Respective Roles of Neurofilaments, Microtubules, MAP1B, and Tau in Neurite Outgrowth and Stabilization

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The respective roles of neurofilaments (NFs), microtubules (MTs), and the microtubule-associated proteins (MAPs) MAP 1B and tau on neurite outgrowth and stabilization were probed by the intracellular delivery of specific antisera into transiently permeabilized NB2a/d1 cells during treatment with dbcAMP. Intracellular delivery of antisera specific for the low (NF-L), middle (NF-M), or extensively phosphorylated high (NF-H) molecular weight subunits did not prevent initial neurite elaboration, nor did it induce retraction of existing neurites elaborated by cells that had been previously treated for 1 d with dbcAMP. By contrast, intracellular delivery of antisera directed against tubulin reduced the percentage of cells with neurites at both these time points. Intracellular delivery of anti-NF-L and anti-NF-M antisera did not induce retraction in cells treated with dbcAMP for 3 d. However, intracellular delivery of antisera directed against extensively phosphorylated NF-H, MAP1B, tau, or tubulin induced similar levels of neurite retraction at this time. Intracellular delivery of monoclonal antibodies (RT97 or SMI-31) directed against phosphorylated NF-H induced neurite retraction in cell treated with dbcAMP for 3 d; a monoclonal antibody (SMI-32) directed against nonphosphorylated NF-H did not induce neurite retraction at this time. By contrast, none of the above antisera induced retraction of neurites in cells treated with dbcAMP for 7 d. Neurites develop resistance to retraction by colchicine, first detectable in some neurites after 3 d and in the majority of neurites after 7 d of dbcAMP treatment. We therefore examined whether or not colchicine resistance was compromised by intracellular delivery of the above antisera. Colchicine treatment resulted in rapid neurite retraction after intracellular delivery of antisera directed against extensively phosphorylated NF-H, MAP1B, or tau into cells that had previously been treated with dbcAMP for 7 d. By contrast, colchicine resistance was not compromised by the intracellular delivery of antisera directed against NF-L, NF-M, or tubulin. These findings support previous studies indicating that MT polymerization mediates certain aspects of axonal neurite outgrowth and suggest that NFs do not directly participate in these events. These findings further suggest that NFs function in stabilization of the axonal cytoskeleton, apparently by interactions among NFs and MTs that are mediated by NF-H and MAPs.

INTRODUCTION

Neuronal differentiation is accompanied by profound morphological alterations, including the elaboration of dendritic and axonal neurites. The relative requirement for plasticity in outgrowing neurites versus stabilized

axons is reflected in their cytoskeletal composition (Burgoyne, 1991). For example, microtubules (MTs) of outgrowing neurites or the rapidly outgrowing areas of neurites (e.g., the growth cone versus the proximal shaft) contain chiefly tyrosinated and unacetylated tubulin, whereas MTs of stabilized neurites contain significantly

higher proportions of tubulin that has been posttranslationally modified by detyrosination and acetylation (Black and Keyser, 1987; Cambray-Deakin and Burgoyne, 1987; Gordon-Weeks and Lang, 1988; Shea and Beermann, 1990; Shea *et al.*, 1990a). "Juvenile" and "adult" tau isoforms are expressed differentially in brain; immunocytochemical studies during brain development and PC12 differentiation have led to the suggestion that juvenile tau isoforms, which do not bind as efficiently as adult isoforms (Kosik *et al.*, 1989), mediate rapid neurite elongation, whereas adult tau isoforms, perhaps along with other MT-associated proteins (MAPs), subsequently stabilize the axon (Lee *et al.*, 1988; Hanemaaijer and Ginzburg, 1991; Takemura *et al.*, 1991). The axonal-specific MAP tau has been shown to be required for the initiation of neurite outgrowth in NB2a/d1 cells (Shea *et al.*, 1992c) and cultured cerebellar neurons (Caceres and Kosik, 1990; Caceres *et al.*, 1991). By contrast, MAP1B, which preferably associates with growing axons (Fischer and Romano-Clarke, 1991), was apparently not required for the early events of neuritogenesis in NB2a/d1 cells (Shea *et al.*, 1992c) but was, by contrast, required in PC12 cells (Brugg *et al.*, 1993). These findings highlight distinct requirements for different MAPs during various stages of neurite outgrowth and point toward differential requirements among neurons.

Although functional roles for the selective and timely stabilization of subsets or select regions of neuritic MTs by posttranslational modification and associated proteins have been demonstrated in vivo and in vitro, the role(s) of neuronal intermediate filaments in neurite outgrowth and stabilization is less well understood. An hierarchical expression of intermediate filament species accompanies neuronal differentiation and neurite outgrowth, including the transitional expression of nestin (Lendahl *et al.*, 1990) and vimentin (Tapscott *et al.*, 1981; Bignami *et al.*, 1982; Cochar and Paulin, 1984), before the eventual expression of neurofilaments (NFs) (reviewed in Nixon and Shea, 1992). NFs themselves undergo a series of progressively stabilizing events. Initial NFs in developing brain are comprised of only the low (NF-L) and middle (NF-M) molecular weight subunits, whereas the appearance of the high (NF-H) molecular weight subunit is delayed (Carden *et al.*, 1987). Extensive phosphorylation of NF-H, which is associated with cytoskeletal stabilization (Julien and Mushynski, 1982; Lewis and Nixon, 1988; Shea *et al.*, 1991b), is further delayed (Foster *et al.*, 1987; Dahl, 1988; Shea *et al.*, 1989; Fischer and Shea, 1991).

NB2a/d1 cells have provided a useful model system for certain aspects of axonal neurite outgrowth. NF-L, NF-M, and hypophosphorylated NF-H are detected within neurites as soon as they are elaborated (<4 h) after treatment with dbcAMP. Extensively phosphorylated NF-H isoforms are first detected in cytoskeletons after 3 d of dbcAMP treatment, and additional phos-

phate-dependent NF-H epitopes appear between 5–7 d of dbcAMP treatment (Shea *et al.*, 1989). Resistance to retraction after colchicine treatment is first detected in some neurites after 3 d and is widespread by 7 d of dbcAMP treatment. These phenomena coincide with the selective accumulation within neurites between days 3–7 of α -tubulin subunits that have been acetylated and detyrosinated—posttranslational modifications associated with MTs that exhibit a relatively slow rate of subunit turnover (Shea *et al.*, 1989, 1993b). The cytoskeletal constituents that mediate the development of axonal neurite stability remain undisclosed. However, this phenomenon cannot be attributed solely to the deposition of phosphorylated NFs, because treatment of these cells with the protein phosphatase inhibitor okadaic acid resulted in a substantial increase in numbers of phosphorylated NFs within dbcAMP-induced neurites, but was also accompanied by a loss of stabilized MTs, and did not hasten the development of resistance of neurites to retraction after colchicine treatment (Shea *et al.*, 1993b).

In the present study, we examined the respective roles of NFs and MTs, including the microtubule-associated proteins MAP1B and tau, in the outgrowth and development of stability of neurites by the intracellular delivery of specific antisera into undifferentiated mouse NB2a/d1 neuroblastoma cells or cells previously treated with dbcAMP for varying lengths of time. Portions of these findings have appeared in abstract form (Shea *et al.*, 1991b).

MATERIALS AND METHODS

Cell Culture and Differentiation

NB2a/d1 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and antibiotics. To induce the outgrowth of neurites displaying some of the characteristics of axons (Shea *et al.*, 1985, 1988, 1991b), the medium was replaced with 1 mM dibutyryl cyclic AMP (dbcAMP) 24 h after plating. All reagents were from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Intracellular Loading of Antibodies

Antibodies were loaded into transiently permeabilized cells as described previously (Shea and Beermann, 1990; Shea *et al.*, 1992c, 1992). Undifferentiated cells and cells that had been previously treated with dbcAMP for 0–7 d in 35-mm² petri dishes were rinsed with serum-free DMEM and treated with 1 ml of prewarmed (37°C) 1.2 M glycerol in phosphate-buffered saline (PBS) pH 7.4. All cultures were 50–75% confluent at the time of loading. The cells were then incubated at 0°C for 10 min by placing the culture plates directly on ice, after which L- α -lysophosphatidylcholine (LPC) (Sigma Chemical) was added at a final concentration of 40 μ g/ml, and the incubation was continued for an additional 8 min. One milliliter of prewarmed serum-free DMEM containing either no antiserum or containing 1:40 dilutions (unless otherwise indicated) in PBS of polyclonal antisera specific for NF-L, NF-M, and extensively phosphorylated NF-H (termed "L3", "M2" and "H3", respectively) (Shea *et al.*, 1992a), MAP1B (gift of Dr. I. Fischer, Medical College of Philadelphia) (Fischer and Romano-Clarke, 1991), or their corresponding preimmune antisera, commercial polyclonal antisera directed against α -tubulin (ICN

Immunobiochemicals, Lisle, IL) and tau (Sigma) or pooled normal rabbit antiserum (Sigma), a monoclonal antibody (RT97) directed against extensively phosphorylated NF-H (Anderton *et al.*, 1982), or monoclonal antibodies directed against phosphorylated (SMI-31) or nonphosphorylated (SMI-32) NF epitopes (Sternberger Monoclonals, Bethesda, MD) were then added to cultures incubating with the glycerol-LPC solution, and the cultures were immediately returned to the 37°C incubator. After a 10-min incubation, cultures received an additional 1 ml of prewarmed DMEM containing 10% serum and dbcAMP, and the incubation was continued for 2 h. Some cultures were treated with 10^{-6} M colchicine for 2 h immediately after antibody loading. Cultures were immediately fixed (4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4) and examined by phase-contrast microscopy. Loaded cells were not monitored for longer periods because of the likelihood of antibody degradation. In all experiments, successful loading of antibody was confirmed by fixation of additional loaded and unloaded cultures, rinsing 3× with tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS), incubation for 1 h with a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, and staining as described below for immunocytochemical analyses. Viable cell numbers were determined by hemacytometer counts of resuspended cells in the presence of trypan blue.

Quantitation of Neurite Outgrowth

Cultures under all conditions were fixed and examined by phase-contrast microscopy 4–72 h after induction of neurite outgrowth. As previously described (Shea *et al.*, 1991a), cells were scored for the presence or absence of one or more neurites of length relative to somal diameter and grouped into those elaborating putative neurites that were <1, 1–2, 2–3 somal diameters in length, 3–4 or >4 somal diameters in length. Calculations were pooled from at least five microscopic fields in duplicate or triplicate cultures from three to four separate experiments. To assay neurite retraction, NB2a/d1 cells were scored for the presence or absence of at least one neurite of length ≥ 1 somal diameter for cells that had been treated with dbcAMP for 0–1 d and for at least one neurite of length ≥ 2 somal diameters in cultures that had been treated with dbcAMP for 3–7 d. These criteria were derived from the typical distribution of neurite lengths at these times (e.g., Figure 1) (see also Shea *et al.*, 1985, 1991a). In cases where short neurite “remnants” were still detectable after a treatment that induced neurite retraction, the cell was scored as negative provided such remnants were shorter than 1 somal diameter for 0–1 or 2 somal diameters for 3–7 d, respectively; as previously described (Shea *et al.*, 1990a), monitoring cultures for longer periods during preliminary experiments indicated that such conditions usually denoted neurites that were in the process of retraction. At least 100 cells were scored in each of two to six cultures from two to four separate experiments for totals of 8–20 cultures for each antiserum. Values among experiments were compiled by first normalizing the extent of neurite outgrowth obtained in unpermeabilized dbcAMP-treated cultures and then adjusting the values obtained for corresponding permeabilized and antibody loaded cultures according to the corresponding factor. Normalized values were then compared statistically using Student's *t* test. A significant difference ($p < 0.0005$) between samples is denoted in graphs by an asterisk. The lack of an error bar for certain individual samples on graphs of neurite quantitation indicates that the SE for that respective sample is too small to be visualized. Values obtained after intracellular delivery of nonimmune serum, and in certain cases, preimmune serum are omitted from some graphs simply for the sake of clarity.

Immunocytochemistry and Immunofluorescence

Cultures were rinsed with cold TBS (pH 7.4) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 15 min. The fixed cultures were rinsed in TBS and incubated with 1:500–1000 dilutions of the above antisera, 1:1000 dilutions of monoclonal antibodies SMI-31 and SMI-32, or simultaneously with a 1:

100 dilution of a monoclonal antibody (6-11B-1, generous gift of Dr. G. Piperno, Rockefeller University) directed against acetylated α -tubulin and a commercial (ICN, Naperville, MD) polyclonal antiserum directed against all forms of α -tubulin, in TBS overnight at room temperature with shaking. Cultures were then rinsed three times, incubated for 2 h with the appropriate goat secondary antibody conjugated to fluorescein or rhodamine, viewed under phase contrast and UV optics or with secondary antibody conjugated with horseradish peroxidase, developed for 5 min with diaminobenzidine in the presence of H_2O_2 , and viewed under phase-contrast and bright field optics (Shea *et al.*, 1988).

Fractionation of Cells into Triton-insoluble Cytoskeletons, Gel Electrophoresis, and Immunoblot Analyses

Undifferentiated cells and cells treated with dbcAMP for 1–7 d were fractionated into Triton-insoluble and -soluble fractions according to the procedure of Chiu and Norton (1982) as previously utilized for NB2a/d1 cells (Shea *et al.*, 1988, 1990b). Cells were rinsed with cold TBS (pH 6.8) then scraped from the plate with a rubber policeman in cold 1% Triton X-100 in 50 mM Tris-HCl (pH 6.8), 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 50 μ g/ml leupeptin. The cells were homogenized in a glass-teflon homogenizer and centrifuged for 10 min at $13\,000 \times g$ at 4°C. Sedimented material, defined as the (Triton-insoluble) cytoskeleton, was resuspended in 1% sodium dodecyl sulfate (SDS) in the above buffer. Equivalent aliquots (100 μ g) received an equal volume of 2× Laemmli treatment buffer and were then boiled for 5 min and electrophoresed on 5, 7, or 10% polyacrylamide SDS-gels. The separated proteins were transferred to nitrocellulose, and the replicas were immunostained with 1:500–1:1000 dilutions of the above polyclonal and monoclonal antibodies as optimized in previous studies (e.g., Shea *et al.*, 1988, 1989, 1990a,b, 1992c, 1993c; Shea and Beermann, 1990; Fischer and Romano-Clarke, 1991) and visualized with the appropriate alkaline phosphatase-conjugated secondary antibodies as described (Shea *et al.*, 1993c).

RESULTS

Alterations in Rate of Outgrowth and Cytoskeletal Composition in Neurites During Treatment with dbcAMP

Treatment of NB2a/d1 cells with dbcAMP induces rapid neurite elaboration (Figure 1) (see also Shea *et al.*, 1985, 1988). In preparation for analyses of the requirements for various cytoskeletal constituents on neurite outgrowth and maintenance, we quantitated the distribution of neurite length at various time points during dbcAMP-induced differentiation (Figure 1). Although neurite length covers a relatively broad distribution range, especially at the later time points, examination of the maximum length in somal diameters that has been obtained by $\geq 10\%$ of the total cells during dbcAMP treatment yields a “maximum relative outgrowth rate” of 1–2 somal diameters after 4 h, 2–3 by 1 d, 3–4 by 3 d, and remaining at 3–4 by 7 d (Table 1). These quantitations and examination of the overall distribution of neurites at individual time points reveal that the rate of neurite outgrowth undergoes a continued progressive decrease to the point where the distribution of neurite lengths at day 3 is markedly similar to that at day 7 (Figure 1). This progressive slowing in outgrowth rate, coupled with our previous demonstrations

Figure 1. Distribution of neurite lengths at various time points during dbcAMP-induced differentiation. Neurite length relative to respective somal diameter was quantitated at various time points during dbcAMP-induced differentiation as described in MATERIALS AND METHODS. Note that the initial rate of neurite outgrowth is substantially more rapid than at later times during differentiation. For example, while the majority of neurites have attained a length of 1–2 somal diameters within 4 h, by 7 d of outgrowth, however, only a minor percentage of neurites are longer than 4 somal diameters. Note also the similarity of distribution of neurite lengths between days 3 and 7 of outgrowth.

of the development of colchicine stability, and the accumulation of additional neurofilament and microtubule isoforms between days 3 and 7 (Figure 2 and Table 1) (see also Shea *et al.*, 1988, 1989, 1990a, 1992c) are likely to reflect significant alterations in cytoskeletal dynamics during dbcAMP-induced differentiation. We addressed this possibility by examining the impact of intracellular delivery of specific antisera at various times during dbcAMP-induced on neurite outgrowth and maintenance.

Intracellular Delivery of Anti-Tubulin, But Not Anti-NF Antisera, Inhibits Neurite Elaboration and Induces Retraction of Neurites After 1 Day of Outgrowth

Intracellular delivery of antisera against any of the NF subunits did not reduce initial neurite elaboration (Figure 3), nor did it induce retraction of existing neurites elaborated by cells that had been treated for 1 d with dbcAMP before permeabilization (Figure 4). By contrast, intracellular delivery of anti-tubulin antiserum reduced the percentage of neurite-bearing cells at both these time points (Figures 3 and 4) (see also Shea and Beermann, 1990).

Intracellular Delivery of Anti-NF-H, Anti-MAP1B, Anti-tau, and Anti-Tubulin Antisera Induces Retraction of Neurites After 3 Days of Outgrowth

Intracellular delivery of anti-NF-L and anti-NF-M antisera did not induce retraction of neurites in cells that

had been treated with dbcAMP for 3 d. However, unlike earlier time points, intracellular delivery of anti-NF-H antiserum after 3 d of dbcAMP treatment induced neurite retraction to an extent similar to that observed after intracellular delivery of anti-tubulin antiserum (Figure 5). Whereas our previous studies have shown that intracellular delivery of anti-MAP1B antiserum did not affect neurites elaborated by cells treated for up to 1 d with dbcAMP (Shea *et al.*, 1992a), intracellular delivery of anti-MAP1B antiserum into cultures treated for 3 d with dbcAMP induced neurite retraction (Figure 5). Intracellular delivery of anti-tau antiserum into cultures treated for 3 d with dbcAMP also induced neurite retraction (Figure 4); previous studies have also shown that, in contrast to MAP1B antiserum, intracellular delivery of anti-tau antiserum both prevented initial neurite outgrowth by dbcAMP and induced retraction of neurites 1 d after dbcAMP treatment (Shea *et al.*, 1992c).

Intracellular Delivery of Monoclonal Antibodies Directed Against Phosphorylated, But Not Nonphosphorylated, NF Epitopes Induce Retraction of Neurites After 3 Days of Outgrowth

Whereas anti-NF-H antiserum reacts specifically extensively phosphorylated NF-H isoforms (Shea *et al.*,

Table 1. Cytoskeletal and physiological characteristics of dbcAMP-induced NB2a/d1 neurites

Cytoskeletal protein ^a	Length of treatment with dbcAMP			
	4 hr	1 d	3 d	7 d
Tubulin	+	+	+	+
tau	+	+	+	+
MAP1B	±	+	+	++
NF-L	+	+	+	+
NF-M	+	+	+	+
Hypophosphorylated NF-H	+	+	+	+
Extensively phosphorylated NF-H	–	–	+	++
Colchicine resistance ^b	–	–	±	+
Maximum elongation rate ^c	1–2	2–3	3–4	3–4

^a Values represent the relative levels of immunoreactivity detected within neurites using well characterized polyclonal and monoclonal antibodies according to the following criteria: –, none detected above background; ±, slight immunoreactivity above background; +, definitive immunocytochemical localization; ++, a substantial continued increase in levels beyond that considered as +. With the exception of the maximum elongation rate (below), the data presented within this table are derived from previous studies as referenced within the text.

^b The ability of neurites to resist retraction after exposure to 10^{-6} M colchicine for 2 h (Shea and Beermann, 1990; Shea *et al.*, 1990) as follows: –, no surviving neurites; ±, a small percentage of surviving neurites; +, the majority of neurites surviving. The presence or absence of neurites was quantitated using the identical criteria for quantitation in determining the initial outgrowth rate and for retraction after intracellular delivery of antisera as described in MATERIALS AND METHODS.

^c Values, expressed in somal diameters, were derived from data presented in Figure 1 as described in RESULTS.

Figure 2. Cytoskeletal constituents of NB2a/d1 cells during dbcAMP-induced differentiation. Immunoblot analyses of Triton-insoluble cytoskeletons during 0–7 d of dbcAMP-induced differentiation as indicated. Only the relevant portions of nitrocellulose replicas are presented; except for low (<40 kDa) breakdown products of tau, NF-H, and MAP1B, none of the antibodies demonstrated cross-reactivity with other cytoskeleton proteins. Although SMI-31 and SMI-32 react with epitopes present on both NF-H and NF-M, these monoclonal antibodies do not demonstrate significant reactivity with NF-M in NB2a/d1 cells (Shea *et al.*, 1988). Note the temporal accumulation of MAP1B, additional high-molecular weight isoforms of tau and phosphate-dependent isoforms of NF-M and NF-H. Because identical amounts of cytoskeletal proteins were loaded in all lanes, it must be considered that the apparent temporal decrease in certain species (e.g., NF-L) may actually be a reflection of displacement because of the substantial increases in other proteins; this line of reasoning is supported by the lack of any detectable decrease in these proteins in cytochemical analyses (Shea *et al.*, 1989).

1992a), it reacts with a range of isoforms that migrate between 190 and 200 kDa on SDS-gels (e.g., Figure 2). Neurites elaborated by NB2a/d1 cells after 3–7 d of dbcAMP treatment contain both extensively phosphorylated and hypophosphorylated NF-H isoforms (Figure 6) (see also Shea *et al.*, 1988, 1989). To address more directly whether or not the development of neurite stability was mediated at least in part by extensively phosphorylated NF-H, monoclonal antibodies directed against phosphorylated NF-H (RT97) or phosphorylated (SMI-31) and nonphosphorylated (SMI-32) NF epitopes were delivered into cells that had been transiently permeabilized after 3 d of dbcAMP treatment. Reductions of ~40 and 31% in the percentage of cells with neurites were observed after the intracellular delivery of RT97 and SMI-31, respectively, whereas no reduction was observed after the intracellular delivery of SMI-32 (Figure 7).

Intracellular Delivery of Anti-NF-H, Anti-MAP1B, and Anti-tau Antisera Compromise Colchicine Resistance in Neurites After 7 Days of Outgrowth

Intracellular delivery of anti-NF-L, anti-NF-M, anti-NF-H, anti-MAP1B, or anti-tubulin antisera (Figure 8) did

not induce retraction of neurites in cells that had been treated with dbcAMP for 7 d. Previous studies have shown that neurites develop resistance to retraction after 7 d of treatment with dbcAMP. Quantitation revealed that $72.9 \pm 2.4\%$ of day 7 cells elaborated ≥ 2 somal diameter neurites; 2-h colchicine treatment did significantly reduce this value ($p < 0.003$, Student's *t* test), but $66.6 \pm 2.1\%$ of the neurites were resistant (Figures 8 and 9) (see also Shea and Beermann, 1990). This extent of survival contrasts dramatically with the rapid (<20 min) retraction of all neurites after 4 h to 1 d of dbcAMP treatment and most neurites after 3 d of dbcAMP treatment (Shea *et al.*, 1990a; Shea and Beermann, 1990). We therefore undertook to determine whether or not the development of resistance to colchicine was mediated by interactions between cytoskeletal constituents. Indeed, neurites surviving colchicine treatment still contain phosphorylated NFs (Figure 10) and, as previously shown, acetylated and detyrosinated MTs (Shea *et al.*, 1990a). To address whether antisera directed against certain cytoskeletal constituents indeed compromised colchicine resistance, we exposed cultures to colchicine after transient permeabilization and intracellular delivery of antisera.

After intracellular delivery of anti-NF-H antiserum, colchicine treatment induced retraction of nearly half of existing neurites (Figures 8 and 11). By contrast, colchicine treatment did not induce retraction of neurites after intracellular delivery of anti-NF-L and anti-NF-M antisera (Figure 8). Colchicine treatment also induced extensive neurite retraction after intracellular delivery of anti-MAP1B and anti-tau antisera but did not induce

Figure 3. Intracellular delivery of anti-tubulin, but not anti-NF antisera, inhibits initial neurite elaboration. Antisera directed against NF-L, NF-M, NF-H, tubulin, and their corresponding preimmune sera were delivered into undifferentiated, transiently permeabilized cells. These cells and unpermeabilized controls then received 1 mM dbcAMP for 2 h, and neurite outgrowth was quantitated. Cultures in which neurite outgrowth differed significantly ($p < 0.0005$, Student's *t* test) between the specific antisera and preimmune are indicated by an asterisk.

Figure 4. Intracellular delivery of anti-tubulin, but not anti-NF antisera, induces retraction of neurites after 1 d of outgrowth. Antisera directed against NF-L, NF-M, NF-H, tubulin, and their corresponding preimmune sera were delivered into transiently permeabilized cells that had been treated with dbcAMP for the previous 24 h. These cells and unpermeabilized controls were then incubated for an additional 2 h in the continued presence of dbcAMP, and neurite outgrowth was quantitated. Cultures in which neurite outgrowth differed significantly ($p < 0.0005$, Student's t test) between the specific antisera and preimmune are indicated by an asterisk.

Figure 5. Intracellular delivery of anti-NF-H, anti-MAP1B, and anti-tubulin antisera induces retraction of neurites after 3 d of outgrowth. Antisera directed against NF-L, NF-M, NF-H, tubulin, MAP1B, tau, and their corresponding preimmune sera were delivered into transiently permeabilized cells that had been treated with dbcAMP for the previous 3 d. These cells and unpermeabilized controls were then incubated for an additional 2 h in the continued presence of dbcAMP, and neurite outgrowth was quantitated. NF-L and NF-M preimmune sera are omitted for clarity only. Cultures in which neurite outgrowth differed significantly ($p < 0.0005$, Student's t test) between the specific antisera and preimmune are indicated by an asterisk.

Figure 6. Extensively phosphorylated and hypophosphorylated NF-H isoforms are differentially distributed within NB2a/d1 cytoskeletons. Immunocytochemical analyses of Triton-extracted NB2a/d1 cells with monoclonal antibodies directed against phosphorylated (SMI-31) and nonphosphorylated (SMI-32) antibodies as indicated. Nuclei (n) are intensely stained by SMI-31 because of shared epitopes with nuclear lamins (see Shea *et al.*, 1989). Note the segregation of SMI-31 immunoreactivity within axonal neurites, whereas nonphosphorylated NF immunoreactivity is present throughout perikarya and neurites.

neurite retraction after the intracellular delivery of anti-tubulin antiserum (Figures 8 and 11) (see also Shea and Beermann, 1991).

DISCUSSION

In the present study, the intracellular delivery of specific antibodies into transiently permeabilized cells has demonstrated sequential requirement for various cytoskeletal constituents in dbcAMP-induced neurite outgrowth and stabilization. We observed that the initial rapid outgrowth phases of neuritogenesis were sensitive to the intracellular delivery of anti-tubulin but not to anti-NF or anti-MAP1B antisera. Conversely, the later stages, which were accompanied by a slowing of outgrowth and the development of neurite stability, were sensitive to the intracellular delivery of anti-NF-H and anti-MAP1B antisera but had developed resistance to the intracellular delivery of anti-tubulin antiserum at a time that paralleled the development of colchicine resistance. Neurites at all stages were sensitive to the intracellular delivery of anti-tau antisera (see also Shea *et al.*, 1992bc). Colchicine resistant neurites were retracted after the intracellular delivery of anti-NF-H, anti-

Figure 7. Intracellular delivery of monoclonal antibodies directed against phosphorylated, but not nonphosphorylated, NF epitopes induce retraction of neurites after 3 d of outgrowth. Monoclonal antibodies directed against extensively phosphorylated NF-H (RT97) (Anderton *et al.*, 1982), phosphorylated NF epitopes (SMI-31), or nonphosphorylated NF epitopes (SMI-32) were delivered into transiently permeabilized cells that had been treated with dbcAMP for the previous 3 d. These cells and cells that had been permeabilized in the absence of antibody were incubated for an additional 2 h in the continued presence of dbcAMP and neurite outgrowth was quantitated. Significant neurite retraction was observed after intracellular delivery of RT97 and SMI-31 ($p < 0.0005$ and 0.002 vs. no antibody, Student's t test) but not SMI-32 ($p < 0.399$ vs. no antibody).

MAP1B, and anti-tau antisera in the presence of colchicine.

Intracellular Delivery of Antisera: Technical Considerations and Hypothetical Effects

Our previous studies have demonstrated that our routine methodologies for transient permeabilization and intracellular delivery of antibodies do not induce extensive neurotoxicity, provided that antisera are utilized at a minimum dilution of 1:40 (as was carried out in the present study), and have confirmed the usefulness of this methodology for inhibiting the function of specific target antigen *in situ* (e.g., Shea and Beermann, 1990; Shea *et al.*, 1991b,c, 1992b,c). Nonspecific neurotoxicity was not associated with any individual antibody utilized in this study, because none of the antibodies induced neurite retraction at all stages of differentiation. Similarly, the combination of colchicine treatment with antibody loading does not result in neurite loss by nonspecific neurotoxicity, because colchicine treatment in combination with the intracellular delivery of antisera directed against tubulin, NF-L, and NF-M did not induce neurite retraction beyond the minor extent observed with colchicine alone. It should be noted that at all time points at least one antibody species induced retraction in each experiment, confirming the relevance of our

permeabilization methodologies for all time points examined.

Unlike the potential ability to monitor reduced substrate modification after effects of intracellular delivery of a neutralizing antiserum directed against an enzyme, we have no direct assay of the effects of our antibodies directed against structural proteins and can only infer effective disruption of antigen function by alterations in neurite physiology. Our working hypothesis is that intracellular antibodies do not induce depolymerization *per se*, but rather inhibit *de novo* polymer formation and/or inhibit the addition of subunits to an existing polymer by binding free subunits; this hypothesis is analogous to the prevention of tubulin addition to MTs by colchicine (Margolis and Wilson, 1977; Sternlicht and Ringel, 1979). Whereas intracellular antisera may indeed not be able to induce direct disassembly of existing MTs

Figure 8. Intracellular delivery of anti-NF-H, anti-MAP1B, and anti-tau antisera compromise colchicine resistance in neurites after 7 d of outgrowth. Antisera directed against NF-L, NF-M, NF-H, tubulin, MAP1B, tau, and their corresponding preimmune sera were delivered into transiently permeabilized cells that had been treated with dbcAMP for the previous 3 d. These cells and unpermeabilized controls then were incubated for an additional 2 h in the continued presence of dbcAMP with inclusion of 10^{-6} M colchicine in alternate cultures under each regimen received during this time, and neurite outgrowth was quantitated. NF-L and NF-M did not differ from their preimmune antisera in the presence or absence of colchicine and are not presented for clarity only. Cultures in which neurite outgrowth differed significantly ($p < 0.0005$, Student's t test) between the specific antisera and preimmune are indicated by an asterisk.

or NFs, those polymers that undergo rapid subunit exchange may be lost while stabilized polymers may persist. However, stabilized polymers are still subject to "coating" by intracellular antibodies, and progressive loss of dynamic interactions between polymers is likely to occur. Thus, for example, stabilized MTs may persist despite the intracellular delivery of anti-tubulin or anti-MAP antibodies, but MT-MT and/or MT-NF interactions will be depleted if they are of a dynamic nature (discussed more fully below).

Neurite Outgrowth Is Dependent upon MT Polymerization

Intracellular delivery of anti-tubulin antiserum prevented neuritogenesis and induced the retraction of neurites after days 1–3 of dbcAMP treatment. Previous studies in these cells have demonstrated that MT-dependent neurite outgrowth can occur after simple relaxation of the submembrane actin network (Shea, 1990). This "driving force", however, may be mediated by the association of tau with MTs, because we (Shea *et al.*, 1992c) and others (Caceres and Kosik, 1990; Caceres *et al.*, 1991) have shown by similar intracellular delivery of anti-tau antisera and antisense oligonucleotides that tau is essential for neurite outgrowth. By contrast, neither MAP1B nor NFs appeared to play a role in initial neurite elaboration or maintenance of neurite after 1 d of dbcAMP treatment. Although we observed no requirement for MAP1B in neurite initiation and outgrowth, it should be noted that antisense oligonucleotide-mediated suppression of MAP1B expression did inhibit PC12 neurite outgrowth (Brugg *et al.*, 1993). Similarly, intracellular delivery of an antibody against phosphorylated NF subunits induced the collapse of the neurofilament network in motor neurons but not in dorsal root ganglion cells (Durham, 1992). These differential findings underscore that different neuronal populations exhibit differential cytoskeletal dynamics; such phenomena may reflect differential requirements of neuronal subpopulations during development *in situ*. That we observed no inhibition of neurite outgrowth after intracellular delivery of anti-NF antisera is consistent with similar findings in other systems using anti-NF antibodies and antisense oligonucleotides (Shaw and Harris, 1991; Troy *et al.*, 1992).

Neurite Outgrowth Rate, Resistance to Colchicine and Cytoskeletal Constituents Alter Drastically During dbcAMP Treatment

Whereas neurites of 1–2 somal diameters are elaborated within 4 h after dbcAMP treatment, neurites continue to elongate at progressively slower rates, to the point that the distribution in neurite lengths in cultures treated with dbcAMP for 3 and 7 d were relatively similar. Although this may partially reflect an inherent maximum

Figure 9. Neurites of NB2a/d1 cells elaborated in response to dbcAMP develop resistance to retraction by colchicine. Cultures were treated with 1 mM dbcAMP for 7 d (A) after which alternate cultures received 10^{-6} M colchicine for 2 h (B). Many neurites remain after this treatment. In many cases, the overall neurite shaft diameter of surviving neurites is reduced after colchicine treatment, although the varicosities remain.

length for dbcAMP-induced NB2a/d1 neurites, it certainly reflects alterations in cytoskeletal dynamics.

We observed a change in the sensitivity of neurites to the intracellular delivery of several antisera after 3 d, and a further change after 7 d of dbcAMP treatment. As at earlier time points, the intracellular delivery of anti-tubulin and anti-tau antisera induced neurite retraction after 3 d of dbcAMP treatment. However, unlike earlier time points, neurites after 3 d of dbcAMP treatment were also induced to retract after the intracellular delivery of anti-NF and anti-MAP1B. Resistance to retraction after colchicine treatment was first observed in some neurites after 3 d and became widespread after 7-d treatment with dbcAMP (see also Shea and Beermann, 1990; Shea *et al.*, 1990a). Anti-tubulin antiserum

Figure 10. Neurites surviving colchicine treatment contain phosphorylated NFs. Cultures were immunostained with a monoclonal antibody (SMI-31) directed against phosphorylated neurofilament epitopes before (A and B) and after 2-h exposure to 10^{-6} M colchicine (C and D). To address whether antisera directed against certain cytoskeletal constituents indeed compromised colchicine resistance, we exposed cultures to colchicine after transient permeabilization and intracellular delivery of antisera.

did not induce retraction of neurites when delivered into cells that had been treated with dbcAMP for 7 d, coinciding with the development of widespread resistance to following colchicine treatment (see also Shea and Beermann, 1990; Shea *et al.*, 1990a). These findings indicate the deposition within neurites of a population of stabilized MTs within 7 d of dbcAMP treatment that, as previously demonstrated in other systems (Margolis and Wilson, 1977; Sternlicht and Ringel, 1979), may undergo slower subunit exchange than the overall cellular MTs. This population is evidenced by the appearance at this time of selectively acetylated and detyrosinated MTs within neurites (Shea *et al.*, 1990a, 1993b). One interpretation that is consistent with all of the above immunocytochemical and physiological observations is that during the period from approximately days 3–7 of dbcAMP treatment, neurites may be undergoing a transition from relatively plastic, rapidly elongating structures to a more stabilized state. Such a transition, along with a decrease in the rate of outgrowth, would be likely to render the maintenance of the developing neurite

more dependent upon interactions between MTs and NFs, rather than continued MT assembly. Importantly, this interpretation is consistent with the *de novo* onset at 3 d of dbcAMP treatment of retraction of neurites after the intracellular delivery of anti-MAP1B, anti-NF-H, RT97, and SMI-31.

The Development of Neurite Stability, as Indicated by Colchicine-Resistance, Is Mediated by Interactions Between Cytoskeletal Polymers

Intracellular delivery of antiserum directed against MAP1B or tau induced retraction of neurites elaborated after 7-d dbcAMP treatment provided cells were subsequently treated with colchicine. These findings support the hypothesis that MAP-mediated MT stabilization may be responsible for the development of colchicine resistance as follows: the intracellular delivery of anti-MAP1B or anti-tau antiserum could lead to MT destabilization by inhibiting continued association of these MAPs with MTs. This is apparently sufficient to promote net MT loss at day 3 as evidenced by neurite retraction; at day 7, colchicine-mediated prevention of tubulin addition to these now destabilized MTs would be expected to lead to net MT loss and eventual neurite retraction. These findings indicate that MAP-mediated MT stabilization is indeed essential for neurite stabilization (for review, see Hirokawa, 1994). However, intracellular delivery of antibodies against extensively phosphorylated NF-H also induce neurite retraction at later times. Thus, it is unlikely that MAP-mediated MT stabilization alone can account for the development of neurite stability and indicate the involvement of phosphorylated NFs in these phenomena. In this regard, bundling of neuronal intermediate filaments has been purported to stabilize the axonal cytoskeleton (Shaw and Hou, 1990). However, treatment of these cells with the phosphatase inhibitor okadaic acid (OA), which increased numbers of phosphorylated NFs yet reduced numbers of stabilized MTs, did not hasten the development of colchicine resistance (Shea *et al.*, 1993b); this finding argues against NF-NF interactions and supports MT-MT and/or MT-NF interactions, as mediating stability. However, the nonspecific nature of OA treatment does not completely rule out certain site-specific NF-NF interactions as contributing to axonal cytoskeletal stability. In addition to binding to MTs, however, MAPs, as well as the protruding sidearm of extensively phosphorylated NF-H (reviewed in Nixon and Shea, 1992), may also mediate critical MT-NF interactions. For example, MAPs bind to spinal cord NFs and mediate MT-NF associations *in vitro*, including inducing the formation of a sedimentable complex (Leterrier *et al.*, 1982; Aamodt and Williams, 1984; Miyata *et al.*, 1986). Indeed, otherwise nonsedimentable, Triton-soluble NF subunits in NB2a/d1 cells (Shea *et al.*, 1990b) and early postnatal mouse brain (Shea *et*

Figure 11. Neurites of NB2a/d1 cells elaborated in response to dbcAMP develop resistance to retraction after treatment with colchicine. Phase-contrast micrographs of cells that had been transiently permeabilized (A) after 2-h incubation with 10^{-6} M colchicine in the absence of intracellular delivery of antiserum (B) or incubation with colchicine after intracellular delivery of anti-tubulin (C), anti-NF-H (D), or anti-MAP1B (E). Note the substantial loss of neurites in colchicine-treated cultures receiving anti-NF-H and anti-MAP1B.

al., 1991a) sedimented when cells were harvested under conditions that stabilized existing MTs. Phosphorylation-dependent associations between NFs and MTs have recently been described (Hisanaga *et al.*, 1993). These findings collectively suggest that interactions between MTs and NFs, perhaps mediated by MAP1B, tau, and/or extensively phosphorylated NF-H isoforms, are crucial for the development of neurite stabilization. It remains possible that multiple specific interactions (i.e., MT-MT, NF-NF, and MT-NF) contribute to stabilization of the axonal cytoskeleton. It should be noted that studies demonstrating no inhibition of neuritogenesis after treatment of PC12 cells or sensory neurons with anti-NF antibodies and antisense oligonucleotides did not assay neurite stability by simultaneous treatments such as colchicine (Shaw

and Harris, 1991; Troy *et al.*, 1992). Accordingly, our finding of a potential role for NFs in neurite stabilization, but not outgrowth, is consistent with these earlier studies. Similarly, that otherwise apparently normal, but NF-deficient, axons are observed in a quail mutant (Osamu *et al.*, 1993) and in transgenic mice (Eyer and Peterson, 1994) is also consistent with the possibility that NFs only serve to stabilize axons under extremely stressful conditions that we have mimicked in culture by colchicine treatment. Indeed, neurites can be elaborated by NB2a/d1 cells even in the virtual absence of microtubules provided cells are cultured on a sufficiently adhesive substrate or are treated with inhibitors of the neuronal surface proteases that normally restrict neuritogenesis (Shea *et al.*, 1992b; Shea and Beermann, 1993).

Although anti-NF-H, RT97, and SMI-31 react specifically with extensively phosphorylated isoforms (Shea *et al.*, 1992a) and our findings therefore implicate those isoforms in mediating axonal cytoskeletal stability, we have not similarly addressed the phosphorylation state of MAP1B, because our anti-MAP1B antiserum reacts with both phosphorylated and nonphosphorylated isoforms (Fischer and Romano-Clarke, 1990). Considerable heterogeneity of phosphorylation of MAP1B accompanies development (Fischer and Romano-Clarke, 1990; Ullola *et al.*, 1993), and phosphorylation at key sites may represent a crucial mechanism governing the interaction of MT with each other and with NFs.

We had previously demonstrated a requirement for tau in the initial elaboration of neurites after dbcAMP-treatment and/or serum-deprivation (Shea *et al.*, 1992c); in the present study we demonstrate that tau also plays a role in the development of neurite maintenance and stabilization. In this regard, juvenile tau isoforms have been purported to stabilize microtubules sufficiently to initiate neuritogenesis while maintaining a degree of cytoskeletal plasticity, whereas the late-appearing forms, by virtue of an additional microtubule-binding domain, provide further stabilization to the axonal cytoskeletal suprastructure (Hanemaier and Ginzburg, 1991; Takemura *et al.*, 1991). Furthermore, phosphorylation of juvenile isoforms may be distinct from that of adult (Kosik *et al.*, 1989). The phosphorylation state of tau affects its binding to microtubules (Lindwall and Cole, 1984), suggesting that phosphorylation may also underlie the differential ability of juvenile and adult tau to promote microtubule stability (Hanemaier and Ginzburg, 1991; Scott *et al.*, 1991). Indeed, in the present study, we observed an increase in the 115-kDa tau isoform between days 3 and 7. Whether or not this or other tau isoforms play distinct roles in the outgrowth and subsequent stabilization of NB2a/d1 neurites, as has been suggested in PC12 cells (Hanemaier and Ginzburg, 1991), remains to be determined.

No attempt has been made in the present study to determine the actual nature of cytoskeletal polymer interconnections or "cross-links" that are purported to impart stability to developing axons. Several hypotheses have been previously advanced to account for the mechanism by which extensively phosphorylated NF-H isoforms may contribute to axonal cytoskeletal stabilization, including 1) steric restrictions to polymer sliding generated by the extensions of NF-H sidearms after extensive phosphorylation (reviewed in Nixon and Sihag, 1991), 2) ionic repulsion between sidearms because of their highly negative charge generated by extensive phosphorylation (Carden *et al.*, 1987), 3) direct physical cross-linking as suggested by ultrastructural analyses (Hirokawa *et al.*, 1987), or 4) various combinations of several of the above. Our observation of the disruption of stabilizing interactions between cytoskeletal polymers after intracellular delivery of antibody

may provide limited insight into the nature of such interactions. Ionic repulsion may be at least partially neutralized by coating with antibody. Steric restrictions between polymers would probably not be blocked but rather would be expected to be either unaffected or perhaps even strengthened somewhat by localized antibody binding, unless the proteins mediating such restrictions underwent dynamic exchange with existing polymers, or the polymers themselves underwent rapid assembly/disassembly cycles. In the latter case, the pool of available subunits to establish new polymers may be depleted. However, neither anti-tubulin, anti-NF-L, nor anti-NF-M antisera perturbed neurite stability. Accordingly, should steric interactions between polymers mediate the development of neurite stability, our results are consistent with the possibility that the cross-linking proteins undergo dynamic exchange with established polymers. Analogous reasoning can be applied in interpreting our results in the context that the stabilizing interactions between polymers are mediated by direct physical connections between cross-linking proteins. The ability of antibody to disrupt direct physical connections would be expected to be a function of the rate at which such interactions are established and severed. Dynamic interactions could be depleted in the presence of antibody, whereas stable interactions may remain unaffected (see above). Therefore, if it is indeed to be concluded that disruption of interactions between MTs and NFs is responsible for the compromise in neurite stability, the findings of the present study are consistent with ionic repulsion between sidearm extensions and/or direct physical crosslinking, provided that such direct crosslinks are relatively dynamic. However, such interpretations remain extremely speculative. Our results are also consistent with the possibility that one or more as yet unidentified NF-associated proteins exist that normally mediate interactions among cytoskeletal polymers, and that intracellular antibodies block the association of such putative associated proteins with NFs and/or MTs. In this regard, NF-associated proteins have been described (Lieska *et al.*, 1985; Yang *et al.*, 1985), the distribution of which may be regulated by phosphorylation (Skalli *et al.*, 1992) and some types of which may mediate NF-MT associations (Tashiro *et al.*, 1984).

The findings of the present study have provided further evidence, and in some cases substantiated earlier hypotheses based solely on morphological and immunocytochemical observations, that NFs and MTs and their associated proteins mediate distinct roles in neuritogenesis and maturation. Previous studies using this methodology, coupled with treatment with antisense oligonucleotides, have revealed transient requirements for vimentin and GAP-43 in the initial stages of neuritogenesis (Shea *et al.*, 1991c, 1993a). We have also previously shown that calcium ionophore- and cytochalasin B-mediated disruption of the submembrane actin skeleton fosters microtubule-dependent neurito-

genesis (Shea, 1990). These findings, coupled with the results of the present study, indicate a sequential requirement of membrane skeletal and cytoskeletal constituents for different aspects of neurite outgrowth and underscore that axonal neurite outgrowth and stabilization, even as examined in a cell culture model system, is a complex series of phenomena.

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