Molecular Biology of the Cell
Vol. 4, 1225–1238, December 1993

Calcineurin Is Associated with the Cytoskeleton of Cultured Neurons and Has a Role in the Acquisition of Polarity

Adriana Ferreira, Randall Kincaid,* and Kenneth S. Kosik

Department of Medicine, Division of Neurology, Harvard Medical School and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, Massachusetts 02115; and
*Laboratory of Molecular and Cellular Neurobiology, Section on Immunology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland 20852

Submitted July 27, 1993; Accepted October 6, 1993

Calcineurin is a calmodulin-dependent serine-threonine phosphatase found in many cell types but most abundant in neurons. To determine its localization in developing neurons, dissociated cultures from embryonic day 15 rat cerebellum were analyzed immunocytochemically after treatment with cytoskeletal-disrupting drugs. During the initial outgrowth of neurites, calcineurin is enriched in growth cones where its localization depends upon the integrity of both microtubules and actin filaments. Treatment with cytochalasin shifts calcineurin from the growth cone to the neurite shaft, and with nocadozole calcineurin translocates to the cell body. Therefore calcineurin is well positioned to mediate interactions between cytoskeletal systems during neurite elongation. By 14 d in culture, when the neurons have developed extensive neuronal contacts and synapses are present, calcineurin is predominantly in the neurite shaft. Incubation of cultured cells with Cyclosporin A or a specific peptide, both of which selectively inhibit calcineurin's phosphatase activity, prevented axonal elongation. Because the microtubule-associated protein tau appears to play a key role in asymmetric neurite elongation, we examined modifications in its phosphorylation state resulting from calcineurin inhibition. In contrast to the normal development of cerebellar macroneurons in which reactivity with the phosphorylation-dependent antibody, tau-1, progressively increases, there was a persistent inhibition of tau-1 reactivity in cells exposed to Cyclosporin A. These findings suggest a role for calcineurin in regulating tau phosphorylation and possibly modulating other steps required for the determination of polarity.

INTRODUCTION

Neurite outgrowth involves the assembly of microtubules into a bundled configuration in the neurite shaft and a splayed, looped, or bundled appearance in the central region of the growth cone (Tanaka and Kirschner, 1991). The asymmetric disposition of microtubules within the growth cone may anticipate the direction of future growth (Sabry *et al.*, 1991). In this distal location microtubules are labile (Bamburg *et al.*, 1986) and not associated with organelles (Cheng and Reese, 1985). In advance of the assembly-competent pool of microtubules lies a region of the growth cone from which filopodia with a core of bundled actin filaments rapidly protrude and retract. Actin-containing filaments are also present in the broad flattened lamellopodia that extend from growth cones and within the cortical rim of the growth cone (Yamada *et al.*, 1971; Hirokawa, 1982; Schnapp and Reese, 1982; Letourneau and Ressler, 1983).

The differentiation of neurites into axons or dendrites occurs in a well-defined sequence of morphological changes leading to the establishment of polarity. In cultures of either hippocampal neurons or cerebellar macroneurons, this sequence involves the extension of several undifferentiated neurites of approximately equal lengths (Dotti *et al.*, 1988; Ferreira and Caceres, 1989). These processes extend and retract in dynamic equilibrium until one of them exceeds the others in length by

10 μm and rapidly elongates to become the axon (Goslin and Banker, 1989). The microtubule-associated protein (MAP), tau, appears to have a role in this asymmetric phase of neurite elongation. Suppression of tau synthesis in cerebellar macroneurons using antisense oligonucleotides resulted in the failure of the cells to selectively elongate an axon, although they retained the ability to elaborate minor neurites (Caceres and Kosik, 1990). In these cells, tau protein is present in all of the processes. However, at the onset of axonal elongation, tau is bound to the microtubules only in the axon as observed by antibody detection after extraction with detergent (Ferreira *et al.*, 1989). One way in which the binding of tau to microtubules is regulated is by its phosphorylation state (Lindwall and Cole, 1984; Drechsel *et al.*, 1992). The *in vivo* tau phosphatases are unknown; however, both calcineurin (protein phosphatase 2B) and protein phosphatase 2A have been suggested on the basis of *in vitro* data (Goto *et al.*, 1985; Yamamoto *et al.*, 1990; Goedert *et al.*, 1992).

Of these two serine-threonine phosphatases, the Ca^{2+} and calmodulin-dependent-enzyme is a candidate for regulating reversible interactions between tau and the microtubules. Calcineurin is the most abundant CaM-binding protein in adult brain (Klee *et al.*, 1979), and its expression increases dramatically during development (Tallant and Cheung, 1983; Polli *et al.*, 1991). Although it is present in many cell types, it is 10–20 times more abundant in the brain. Immunocytochemical studies of tissue sections from various brain regions have localized calcineurin to neurons (Wood *et al.*, 1980; Goto *et al.*, 1986; Kincaid *et al.*, 1987) where it exhibits staining both in the processes and the cell soma. A more detailed immunocytochemical localization in tissue sections is difficult because of the density of processes in the neuropil. The holoenzyme consists of an A and a B subunit forming a heterodimeric structure in 1:1 stoichiometric proportions (reviewed in Klee *et al.*, 1988). Calcineurin A, the 60-kDa catalytic and calmodulin-binding subunit, exists in multiple isoforms resulting from alternative splicing of three distinct genes (Guerini and Klee, 1989; Kincaid *et al.*, 1990; Muramatsu *et al.*, 1992). Calcineurin B, the 19-kDa calcium-binding regulatory subunit is encoded by two genes (Ueki *et al.*, 1992). In contrast to other serine-threonine phosphatases, calcineurin has a relatively restricted substrate specificity. Several neuronal phosphoproteins that are phosphorylated by cyclic nucleotide-dependent protein kinases appear to be good substrates *in vitro* (King *et al.*, 1984).

Although the *in vivo* relationship to calcineurin is unknown, both Ca^{2+} and calmodulin are known to have effects on the phosphorylation state of the cytoskeleton and consequently on cytoskeletal organization. Microtubule assembly is affected by CaM, either through direct interactions with tubulin (Kumagai *et al.*, 1982), MAPs (Lee and Wolff, 1984), or via phosphorylation-based mechanisms (Yamamoto *et al.*, 1985). In addition, two CaM binding proteins, fodrin and caldesmon, are also cytoskeletal binding proteins that may link membrane and actin-based structures to changes in local Ca^{2+} concentrations (Sobue *et al.*, 1981; Carlin *et al.*, 1983; Owada *et al.*, 1984; Zagon *et al.*, 1986). By exhibiting Ca^{2+}-dependent associations with MAPs and actin-associated proteins, CaM may mediate reversible states of self-assembly in cytoskeletal structures (Sobue *et al.*, 1983, 1985). Significantly, CaM is thought to play a central role in the regulation of growth cone development possibly through its interaction with the growth-associated protein, GAP-43 (Meiri *et al.*, 1986). The interaction between GAP-43 and CaM *in vitro* depends upon the phosphorylation state of GAP-43, a modification that may be under the control of calcineurin (Alexander *et al.*, 1987; Liu and Storm, 1989).

In this study we have used cerebellar macroneurons grown in culture to determine the localization of calcineurin in relation to components of the cytoskeleton and have examined the functional consequences of its inhibition during neuronal development. We report here that calcineurin is localized in growth cones, and this localization depends upon intact actin filaments and microtubules. In addition, inhibitors of calcineurin's phosphatase activity induce changes in the phosphorylation of tau protein and prevent axonal elongation, suggesting that the phosphorylation state of tau and its modulation via calcineurin have a role in the acquisition of polarity.

MATERIALS AND METHODS

Culture of Cerebellar Macroneurons

Dissociated cultures of cerebellar macroneurons were prepared from the cerebellar primordia of embryonic day 15 rat embryos as described previously (Ferreira *et al.*, 1989). Cells were plated onto polylysine-coated glass coverslips at densities ranging from 100 000 to 150 000 cells per 60-mm dish and maintained with Eagle's minimum essential medium (MEM) plus 10% horse serum for 4 h. The coverslips with the attached cells were then transferred to 60-mm Petri dishes containing an astroglial monolayer. All cultures were maintained in MEM containing the N2 supplements (Bottenstein and Sato, 1979) plus ovalbumin (0.1%) and pyruvate (0.1 mM).

Antibodies

Two different calcineurin antibodies were used in these experiments. Goat anti-calcineurin antibodies were prepared by affinity chromatography on calcineurin Sepharose and further fractionated into an IgG fraction using Protein A-Sepharose (Kincaid, 1988). This antibody preparation recognizes both the catalytic and regulatory subunits of calcineurin and has been extensively characterized for immunocytochemical studies in adult and developing neural tissue (Kincaid *et al.*, 1987; Polli *et al.*, 1991). To control for nonspecific immunoreactivity, portions of the antibody (0.4 mg IgG/ml) were preabsorbed with a twofold molar excess of purified bovine brain calcineurin (Kincaid *et al.*, 1984) for 10 h at 4°C before immunofluorescence experiments; this treatment eliminated specific immunofluorescence. Sham incubations in which antibody was preabsorbed with bovine serum albumin (BSA) were also carried out, showing no change in specific immunoreactivity. A second calcineurin antibody (unpublished data) was prepared in rabbit against a calcineurin peptide from a region

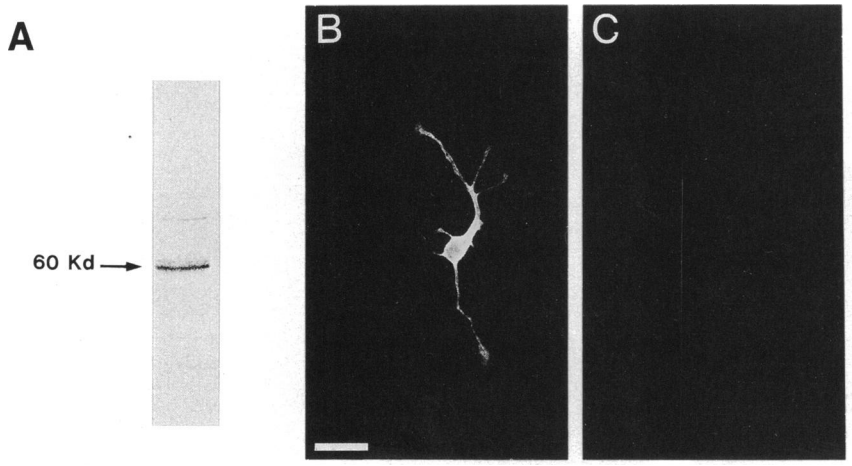


Figure 1. Specificity of the goat anti-calcineurin antibody. (A) A whole cell homogenate of an embryonic day 15 cerebellar culture 1 d after plating was separated by SDS-polyacrylamide gel electrophoresis (40 μg/lane), transfered to Immobilon membrane, and reacted with the calcineurin antibody. The stained 60-kDa band represents the catalytic subunit of calcineurin. (B) Cerebellar macroneuron stained with tubulin antibody and double labeled with calcineurin antibody that has been preadsorbed with calcineurin. (C) Bar, 20 μm.

near the amino terminus of the catalytic subunit, was purified by affinity chromatography, and was used to confirm the results. The goat antibody was used at a dilution of 1:250 and the rabbit antibody at 1:500. In addition, a monoclonal antibody (mAb) against β-tubulin (clone DM1B, Boehringer, Indianapolis, IN) diluted 1:1000, a mAb against acetylated α-tubulin (clone 6-11B-1) (Piperno *et al.*, 1987) diluted 1:100, a polyclonal antibody against tubulin (Sigma, St. Louis, MO) diluted 1:100, and three mAbs against tau protein designated clone 5E2 (Kosik *et al.*, 1988), tau-1 (Kosik *et al.*, 1988), and AT8 (Biernat *et al.*, 1992) were used. The following secondary antibodies were used: biotinylated horse anti-mouse IgG (1:1000), avidin conjugated to rhodamine (1:500) (Vector Laboratories, Norwalk CT), swine anti-goat IgG conjugated to fluorescein isothiocyanate (FITC), and goat anti-mouse IgG conjugate to FITC or rhodamine (Boehringer) diluted 1:100.

Immunofluorescence Microscopy

Cultures were fixed for 30 min with 4% formaldehyde in phosphate-buffered saline (PBS) and 0.12 M sucrose. They were then permeabilized in 0.3% Triton X-100 in PBS and rinsed twice in PBS. In some experiments the cells were extracted for 2 min with 0.2% Triton X-100 in a microtubule-stabilizing buffer (130 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2 mM MgCl,. 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid pH 6.9), rinsed in the same buffer, and then fixed. Cultures were then incubated with 10% BSA/PBS for 30 min and exposed to primary antibodies (diluted in 1% BSA/PBS) overnight at 4°C. After exposure to primary antibodies, the cultures were rinsed in PBS and incubated for 1 h at 37°C with the secondary antibodies. In some experiments rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) was included with the secondary antibody to visualize filamentous actin.

Treatment with Cytoskeletal-disrupting Drugs

To assess the association of calcineurin with microtubules and microfilaments, cultures were treated with nocodazole or cytochalasin D, respectively. For these drug treatments, fresh culture medium with 10 μg/ml nocodazole (Sigma) or 5 μg/ml cytochalasin D (Sigma) was added. The drugs were prepared as stock solutions in dimethylsulfoxide (DMSO) and added to the culture medium so that the concentration of DMSO did not exceed 0.1% DMSO. DMSO (0.1%) was added to the culture medium of control cells. The cultures were incubated for 1 h, rinsed twice, fixed or placed in fresh medium, and returned to the incubator for 2 h.

Treatment with Calcineurin Inhibitors

To inhibit calcineurin function, cultures were treated with cyclosporin A and the autoinhibitory peptide, ITSFEEAKGLDRINERMP-PRRDAMP (Hashimoto *et al.*, 1990). Two hours after plating, Cyclosporin A (Sandoz, East Hanover, NJ) or the autoinhibitory peptide were added directly to the culture medium to reach a final concentration of 1 μM or 50 μM, respectively. The cells were either fixed or scraped into Laemmli buffer 24 h later. For dose-response experiments cyclosporin A was added at 1 μM, 500 nM, and 50 nM final concentrations.

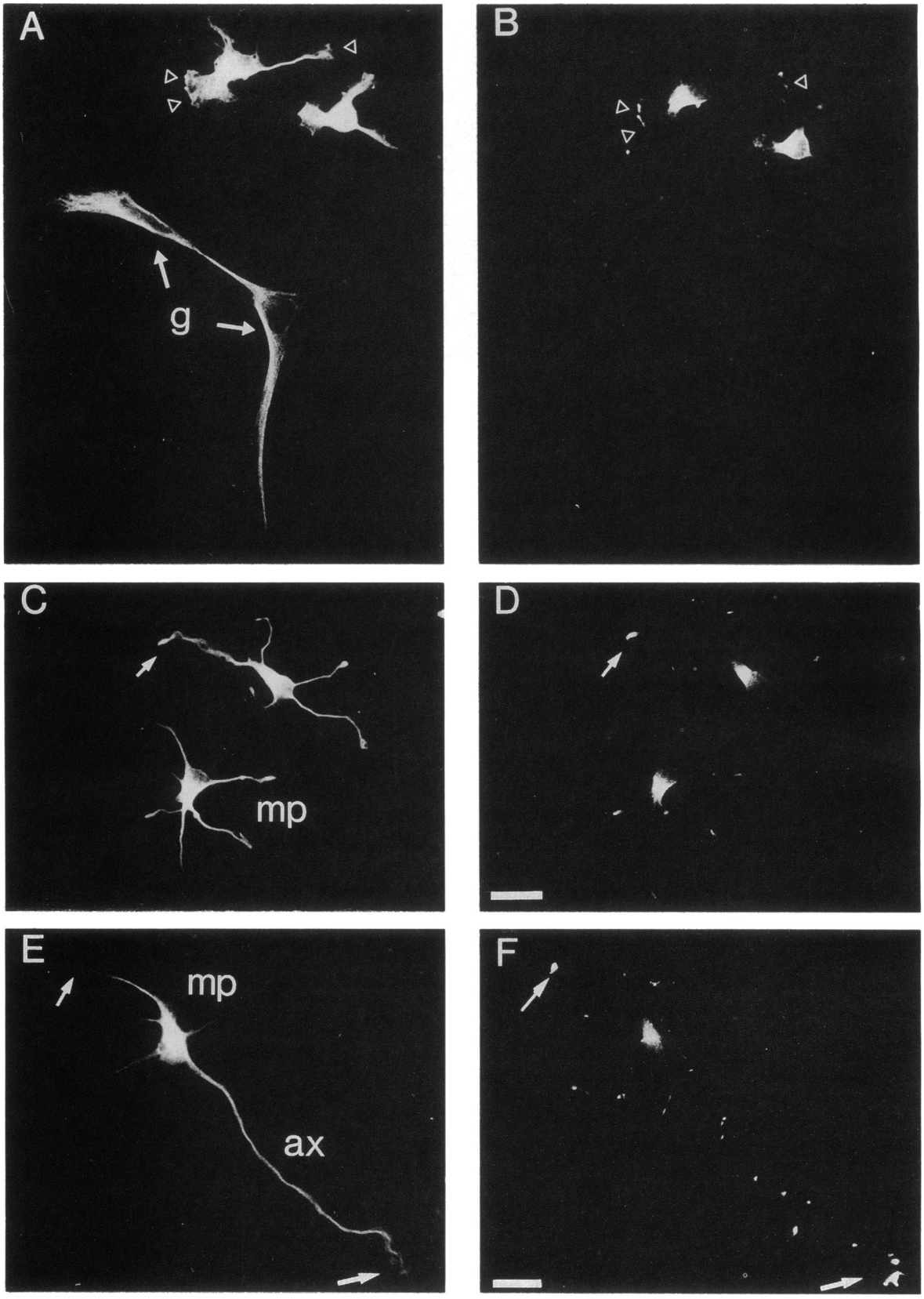
Protein Determination, Electrophoresis, and Immunoblotting

Cultures were rinsed twice in warmed PBS, scraped into Laemmli buffer, homogenized in a boiling water bath for 5 min, and centrifuged at 33 000 rpm. The supernatant was removed and stored at −80°C until use. Protein concentration was determined by the method of Lowry *et al.* (1951) as modified by Bensadoun and Weinstein (1976). Sodium dodecyl sulfate (SDS)-polyacrylamide gels were run according to Laemmli (1970). Transfer of proteins to Immobilon membranes (Millipore, Bedford, MA) and immunodetection were performed according to Towbin *et al.* (1979), as modified by Ferreira *et al.* (1989).

RESULTS

Specificity of Calcineurin Antibody in Cerebellar Tissue

The specificity of the antibody pattern labeled by calcineurin antibodies in this study was determined in several ways. The goat anti-calcineurin was used to immunoblot whole cell homogenates from cerebellar cultures 1 d after plating. Figure 1A shows the specificity of the antibody that recognizes a band at 60 kDa, the apparent molecular weight of the calcineurin catalytic subunit. The specificity was further confirmed by the preadsorption experiment described in MATERIALS AND METHODS. Figure 1, B and C show that immunoreactivity was eliminated by adsorbing the antibody with purified calcineurin. Finally, the pattern of staining was reproduced by a second independent calcineurin antibody, which reacts specifically with the catalytic subunit of this phosphatase (see rabbit antibody in MATERIALS AND METHODS).


A
g
B
C
mp
D
E
mp
ax
F

Localization of Calcineurin to the Tips of Growing Neurites

Calcineurin antibodies were used to label cerebellar macroneurons undergoing neurite elongation in culture. These neurons develop in a predictable fashion through a series of well-characterized stages (Ferreira and Caceres, 1989). Shortly after plating, the neurons elaborate ,R,I0na lammellopodial veil (stage I, Figure 2A). Within the first 12 h the veil consolidates to form minor neurites (stage II, Figure 2C). By 24 h one of the minor neurites elongates to form an axon-like process, and there is a modest narrowing of the remaining minor neurites (stage III, Figure 2E). At 72 h the minor neurites begin to undergo dendritic differentiation (stage IV).

During stage I, calcineurin immunofluorescence was detected in the cell body and within a discrete rim along the distal edge of the lammellopodial veil (Figure 2B). The occasional glial cells observed in these cultures were not labeled by the calcineurin antibody (Figure 2B). At stage II, calcineurin staining diminished somewhat in the cell body and was localized to the tips of the minor neurites whether or not a spread and flattened growth cone-like structure was present at the tip. Most of the cerebellar macroneurons have four to five minor neurites, and calcineurin immunoreactivity was usually detectable in three to four of them (Figure 2D). At stage III, there was a further diminution of immunoreactivity within the cell body, whereas staining at the process tips remained intense (Figure 2F). Occasionally an isolated point of labeling was observed at a site along the axon shaft; such sites may anticipate collateralization. At stage IV, cell body labeling remained weak, and labeling at the tips of both axonal and dendritic processes was apparent (Figure 3B). At 14 d after plating when the neurons had developed extensive neuronal contacts and synapses were present (stage V, Figure 3C), calcineurin immunofluorescence shifted from the neurite tip to the shaft of both axons and dendrites (Figure 3D).

Association of Calcineurin with the Neuronal Cytoskeleton

To determine whether calcineurin is associated with cytoskeletal elements in the growth cone, after 5 d in culture, neurons were extracted with 0.2% Triton for 2 min and then double stained with tubulin (Figure 4A) and calcineurin antibodies (Figure 4B). This treatment did not alter the ability of calcineurin antibodies to react with the phosphatase in neurons (Figure 4) indicating that calcineurin was tightly associated with the Triton-resistant cytoskeleton.

To examine the influence of microtubule-disrupting agents, cerebellar macroneurons after 3 d in culture were treated with nocadozole for 1 h. After nocadozole treatment, there was a redistribution of calcineurin staining to the region of the cell body (Figure 4D), whereas nocadozole-resistant stable microtubules remained intact (Figure 4C). Thus the localization of calcineurin to the growth cone depends upon intact microtubules, particularly the more labile microtubules disrupted by nocadozole and found in the growth cone. No changes were observed in F-actin after nocadozole treatment (Figure 4, E and F).

The preferential localization of calcineurin in the growth cones of cerebellar macroneurons and its association there with the cytoskeleton prompted us to carry out double-labeling experiments with rhodamine-tagged phalloidin and calcineurin antibodies. The rhodamine-tagged phalloidin revealed the expected pattern of intense staining at the tips of growth cones where there are bundles of filamentous actin, and this staining colocalized with that for calcineurin (Figure 5).

When the cells were treated with cytochalasin D (5 μg/ml) after 3 d in culture, the pattern of labeling with the calcineurin antibody shifted from the growth cones to the shafts of the neurites (Figure 5D). The dislocation of calcineurin from growth cones was accompanied by a dissociation of calcineurin from the disrupted actin filaments as revealed by double-labeling of the cells with rhodamine-tagged phalloidin, which no longer colocalized with calcineurin (Figure 5). This observation suggests that the compartmentalization of calcineurin in growth cones depends upon intact actin filaments. After the cytochalasin treatment, the rhodamine-phalloidin label shifted from the entire growth cone to a small region concentrated at the edge, as observed previously in *Aplysia* growth cones (Forscher and Smith, 1988).

Effect of Calcineurin Inhibition on Cerebellar Macroneurons

The immunocytochemical correlations observed for calcineurin and the cytoskeletal components in the growth cone do not demonstrate, *a priori*, a functional relationship. To assess the function of calcineurin in neurons, we exposed the cells to reagents that selectively inhibit this phosphatase. One of these, the immunosuppressant Cyclosporin A, binds to its cytosolic receptor, cyclophilin (Swanson *et al.*, 1992), forming a complex that is capable of inhibiting calcineurin *in vitro* and *in vivo* (Liu *et al.*, 1991; Clipstone and Crabtree, 1992;

Figure 2. Double labeling of cerebellar macroneurons with tubulin (A, C, and E) and calcineurin (B, D, and F) antibodies at 3 h (A and B), 8 h (C and D), and 24 h (E and F) after plating. The calcineurin antibody labels cell bodies, the rim of lamellopodial veil (open arrow heads), and the growing tips (arrows). Axons at 24 h (F) have calcineurin immunoreactivity along the shaft consistent with filopodial spikes occuring abundantly early in development. Note the absence of calcineurin immunostaining in glial cells (g). Minor processes (mp) and axons (ax) are indicated. Bar, 20 μm.

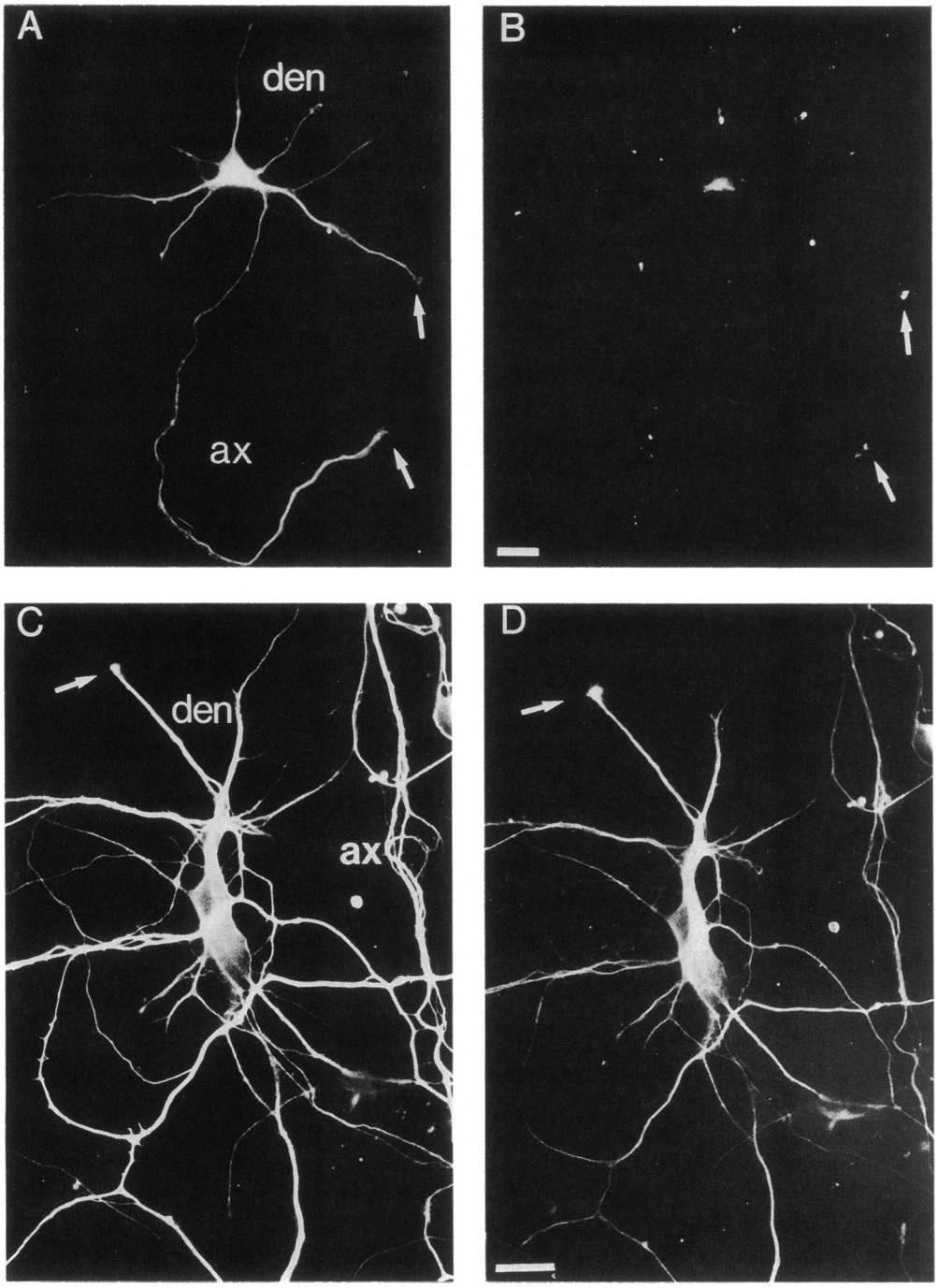


Figure 3. Double labeling of cerebellar macroneurons 7 d (A and B) and 14 d (C and D) after plating stained with tubulin (A and C) and calcineurin (B and D) antibodies. Calcineurin labels axonal (ax) and dendritic (den) growth cones at 7 d after plating. In the more mature cultures calcineurin staining shifts from the growth cones (arrows) to the axonal and dendritic processes. Bar, 20, μm.

Fruman *et al.*, 1992; O'Keefe *et al.*, 1992). To test its effect on neuronal phenotype, cerebellar macroneurons were exposed to various doses of Cyclosporin A ranging from 50 nM to 1000 nM for the first 24 h after plating. At 24 h the cells were fixed, immunolabeled (Figure 6), and analyzed with regard to their development of polarity (Figure 7). The numbers of cells with a symmetric array of minor neurites, e.g., stage II, were compared to those exhibiting a single process longer than the others, e.g., stage III. The numbers of stage II cells increased, and the numbers of stage III cells decreased as a function of the Cyclosporin dose. Thus neurite formation was unaffected by Cyclosporin A; instead, the selective elongation of a single neurite to form the axon was impaired. To confirm that this effect was mediated through inhibition of calcineurin, a synthetic peptide corresponding to the autoinhibitory domain of calcineurin (Hashimoto *et al.*, 1990) was added to the culture me-

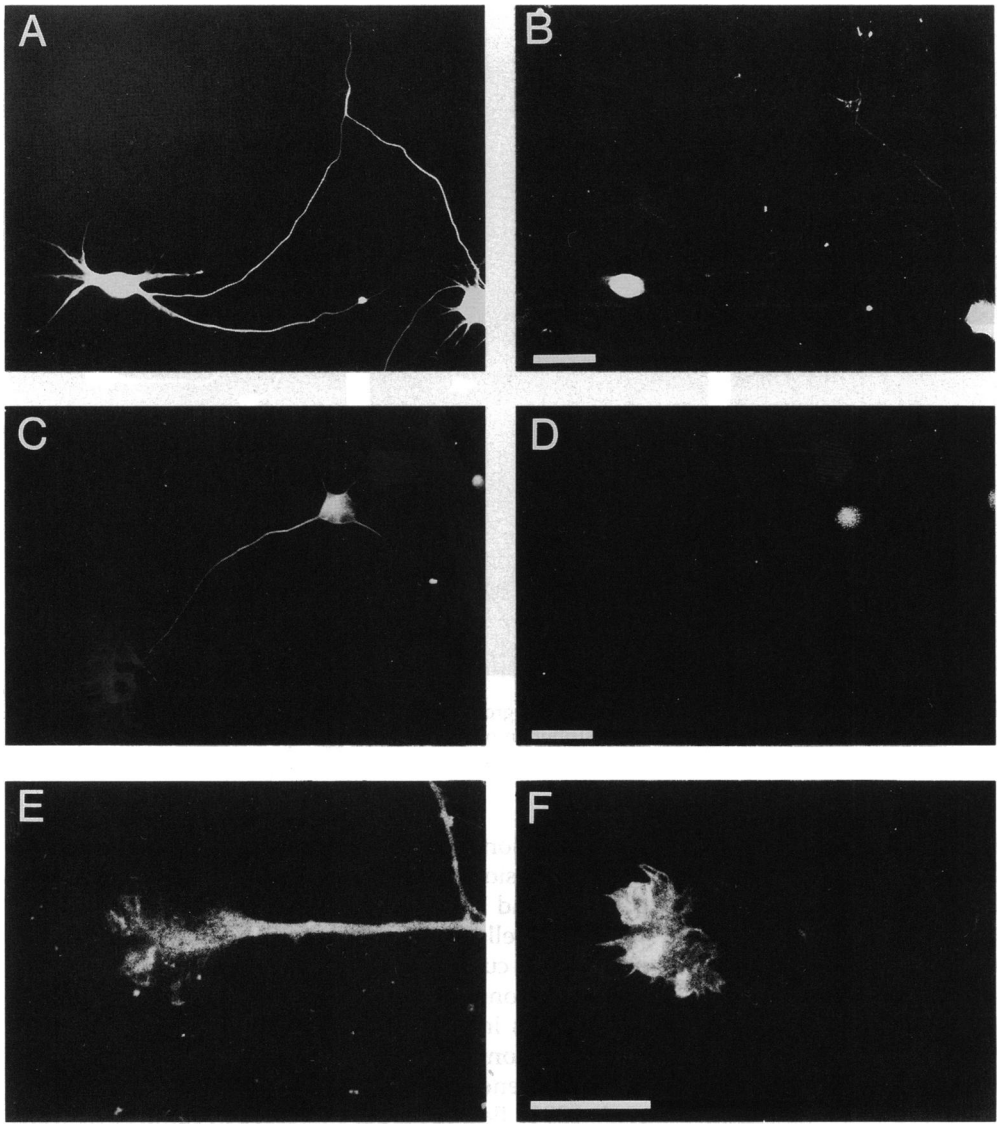


Figure 4. The association of calcineurin with the cytoskeleton in cerebellar macroneurons 5 d after plating. Extraction with Triton X-100 before fixation and double labeling with tubulin (A) and calcineurin (B) antibodies. Calcineurin staining is retained in the tips after extraction. Depolymerization of microtubules by nocadozole resulted in the redistribution of calcineurin from the growth cone to the cell body seen with calcineurin antibodies in D and the same cells double labeled with an antibody against acetylated microtubules (C) indicating the population of stable microtubules that remain after nocadozole. (E and F) The distribution of F-actin visualized with rhodamine-phalloidin is not altered by nocadozole. Bar, 20 μm.

dium. At 50 μM, this peptide also inhibited the stage II to stage III transition (Figures 6 and 7).

We had previously shown that suppression of the MAP tau, using antisense oligonucleotides, also blocked the stage II to stage III transition (Caceres and Kosik, 1990), and we hypothesized that emergence of the axon in stage III involves the binding of tau to microtubules within the rapidly elongating process. Because the phosphorylation state of tau regulates its binding to microtubules, we used antibodies directed against a specific phosphorylation site in tau under conditions of calcineurin inhibition. The mAbs tau-1 (Kosik *et al.*, 1988) and AT8 (Biernat *et al.*, 1992) are directed against a region of tau that spans approximately amino acid 131–149. When the site is phosphorylated, tau protein reacts with AT8, and when the site lacks phosphate, tau is reactive with tau-1 (Binder *et al.*, 1985; Biernat *et al.*, 1992). Cerebellar macroneurons after 24 h in culture were scraped and analyzed by immunoblots. Under control conditions the cells were reactive with tau-1 but not with AT8, indicative of the tau dephosphorylation that occurs as a function of development (Figure 8). Cells treated with Cyclosporin A as described above were not reactive with tau-1 but were reactive with AT8 (Figure 8). No changes were observed in the total tubulin levels and total tau levels as revealed by a mAb against a tau nonphosphorylated epitope (Figure 8).

The acquisition of tau-1 reactivity during development and the reduced reactivity on immunoblots with this antibody after Cyclosporin treatment was paralleled by altered tau-1 immunohistochemistry on the cultured cells. Under control conditions, tau-1 stains neurons and their minor neurites at stage II. Generalized cell staining is apparent at stage III (Figure 9, A and B); however, at

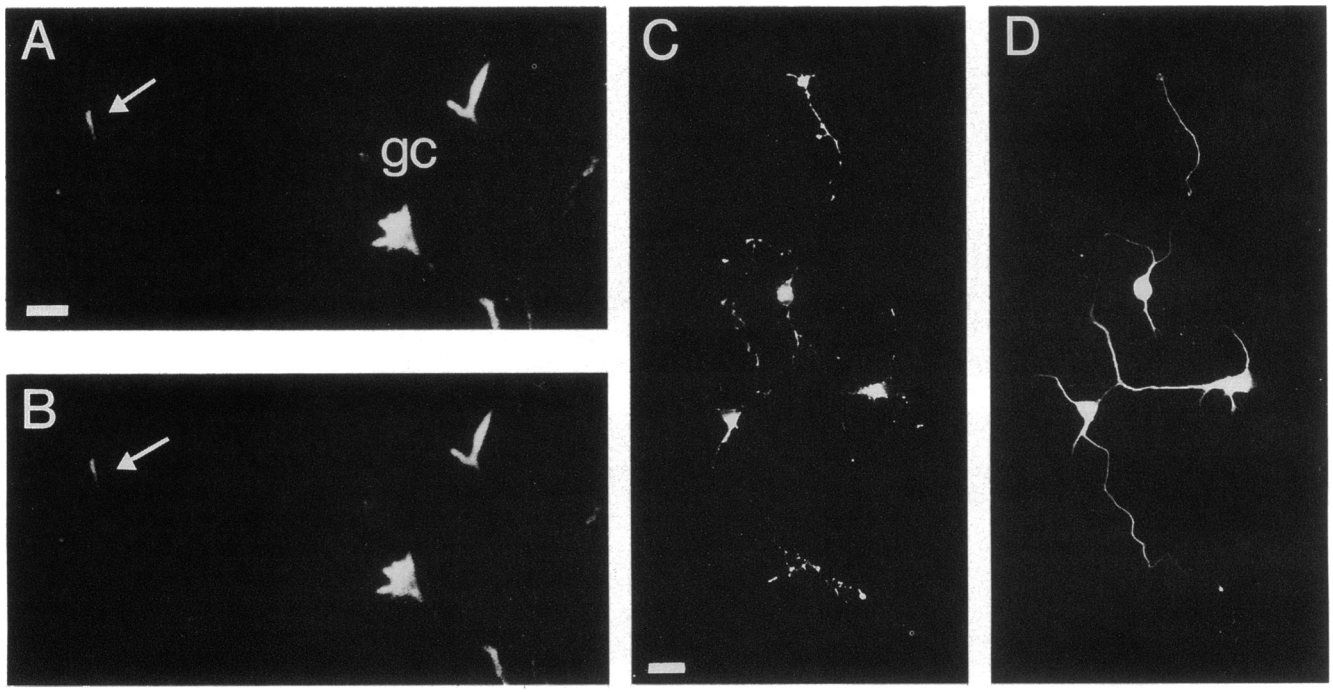


Figure 5. Colocalization of rhodamine-tagged phalloidin (A) and calcineurin (B) in growth cones (gc) and the tips of processes (arrow). Treatment of cerebellar macroneurons with cytochalasin D resulted in the redistribution of rhodamine-tagged phalloidin (C) and calcineurin (D) to the neurite processes. Bar, 20 μm.

this stage extraction of the cells with Triton reveals tau-1 staining only in the axonal process (Ferreira *et al.*, 1989). Cyclosporin treatment completely inhibits tau-1 staining of all the cells regardless of their stage (Figure 9, C and D). One role of phosphorylated tau involves the stabilization of microtubules, particularly that population of microtubules undergoing rapid assembly in the elongating axon. Stable microtubules in neurons are acetylated on the α-tubulin, a modification that can be detected with the specific antibody, 6-11B-1 (Piperno *et al.*, 1987). Because acetylation is inhibited when tau expression is suppressed by antisense oligonucleotides (Caceres *et al.*, 1992), we determined whether Cyclosporin treatment alters acetylation of the tubulin. After a 24-h treatment with the drug, cerebellar macroneurons were reactive with antibody 6-11B-1. Typically the Cyclosporin-treated cells had a symmetric array of minor neurites (see above). Only one of these neurites was reactive with 6-11B-1 (Figure 9, F and G), suggesting that the treated cells were competent to develop an asymmetry in the distribution of stable microtubules, a population of microtubules that very likely anticipates rapid elongation during the acquisition of polarity.

DISCUSSION

Tau Phosphorylation and Neuronal Polarity

Studies using a variety of techniques suggest that tau protein functions in the rapid elongation phase of axonal differentiation. Evidence for this role comes from both tau suppression studies using antisense oligonucleotides (Caceres and Kosik, 1990) and from the expression of tau in Sf9 cells (Knops *et al.*, 1991). Suppression of tau in neuronal cultures prevents axonal elongation despite the formation of minor neurites. Tau expression in Sf9 cells results in the elaboration of long, usually single processes containing bundles of microtubules as well as a more general reorganization of the cytoplasm (Baas *et al.*, 1991). Because tau is expressed in cultured neurons before axonal elongation occurs, mechanisms other than those relying on altered expression of the tau protein must exist to regulate tau function. One of these mechanisms is likely to be the phosphorylation state of tau. Although the *in vivo* kinases and phosphatases for tau have not been identified, one candidate control point based on our data is the Ca^{2+}-sensitive phosphatase, calcineurin.

Recent studies have identified calcineurin as the primary target of the immunosuppressive drugs Cyclosporin A and FK-506 in T cells. The drug-induced conformations of the receptor proteins or "immunophilins" specifically bind to calcineurin inhibiting its protein phosphatase activity (Liu *et al.*, 1991). This, in turn, prevents the activation of Ca^{2+}-dependent signaling pathways for transcription factors (e.g., NFAT and NFIL2A) involved in lymphokine gene expression. Because of the ubiquitous distribution of cyclophilins, the

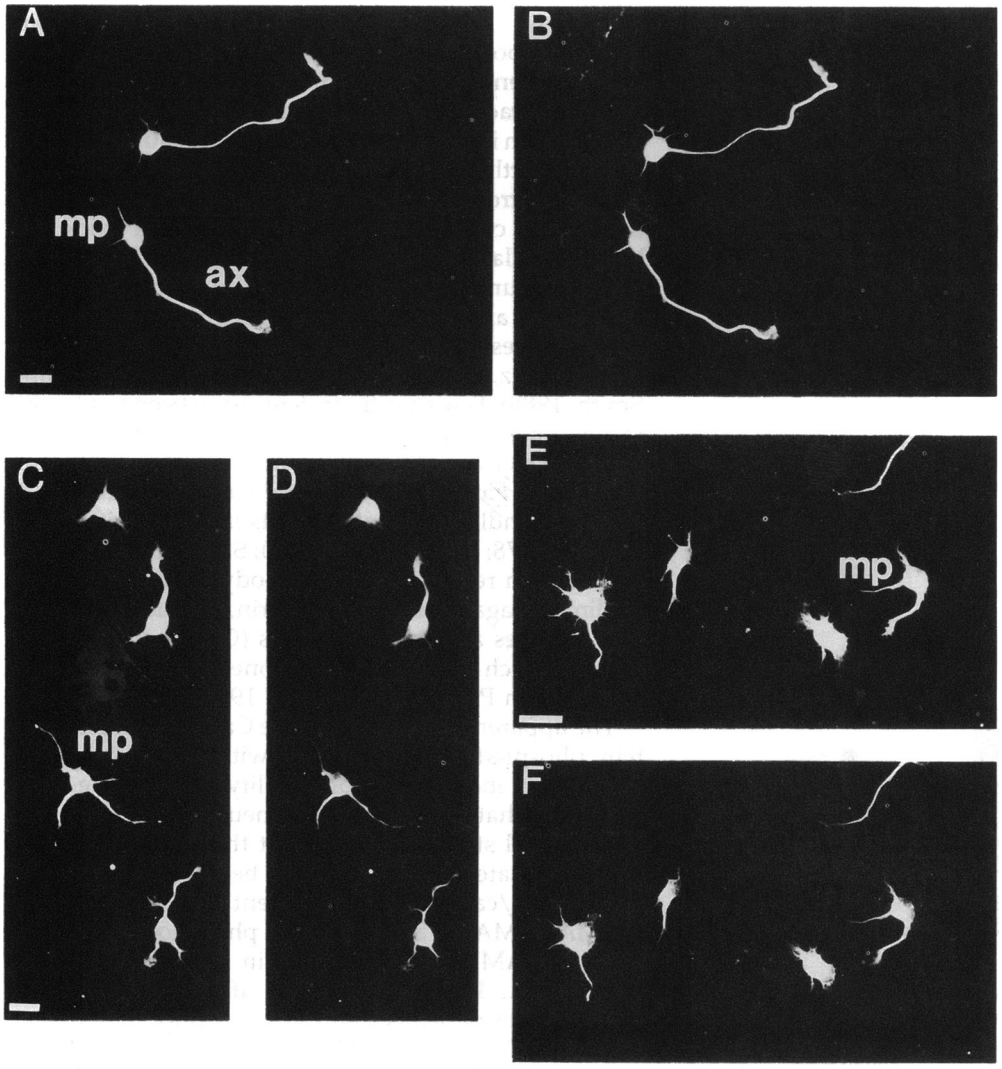


Figure 6. Treatment of cerebellar macroneurons with calcineurin inhibitors. Twenty-four hours after plating cerebellar macroneurons have elongated several minor processes (mp) and an axon (ax) (A and B). Treatment with cyclosporin (C and D) or the calcineurin autoinhibitory peptide (E and F) resulted in the failure to elaborate an axon despite the presence of minor processes. The cells were double labeled with tubulin (A, C, and E) and tau (monoclonal 5E2) (B, D, and F) antibodies. Bar, 20 μm.

effects of Cyclosporin A may provide a powerful tool to investigate the role of calcineurin in mediating Ca^{2+}-dependent events in nonlymphoid cells. Indeed, the phenotypic consequences of drug treatment in cultured macroneurons were mimicked by a peptide inhibitor of this phosphatase, supporting the notion that these events reflect functional inhibition of calcineurin. The effect of cyclosporin on the tau phosphorylation state suggests a biochemical mechanism by which calcineurin may regulate cytoskeletal events in cultured neurons. Because inhibition of calcineurin resulted in the loss of the tau-1 epitope and the acquisition of the AT8 epitope, it appears that a key dephosphorylation event was blocked. However, from the data presented here it is impossible to say whether the effect is direct, with tau serving as a substrate for calcineurin, or whether the effect is mediated through calcineurin's regulation of upstream kinases.

It seems likely that the altered phosphorylation state of tau induced by calcineurin inhibition resulted in the failure to elongate an axon. When cerebellar macroneurons are extracted with Triton X-100 at stage III, tau immunoreactivity is confined to the axon (Ferreira *et al.*, 1989) suggesting that the binding of tau to microtubules within the single process destined for axonal differentiation is a crucial precondition. When cerebellar macroneurons are treated with Cyclosporin and extracted with Triton X-100, the tau-immunoreactivity is lost suggesting that under the conditions of drug treatment tau is weakly bound to microtubules. Therefore phosphorylation of the tau-1 site at a minimum is associated with decreased binding of tau to microtubules. Furthermore, rapid elongation of the axon requires not only tau expression (Caceres and Kosik, 1990) but also the correct modifications of tau so that it binds to microtubules in a way that promotes neurite elongation.

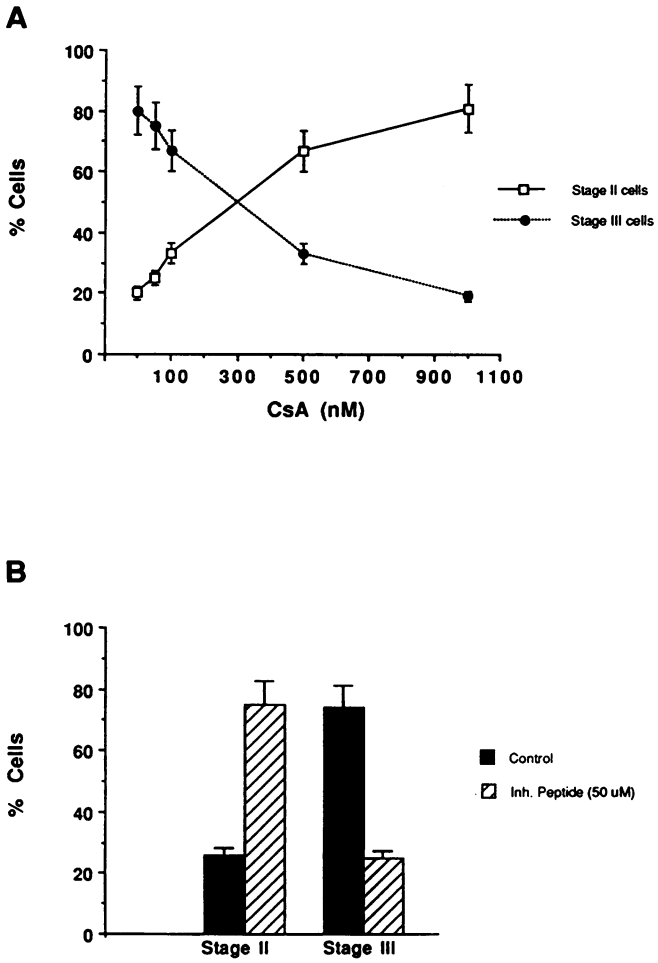


Figure 7. Effect of cyclosporin (A) and the calcineurin autoinhibitory peptide (B) on neurite outgrowth in cerebellar macroneurons after 1 d in culture. Both inhibitors affect the transition between stages II and III. A total of 300 cells from three different cultures were analyzed for each condition. Each value represents the mean ± SEM. For all the conditions analyzed, comparisons with the controls were significant with $P < 0.005$.

Although the Cyclosporin-treated neurons do not elaborate an axon, microtubules in drug-treated cells become acetylated, first within a single neurite. Under control conditions, acetylation occurs only in the axon of stage III cerebellar macroneurons (Ferreira and Caceres, 1989) and later appears in all neurites. Acetylation, therefore, is a useful marker for incipient polarity. After 24 h, cultures treated with Cyclosporin contain acetylated microtubules in a single process despite the absence of an axon. Thus a minor neurite can acquire some features of an axon without undergoing rapid elongation.

Possible Control Points for Calcineurin

As shown here calcineurin is highly concentrated in growth cones during the initial phases of neurite development, and its localization within the growth cone depends upon the integrity of both microtubules and microfilaments. Several studies have provided evidence for an interaction between microtubules and the actin cytoskeleton in the growth cone. Letourneau and Ressler (1983) directly visualized close associations between the ends of microtubules and bundles of actin filaments in the growth cones of cultured neurons from chick embryos. Similarly the reorganization that follows treatment of neuronal-like cells in culture with either cytochalasin D and/or colchicine points to an interaction between these systems (Bray *et al.*, 1978; Solomon and Magendantz, 1981; Joshi *et al.*, 1985; Forscher and Smith, 1988). Few specific proteins have been implicated in this interaction. One putatively involved protein is MAP2, which can be observed in neuronal growth cones (Kosik and Finch, 1987) and induces the formation of discrete bundles of actin filaments *in vitro* (Griffith and Pollard, 1978; Nishida *et al.*, 1980; Sattilaro *et al.*, 1981). The antigen recognized by antibody 13H9, thought to be directed against the protein ezrin, interacts with both microtubules and microfilaments (Goslin *et al.*, 1989). Another such protein may the one recognized by the mAb 2E4 in PC-12 cells (Bearer, 1992).

The apparent association of the CaM-dependent protein phosphatase, calcineurin, with cytoskeletal elements may increase the probability of its acting upon substrates that are important for neurite outgrowth. Enzymological studies showed that this enzyme can dephosphorylate tubulin that has been phosphorylated by calcium/calmodulin-dependent kinase, as well as the MAPs, MAP-2 and tau, when phosphorylated either by cyclic AMP or Ca-calmodulin dependent kinases (Goto *et al.*, 1985). Because the affinity of MAPs for microtubules depends upon the phosphorylation state of the MAP (Brugg and Matus, 1991), calcineurin may be involved in neurite elongation by regulating the binding of MAPs to microtubules. As suggested by our findings, calcineurin may regulate the state of tau phosphorylation.

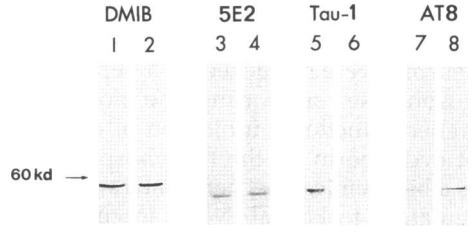


Figure 8. Immunoblot analysis of whole cell homogenates after 24 h in culture labeled with antibodies against tubulin (lanes 1 and 2) and tau proteins (lanes 3–8). Lanes 1, 3, 5, and 7 are from control cerebellar macroneurons, and lanes 2, 4, 6, and 8 are treated with cyclosporin for 24 h. Ten micrograms of protein was loaded in lanes 1 and 2 and 50 μg in lanes 3–8. Tau-1 reactivity is lost as a result of cyclosporin treatment (lane 6), and AT8 reactivity is gained (lane 8). DM1B is a mAb against β-tubulin, and 5E2 is a mAb against tau protein.

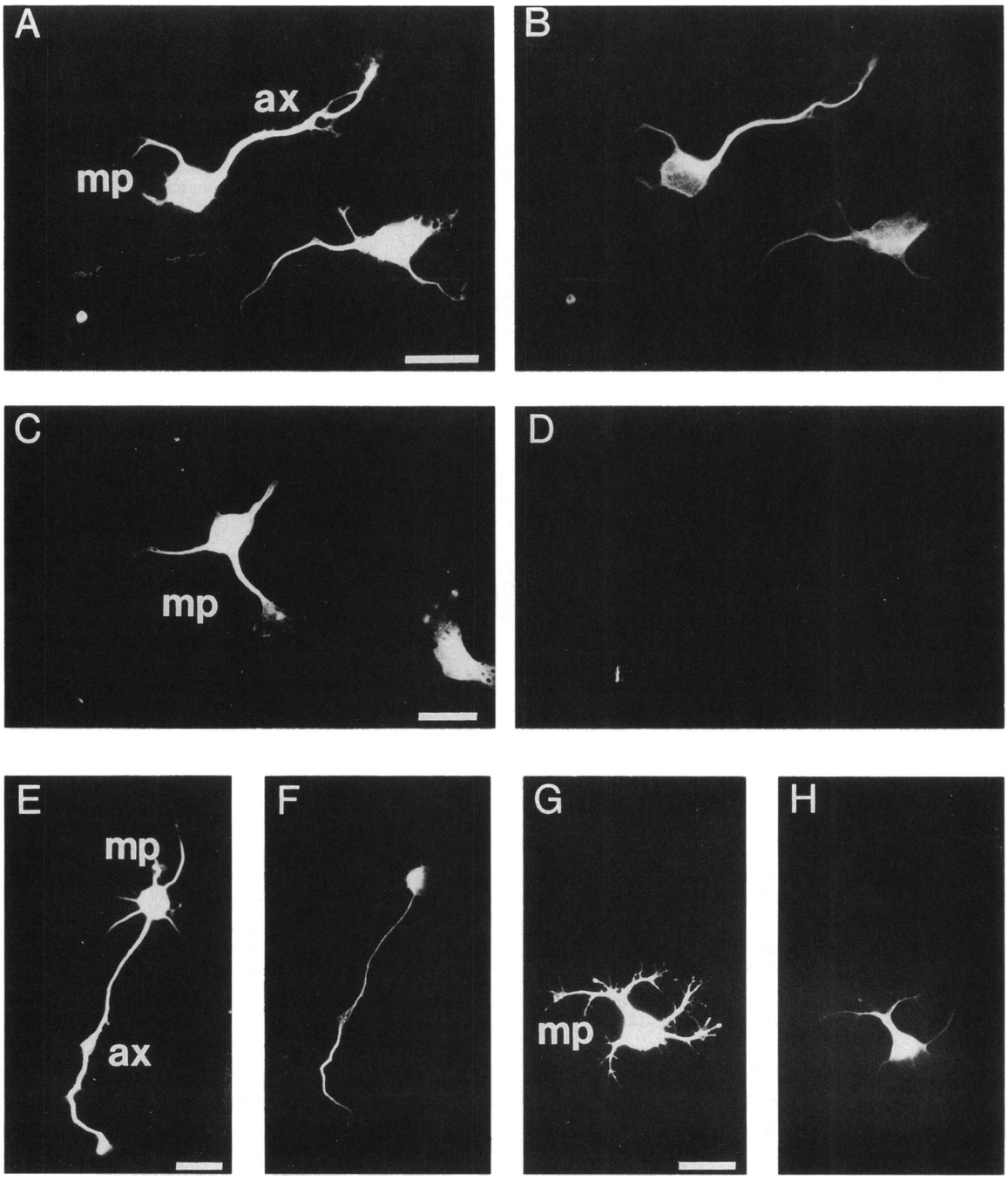


Figure 9. The distribution of tau-1 immunoreactivity (B and D) and acetylated microtubules (F and H) in control (A, B, E, and F) and cyclosporine-treated (C, D, G, and H) cerebellar macroneurons 1 d after plating. No tau-1 immunoreactivity was detected in cyclosporin-treated neurons (D). The cells were double labeled with a tubulin antibody (A, C, E, and G). Minor processes (mp) and axons (ax) are indicated. Bar, 20 μm.

Although present in almost all growth cones, calcineurin may be selectively activated by binding the Ca^{2+}-liganded form of CaM in those growth cones that, because of environmental factors, permit calcium entry. As a calcium "sensor", local variations in environmental Ca^{2+} could lead to the differential activation of calcineurin among neurites and subsequently polarity. Filopodia protruding from the growth cone may arise from one portion of the membrane in response to a focal rise in intracellular calcium (Goldberg, 1988). This type of

focal behavior may be accomplished by preventing the diffusion of regulatory gene products through the cell by linking these elements to the cytoskeleton. Labile microtubule populations may target calcineurin to specific regions within the growth cone to control the direction in which microtubule elongation occurs. The source of the plasma membrane from which new growth cone formation derives may be the stream of membrane-bound organelles moving through the region just behind the advancing tip and sometimes becoming trapped at the most distal edges of growth cones (Sheetz *et al.*, 1990). The finding that calcineurin may regulate organelle directionality in melanophores by switching their translocation from anterograde to retrograde (Thaler and Haimo, 1990) suggests that it may serve to control the flow of membrane to the growth cone and consequently neurite elongation. Thus, there may be several potential control points that might impinge on the activity and/or accessability of this phosphatase to potential substrates in growth cones. Such regulation may be important to preserve flexibility in "decision-making" during determination of neuronal polarity.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants AG-06601 and NS-29031 to K.S.K.

REFERENCES

Alexander, K.A., Cimler, B.M., Meier, K.E., and Strom, D.R. (1987). Regulation of calmodulin binding to P-57. J. Biol. Chem. *262*, 6108–6113.

Baas, P.W., Pienkowski, T.P., and Kosik, K.S. (1991). Processes induced by tau expression in Sf9 cells have an axon-like microtubule organization. J. Cell Biol. *115*, 1333–1344.

Bamburg, J.R., Bray, D., and Chapman, K. (1986). Assembly of microtubules at the tip of growing axons. Nature *321*, 788–790.

Bearer, E.L. (1992). An actin-associated protein present in the microtubule organizing center and the growth cones of PC-12 cells. J. Neurosci. *12*, 750–761.

Bensadoun, A., and Weinstein, R. (1976). Assay of protein in the presence of interfering materials. Anal. Biochem. *70*, 241–250.

Biernat, J., Mandelkow, E.-M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M., and Mandelkow, E. (1992). The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. EMBO J. *11*, 1593–1597.

Binder, L.I., Frankfurter, A., and Rebhun, L.I. (1985). The distribution of tau in the mammalian central nervous system. J. Cell Biol. *103*, 545–557.

Bottenstein, J.E., and Sato, G.H. (1979). Growth of a rat neuroblastoma cell line in serum-free supplemented media. Proc. Natl. Acad. Sci. USA *76*, 514–517.

Bray, D., Thomas, C., and Shaw, G. (1978). Growth cone formation in cultures of sensory neurons. Proc. Natl. Acad. Sci. USA *75*, 5226–5229.

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

Caceres, A., and Kosik, K.S. (1990). Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. Nature *343*, 461–463.

Caceres, A., Mautino, J., and Kosik, K.S. (1992). Suppression of MAP-2 in cultured cerebellar macroneurons inhibits minor neurite formation. Neuron *9*, 607–618.

Carlin, R.K., Bartlett, D.C., and Siekevitz, P. (1983). Identification of fodrin as a major calmodulin-binding protein in postsynaptic density preparations. J. Cell Biol. *96*, 443–448.

Cheng, T.P.O., and Reese, T.S. (1985). Polarized compartmentalization of organelles in growth cones from developing optic tectum. J. Cell Biol. *101*, 1473–1480.

Clipstone, N.A., and Crabtree, G.R. (1992). Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. Nature *357*, 695–697.

Dotti, C.G., Sullivan, C.A., and Banker, G.A. (1988). The establishment of polarity by hippocampal neurons in culture. J. Neurosci. *8*, 1454–1468.

Drechsel, D.N., Hyman, A.A., Cobb, M.H., and Kirschner, M.W. (1992). Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. Mol. Biol. Cell *3*, 1141–1154.

Ferreira, A., Busciglio, J., and Caceres, A. (1989). Microtubule formation and neurite growth in cerebellar macroneurons which develop in vitro: evidence for the involvement of the microtubule associated proteins, MAP-1a, HMW-MAP2, and tau. Dev. Brain Res. *49*, 215–228.

Ferreira, A., and Caceres, A. (1989). The expression of acetylated microtubules during axonal and dendritic growth in cerebellar macroneurons which develop *in vitro*. Dev. Brain Res. *49*, 204–213.

Forscher, P., and Smith, S.J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J. Cell Biol. *107*, 1505–1516.

Fruman, D.A., Klee, C.B., Bierer, B.E., and Burakoff, S.J. (1992). Calcineurin phosphatase activity in T lymphocytes is inhibited by FK506 and Cyclosporin A. Proc. Natl. Acad. Sci. USA *39*, 3686–3690.

Goedert, M., Cohen, E.S., Jakes, R., and Cohen, P. (1992). p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase $2A_1$. FEBS Lett. *312*, 95–99.

Goldberg, D.J. (1988). Local role of $Ca2^+$ in formation of veils in growth cones. J. Neurosci. *8*, 2596–2605.

Goslin, K., and Banker, G. (1989). Experimental observations on the development of polarity by hippocampal neurons in culture. J. Cell Biol. *108*, 1507–1516.

Goslin, K., Birgbauer, E., Banker, G., and Solomon, F. (1989). The role of cytoskeleton in organizing growth cones: a microfilament-associated growth cone component depends upon microtubules for its localization. J. Cell Biol. *109*, 1621–1631.

Goto, S., Matsukado, Y., Mihara, Y., Inoue, N., and Miyamoto, E. (1986). Calcineurin in human brain and its relation to extrapyramidal system. Acta Neuropathol. *72*, 150–156.

Goto, S., Yamamoto, H., Fukunaga, K., Iwasa, T., Matsukado, Y., and Miyamoto, E. (1985). Dephosphorylation of microtubule associated protein 2, tau factor and tubulin by calcineurin. J. Neurochem. *45*, 276–283.

Griffith, L.M., and Pollard, T.D. (1978). Evidence for actin-filament-microtubule interaction mediated by microtubule-associated proteins. J. Cell Biol. *78*, 958–965.

Griffith, L.M., and Pollard, T.D. (1982). The interaction of actin filaments with microtubules and microtubule-associated proteins. J. Biol. Chem. *257*, 9143–9151.

Guerini, D., and Klee, C.B. (1989). Cloning of human calcineurin A: evidence for two isozymes and identification of a polyproline structural domain. Proc. Natl. Acad. Sci. USA *86*, 9183–9187.

Hashimoto, Y., Perrino, B.A., and Soderling, T.R. (1990). Identification of an autoinhibitory domain in calcineurin. J. Biol. Chem. *265*, 1924–1927.

Hirokawa, N. (1982). Cross-linker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by the quick freeze, deep-etching method. J. Cell Biol. *94*, 129–142.

Joshi, H., Chu, D., Buxbaum, R., and Heidemann, S.R. (1985). Tension and compression in the cytoskeleton of PC12 neurites. J. Cell Biol. *101*, 697–705.

Kincaid, R.L. (1988). Preparation, characterization, and properties of affinity-purified antibodies to calmodulin-dependent cyclic nucleotide phosphodiesterase and protein phosphatase (calcineurin). Methods Enzymol. *159*, 627–651.

Kincaid, R.L., Balaban, C.D., and Billingsley, M.L. (1987). Differential localization of calmodulin-dependent enzymes in rat brain: evidence for selective expression of cyclic nucleotide phosphodiesterase in specific neurons. Proc. Natl. Acad. Sci. USA *84*, 1118–1122.

Kincaid, R.L., Giri, P.R., Higuchi, S., Tamura, J., Dixon, S., Marietta, C.A., Amorese, D.A., and Martin, B.M. (1990). Cloning and characterization of molecular isoforms of the catalytic subunit of calcineurin using nonisotopic methods. J. Biol. Chem. *265*, 11312–11319.

Kincaid, R.L., Manganiello, V.C., Odya, C.E., Osborne, J.C., Jr., Stith-Coleman, I.E., Danello, M.A., and Vaughan, M. (1984). Purification and properties of calmodulin-stimulated phosphodiesterase from mammalian brain. J. Biol. Chem. *259*, 5158–5166.

King, M.M., Huang, C.Y., Chock, P.B., Nairn, A.C., Hemmings, H.C., Jr., Chan, J., and Greengard, P. (1984). Mammalian brain phosphoproteins as substrates for calcineurin. J. Biol. Chem. *259*, 8080–8083.

Klee, C.B., Crouch, T.H., and Krinks, M.H. (1979). Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. USA *76*, 6270–6273.

Klee, C.B., Draetta, G.F., and Hubbard, M.J. (1988). Calcineurin. Adv. Enzymol. Relat. Areas Mol. Biol. *61*, 149–200.

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

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

Kosik, K.S., Orecchio, L.D., Binder, L.I., Trojanowski, J., Lee, V., and Lee, G. (1988). Epitopes that span the tau molecule are shared with paired helical filaments. Neuron *1*, 817–825.

Kumagi, H., Nishida, E., and Sakai, H. (1982). The interaction of calmodulin and microtubule proteins. IV. Quantitative analysis of the binding between calmodulin and tubulin dimer. J. Biochem. *91*, 1329–1336.

Laemmli, U.K. (1970). Cleavage of structural protein during the assembly of the head of the bacteriophage T4. Nature *227*, 680–685.

Letourneau, P.C., and Ressler, A.H. (1983). Differences in the organization of actin in the growth cones compared with the neurites of culture neurons from chick embryos. J. Cell Biol. *97*, 963–973.

Lindwall, G., and Cole, R.D. (1984). Phosphorylation affects the ability of tau protein to promote microtubule assembly. J. Biol. Chem. *259*, 5301–5305.

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807–815.

Liu, Y., and Storm, D.R. (1989). Dephosphorylation of neuromodulin by calcineurin. J. Biol. Chem. *264*, 12800–12804.

Lowry, O.H., Resebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurements with the folin phenol reagent. J. Biol. Chem. *193*, 265–275.

Meiri, K.F., Pfenninger, K.H., and Willard, M. (1986). Growth associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. Proc. Natl. Acad. Sci. USA *83*, 3537–3541.

Muramatsu, T., Rathna Giri, P., Higuchi, S., and Kincaid, R.L. (1992). Molecular cloning of a calmodulin-dependent phosphatase from murine testis: identification of a developmentally-expressed nonneural isoenzyme. Proc. Natl. Acad. Sci. USA *89*, 529–533.

Nishida, E., Kuwake, T., and Sakai, H. (1980). Phosphorylation of microtubule associated proteins (MAPs) and pH of the medium control interaction between MAPs and actin filaments. J. Biochem. *90*, 575–578.

O'Keefe, S.J., Tamura, J., Kincaid, R.L., Tocci, M.J., and O'Neill, E.A. (1992). FK-506 and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature *357*, 692–694.

Owada, M.K., Hakura, A., Ilda, K., Yahara, I., Sobue, K., and Kakiuchi, S. (1984). Occurrence of caldesmon (a calmodulin-binding protein) in cultured cells: comparison of normal and transformed cells. Proc. Natl. Acad. Sci. USA *81*, 3133–3137.

Piperno, G., Le-Dizet, M., and Chang, X. (1987). Microtubules containing acetylated alpha tubulin in mammalian cells in culture. J. Cell Biol. *104*, 289–302.

Polli, J.W., Billingsley, M.L., and Kincaid, R.L. (1991). Expression of the calmodulin-dependent phosphodiesterase, calcineurin, in rat brain: developmental patterns and the role of nigrostriatal innervation. Dev. Brain Res. *61*, 105–119.

Sabry, J.H., O'Conner, T.P., Evans, L., Toroian-Raymond, A., Kirschner, M., and Bentley, D. (1991). Microtubule behavior during guidance of pioneer neuron growth cones *in situ*. J. Cell Biol. *115*, 381–395.

Sattilaro, R.F., Dentler, W.L., and LeCluyse, E.L. (1981). Microtubule associated proteins (MAPs) and the organization of actin filaments *in vitro*. J. Cell Biol. *90*, 467–473.

Schnapp, B.J., and Reese, T.S. (1982). Cytoplasmic structure in rapid-frozen axons. J. Cell Biol. *102*, 1020–1031.

Sheetz, M.P., Baumrind, N.L., Wayne, D.B., and Pearlman, A.L. (1990). Concentration of membrane antigens by forward transport and trapping in neuronal growth cones. Cell *61*, 231–241.

Sobue, K., Kanda, K., Adachi, J., and Kakiuchi, S. (1983). Calmodulin-binding proteins that interact with actin filaments in a Ca^{2+}-dependent flip-flop manner: survey in brain and secretory tissues. Proc. Natl. Acad. Sci. USA *80*, 6868–6871.

Sobue, K., Muramoto, Y., Fujita, M., and Kakiuchi, S. (1981). Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. Proc. Natl. Acad. Sci. USA *78*, 5652–5655.

Sobue, K., Tanaka, T., Ahino, N., and Kakiuchi, S. (1985). Ca^{2+} and calmodulin regulate microtubule-associated protein-actin interaction in a flip-flop switch mechanism. Biochim. Biophys. Acta *845*, 366–372.

Solomon, F., and Magendantz, M. (1981). Cytochalasin separates microtubule disassembly from loss of asymmetric morphology. J. Cell Biol. *89*, 157–161.

Swanson, S.K.H., Born, T., Zydowsky, L.D., Cho, H., Chang, H.Y., Walsh, C.T., and Rusnak, F. (1992). Cyclosporin-mediated inhibition of bovine calcineurin by cyclophilins A and B. Proc. Natl. Acad. Sci. USA *89*, 3741–3745.

Tallant, E.A., and Cheung, W.Y. (1983). Calmodulin-dependent protein phosphatase: a developmental study. Biochemistry *22*, 3630–3635.

Tanaka, E.M., and Kirschner, M.W. (1991). Microtubule behavior in the growth cones of living neurons during axon elongation. J. Cell Biol. *115*, 345–363.

Thaler, C.D., and Haimo, L.T. (1990). Regulation of organelle transport in melanophores by calcineurin. J. Cell Biol. *111*, 1939–1948.

Towbin, H., Staehelein, T., and Gordon, J. (1979). Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4354–4356.

Ueki, K., Muramatsu, T., and Kincaid, R.L. (1992). Structure and expression of two isoforms of murine calmodulin-dependent protein phosphatase regulatory subunit (calcineurin B). Biochem. Biophys. Res. Commun. *187*, 537–543.

Wood, J.G., Wallace, R.W., Whitaker, J.N., and Cheung, W.Y. (1980). Immunocytochemical localization of calmodulin and a heat-labile calmodulin-binding protein ($CaM\text{-}BP_{80}$) in basal ganglia of mouse brain. J. Cell Biol. *84*, 8687–8691.

Yamada, K.M., Spooner, B.S., and Wessells, N.K. (1971). Ultrastructure and function of growth cones and axons of cultured nerve cells. J. Cell Biol. *49*, 614–635.

Yamamoto, H., Fukunaga, K., Goto, S., Tanaka, E., and Miyamoto, E. (1985). Ca^{2+}, calmodulin-dependent regulation of microtubule formation via phosphorylation of microtubule-associated protein 2, tau factor, and tubulin, and comparison with the cyclic AMP-dependent phosphorylation. J. Neurochem. *44*, 759–768.

Yamamoto, H., Saitoh, Y., Yasugawa, S., and Miyamoto, E. (1990). Dephosphorylation of tau factor by protein phosphatase 2A in synaptosomal cytosol fractions, and inhibition by aluminum. J. Neurochem. *55*, 683–690.

Zagon, I.S., Higbee, R., Rieder, B.M., and Goodman, C. (1986). Spectrin subtypes in mammalian brain: an immunoelectron microscopic study. J. Neurosci. *10*, 2977–2986.