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Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain

(neurite outgrowth/adhesive specificity/cell adhesion molecules/immunohistochemistry)

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ABSTRACT **The nerve growth factor-inducible large external glycoprotein (NILE) has been found only on the surface of neuronal cells and Schwann cells. Since NILE seems to be concentrated on neurites, we have investigated its possible role in the development of neurites in primary cultures of rat brain. Cultures of embryonic day 14 (E14) whole brain and cultures of postnatal day 5 (P5) cerebellum were grown in the presence of Fab′ fragments of antibody against NILE in an attempt to perturb the normal pattern of neurite development. For comparison, cultures were treated with two other reagents that recognize neuronal cell surface molecules: tetanus toxin, which binds to the G_{D1b} and G_{T1} gangliosides, and Fab′ fragments of antibody against neural cell adhesion molecule (N-CAM). Under the conditions used, none of the exogenous reagents affected neurite outgrowth, but specific effects on neurite fasciculation were observed. Anti-NILE inhibited fasciculation in cultures of E14 whole brain but had no effect on fasciculation in cultures of P5 cerebellum. Conversely, anti-N-CAM inhibited fasciculation in cultures of P5 cerebellum, which contain the adult form of N-CAM, but had little effect on fasciculation in cultures of E14 whole brain, which contain the embryonic form of N-CAM. Tetanus toxin had no effect on fasciculation in either culture system. Our results imply that NILE-mediated neurite–neurite interactions are stronger than N-CAM (embryonic)-mediated interactions in the E14 brain cultures, whereas N-CAM (adult)-mediated interactions are stronger than NILE-mediated interactions in the P5 cerebellar cultures.**

The nerve growth factor-inducible large external glycoprotein (NILE) was originally identified as a cell surface component of PC12 cells (1) which incorporated increased amounts of fucose and glucosamine upon exposure of the cells to nerve growth factor (2). Subsequently, it was shown that this 230-kDa glycoprotein was one of the components recognized by polyclonal antisera raised against PC12 cells (3). Closely related glycoproteins have now been found on the surfaces of all neuronal cell lines examined and all neurons in primary culture, including neurons of central, sensory, and sympathetic origin (4–7). Schwann cells also express a NILE-related component (5, 6). Although they are immunologically cross-reactive, these neuronal glycoproteins are not identical. Depending on the neuronal cell type from which they are derived, glycoproteins of the NILE family range in size from 215 to 230 kDa as judged by their mobility in $NaDodSO_4$/PAGE (5–7).

Anti-NILE antibodies are useful not only for biochemical studies of this family of neuronal glycoproteins but also for immunohistochemical identification of neuronal cells in primary culture (5, 6) and in tissue sections (8). In central nervous system tissue, astrocytes, oligodendrocytes, and fibroblasts do not express a NILE-related component, so that the anti-NILE antibody can be used with confidence to identify neurons. In our hands, NILE-related glycoproteins appear to be distributed nonuniformly over the neuronal cell surface, being found predominantly on neurites and to a much lesser degree on the cell body. This is true of neurons both in culture (5) and in tissue sections (8). This suggested to us a possible involvement of NILE in some aspect of neurite formation or function. Primary cell cultures provide an initial means of testing these possibilities because antibodies against neuronal cell surface molecules can alter the normal pattern of neurite development in culture (9). In this study we have concentrated on the processes of neurite outgrowth and neurite fasciculation in cultures of neurons from the rat central nervous system. We have investigated the manner in which these processes are affected by antibody against NILE, and we have compared the effects of this antibody with the effects of two other reagents that recognize different neuronal cell surface molecules: antibody against a neural cell adhesion molecule (N-CAM) (10, 11), and tetanus toxin, which binds to the G_{D1b} and G_{T1} gangliosides (12). The three reagents have very different effects on the patterns of neurite development in tissue culture.

MATERIALS AND METHODS

Primary Cultures. Cultures of embryonic day 14 (E14) rat whole brain and postnatal day 5 (P5) cerebellum were prepared as described previously (13). Dissociated cells were seeded, at a density of 4×10^4 per cm^2 in Dulbecco's modified Eagle's medium containing 25 mM KCl and 10% fetal calf serum, on three types of surfaces: untreated 35-mm tissue culture dishes (Falcon), tissue culture dishes coated with poly(L-lysine) (Sigma), and tissue culture dishes coated with poly(D-lysine) (Sigma). 1-β-D-Arabinofuranosylcytosine (10 μM) was added during the fourth and fifth days in culture to inhibit the overgrowth of fibroblasts and astrocytes.

Before toxin or Fab′ fragments of antibodies were added to these cultures, the dissociated cells first were allowed to recover overnight from the trypsinization in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. On the next day, they were dissociated again by pipetting and seeded on the various types of 35-mm dishes in the presence or absence of the desired antibodies or toxin. Thus, the agents were present from the start of the culture period and were replenished each time the cultures were fed. Fab′ fragments of anti-NILE antibody and anti-N-CAM antibody

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Abbreviations: NILE, nerve growth factor-inducible large external glycoprotein; N-CAM, neural cell adhesion molecule; E14, embryonic day 14; P5, postnatal day 5.

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were used at a concentration of 25 μg/ml. Tetanus toxin was used at 10 μg/ml. These concentrations were chosen after incubating cultures overnight with serial 2-fold dilutions of each of the reagents. Binding of the reagent to neurons in the cultures was assessed by immunofluorescent staining (see below) so that saturating doses could be determined. The concentrations given above are double the saturating doses determined in these immunofluorescence experiments.

Antisera. As described previously (5), rabbit antibody against NILE was prepared by adsorbing rabbit antibody against PC12 cells with non-neuronal cell lines B9, B92 (14), and PCG2 (15) and with rat adrenal tissue. As shown in Fig. 3 *Left*, this antibody is quite specific even though it was prepared by adsorption of a heterogeneous antiserum rather than by immunization with a purified antigen. Monovalent Fab' fragments of the anti-NILE antibody were prepared from a purified IgG fraction according to Brackenbury *et al.* (16).

Rabbit antibody against mouse N-CAM and Fab' fragments of this antibody were generously provided by G. Edelman and C.-M. Chuong (Rockefeller University). This antibody also recognizes rat N-CAM with high efficiency (17). Tetanus toxin and rabbit antitoxin were gifts from R. Thomson (Wellcome). Rabbit antibody against the glial fibrillary acidic protein was a gift from D. Dahl.

Immunofluorescence. Binding of anti-NILE, anti-N-CAM, tetanus toxin, and antibody to glial fibrillary acidic protein to cells in primary culture was detected by immunofluorescence as described previously (5, 13). For experiments in which neurite outgrowth or fasciculation was to be quantitated, control cultures or cultures that had been incubated in the presence of tetanus toxin, anti-NILE or anti-N-CAM were stained by using sequential 30-min incubations with tetanus toxin, rabbit antitoxin, and fluorescein-labeled goat antibody against rabbit immunoglobulin. This protocol was followed to ensure that all neurites, whether in bundles or isolated as individual processes, would be stained by the fresh application of tetanus toxin, which binds to the G_{D1b} and G_{T1} gangliosides on most, if not all, neurons in culture (12, 18). Quantitation of neurite outgrowth and fasciculation is described in the legends to Fig. 1 and Table 1, respectively.

Immunoprecipitation and Gel Electrophoresis. Immunoprecipitation from detergent extracts of radioiodinated cultures was carried out as described previously (5). Immunoprecipitates were analyzed by $NaDodSO_4$/7.5% PAGE (19).

RESULTS

Neurite Outgrowth and Fasciculation. To test the effects of anti-NILE, anti-N-CAM, and tetanus toxin on both the outgrowth and fasciculation of neurites, we defined conditions under which neurite outgrowth occurred with a suitable level of fasciculation. Since fasciculation is influenced by the surface upon which cells are plated, three substrata were tested: (*i*) untreated tissue culture dishes, (*ii*) tissue culture dishes coated with poly(L-lysine), and (*iii*) tissue culture dishes coated with poly(D-lysine). Dissociated cells from E14 rat brain formed large aggregates on untreated tissue culture dishes during the first 2 days in culture, and this ultimately resulted in the majority of neurites being included in very thick bundles connecting the clumps of cells. E14 cells plated on poly(L-lysine)-coated dishes produced a mixture of smaller aggregates and single cells, resulting in the formation of small to intermediate size fascicles as well as a network of single neurites. E14 cells plated on poly(D-lysine)-coated dishes remained largely as single cells which elaborated a dense network of individual neurites. The poly(L-lysine)-coated dishes were judged to be optimal for our experiments with cells from E14 whole brain. In the case of P5 cerebellar cells, an optimal mixture of single and fasciculated neurites occurred on untreated tissue culture dishes. Cerebellar cells on either poly(L-lysine)- or poly(D-lysine)-coated dishes produced substantially lower levels of fasciculation.

Effects of Antisera on Neurite Growth Patterns. Neurons in cultures of E14 whole brain and P5 cerebellum expressed all three of the surface components being studied (NILE, N-CAM, and gangliosides G_{D1b} and G_{T1}) as shown by the ability of anti-NILE, anti-N-CAM, and tetanus toxin, respectively, to stain these cells in an immunofluorescence assay (not shown). Thus, it was possible to mask the three types of molecules with saturating levels of the respective antibodies or toxin and determine the effect of this masking on the pattern of neurite outgrowth and fasciculation.

The effect of the three reagents on neurite outgrowth was quantitated according to the protocol outlined in the legend to Fig. 1. Neurite outgrowth was highly variable in the cultures of both E14 brain and P5 cerebellum. Nevertheless, over the first 4 days in culture, there was an obvious trend in the direction of longer and more numerous processes. The example of E14 brain cultures is presented in Fig. 1. Between days 1 and 3 in culture, there was a decrease in the number of cells without neurites and an increase in the number of cells having neurites longer than 60–70 μm. This trend was not changed by the addition of anti-NILE Fab' fragments to the cultures. Anti-N-CAM Fab' fragments and teta-

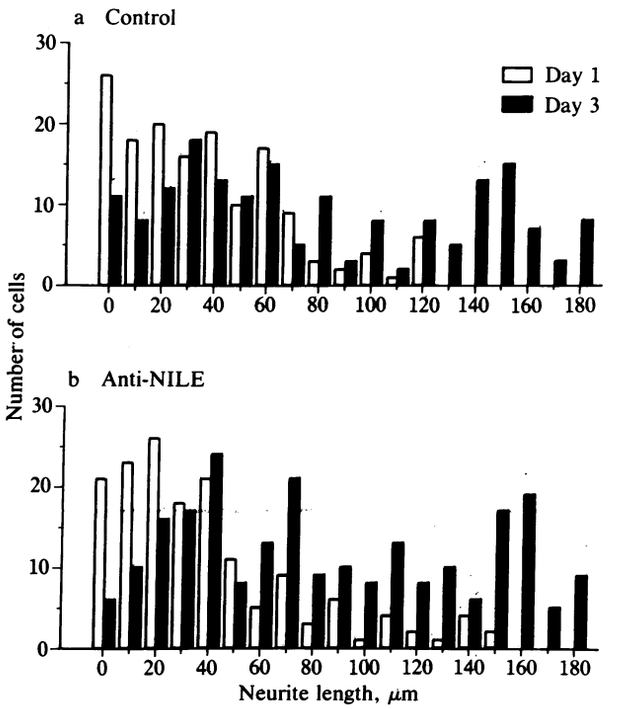

FIG. 1. Neurite outgrowth in rat brain cultures. Control cultures of E14 rat brain and P5 cerebellum, as well as parallel cultures treated with tetanus toxin, anti-N-CAM Fab' fragments, and anti-NILE Fab' fragments, were stained by indirect immunofluorescence with tetanus toxin after 1, 2, 3, and 4 days *in vitro*. Using the fluorescence microscope and a reticle, we then determined the lengths of neurites growing from neuronal cell bodies. At least 150 cells were sampled in each case. Data were put in the form of bar graphs showing the number of cells having neurites of a given length. Because of the variability of neurite outgrowth, no attempt was made to analyze these results statistically. However, the experiments were repeated several times, and very similar conclusions were reached in each case—i.e., there was no noticeable effect of toxin or Fab' fragments on the rate of neurite outgrowth. Data are presented only for E14 brain cultures at days 1 and 3 with (*b*) and without (*a*) anti-NILE antibody.

nus toxin also failed to affect the rate of neurite outgrowth in the E14 brain cultures (data not shown). Similar observations were made in the P5 cerebellar cultures; i.e., no effect on neurite outgrowth was produced by adding tetanus toxin, anti-NILE, or anti-N-CAM.

On the other hand, some significant effects of antibodies were seen in the process of neurite fasciculation. In control cultures of either E14 whole brain or P5 cerebellum, some fascicles were usually evident by day 6 or 7 *in vitro*, and by day 10 *in vitro* networks of fascicles were commonly found throughout the cultures (Fig. 2 *a* and *e*). In the P5 cerebellar cultures, anti-N-CAM reduced both the size and number of neurite fascicles (Fig. 2*c*). In contrast, neither tetanus toxin nor anti-NILE appeared to affect fasciculation in the P5 cerebellar cultures (Fig. 2 *b* and *d*). Although fasciculation is difficult to quantitate, we have devised a semiquantitative means of comparing fasciculation in the various cultures. These results and the protocol by which they were obtained are presented in Table 1. For the P5 cerebellar cultures, the index of fasciculation (percent of aggregates with fascicles) in the anti-N-CAM-treated cultures is roughly one-third that found in control cultures or in cultures treated with either tetanus toxin or anti-NILE antibody.

The antibodies had quite different effects on fasciculation in the cultures of E14 whole brain. Although anti-N-CAM again caused some inhibition of fasciculation, the effect (Fig. 2*g*) was much smaller than in the case of P5 cells (Fig. 2*c*). Anti-NILE antibody, on the other hand, almost completely abolished the formation of these neurite fascicles (Fig. 2*h* and Table 1). The large majority of neurites seen in these anti-NILE-treated cultures were present in the form of single fibers. Tetanus toxin once again had no effect on the formation of fascicles (Fig. 2*f*).

We performed two additional control experiments. First, we tested anti-N-CAM and anti-NILE antibodies that had been adsorbed with PC12 cells, which express both of these antigens. These preadsorbed antibodies failed to stain either of the two types of cultures in an immunofluorescence assay and did not affect fasciculation in the cultures (data not shown). Second, since it has been reported that neurite fasciculation is greatly influenced by glial cells on the substratum (20), we stained both control and experimental cultures with rabbit antibody against glial fibrillary acidic protein to investigate the unlikely possibility that antibody or toxin treatment affected the growth patterns of astrocytes, which might in turn affect fasciculation in the cultures. As expected, the growth and distribution of astrocytes in both E14 brain cultures and P5 cerebellar cultures was not affected by tetanus toxin, anti-N-CAM Fab′ fragments, or anti-NILE Fab′ fragments (data not shown).

Identification of NILE and N-CAM Components by Immunoprecipitation and Gel Electrophoresis. Fig. 3 *Right* shows that the NILE species present in the E14 brain and P5 cerebellar cultures are very similar but not quite identical. Both have an apparent molecular mass of about 215 kDa. In contrast, the N-CAM components differ greatly in the two cultures. The cultures of E14 brain contain the embryonic form of N-CAM which migrates as a broad band in the range of

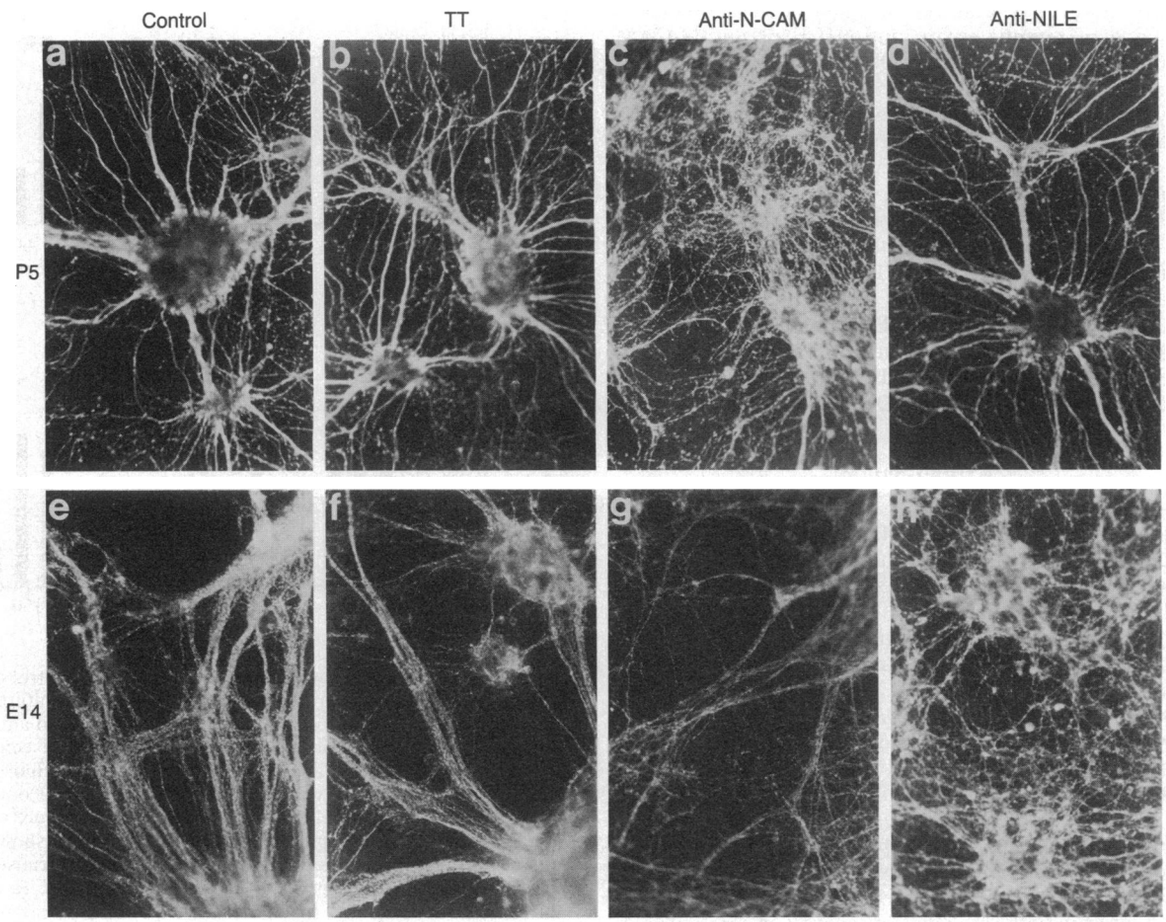

FIG. 2. Inhibition of neurite fasciculation in rat brain cultures. Primary cultures of P5 cerebellum and E14 whole brain were grown for 10 days in the absence of exogenous toxin or antibody (control) or in the presence of tetanus toxin (TT), Fab′ fragments of rabbit anti-N-CAM, or Fab′ fragments of rabbit anti-NILE. For evaluation, the cultures were stained by indirect immunofluorescence with tetanus toxin as described in *Materials and Methods* and scored as described in the legend to Table 1. (×280.)

Table 1. Effect of antibodies and tetanus toxin on neurite fasciculation

Culture type	% aggregates with neurite fascicles*			
	Control	Tetanus toxin	Anti-N-CAM Fab'	Anti-NILE Fab'
P5 cerebellum	66 ± 9	60 ± 14	21 ± 10	72 ± 12
E14 whole brain	71 ± 15	79 ± 8	44 ± 11	9 ± 5

*Analysis of cultures depends on the observation that, when fasciculation occurs, it almost invariably involves neurites that emerge from an aggregate of neuronal cell bodies. In each of the four culture conditions, we determined the percentage of aggregates that had neurite fascicles emerging from them. Since individual neurites are extremely difficult to distinguish from small fascicles, we were very conservative in our definition of a fascicle. We scored fascicles as any bundle of neurites >3 μm in diameter. Since these large bundles take a long time to form in culture, our scoring procedure did not allow us to evaluate fasciculation at early time points. For the experiment summarized here, three identical cultures of each type were prepared for each of the four conditions, and fasciculation was scored on day 10 *in vitro* after staining with tetanus toxin; ≥50 aggregates were scored in each of the three sister cultures. Each percentage value is the mean ± SD for the three cultures. In several similar experiments, different quantitative values for fasciculation were obtained, but the qualitative results were the same: tetanus toxin had no effect on fasciculation in either type of culture, but rabbit anti-NILE had a very large effect on E14 cultures, and rabbit anti-N-CAM had a large effect on P5 cultures.

180–230 kDa. The cultures of P5 cerebellum contain adult forms of N-CAM migrating at 190, 140, and 120 kDa (see refs. 10 and 11).

DISCUSSION

We have used cultures of dissociated rat brain cells to investigate the possible involvement of NILE in the outgrowth and fasciculation of neurites. Cultures were treated with Fab' fragments of anti-NILE in an attempt to perturb the normal pattern of neurite development. To test the specificity of the effects of anti-NILE, we treated parallel cultures with tetanus toxin and Fab' fragments of anti-N-CAM. In this way the effects of masking different neuronal cell surface molecules could be compared. Immunofluorescence experiments confirmed the fact that all three reagents bound to the neurons in our cultures and that the reagents were present in concentrations sufficient to saturate the respective surface antigens. The intensity of staining was greatest in the case of tetanus toxin, with NILE and N-CAM staining being somewhat less intense but similar to each other in both the P5 cerebellar and E14 whole brain cultures. Thus, the levels of cell surface NILE and N-CAM are probably roughly comparable in our two culture systems.

Neither tetanus toxin nor the Fab' antibody fragments had an effect on neurite outgrowth in either of the two culture systems. Neurite sprouting and outgrowth took place in normal fashion over the first 4 days in culture. Rutishauser *et al.* (9) also found that anti-N-CAM had no measurable effect on neurite outgrowth from embryonic chick spinal ganglia in culture, and Salton *et al.* (6) noted no effect of anti-NILE antibody on nerve growth factor-stimulated neurite outgrowth from PC12 cells.

In contrast, we observed some marked effects of antibodies on the formation of neurite bundles. As summarized in Table 1 and illustrated in Fig. 2, fasciculation in cultures of E14 rat brain and P5 rat cerebellum treated with tetanus toxin was indistinguishable from that seen in control cultures. Fasciculation in P5 cerebellar cultures also was not measurably affected by treatment with anti-NILE but was significantly inhibited by the addition of anti-N-CAM. Conversely,

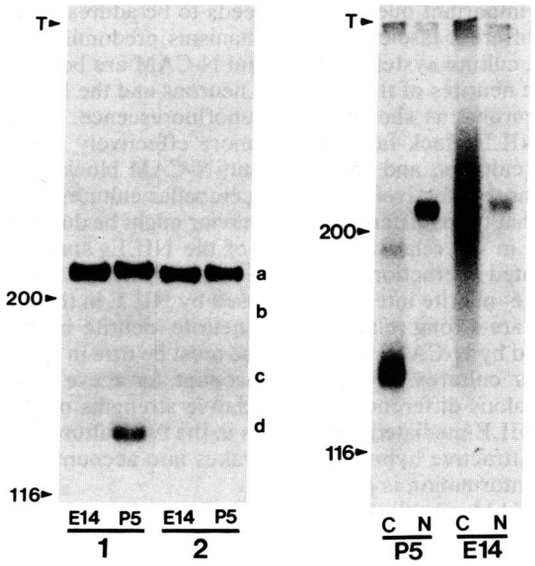

FIG. 3. Molecular forms of NILE and N-CAM expressed in culture. Immunoprecipitates from detergent extracts of ^{125}I-labeled P5 cerebellar cultures and E14 whole brain cultures were prepared, using rabbit anti-N-CAM (C) and rabbit anti-NILE (N) antibodies, and analyzed by NaDodSO$_4$/PAGE and autoradiography. Positions of myosin heavy chain (200 kDa), β-galactosidase (116 kDa), and the top (T) of the 7.5% separation gel are indicated. Only relevant portions of gels are shown; no bands were seen below 116 kDa. (*Left*) Specificity of anti-NILE. Lanes 1: after adsorption with three nonneuronal cell lines but before adsorption with adrenal tissue, the antibody immunoprecipitates components a–d. Lanes 2: after adsorption with adrenal tissue, the antibody still precipitates the 215-kDa NILE-related glycoproteins from both E14 whole brain and P5 cerebellar cultures but no longer recognizes components b (190 kDa) and d (140 kDa); >90% of the radioactivity in the immunoprecipitate migrates with the 215-kDa component a. Component c (160 kDa) is occasionally seen in these immunoprecipitates (note its absence in gel at *Right*) and may be a partially degraded form of NILE. (*Right*) Comparison of NILE and N-CAM species. E14 brain cultures contain the embryonic form of N-CAM (180–230 kDa) but P5 cerebellar cultures express the adult forms of N-CAM (190, 140, and 120 kDa). NILE components derived from E14 and P5 cultures are similar but not quite identical. There is a certain amount of variability in the mobility of the NILE components; the E14 species sometimes migrates faster than the P5 species (see *Left*) but sometimes migrates slower than the P5 species (*Right*). This variability may be due to changes in glycosylation of the polypeptides.

fasciculation in cultures of E14 rat brain was reduced only slightly by addition of anti-N-CAM but was almost completely abolished by the addition of anti-NILE.

We can draw two important conclusions from the differences in the effects of tetanus toxin, anti-N-CAM, and anti-NILE on neurite bundling in the two culture systems. First, the fact that fasciculation is not affected in every case shows that inhibition, when it occurs, must be caused by the masking of specific molecules and does not necessarily result from nonspecific steric interference due to the binding of large, exogenous polypeptides to the neurites. Second, two types of mechanisms influencing neurite fasciculation must be operating in these cultures. One mechanism involves N-CAM and is predominant in the cultures of P5 cerebellum. The other mechanism involves NILE and is predominant in the cultures of E14 brain. The importance of N-CAM in mediating adhesive interactions in the nervous system has been demonstrated (10, 11), but the involvement of an additional mechanism should not be surprising. Several other cell surface molecules and adhesion factors have also been implicated in various types of neural recognition and adhesive processes (21–26).

An important question that needs to be addressed is why two different fasciculation mechanisms predominate in different culture systems. NILE and N-CAM are both present on the neurites of the E14 brain neurons and the P5 cerebellar neurons, as shown by immunofluorescence. Why does anti-NILE block fasciculation more effectively in the E14 brain cultures, and why does anti-N-CAM block fasciculation more effectively in the P5 cerebellar cultures? We suggest that these differences in behavior might be due to differences in the relative strengths of the NILE- and N-CAM-mediated interactions in the two culture systems. That is, the neurite–neurite interactions caused by NILE in the E14 cultures are strong relative to the neurite–neurite interactions caused by N-CAM. The converse must be true in the P5 cerebellar cultures. What could account for these seemingly anomalous differences in the relative strengths of N-CAM- and NILE-mediated interactions in the two culture systems? One attractive hypothesis that takes into account the available information is given below.

N-CAM is known to exist in two forms: a highly sialylated embryonic (E) form and a less sialylated adult (A) form. The interaction between the A forms of the N-CAM molecule has been shown to be much stronger than interaction between the E forms, largely due to steric or ionic interference of the sialic acid-containing portion of the E form (10, 11). Our cultures of E14 brain express the E form of N-CAM, whereas the cultures of P5 cerebellum express the A form. If we assume that N-CAM(A)-mediated interactions are stronger than NILE-mediated interactions in the P5 cerebellar cultures but NILE-mediated interactions are stronger than N-CAM(E)-mediated interactions in the E14 brain cultures, we have a basis for explaining why N-CAM-mediated fasciculation predominates in the P5 cerebellar cultures and NILE-mediated fasciculation predominates in the E14 brain cultures.

It seems likely that both N-CAM- and NILE-mediated interactions are important for controlling the fasciculation of any given population of neuronal processes. Although parallel studies of the distribution of NILE and N-CAM during embryogenesis of a single species have not been conducted, it appears that the timing of the expression of the two molecules may be quite different. In the chick, N-CAM is found on cells of the neural plate and neuroepithelium as well as on pre- and post-migratory neural crest cells (27). In the rat, NILE immunoreactivity does not seem to be present on precursor cells of the neural plate and neuroepithelium. NILE glycoproteins are first found at embryonic day 11 on the axonal processes of post-mitotic neurons in fiber tracts of the spinal cord and rhombencephalon. At later stages of development, progressively more NILE immunoreactivity is seen in fiber tracts in other regions of the brain (8). Thus, the relative contributions of the NILE- and N-CAM-mediated interactions may change during the course of neuronal differentiation and fiber-tract formation, and modulating the relative strength of the two types of interactions could provide a means of controlling the strength and specificity of neurite–neurite interactions over a given developmental time span.

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