization of biotin-tau (*A*), -MAP2 (*B*), and -MAP2C in spinal cord neurons that were permeabilized and then fixed. (*A*) Anti-biotin staining of a cell permeabilized and fixed 4 h after injecting biotin-tau. Labeled tau exists in the axon (*short arrows*) and dendrites (*long arrows*). (*B*) Anti-biotin staining of a cell permeabilized and fixed 4 h after injecting biotin-MAP2. Labeled MAP2 exists in the dendrites (*long arrows*), but is not detected in the axon. (*C*) Anti-biotin staining of cells permeabilized and fixed 4 h after injecting biotin-MAP2C. Labeled MAP2C is only detected in dendrites. Bars, 100 mm.
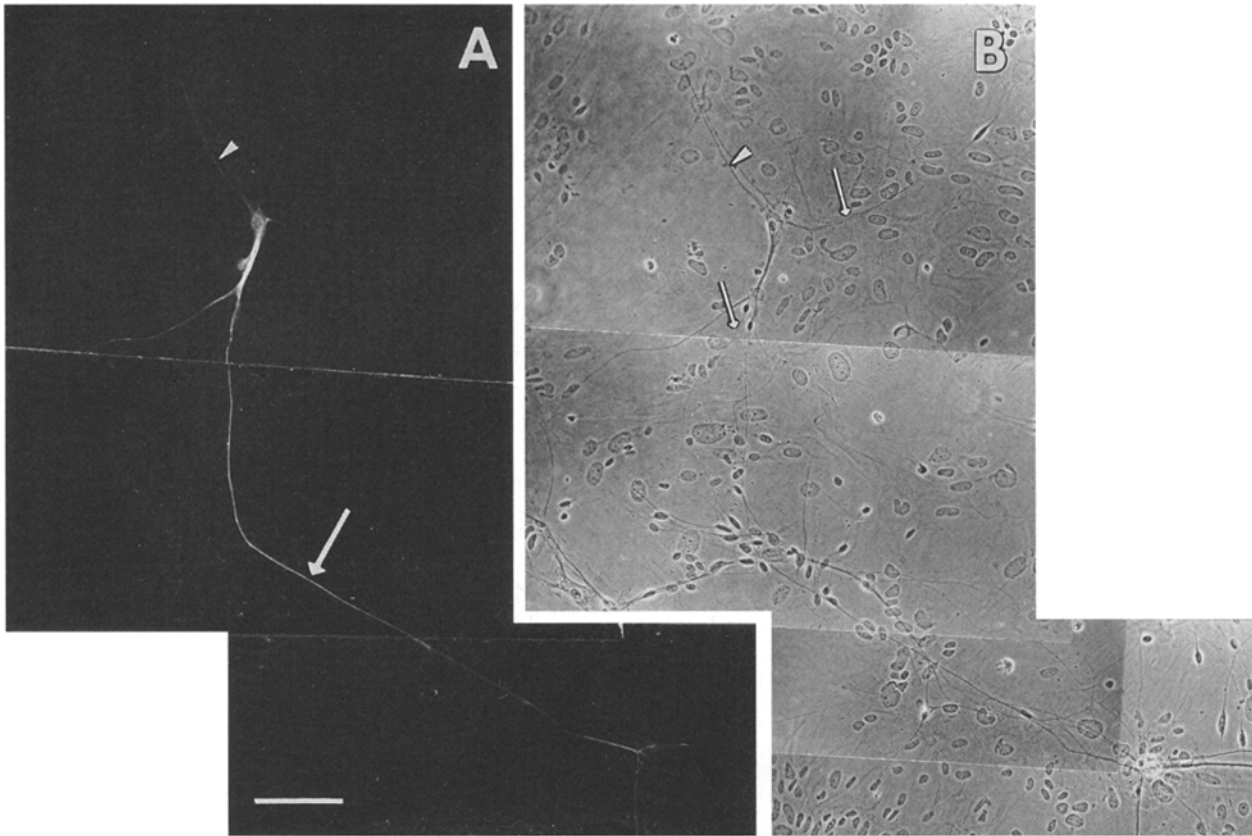
Figure 4. (*A*) Immunofluorescence localization of biotin-tau in a spinal cord neuron that was permeabilized and fixed 24 h after inject-ing biotin-tau. Labeled tau is detected clearly in the axon (*arrow*) for a long distance, whereas staining in dendrites is much weaker (*ar-rowhead*) and in some dendrites staining is not detected (*B, thin arrows*). (*B*) The DIC image of the same field as *A*. Bar, 100 mm.

However, in permeabilized cells, tau, MAP2, and MAP2C were mainly localized in MT domains, and gold particles were especially observed between or on MTs in these per-meabilized cells (Figs. 5, *B–C* and 6 *A*), while in the axon of cells injected with MAP2 only a few gold particles were found in MT domains (Fig. 6 *B*). This pattern of localiza-tion of gold particles resembles that of the immunocy-tochemical localization of MAPs detected with gold-labeled second antibodies in permeabilized neurons in vivo (Shio-mura and Hirokawa, 1987*a*, *b*; and Hirokawa et al., 1988*b*).

3 d after injecting the cells fixed without permeabiliza-tion, the localization of injected tau, MAP2, and MAP2C in the axons was notably different. Although MAP2 tended to vanish in axons or was detectable only in their proximal region (i.e., 19% of the cells examined in axons, cell bodies, and dendrites, but 81% in the cell bodies and dendrites) (Fig. 7 *B*), the presence of tau persisted for a considerable length in axons, namely, 53% in axons, cell bodies, and dendrites, and 47% in axons (Fig. 7 *A*). In contrast, MAP2C was localized in cell bodies and den-drites at 50%, and in axons, cell bodies, and dendrites at 50% (Fig. 7 *C*).

4 d after the injection, however, tau had almost disap-peared from cell bodies and dendrites, though it persisted in axons to a remarkable length: 94% in axons, and 6% in axons, cell bodies, and dendrites (Fig. 8 *A*). In fact, at this stage we still found MAP2 at 80% in cell bodies and den-drites, and at 20% in axons, cell bodies, and dendrites

(Fig. 8 *B*), although its staining tended to be weaker than at day 3 of postinjection. At this stage the injected MAP2C was localized in the axons, cell bodies, and dendrites at 33%, while it was found at 67% in cell bodies and den-drites (Fig. 8 *C*). The localization of biotin -tau, -MAP2, and -MAP2C in nonpermeabilized cells at several time points is summarized in Fig. 9.

Existence of MAP2 in Peripheral Nerve Detected by Immunoblotting In Vivo

Whether or not MAP2 exists in axons in vivo remains a controversy. Although several immunofluorescence stud-ies did not detect MAP2 in axons (Bernhardt and Matus, 1984; Cumming et al., 1984; Caceres et al., 1984; De Cam-illi et al., 1984), Vallee (1982) reported its existence in white matter by a biochemical study. Other immunohis-tochemical studies also indicated that MAP2 is localized in the axons of peripheral nerves, although only in limited quantities (Papasozomenos et al., 1985; Hirokawa et al., 1985). Thus, to determine if MAP2 really exists in axons, we used immunoblotting of homogenates of peripheral nerves by anti-MAP2 antibodies. Anti-MAP2 antibodies clearly stained a band of ~280 kD, thus supporting the possibility that a small amount of MAP2 is actually present in these nerves in vivo (Fig. 10). For comparison, the same materials were immunoblotted using anti-MAP1A antibodies, anti-tau antibodies, and anti-tubulin antibodies
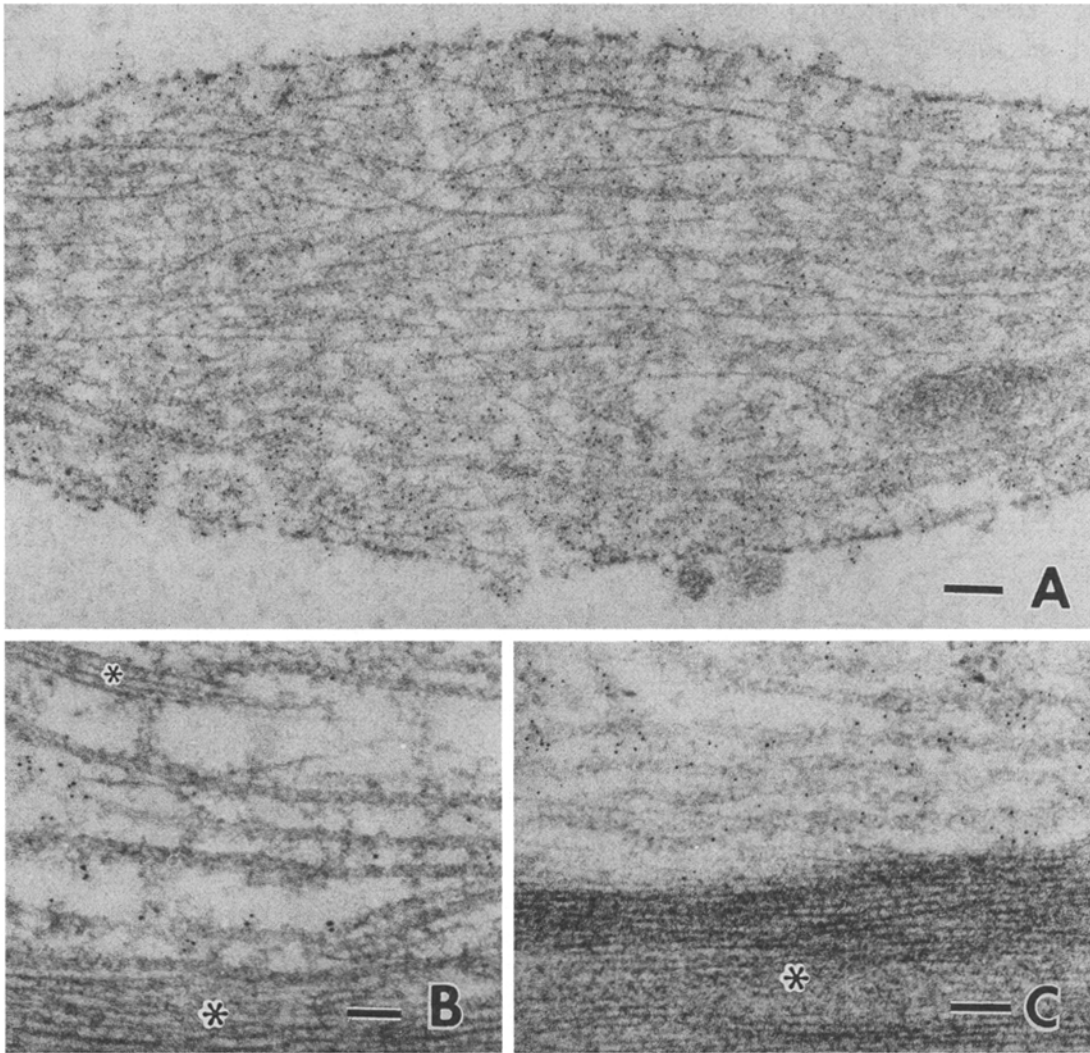
Figure 5. Electron micrographs of neurons fixed and then permeabilized (*A*) or permeabilized and then fixed (*B* and *C*) 4 h after micro-injection of biotin-tau. *A* shows a varicosity in an axon. Gold particles are scattered in the cytoplasm. *B* and *C* show axons in a permeabilized cell, where gold particles tend to be localized either between or on the microtubules, though they are not localized in the neurofilament domains (*). Bar, 100 nm.

(Fig. 10). As another experiment to quantify the amount of MAP2 in sciatic nerves relative to tubulin, we immunoblotted MT pellets, purified MAP2 and tubulin using anti-MAP2 and anti-tubulin antibodies, and then quantitated the amount to have a 1:200 weight ratio of MAP2 to tubulin.

Discussion

Cultured Spinal Cord Neurons Develop Compartmentalization of Tau, MAP2, and MAP2C

Cultured spinal cord neurons (~3 wk) revealed a characteristic staining pattern with the tau-1 antibody and anti-MAP2 antibodies similar to that for nerve cells in vivo and other mature neurons in vitro; namely, tau-1 stains the axon, while anti-MAP2 stains dendrites. In terms of tau localization, Papasozomenos and Binder (1987) suggested that tau protein phosphorylated at the tau-1 site also exists in some dendrites. Other studies, however, showed com-

partmentalized localization of tau in the axon with anti-tau polyclonal antibodies (Kosik and Finch, 1987; Brion et al., 1988), and recently it was shown that although differently phosphorylated tau exists in dendrites in developing neurons, it tends to be compartmentalized in the axon and cell body in mature neurons (>2 wk in culture) (Mandel and Banker, 1995). In fact, our immunofluorescence data of spinal cord neurons treated with alkaline-phosphatase also indicated that in our system tau is mainly compartmentalized in the axon, although tau proteins phosphorylated at tau-1 site are also localized in small amounts in some proximal dendrites and cell bodies.

Concerning the localization of MAP2C in juvenile neurons, it has been proven by two approaches to be localized in axons: (*i*) immunofluorescence microscopy using one antibody which stains both MAP2A/2B and MAP2C, and another antibody which stains only MAP2A/2B (Tucker et al., 1988*a*), and (*ii*) transfection of tagged MAP2C cDNA into cultured neurons (Meichsner et al., 1993). On the other hand, Tucker et al. (1988*b*) showed that MAP2C
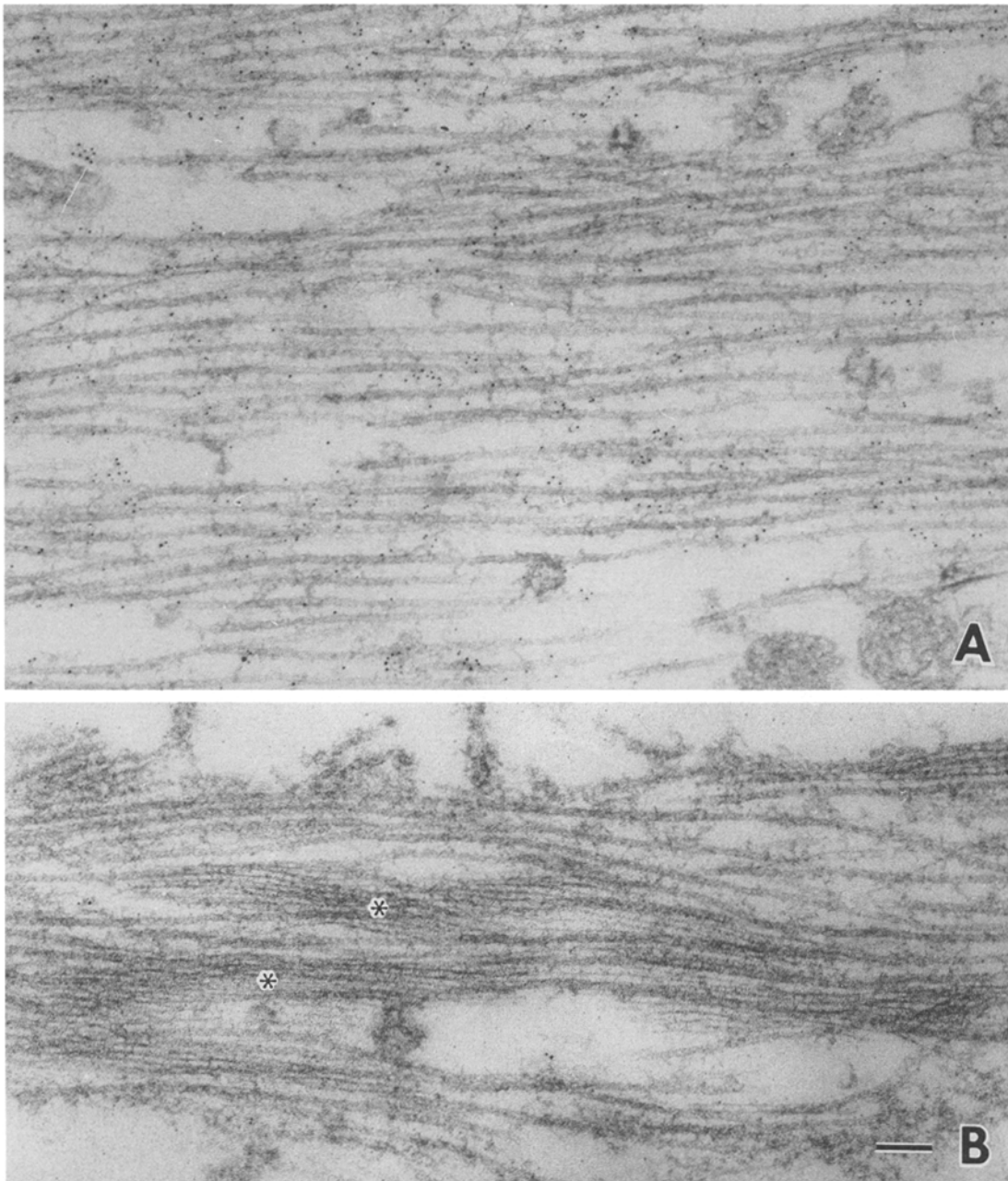
Figure 6. Electron micrographs of a neuron permeabilized and then fixed 4 h after microinjection of biotin-MAP2. *A* shows a dendrite. Gold particles are localized between or on the microtubules in the dendritic microtubule domain. *B* shows the axon of the same cell shown in *A.* *, neurofilament domains characteristic in the axon. Only a few gold particles are observed in the axon. Bar, 100 nm.

was not localized immunofluorescently in the axon of mature neurons, and we too did not detect it in the axon of cultured spinal cord neurons. However, because our immunoblotting study indicated the existence of MAP2C in cultured spinal cord cells, and because only cell bodies and dendrites of neurons were stained with antibodies that recognize MAP2A/2B and MAP2C, it is reasonable to conclude that cultured mature spinal cord neurons compartmentalize MAP2C in cell bodies and dendrites. Thus, judging from the characteristic staining pattern we obtained using anti-tau and anti-MAP2 antibodies, we consider our cultured spinal cord neurons as a model system of mature neurons.

Biotin-labeled MAPs Progressively Moved in the Axon at a Slow Transport Rate

After biotinylation, tau, MAP2, and MAP2C effectively bound to MTs both in vitro and in vivo. In addition, double staining with anti-biotin antibody and anti-MAP2 antibody showed that labeled MAP2 is conveyed in the axon. Although there may be some technical limitation in determining the rate of axonal transport of cytoskeletal proteins by the microinjection method, our data (tau, MAP2, and MAP2C moved 2.30 ± 0.85 mm, 1.58 ± 0.49 mm, and 1.7 ± 0.42 mm, respectively, in 24 h) indicate that the rates for the biotin-labeled proteins are in the range of slow ax-
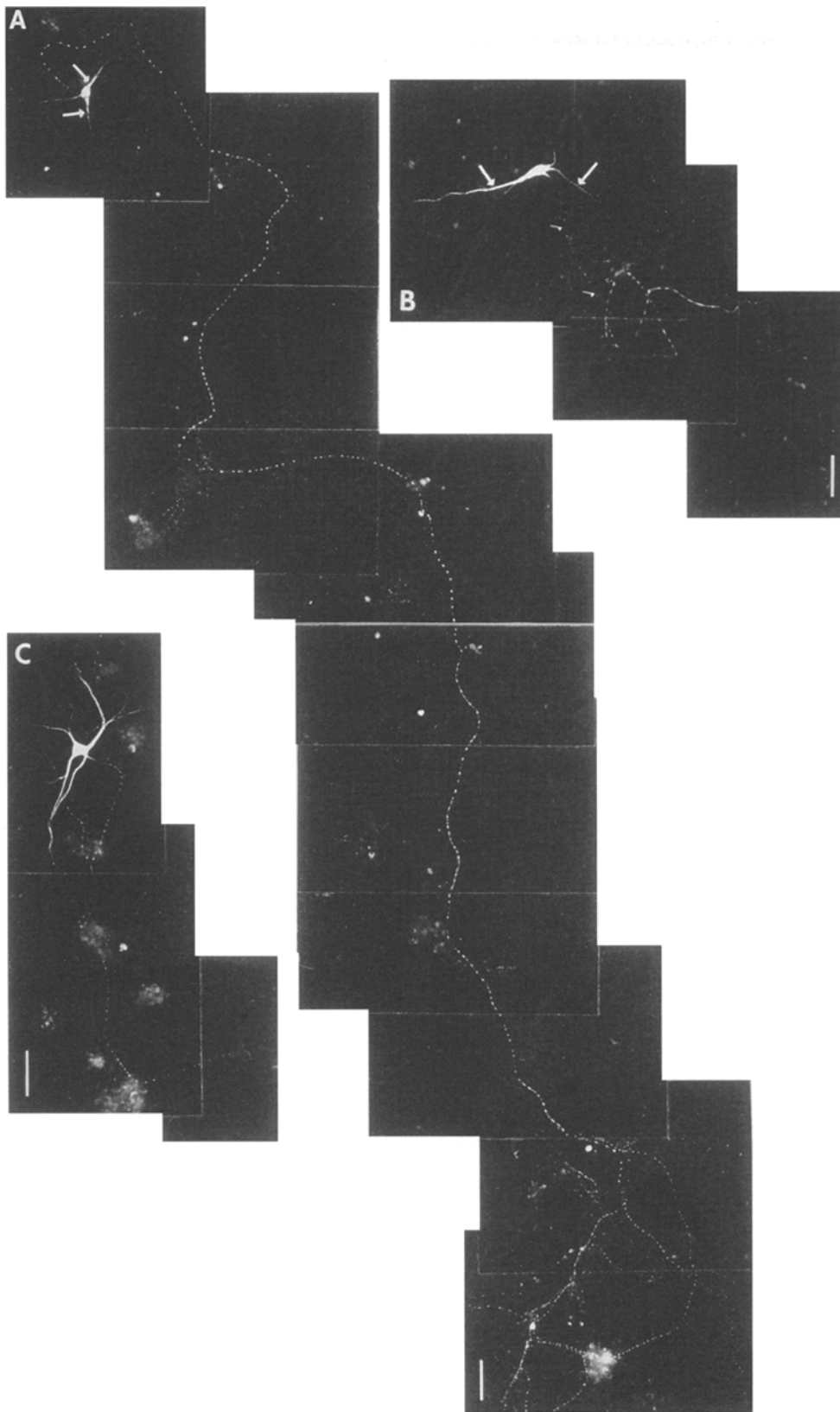
674

Figure 7. Immunofluorescence localization of biotinylated tau (*A*), biotinylated MAP2 (*B*), and biotinylated MAP2C (*C*) in spinal cord neurons fixed 3 d after microinjection. (*A*) Tau exists in the axon and dendrites (*arrows*). Although tau is detected clearly for long distance in the axon, dendritic staining is weak (*arrows*). Bar, 100 mm. (*B*) MAP2 persists in the cell body and dendrites (*long arrows*), but is localized only in the proximal region of the axon (*short arrows*). (*C*) MAP2C also persists in the cell body and dendrites, although in a much shorter proximal segment in the axon compared to that of tau. Bar, 100 mm.

onal transport (Grafstein and Forman, 1980; Tytell et al., 1984). In this regard, Nixon et al. (1990) recently showed that MAP1A is transported in the axon of mouse retinal ganglion cells at a rate of 1.0–1.2 mm/d. Because the labeled MAPs reached >1 mm in the axon from the cell body, and judging from the rates for the transport of labeled MAPs, the movement of these MAPs could not be explained by simple diffusion (Lasek, 1988; Sabry et al., 1995). Taken together, these results suggest that biotin-tau, -MAP2, and -MAP2C behave normally in injected
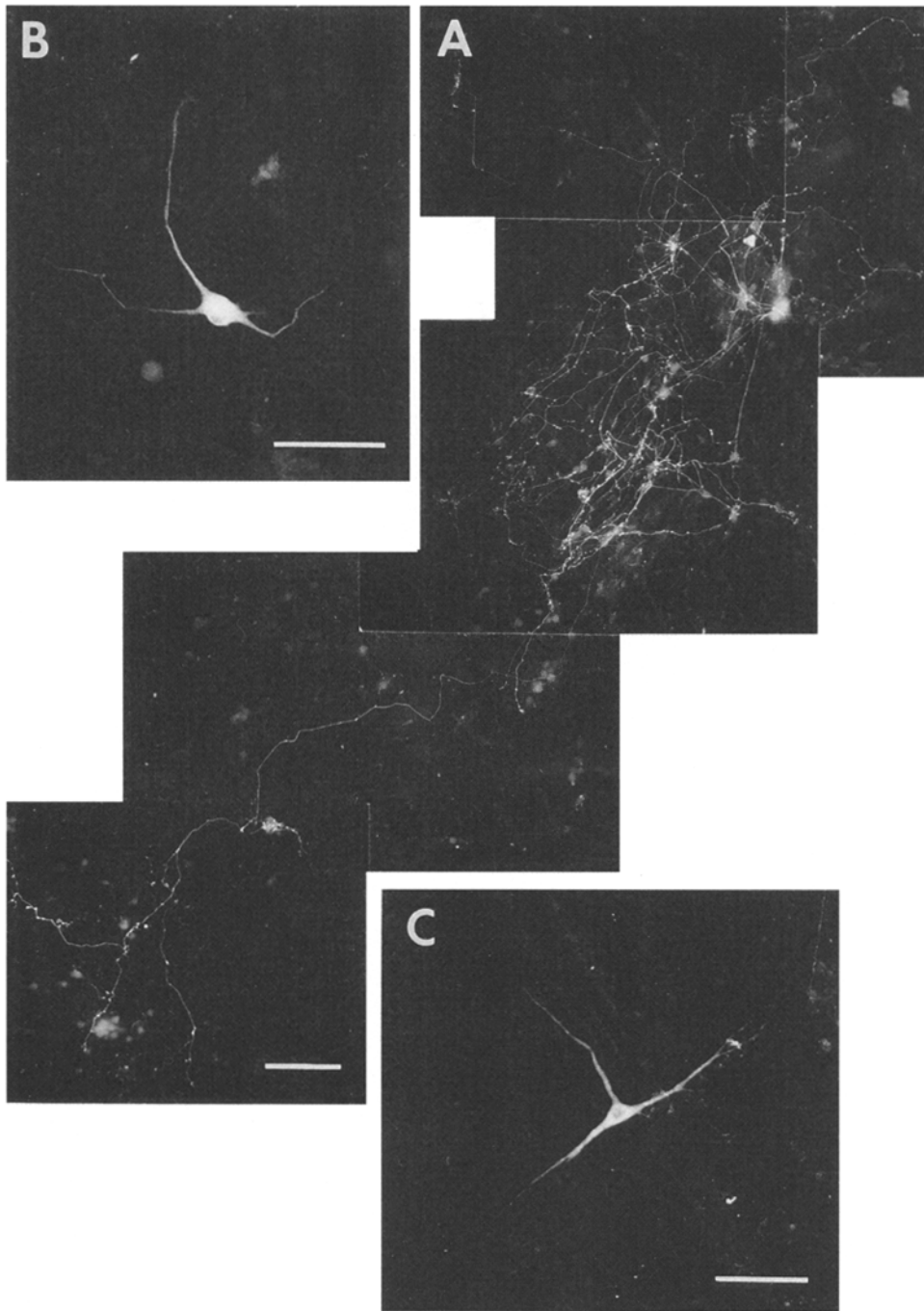
Figure 8. Immunofluorescence localization of biotin-tau (*A*), -MAP2 (*B*), and -MAP2C (*C*) in neurons fixed 4 d after microinjection. (*A*) Tau still exists over a long distance along the axon. Since cell bodies and dendrites are not stained, only axons are recognizable by fluorescence microscopy. (*B*) MAP2 still exists in the cell body and dendrites, but vanishes in the axon. (*C*) MAP2C again exists in the cell body and dendrites, whereas it tends to vanish in the axon. Bars, 100 mm.

nerve cells and are transported down the axon. However, in contrast, our recent transfection studies of cDNAs of tagged MAPs (tau, MAP2, MAP2C) into cultured mature spinal cord neurons revealed that, although tau and MAP2C are detected in the axons over a long distance from the cell body, MAP2 is only localized at the short proximal segment of axons (Kanai and Hirokawa, 1995). Obviously then, our transfection study suggests the suppression of the transit of MAP2 into axons, which is seemingly in contradiction with the results we previously obtained after microinjection of biotin-MAP2 (Okabe and Hirokawa, 1989) as well as the present results. Nevertheless, because previous studies have revealed the existence of MAP2 in axons in vivo immunocytochemically (Papaso-

zomenos et al., 1985; Hirokawa et al., 1985) and in white matter of brains biochemically (Vallee, 1982), and because our present Western blotting data for peripheral nerves convincingly proved that MAP2 exists in them, albeit in limited amounts, we suggest that a small amount of MAP2 is probably transported down the axon in native neurons but is unstable so as to become undetectable more distally in the axon.

Differential Affinity of Tau, MAP2, and MAP2C with MTs in the Axon vs Dendrites

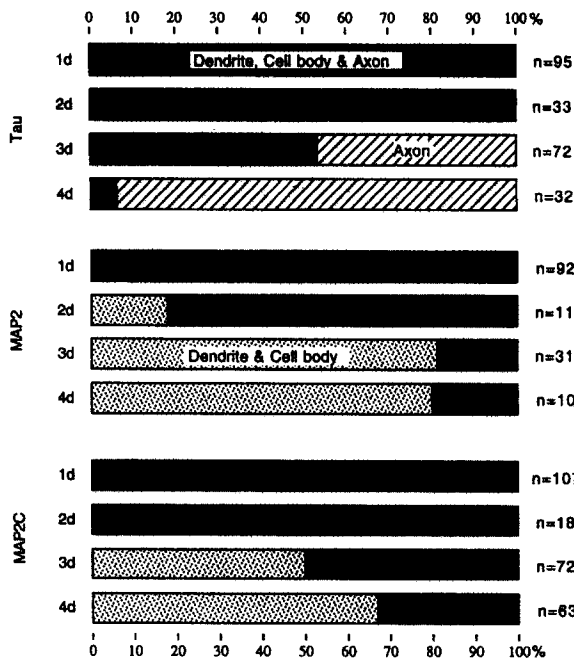Our results also indicate that microinjected tau, MAP2, and MAP2C have two different forms in the neuronal cy-

Figure 9. Relative population of neurons which localize biotin-tau, -MAP2, and -MAP2C in particular regions of neurons at various days after microinjection. The neurons are fixed and then permeabilized at certain time periods after microinjection of labeled proteins. Percentages refer to percentage of cells with the indicated fluorescence distribution and are based on a total area of fluorescence above background. (*Dendrite, Cell body & Axon*) The injected proteins are detected in dendrites, cell bodies, and axons. The injected proteins are localized along axons for a much longer distance than dendrites. (*Axon*) The injected proteins are only detected in axons. Neurons localize injected proteins in axons at a longer distance than dendrites. (*Dendrite & Cell body*) The injected proteins are detected in dendrites and cell bodies. Neurons localizing injected proteins in axons at the same or a shorter distance from the cell body in comparison with dendrites are also included.

Figure 11. Schematic drawing of the fates of microinjected tau, MAP2C, and MAP2 based on the present and previous studies (Okabe and Hirokawa, 1989). These results clearly indicated differential turnover and selective stabilization of tau, MAP2C, and MAP2.

toplasm, MT-nonbound and MT-bound forms. Because phosphorylation is known to critically control the binding of MAPs to MTs, the balance between phosphatases and kinases controls which form occurs. The kinase candidates localized in neurons that phosphorylate these MAPs are proline-directed kinases such as MAP kinase, glycogen
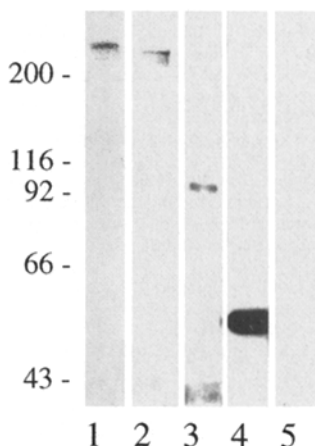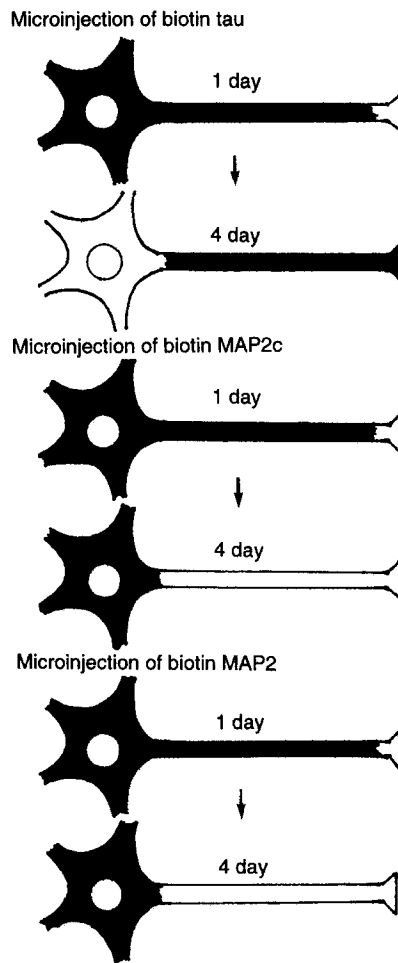

Figure 10. Immunoblot analysis of mouse sciatic nerves by anti-MAPs and anti-tubulin antibodies. The cytosolic extract from mouse sciatic nerves was separated by SDS-PAGE (7.5% running gel), transferred onto a nitrocellulose sheet, and immunoblotted using anti-MAP1A mAb (lane *1*), anti-MAP2 mAb (lane *2*), anti-tau mAb (lane *3*), anti-alpha tubulin mAb (lane *4*), and normal serum (lane *5*). Molecular size standards (*left*).

synthase kinase 3, or members of the cyclin-dependent kinase family, and other kinases such as protein kinase A, protein kinase C, and Ca^{2+} calmodulin kinase II. By comparing the localization of injected MAPs in dendrites of permeabilized cells to that in axons, we found that biotin-tau binds to MTs in the axon and dendrites, whereas MAP2 and MAP2C tend to more firmly bind to MTs in dendrites and cell bodies in comparison with MTs in the axon 4 h after microinjection. However, 24 h after microinjection, the affinity of biotin-tau to MTs in dendrites tends to become much weaker than in the axon. Although we purified biotin-labeled MAPs by MT binding so that they would undergo this activity before the injection, there was a tendency for biotin-MAP2 and -MAP2C to have weak affinity for MTs in the axon 4 h after microinjection. In the case of tau, although it took more time, biotin-tau in dendrites inclined to lose its affinity for MTs 24 h after microinjection. Such phenomena may be caused by (*a*) the difference between MTs in the axon vs those in dendrites, (*b*) the differential phosphorylation of MAPs in the axon vs that in dendrites, or (*c*) the differential sorting of distinct isoforms of MAPs, especially tau, in the axon vs that in dendrites. Concerning (*a*), the expression of differ-

ent β tubulin isotypes in different regions of nerve cells might be related. Joshi and Cleveland (1989) revealed that isotype V is partially excluded from neurites of PC12 cells differentiated by NGF. However, until now, no evidence has been available to suggest that the β tubulin isotype is different between MTs in axons and dendrites. This is also the case for (c), as there is no evidence to indicate that certain isoforms of tau are preferentially localized in the axon. Therefore, the distinct MT-binding activity of injected MAPs in the axon vs. that in the cell body and dendrites could be mainly controlled by the phosphorylation of MAPs. Because microinjected MAPs were derived from whole brains, they contain several differentially phosphorylated forms (Matsuo et al., 1994). Therefore, the time difference for the injected MAPs to acquire the ability of MT binding related to their selective location in certain compartments of neurons may be caused by the degree of the presence of several distinctly phosphorylated forms in the injected MAPs. The MT-binding activity of microinjected MAP2 and MAP2C in spinal cord neurons basically revealed a similar tendency, and consequently, the critical sites for phosphorylation of MAP2 and MAP2C, which regulate microtubule binding, could reside in common MAP2C structures, namely in the short NH$_2$-terminal region, and most probably in the MT-binding, poline-rich, and repeat regions at the COOH-terminus. Concerning the control of MT-binding activity of MAPs, phosphorylation of MAP2 and tau with kinases such as protein kinase A, Ca^{2+} calmodulin kinase II, MAP kinase (reviewed by Wiche et al., 1991; Drewes et al., 1992; Goedert et al., 1992b), glucose synthase kinase 3 (Mandelkow and Mandelkow, 1995), CDK5 (Bauman et al., 1993; Kobayashi et al., 1993; Paudel et al., 1993), CDC2 kinase (Ledesma et al., 1992), and proline-directed kinase (Vulliet et al., 1992) induced dissociation of MAPs from MTs, whereas in another case, phosphorylation in vivo by unknown kinases induced microtubule binding (Brugg and Matus, 1991). There is absolutely no argument that the overall process of the regulation of MT binding of MAPs by kinases is very complex indeed, and that extensive research will be required for its eventual clarification.

Multiple Sorting Mechanisms of Tau and MAP2 in Neurons

Concerning the other possible mechanisms for the compartmentalization of MAPs, our previous study using the transfection of cDNAs of tau, MAP2, MAP2C, and their various deletion mutants into primary-cultured spinal cord neurons, the same system as the one used in the present study, indicated that transit of MAP2 into the axon was inhibited, while tau and MAP2C were transported for a long distance in the axon (Kanai and Hirokawa, 1995). This study clearly showed that the suppression of MAP2 transit into the axon plays an important role, whereas it also suggests that mere suppression of the transit of MAPs is not enough to determine their selective localization as MAP2C is restricted to localization in dendrites and cell bodies despite its transit into the axon for a long distance. On the other hand, the differential localization of mRNA may work for MAP2 and tau as another mechanism for the compartmentalization of MAPs (Garner et al., 1988; Lit-

man et al., 1993). However, in the case of MAP2C, a previous study by Garner et al. (1988) showed that MAP2C mRNA is confined to the cell body in a different manner from MAP2 mRNA, which is extended into dendrites, whereas MAP2C is compartmentalized in the cell body and dendrites in mature spinal cord neurons similarly to MAP2. Obviously, localization of MAP2C mRNA in itself cannot explain this final localization of MAP2C. This also highlights the differential turnover of MAP2C as an important mechanism for its final compartmentalization.

Based on the present study and previous studies, it is reasonable to conclude that multiple mechanisms exist that cause the selective localization of tau in the axon and MAP2 and MAP2C in dendrites in mature neurons in vivo. Some of these mechanisms are as follows: (a) the extended localization of mRNA of MAP2 in dendrites (Garner et al., 1988) and mRNA of tau in the initial segment of the axon (Litman et al., 1993), (b) the differential turnover and selective stabilization of tau, MAP2, and MAP2C (Okabe and Hirokawa, 1989, and present study), and (c) the decreased transit of MAP2 into axons (Kanai and Hirokawa, 1995). Mechanisms (a–c) probably work in concert and lead to the final characteristic compartmentalization of tau, MAP2, and MAP2C in mature neurons.

References

Bauman, K., E.-M. Mandelkow, J. Biernat, H. Piwnica-Worms, and E. Mandelkow. 1993. Abnormal Alzheimer's-like phosphorylation of tau protein by cyclin-dependent cdK2 and cdK5. FEBS (Fed. Eur. Biochem. Soc.) Lett. 336: 417–424.

Bernhardt, R., and A. Matus. 1984. Light and electron microscopic studies of the distribution of micotubule-associated protein 2 in rat brain: a difference between dendritic and axonal cytoskeleton. J. Comp. Neurol. 226:203–211.

Binder, L.I., A. Frankfurter, and L.M. Rebhun. 1985. The distribution of tau in the mammalian central nervous system. J. Cell Biol. 101:1371–1378.

Brion, J.P., J. Guilleminot, J. Couchie, J. Flament-Durand, and J. Nunez. 1988. Both adult and juvenile tau microtubule-associated proteins are axon specific in the developing and adult rat cerebellum. Neuroscience. 25:139–146.

Brugg, B., and A. Matus. 1991. Phosphorylation determines the binding of microtubule-associated protein 2 (MAP2) to microtubules in living cells. J. Cell Biol. 114:735–743.

Caceres, A., L.I. Banker, M.R. Payne, P. Bender, L. Rebhun, and O. Steward. 1984. Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. J. Neurosci. 4:394–410.

Chen, J., Y. Kanai, N.J. Cowan, and N. Hirokawa. 1992. Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. Nature (Lond.). 360:674–677.

Cleveland, D.W. 1990. Microtubule MAPing. Cell. 60:701–702.

Cumming, R., R.D. Burgoyne, and N.A. Lytton. 1984. Immunofluorescence distribution of alpha tubulin, beta tubulin and microtubule-associated protein 2 during in vitro maturation of cerebellar granule cell neurons. Neuroscience. 12:775–782.

De Camilli, P., P.E. Miller, F. Navone, W.E. Theurkauf, and R.B. Vallee. 1984. Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. Neuroscience. 11:819–846.

Dotti, C.G., and G.A. Banker. 1987. Experimentally induced alteration in the polarity of developing neurons. Nature (Lond.). 330:254–256.

Drewes, G., B. Lichtenberg-Kragg, F. Doring, E.-M. Mandelkow, J. Bienart, M. Doree, and E. Mandelkow. 1992. Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. EMBO (Eur. Mol. Biol.

Organ.) J. 11:2131–2138.

Garner, C.C., R.P. Tucker, and A. Matus. 1988. Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature (Lond.).* 336:674–677.

Graessmann, A., M. Graessmann, and C. Mueller. 1980. Microinjection of early SV 40 DNA fragments and T antigen. *Methods Enzymol.* 65:816–825.

Grafstein, B., and D.S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.

Goedert, M., R.A. Crowther, and C.C. Garner. 1991. Molecular characterization of microtubule-associated proteins tau and MAP2. *TINS (Trends Neurosci.).* 14:193–199.

Goedert, M., E.S. Cohen, R. Jakes, and P. Cohen. 1992. p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase 2A1. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 312:95–99.

Gorbsky, G.J., P.J. Sammak, and G.G. Borisy. 1987. Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. *J. Cell Biol.* 104:9–18.

Harada, A., K. Oguchi, S. Okabe, J. Kuno, S. Terada, T. Ohshima, R. Sato-Yoshitake, Y. Takei, T. Noda, and N. Hirokawa. 1994. Targeted disruption of the tau gene results in alteration of microtubule organization and stabilization in small caliber axons. *Nature (Lond.).* 36:488–491.

Hirokawa, N. 1994. Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr. Opin. Cell Biol.* 6:74–82.

Hirokawa, N., G.B. Bloom, and R.B. Vallee. 1985. Cytoskeletal architecture and immunocytochemical localization of microtubule-associated proteins in regions of axons associated with rapid axonal transport: the β, β′-iminodipropionitrile-intoxicated axon as a model system. *J. Cell Biol.* 101:227–239.

Hirokawa, N., Y. Shiomura, and S. Okabe. 1988a. Tau proteins: the molecular structure and mode of binding on microtubules. *J. Cell Biol.* 107:1449–1459.

Hirokawa, N., S. Hisanaga, and Y. Shiomura. 1988b. MAP2 is a component of crossbridges between microtubules and neurofiaments in vivo and in vitro. Quick-freeze, deep etch immunoelectron microscopy and reconstitution studies. *J. Neurosci.* 8:2769–2779.

Joshi, H.C., and D.W. Cleveland. 1989. Differential utilization of β-tubulin isotypes in differentiating neurites. *J. Cell Biol.* 109:663–673.

Kanai, Y., and N. Hirokawa. 1995. Sorting mechanisms of tau and MAP2 in neuron: suppressed axonal transit of MAP2 and locally regulated microtubule bindings. *Neuron.* 14:421–432.

Kanai, Y., R. Takemura, T. Oshima, H. Mori, T. Ihara, M. Yanagisawa, T. Masaki, and N. Hirokawa. 1989. Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* 109:1173–1184.

Knops, J., K.S. Kosik, G. Lee, J.D. Pardee, L. Cohen-Gould, and L. McConlogue. 1991. Overexpression of tau in a nonneuronal cell induces long cellular processes. *J. Cell Biol.* 114:725–733.

Kobayashi, S., K. Ishiguro, A. Omori, M. Takamatsu, K. Arioka, K. Imahori, and T. Uchida. 1993. A cdc-related kinase PSSALRE/cdK5 is homologous with the 30kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 335:171–175.

Kosik, K.S., and E.A. Finch. 1987. MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: an immunocytochemical study of cultured rat cerebrum. *J. Neurosci.* 7:3142–3153.

Kristofferson, D., T. Mitchison, and M. Kirschner. 1986. Direct observation of steady-state microtubule dynamics. *J. Cell Biol.* 102:1007–1019.

Lasek, R.J. 1988. Studying the intrinsic determinants of neuronal form and function. *In* Intrinsic Determinants of Neuronal Form and Function. R. Lasek, and M. Black, editors. Alan R. Liss, Inc., New York. 1–60.

Ledesma, M.D., L. Correas, J. Avila, and J. Diaz-Nido. 1992. Implication of brain cdc2 and MAP kinases in the phosphorylation of tau protein in Alzheimer's disease. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 308:218–224.

Lewis, S.A., I.E. Ivanov, G.H. Lee, and N.J. Cowan. 1989. Organization of microtubules in dendrites and axons is determined by a short hydrophobic zipper in microtubule-associated proteins MAP2 and tau. *Nature (Lond.).* 342:498–505.

Litman, P., J. Barg, L. Rindzooski, and I. Ginzburg. 1993. Subcellular localization of tau mRNA in differentiating neuronal cell culture: implications for neuronal polarity. *Neuron.* 10:627–638.

Mandel, J.W., and G.A. Banker. 1995. The microtubule cytoskeleton and the development of neuronal polarity. *Neurobiol. Aging.* 16:229–238.

Mandelkow, E., and E.-M. Mandelkow. 1995. Microtubules and microtubule-associated proteins. *Curr. Opin. Cell Biol.* 7:72–81.

Matsuo, S., R.-W. Shin, M.L. Billingsley, A.V. de Voorde, M. O'Conner, J.Q. Trojanowski, and V.M.-Y. Lee. 1994. Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. *Neuron.* 13:989–1002.

Meichsner, M., T. Doll, B. Weisshaar, and A. Matus. 1993. The low molecular weight form of microtubule-associated protein 2 is transported into both axons and dendrites. *Neuroscience.* 54:873–880.

Nixon, R.A., I. Fischer, and S.E. Lewis. 1990. Synthesis, axonal transport, and turnover of the high molecular weight microtubule-associated protein MAP1A in mouse retinal ganglion cells: tubulin and MAP1A display distinct transport kinetics. *J. Cell Biol.* 110:437–448.

Okabe, S., and N. Hirokawa. 1988. Microtubule dynamics in nerve cells: analysis using microinjection of biotinylated tubulin into PC12 cells. *J. Cell Biol.* 107:651–664.

Okabe, S., and N. Hirokawa. 1989. Rapid turnover of microtubule-associated protein 2 in the axon. *Proc. Natl. Acad. Sci. USA.* 86:4127–4131.

Papasozomenos, S.C., and L.I. Binder. 1987. Phosphorylation determines two different species of tau in the central nervous system. *Cell Motil. Cytoskeleton.* 8:210–226.

Papasozomenos, S.C., L.I. Binder, P.K. Bender, and M.R. Payne. 1985. Microtubule-associated protein 2 within axons of spinal motor neurons: associations with microtubules and neurofilaments in normal and β, β′-iminodipropionitrile-treated axons. *J. Cell Biol.* 100:74–85.

Paudel, H.K., J. Lew, A. Zenobia, and J.H. Wang. 1993. Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. *J. Biol. Chem.* 268:23512–23518.

Peng, I., L.I. Binder, and M.M. Black. 1986. Biochemical and immunological analyses of cytoskeletal domains of neurons. *J. Cell Biol.* 102:252–262.

Ranson, B.R., E. Neale, M. Henkart, P.N. Bullock, and P.G. Nelson. 1977. Mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties. *J. Neurophysiol. (Bethesda).* 40:1132–1150.

Sabry, J., T.P. O'Conner, and M.W. Kirschner. 1995. Axonal transport of tubulin in Ti1 pioneer neurons in situ. *Neuron.* 14:1247–1256.

Sato, R., Y. Shiomura, H. Miyasaka, and N. Hirokawa. 1989. Molecular structure and localization of microtubule-associated protein 1B and its phosphorylation-dependent expression in the developing neurons. *Neuron.* 3:229–238.

Shelanski, M.L., F. Gaskin, and C.R. Cantor. 1973. Microtubule assembly in the absence of added nucleotide. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Shiomura, Y., and N. Hirokawa. 1987a. The molecular structure of microtubule associated proteins 1A (MAP1A) in vivo and in vitro. An immunoelectron microscopy and quick-freeze deep-etch study. *J. Neurosci.* 7:1461–1469.

Shiomura, Y., and N. Hirokawa. 1987b. Colocalization of microtubule-associated protein 1A and microtubule-associated protein 2 on the neuronal microtubules in situ revealed with double-label immunoelectron microscopy. *J. Cell Biol.* 104:1575–1578.

Takeuchi, M., S. Hisanaga, T. Umeyama, and N. Hirokawa. 1992. The 72kD microtubule-associated protein from porcine brain. *J. Neurochem.* 58:1510–1516.

Tucker, R.P., L.I. Binder, and A.I. Matus. 1988a. Neuronal microtubule-associated proteins in the embryonic avian spinal cord. *J. Comp. Neurol.* 271:44–55.

Tucker, R.P., L.I. Binder, C. Viereck, B.A. Hemmings, and A.I. Matus. 1988b. The sequential appearance of low- and high-molecular weight forms of MAP2 in the developing cerebellum. *J. Neurosci.* 12:4503–4512.

Tytell, M., S.T. Brady, and R.T. Lasek. 1984. Axonal transport of a subclass of tau proteins: evidence for the regional differentiation of microtubules in neurons. *Proc. Natl. Acad. Sci. USA.* 81:1570–1574.

Vallee, R.B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubules-associated proteins (MAPs). *J. Cell Biol.* 92:435–442.

Vulliet, R., S.M. Halloran, R.K. Baun, A.J. Smith, and G. Lee. 1992. Proline-directed phosphorylation of human tau protein. *J. Biol. Chem.* 267:22570–22574.

Wiche, G., C. Oberkannins, and A. Hinnler. 1991. Molecular structure and function of microtubule-associated proteins. *Int. Rev. Cytol.* 124:217–273.