

Differential Contributions of Ng-CAM and N-CAM to Cell Adhesion in Different Neural Regions

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Abstract. Individual neurons can express both the neural cell adhesion molecule (N-CAM) and the neuron-glia cell adhesion molecule (Ng-CAM) at their cell surfaces. To determine how the functions of the two molecules may be differentially controlled, we have used specific antibodies to each cell adhesion molecule (CAM) to perturb its function, first in brain membrane vesicle aggregation and then in tissue culture assays testing the fasciculation of neurite outgrowths from cultured dorsal root ganglia, the migration of granule cells in cerebellar explants, and the formation of histological layers in the developing retina. Our strategy was initially to delineate further the binding mechanisms for each CAM. Antibodies to Ng-CAM and N-CAM each inhibited brain membrane vesicle aggregation but the binding mechanisms of the two CAMs differed. As expected from the known homophilic binding mechanism of N-CAM, anti-N-CAM-coated vesicles did not co-aggregate with uncoated vesicles. Anti-Ng-CAM-coated vesicles readily co-aggregated with uncoated vesicles in accord with a postulated heterophilic binding mechanism. It was also shown that N-CAM was not a ligand for Ng-CAM. In contrast to assays with brain membrane vesicles, cellular systems can reveal functional differences for each CAM reflecting its relative amount (prevalence

modulation) and location (polarity modulation). Consistent with this, each of the three cellular processes examined in vitro was preferentially inhibited only by anti-N-CAM or by anti-Ng-CAM antibodies. Both neurite fasciculation and the migration of cerebellar granule cells were preferentially inhibited by anti-Ng-CAM antibodies. Anti-N-CAM antibodies inhibited the formation of histological layers in the retina. The data on perturbation by antibodies were correlated with the relative levels of expression of Ng-CAM and N-CAM in each of these different neural regions. Quantitative immunoblotting experiments indicated that the relative Ng-CAM/N-CAM ratios in comparable extracts of brain, dorsal root ganglia, and retina were respectively 0.32, 0.81, and 0.04. During culture of dorsal root ganglia in the presence of nerve growth factor, the Ng-CAM/N-CAM ratio rose to 4.95 in neurite outgrowths and 1.99 in the ganglion proper, reflecting both polarity and prevalence modulation. These results suggest that the relative ability of anti-Ng-CAM and anti-N-CAM antibodies to inhibit cell-cell interactions in different neural tissues is strongly correlated with the local Ng-CAM/N-CAM ratio. Differential cell surface modulation of CAMs having different binding mechanisms may play a major role in shaping different neural regions.

APPPLICATION of various immunologically based cell adhesion assays (4, 13, 20, 21, 25, 29, 32) has led to the identification of a number of cell adhesion molecules (CAMs),¹ the amounts and distributions of which are modulated during development (10, 14, 43). Two of the well-characterized CAMs are expressed by neurons: neural CAM (N-CAM) and neuron-glia CAM (Ng-CAM). In addition to its expression in the nervous system throughout life, N-CAM is also expressed during early embryogenesis at many key

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; GA3, glial antigen 3; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM; NILE glycoprotein, nerve growth factor-inducible large external glycoprotein; TCS N-CAM, partially purified preparation of N-CAM from retinal tissue culture supernatants.

morphogenetic sites (10); for this reason it is called a primary CAM (15, 16). In contrast, Ng-CAM is a secondary CAM which is expressed only on postmitotic neurons during histogenesis of the nervous system (14, 42, 43). This CAM, which appears to be the same as the L1 antigen (22, 32) and the nerve growth factor-inducible large external (NILE) glycoprotein (1, 18, 39) seen in the mouse, is involved in both neuron-glia and neuron-neuron binding (21, 22). In addition to their functions in cell adhesion assays, N-CAM and Ng-CAM appear to be involved in developmental processes that can be studied in vitro including neurite fasciculation (37, 40, 41), cell migration (22, 32), and the formation of histological layers (5).

Both N-CAM and Ng-CAM can be expressed on a single

neuron (24), raising the important issue of their differential roles in adhesion as each CAM is spatially and temporarily modulated at the cell surface. N-CAM-mediated adhesion is homophilic, i.e., the N-CAMs on one cell bind to N-CAMs on an adjacent cell. In contrast, neuron–glia adhesion mediated by Ng-CAM appears to be heterophilic inasmuch as the molecule is not expressed to any large extent on glial cells of the central nervous system (21, 39). Results from physicochemical experiments on the binding of N-CAM in reconstituted vesicles (27), immunohistochemical experiments (14, 44), and perturbation experiments (3, 9, 35) have all previously suggested that alterations in the amount of N-CAM at the cell surface (prevalence modulation) play a major role in regulating its cellular binding functions. A striking example of prevalence modulation is seen in the migration of neural crest cells to form dorsal root ganglia: N-CAM is present on the surface of crest cells before migration, is not detected during their motion, and reappears upon subsequent aggregation and differentiation to form ganglia (44). Another form of cell surface modulation (polarity modulation) has been observed for Ng-CAM which in the central nervous system is found on extending neurites but only in small amounts on cell bodies (14, 42, 43). However, when migration of granule cell bodies on Bergmann glia (and possibly on other neurons) occurs, the relative amount of Ng-CAM appears to be increased at the surface of the soma, specifically on the leading process. These observations suggest that in order to understand the role of a CAM in cell adhesion, it is necessary to know both its physicochemical binding mechanism as well as how its binding function can be modulated by the cell.

In the present experiments, we have extended our studies on the molecular mechanism of adhesion (27) using membrane vesicle aggregation assays to compare the mechanism of N-CAM binding in neurons to that of Ng-CAM. We found that Ng-CAM binding must be heterophilic in neuron–neuron adhesion as it appears to be in neuron–glia adhesion (24). We then searched for differential modulation of both CAMs by determining the relative ability of specific antibodies to Ng-CAM and N-CAM to inhibit cell adhesion *in vitro* during a variety of different morphogenetic processes. These included neurite fasciculation in dorsal root ganglia, cell migration on Bergmann glia in the cerebellum, and formation of histological layers in the retina. Correlation with data on the levels of expression of Ng-CAM and N-CAM in the relevant tissues as determined by quantitative immunoblotting suggested that the relative contribution of each neuronal CAM to adhesive functions is a reflection of the local concentration of that CAM. The present findings support the idea that differential cell surface modulation of CAMs of different specificity can sharply alter their relative contributions to the adhesive properties of cells during neural histogenesis.

Materials and Methods

Animals

All experiments were performed on White Leghorn chicken embryos (H and R Poultry Farm, Cochection, NY).

Antigens and Antibodies

N-CAM and Ng-CAM were purified using specific monoclonal antibodies coupled to Sepharose CL-2B (24, 28). Polyclonal and monoclonal antibod-

ies against N-CAM (28) and Ng-CAM (24) were prepared as described. Polyclonal anti-Ng-CAM antibodies were routinely preincubated with N-CAM coupled to Sepharose CL-2B to remove any antibodies cross-reactive with N-CAM. Anti-brain membrane antibody was prepared as previously described (21) and was similarly depleted of anti-N-CAM and anti-Ng-CAM activity using the immobilized CAMs. Monoclonal antibody GA3 was prepared from mice that were immunized with 9-d chicken embryo glial cells; this monoclonal antibody was selected because of its ability to bind to glial cells but not to neurons or meningeal cells. Fab' fragments and IgG were prepared (4) and dialyzed against the specified buffers or media.

Membrane Vesicle Aggregation

Brain membrane vesicles (from 14-d chicken embryos) and reconstituted vesicles containing the embryonic form of N-CAM were prepared and their rate of aggregation was analyzed as described (27). Brain membrane vesicles were antibody-coated by incubating excess amounts of Fab' fragments (5 mg) with the vesicles (0.5 mg) for 1 h at 4°C. The vesicles were then washed three times by centrifugation (15,000 g, 10 min), and used at the indicated concentrations. In the studies based on this methodology, we address the question of whether (a) Ng-CAM is directly involved in cell adhesion as a ligand or (b) Ng-CAM is indirectly involved in cell adhesion due to interactions between Ng-CAM and actual ligands (such as N-CAM) on the same cell. We refer to the first of these possibilities as *trans* interaction and to the second as *cis* interaction. (A diagrammatic representation is given in Fig. 8 in conjunction with the discussion of our data.)

Neurite Fasciculation Assay

Dorsal root ganglia from the lumbar region of 10-d chick embryos were explanted on a collagen gel (2, 17) in 35-mm tissue culture polystyrene dishes (Corning Glass Works, Corning, NY). Five ganglia were explanted on each dish and grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Gibco, Grand Island, NY) and 15 µg/ml of 2.5 S nerve growth factor (Collaborative Research, Lexington, MA). Cultures were kept at 37°C and equilibrated with 10% CO₂.

After 2 d of culture, the ganglia were fixed with 4% formaldehyde/1% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) and stained with toluidine blue (0.5%). To facilitate measurements, the three-dimensional neurite outgrowth was collapsed on the surface of the dish by air drying. For each ganglion, the fascicle diameter distribution was estimated from a sample consisting of all fascicles crossing four lines (200 µm long) drawn midway between the edge of the ganglion and the advancing border of the neurite outgrowth at right angles to two orthogonal diameters with the ganglion at the center. Fascicle diameters were measured using an eyepiece reticle under oil immersion optics. The average diameter of fascicles of each ganglion (more than 30 fascicles were measured) was obtained and used as a measure of the degree of fasciculation; values shown represent the average from the five ganglia explanted per dish.

Cell Migration Assay

Chicken cerebellum explants were cultured using methods modified from published procedures (32, 33). Cerebella from 16-d chicken embryos were removed, their cortices were dissected into 0.5 × 2 × 5 mm pieces, and ~20 of these pieces were biosynthetically labeled in 5 ml of medium (Dulbecco's modified Eagle's medium/0.1% insulin transferrin selenium premix [Collaborative Research, Lexington, MA]/0.02 mM L-glutamine) containing 1 µCi/ml of [³H]thymidine (sp act = 6.7 Ci/mmol; New England Nuclear, Boston, MA) for 90 min. After washing, three pieces of explant were cultured in 1.5 ml of minimum essential medium with Earle's salt (Gibco, Grand Island, NY), supplemented with NaHCO₃ (2.2 g/l), Hepes (20 mM), dextrose (2.6 g/l), glutamine (2 mM), and fetal calf serum (10%). The cultures were gassed with 95% O₂/5% CO₂ mixture, tightly sealed in a tissue culture flask (50-ml size; Costar, Cambridge, MA), and shaken at 50 rpm at 37°C.

After 3 d in culture, the explants were fixed with 2% glutaraldehyde/2.5% paraformaldehyde/100 mM phosphate buffer (pH 7.4) overnight, equilibrated sequentially with 30% sucrose and M-1 embedding matrix (Lipshaw, Detroit, MI), then sectioned (10 µm) at -20°C. Autoradiography was performed (31) using NTB 2 emulsion (Eastman Kodak Co., Rochester, NY). The slides were counter-stained with thionin to allow the histological localization of labeled cells. The degree of cell migration was measured by counting all the tritiated thymidine-labeled cells in the external granule cell layer, molecular layer, and internal granule cell layer. A cell was scored as thymidine-labeled if it contained more than 10 silver grains, and more than 200 labeled cells were scored per explant. The percentage of tritiated

thymidine-positive cells in each cell layer was then calculated. In each experiment, data from three explants were averaged; overall results represent an average of values from 2–6 independent experiments. Immunofluorescent localization to detect the presence of Fab' in sections was performed as previously described (7) using FITC-labeled goat anti-rabbit Fab' (Miles Laboratories, Inc., Elkhart, IN) as the second antibody.

Retinal Tissue Culture

Neural retinas were dissected from 6-d chicken embryos. Tissue fragments ($\sim 2 \times 2$ mm) from the central retina were cultured on 8- μ m pore-size Nucleopore filters (Pleasanton, CA) in Falcon (Cockeysville, MD) organ culture dishes (Catalog Number 3037). After culture in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum for 4 d at 37°C in a 10% CO₂ incubator, tissue fragments were fixed in 4% paraformaldehyde/1% glutaraldehyde/0.5 \times Hank's balanced salt solution/100 mM sodium cacodylate (pH 7.4) and embedded in JB-4 (Polysciences, Inc., Warrington, PA). Sections (1.5 μ m) were cut and were stained with toluidine blue.

Immunoblotting

Purified CAMs or tissue and membrane extracts were resolved by SDS PAGE (30) on minigels (7.5% acrylamide, 8 cm \times 6 cm \times 1.5 mm gels containing 15 sample wells). Extraction buffer was phosphate-buffered saline/0.5% Nonidet P-40/1 mM EDTA/2.5% (vol/vol) Trasylol (Mobay Chemical Co., New York, NY). Gel samples were equivalent to the extract from one-half of a 6-d retina, eighteen 10-d dorsal root ganglia, or from plasma membranes from one-two hundredth of a 14-d brain. Dorsal root ganglia (36 per 35-mm dish) were also cultured on collagen gels for 2–4 d as described above and washed three times with Hank's balanced salt solution plus 2.5% (vol/vol) Trasylol. Plugs containing the regions of the original explants were then dissected from the neurite outgrowths which remained embedded in the collagen gel (46). The two fractions were collected in 1.5 ml Eppendorf tubes, washed once, and each fraction was homogenized in 100 μ l extraction buffer using a 100- μ l capacity Dounce homogenizer. Extracts were clarified by centrifugation for 10 min at 15,000 g. The supernatant fractions were then dried, resuspended in SDS sample buffer, and boiled 3 min before electrophoresis. Freshly dissected 10-d dorsal root ganglia and 6-d retinas were similarly extracted and prepared for SDS PAGE.

After SDS PAGE, polypeptides were transferred to nitrocellulose (45), incubated with 50 μ g of polyclonal rabbit anti-N-CAM or anti-Ng-CAM antibodies, washed, and incubated with 3 μ g of [¹²⁵I]-protein A (1 \times 10⁶ cpm). After autoradiography, the *M_r* 135,000–200,000 regions of lanes blotted with anti-Ng-CAM and the *M_r* 140,000–250,000 regions of lanes blotted with anti-N-CAM were cut from the nitrocellulose and ¹²⁵I was quantitated by gamma spectroscopy. Bound protein A was calculated using its known specific activity after subtracting background levels of ¹²⁵I from blank lanes. In control experiments using purified CAMs, the amount of [¹²⁵I]-protein A bound was found to be directly proportional to the sample concentration. To calculate the Ng-CAM/N-CAM ratio in the various tissues, the amount of [¹²⁵I]-protein A bound to anti-Ng-CAM immunoblots was divided by the amount of [¹²⁵I]-protein A bound to anti-N-CAM immunoblots of duplicate aliquots of the same samples. While these Ng-CAM/N-CAM ratios allow a comparison of the relative levels of the CAMs in the various tissues, they should not be taken as determinations of absolute CAM amounts or ratios because the polyclonal antibodies used for immunoblotting may differ both in the number of antigenic sites per molecule of CAM that they recognize and in their binding affinities.

Results

Previous studies have indicated that N-CAM-mediated adhesion is homophilic (N-CAM to N-CAM) and highly dependent on the local surface concentration of the molecule (27), and that both N-CAM and Ng-CAM are involved in neural cell adhesion and are present on the same individual neurons (22, 24). To evaluate whether N-CAM and Ng-CAM have different roles in development, we have now used antibodies to these molecules to investigate the molecular mechanism of Ng-CAM-mediated adhesion and to determine the contributions of N-CAM and Ng-CAM to the

processes of neurite fasciculation in cultured dorsal root ganglia, external granule cell migration in cerebellum explant cultures, and the formation of histological layers in retinas cultured in vitro. Finally, the ratio of Ng-CAM to N-CAM in each neural region has been determined to evaluate whether the relative levels of CAMs are correlated with their relative contribution to adhesion (as suggested by antibody perturbation).

Membrane Vesicle Aggregation

In previous studies, both anti-N-CAM and anti-Ng-CAM Fab' fragments inhibited neuronal cell aggregation; in contrast, only anti-Ng-CAM antibodies inhibited neuron-glia cell binding in cells from the central nervous system taken at days 9 to 14 (21, 22). To analyze further the roles of N-CAM and Ng-CAM in neuronal cell aggregation, and to determine whether Ng-CAM binding was heterophilic in neurons, a quantitative membrane vesicle aggregation assay was used. This assay has been successfully used to demonstrate N-CAM-dependent aggregation of plasma membrane vesicles prepared from brain homogenates and of reconstituted vesicles containing only purified N-CAM and lipids (27). Fab' fragments of rabbit antibodies directed either against N-CAM or Ng-CAM were found to inhibit the aggregation of plasma membrane vesicles prepared from embryonic chicken brains (Fig. 1, A and B). At saturating doses, anti-N-CAM was somewhat more effective than anti-Ng-CAM (>95% inhibition vs. 75% inhibition). That N-CAM and Ng-CAM are involved in independent adhesive mechanisms was suggested by the observation that doses of anti-N-CAM and anti-Ng-CAM that only partially inhibited adhesion were additive in their ability to inhibit adhesion when mixed (data not shown). Inhibition of aggregation was not simply due to the coating of vesicles with antibody, as evidenced by the observation that rabbit antibodies prepared against the membrane vesicles and depleted of anti-N-CAM and anti-Ng-CAM were still bound to the vesicles, but inhibited aggregation only marginally (Fig. 1 A).

The ability of different monoclonal anti-N-CAM and anti-Ng-CAM antibodies to inhibit neuronal plasma membrane vesicle aggregation varied greatly (Fig. 1, A and B). Fab' fragments and IgG of anti-N-CAM No. 1 (which recognizes the binding region of N-CAM [12]) each inhibited aggregation $\sim 80\%$ even at very low doses. In contrast, fivefold higher doses of Fab' fragments of anti-N-CAM No. 2 (which recognizes a polypeptide antigen in the polysialic acid-rich middle domain of N-CAM [11]) only marginally inhibited vesicle aggregation. Three distinct anti-Ng-CAM monoclonal antibodies (3G2, 10F6, and 16F5, respectively) inhibited the rate of vesicle aggregation $51 \pm 6\%$, $33 \pm 4\%$, and $16 \pm 5\%$ (mean \pm SD in four experiments). These variations suggest that interpretation of antibody effects in perturbation experiments in vitro must be assessed in terms of the relative efficacies determined beforehand by means of simpler binding assays.

To analyze the separate mechanisms of N-CAM and Ng-CAM binding by means of vesicle aggregation, vesicles were precoated with anti-CAM Fab' fragments and were then washed free of excess antibodies. The aggregation of such precoated vesicles and their ability to co-aggregate with uncoated vesicles was then examined. The effects on adhesion of precoating with Fab' fragments were similar to those ob-

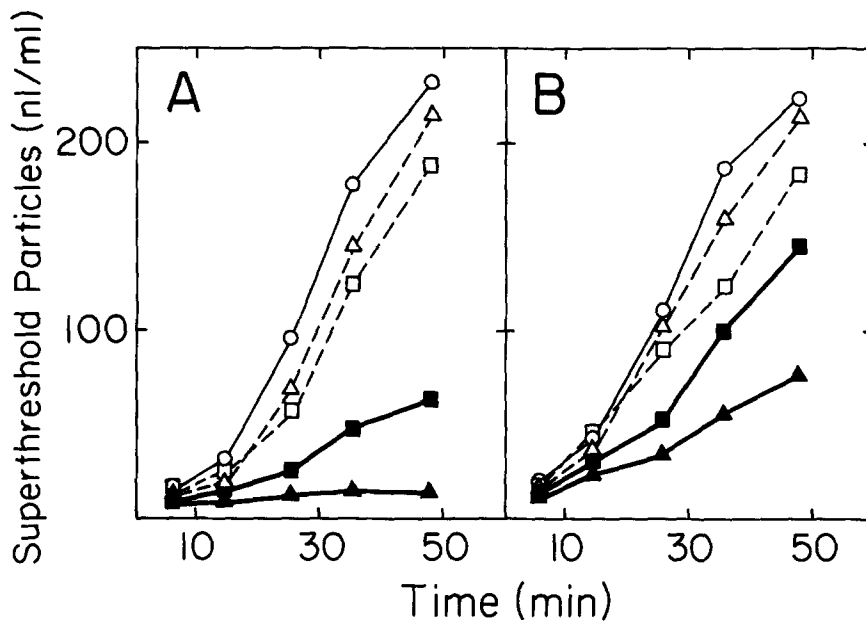


Figure 1. Antibody inhibition of brain membrane vesicle aggregation. Aggregation of membrane vesicles (38 $\mu\text{g/ml}$) in the presence of the indicated antibodies was performed and analyzed as described (26). The concentration of superthreshold particles was plotted as a function of time. (A) \circ , 100 $\mu\text{g/ml}$ non-immune Fab'; Δ , 100 $\mu\text{g/ml}$ anti-brain membrane Fab' depleted of anti-N-CAM and anti-Ng-CAM antibodies; \square , 10 $\mu\text{g/ml}$ Fab' from monoclonal antibody anti-N-CAM clone No. 2; \blacksquare , 2 $\mu\text{g/ml}$ Fab' from monoclonal antibody anti-N-CAM clone No. 1; \blacktriangle , 100 $\mu\text{g/ml}$ anti-N-CAM Fab'. (B) \circ , 100 $\mu\text{g/ml}$ non-immune Fab'; Δ , 10 $\mu\text{g/ml}$ IgG from monoclonal antibody 16F5 (anti-Ng-CAM); \square , 10 $\mu\text{g/ml}$ IgG from monoclonal antibody 10F6 (anti-Ng-CAM); \blacksquare , 10 $\mu\text{g/ml}$ IgG from monoclonal antibody 3G2 (anti-Ng-CAM); \blacktriangle , 100 $\mu\text{g/ml}$ anti-Ng-CAM Fab'.

tained in the presence of excess Fab'. Coating the membrane vesicles with saturating doses of anti-N-CAM antibodies (Fig. 2 A, open triangles) inhibited the rate of membrane vesicle aggregation >95%, while coating with anti-Ng-CAM antibodies inhibited aggregation ~75% (Fig. 2 B, open triangles). It is clear that while the inhibition was complete for anti-N-CAM and only partial for anti-Ng-CAM, both anti-N-CAM and anti-Ng-CAM antibodies effectively inhibited neuronal vesicle aggregation in this assay.

The fact that the rate of vesicle aggregation is proportional to the second power of vesicle concentration (27) allowed us previously to devise a quantitative assay indicating whether vesicle preparations were competent to co-aggregate (26). In this assay, the rate of aggregation of a mixture of vesicles from chick brain and liver was similar to the sum of the rates for each sample alone and we therefore concluded that these vesicles did not co-aggregate. In contrast, the rate of aggregation of a mixture of an aliquot of chick brain vesicles and an aliquot of mouse brain vesicles was significantly greater than the sum of the individual rates (and equal to the rate observed for a double aliquot of chick brain vesicles); we therefore concluded that these vesicles co-aggregated.

Using the co-aggregation assay, strikingly different results were obtained when anti-N-CAM-coated vesicles or anti-Ng-CAM-coated vesicles were co-incubated with uncoated vesicles. The rate of aggregation of a mixture of anti-N-CAM-coated vesicles and uncoated vesicles (Fig. 2 A, solid squares) was similar in value to the sum of the rates of each sample alone. This indicates that no co-aggregation occurred between anti-N-CAM-blocked and uncoated vesicles and that, as expected in homophilic N-CAM binding, blockade of one partner is sufficient. The rate of aggregation of a mixture of anti-Ng-CAM-coated vesicles and uncoated vesicles (Fig. 2 B, solid squares) was, however, much greater than the sum of the rates for each sample alone, indicating that extensive co-aggregation had occurred. These results suggest that Ng-CAM is not a homophilic ligand because if it were, in analogy with N-CAM, blockade of one partner should have inhibited co-aggregation. While these findings are consistent

with the possibility that a large proportion of the vesicles (~50%) may not express Ng-CAM and that the aggregation of the remaining 50% may have been completely inhibited by the antibody, this did not appear to be the case: control immunofluorescence experiments indicated that >90% of the membrane vesicles contained Ng-CAM. The most reasonable explanation for these data is therefore that Ng-CAM is not a homophilic ligand.

To evaluate the possibility that N-CAM may be the heterophilic receptor for Ng-CAM in *trans* binding, reconstituted vesicles containing only N-CAM and lipid were co-incubated with plasma membrane vesicles coated with anti-N-CAM or anti-Ng-CAM antibodies. The reconstituted N-CAM vesicles co-aggregated with anti-Ng-CAM-coated membrane vesicles (Fig. 2 C, solid squares) but not with anti-N-CAM-coated membrane vesicles (Fig. 2 C, solid triangles). The fact that anti-N-CAM-coated membrane vesicles (which contain Ng-CAM and bind to glial cells by an Ng-CAM-dependent mechanism [21]) do not co-aggregate with reconstituted pure N-CAM vesicles suggests that N-CAM is not a heterophilic *trans* receptor for Ng-CAM.

The remaining possibilities are as follows: (a) Ng-CAM is a heterophilic ligand in neuronal cell adhesion binding *trans* to receptor(s) (or other CAMs) on neurons and glia; (b) Ng-CAM is not actually a ligand and the effect of anti-Ng-CAM antibodies on neural cell adhesion is indirect, resulting for example from *cis* interactions between N-CAMs and Ng-CAMs on the same membrane and steric effects of anti-Ng-CAM on N-CAM binding; or (c) a combination of both *trans* and *cis* mechanisms occurs. Inasmuch as operation of either or both of these mechanisms would depend upon the relative amounts of the CAMs at the cell surface, we turned to experiments using *in vitro* assays in which differential modulation of the two CAMs might be observed.

Neurite Fasciculation

Because previous investigations have suggested that N-CAM and Ng-CAM are involved in neurite fasciculation (18, 37, 38,

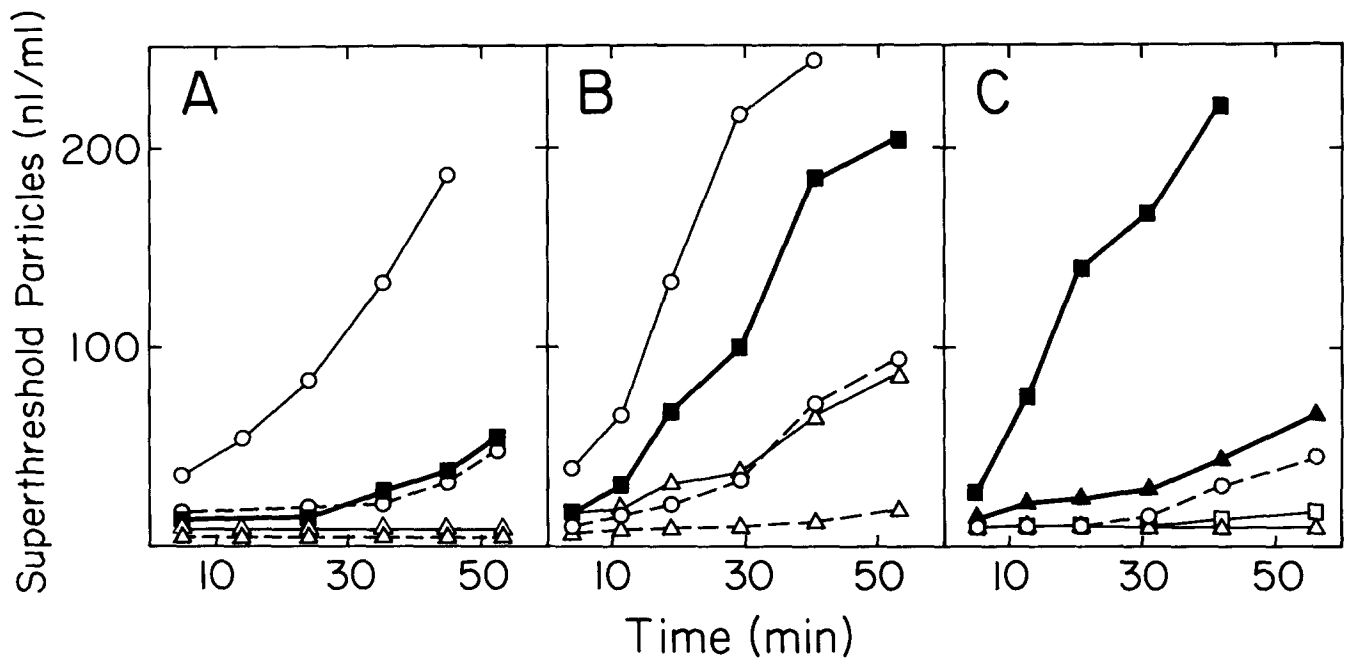


Figure 2. Aggregation behavior of antibody-coated brain membrane vesicles. Aggregation of membrane vesicles was performed and analyzed as described (26). Vesicles were coated with Fab' as described in the Materials and Methods. The concentration of superthreshold particles was plotted as a function of time. (A) ○ with solid line, 34 µg/ml uncoated vesicles; ○ with broken line, 17 µg/ml uncoated vesicles; △ with solid line, 38 µg/ml anti-N-CAM-coated vesicles; △ with broken line, 19 µg/ml anti-N-CAM-coated vesicles; ■, 17 µg/ml uncoated vesicles plus 19 µg/ml anti-N-CAM-coated vesicles. Note that the rate of aggregation of a mixture of anti-N-CAM-coated vesicles (19 µg/ml) and uncoated vesicles (17 µg/ml) was no greater than the sum of the individual rates of aggregation and much less than the rate of aggregation of twice the concentration of uncoated vesicles. (B) ○ with solid line, 40 µg/ml uncoated vesicles; ○ with broken line, 20 µg/ml uncoated vesicles; △ with solid line, 41 µg/ml anti-Ng-CAM-coated vesicles; △ with broken line, 20.5 µg/ml anti-Ng-CAM-coated vesicles; ■, 20 µg/ml uncoated vesicles plus 20.5 µg/ml anti-Ng-CAM-coated vesicles. Note that the rate of aggregation of a mixture of anti-Ng-CAM-coated vesicles (20.5 µg/ml) and uncoated vesicles (20 µg/ml) was significantly greater than the sum of the individual rates of aggregation and approached the rate of aggregation of twice the concentration of uncoated vesicles. (C) △, 17 µg/ml anti-N-CAM-coated vesicles; □, 21 µg/ml anti-Ng-CAM-coated vesicles; ○, artificial N-CAM vesicles (10.5 µg N-CAM and 0.7 mg lipid/ml); ▲, 17 µg/ml anti-N-CAM-coated vesicles plus artificial N-CAM vesicles (10.5 µg N-CAM and 0.7 mg lipid/ml); ■, 21 µg/ml anti-Ng-CAM-coated vesicles plus artificial N-CAM vesicles (10.5 µg/ml N-CAM and 0.7 mg lipid/ml). Note that the rate of aggregation of a mixture of anti-Ng-CAM-coated membrane vesicles and artificial N-CAM vesicles was significantly greater than the sum of the individual rates of aggregation while the rate of aggregation of a mixture of anti-N-CAM-coated membrane vesicles and artificial N-CAM vesicles was similar to the sum of the individual rates of aggregation.

Table I. Effects of Anti-N-CAM and Anti-Ng-CAM on Neurite Outgrowths in Cultured Dorsal Root Ganglia

Antibody (µg/ml)*	Diameter of Fascicles (µm)‡	% Inhibition§	Length of Outgrowth (mm)	% Increase
Polyclonal				
Non-immune (750)	1.29 ± 0.15	—	0.63 ± 0.03	—
Anti-Ng-CAM (10)	0.60 ± 0.04	53	0.90 ± 0.05	42
Anti-Ng-CAM (2)	0.80 ± 0.04	38	0.83 ± 0.06	30
Anti-Ng-CAM (0.9)	0.96 ± 0.06	26	0.75 ± 0.06	17
Anti-N-CAM (750)	0.74 ± 0.05	43	0.75 ± 0.05	17
Anti-N-CAM (100)	0.86 ± 0.04	33	0.68 ± 0.06	7
Anti-N-CAM (44)	1.04 ± 0.05	19	0.75 ± 0.05	17
Anti-Ng-CAM (10) + Anti-N-CAM (750)	0.65 ± 0.06	50		
Monoclonal				
Anti-N-CAM No. 1 (1000)	1.25 ± 0.05	3		
Anti-Ng-CAM 3G2 (1000)	0.80 ± 0.06	38		
Anti-Ng-CAM 10F6 (1000)	0.76 ± 0.03	41		
Anti-Ng-CAM 16F5 (1000)	0.76 ± 0.03	41		

* Polyclonal antibodies and monoclonal antibody anti-N-CAM No. 1 were used as Fab' fragments. Other monoclonal antibodies were used as IgG.

‡ Mean ± SEM (from five ganglia).

$$\% \text{ Inhibition} = \frac{\text{diam (non-immune)} - \text{diam (antibody)}}{\text{diam (non-immune)}} \times 100.$$

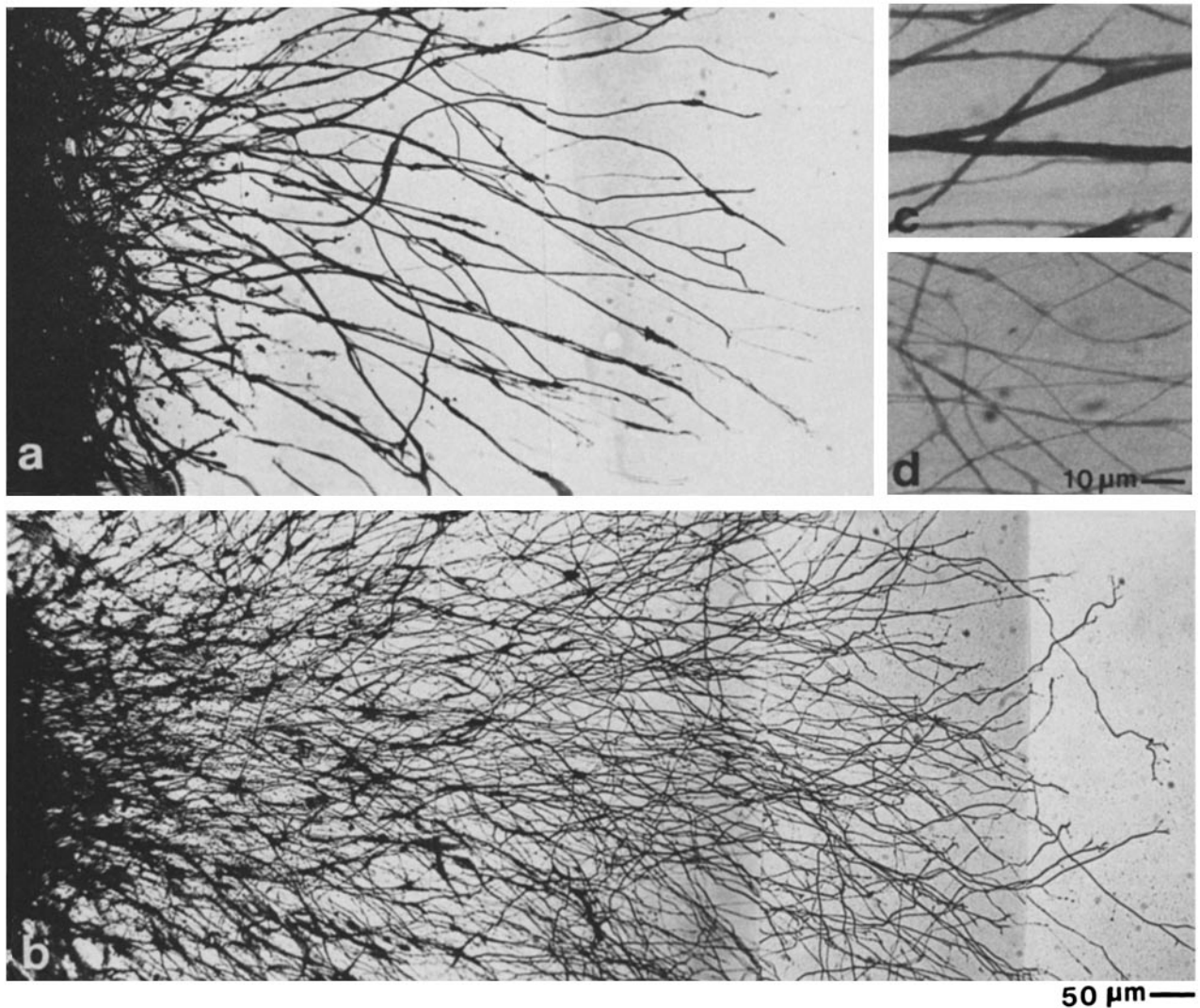


Figure 3. Effects of anti-Ng-CAM antibody on the fasciculation of neurite outgrowths from cultured dorsal root ganglia. Ganglia were cultured for 2 d as described in the Materials and Methods in the presence of 750 $\mu\text{g/ml}$ non-immune Fab' (*a* and *c*) or 10 $\mu\text{g/ml}$ anti-Ng-CAM Fab' (*b* and *d*). The cell body region is at the left in *a* and *b*. *c* and *d* are higher magnification photographs of neurites taken about midway between the margin of the ganglion and the furthest extent of the neurite outgrowth, i.e., the area where diameters of fascicles were measured (see Table I for quantitative measurements).

40, 41), we compared the ability of antibodies to these molecules to inhibit this process. Within 2 d in culture in nerve growth factor, dorsal root ganglia exhibited a rich neuritic outgrowth in the form of thick fascicles on top of and within a supporting three-dimensional collagen gel. The inclusion of low doses of polyclonal antibodies to Ng-CAM in these cultures decreased fascicle diameter and increased fascicle length and number (Fig. 3, Table I). The length of fascicles probably increases because the side-to-side adhesion among neurites is inhibited while neurite-substratum adhesion is not inhibited by the antibody. In contrast, antibodies to N-CAM had similar effects on fasciculation only at 50-fold higher doses, raising the possibility that Ng-CAM was the main adhesion molecule involved in fasciculation under the conditions used. This was consistent with the observation that monoclonal antibodies directed against the polypeptide portion of Ng-CAM inhibited fasciculation, while an anti-N-CAM monoclonal antibody (anti-N-CAM No. 1) that almost

completely inhibits brain membrane vesicle adhesion (Fig. 1) had no effect on fasciculation.

In previous studies performed at a time when Ng-CAM had not yet been identified, an antibody against a partially purified preparation of N-CAM from retinal tissue culture supernatants (TCS N-CAM) was found to inhibit neurite fasciculation (37). To reconcile the previous and present results, particularly in view of the known antigenic relationship of N-CAM and Ng-CAM (24), we carefully examined the specificity of polyclonal antibodies prepared against TCS N-CAM and immunoaffinity-purified N-CAM and Ng-CAM. Each antibody was used to immunoblot purified N-CAM and Ng-CAM (Fig. 4 *A*). After autoradiography, the amount of bound antibody was quantitated (Table II) by excising the appropriate pieces of nitrocellulose and subjecting them to gamma spectroscopy (to detect [^{125}I]-protein A bound to the antibody). Anti-Ng-CAM and anti-N-CAM were highly specific for their respective immunogens, although a weak

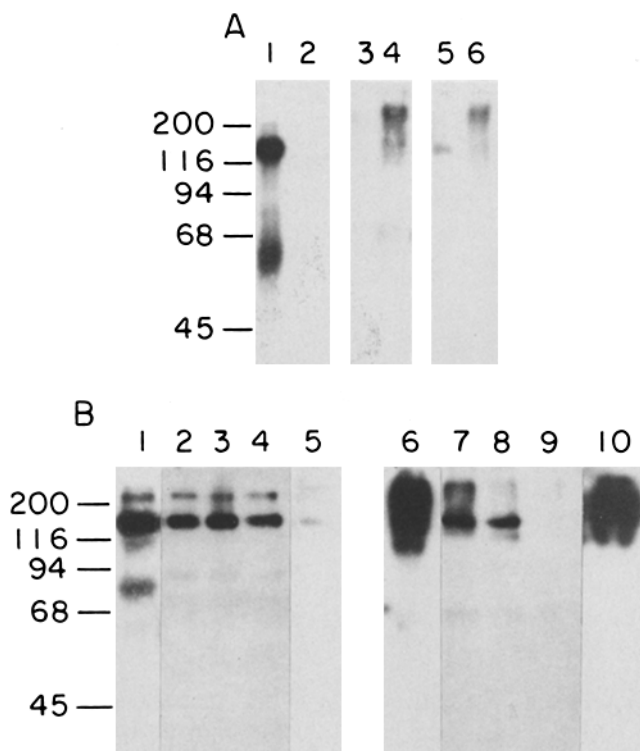


Figure 4. Immunoblots of purified CAMs and tissue extracts. (A) Evaluation of antibody specificity. 0.3- μ g aliquots of purified Ng-CAM (lanes 1, 3, and 5) and N-CAM (lanes 2, 4, and 6) were resolved by SDS PAGE, transferred to nitrocellulose, and incubated with anti-Ng-CAM IgG (lanes 1 and 2), anti-N-CAM IgG (lanes 3 and 4), or anti-TCS N-CAM IgG (lanes 5 and 6). (B) Relative levels of Ng-CAM and N-CAM in various neural tissues. Duplicate aliquots of extracts of 14-d embryonic brain membranes (lanes 1 and 6), 10-d embryonic dorsal root ganglia (lanes 2 and 7), the region of the original explant from cultured dorsal root ganglia (lanes 3 and 8), neurite outgrowths from cultured dorsal root ganglia (lanes 4 and 9), and 6-d embryonic retinas (lanes 5 and 10) were prepared as described in Materials and Methods, resolved by SDS PAGE, transferred to nitrocellulose, and incubated with anti-Ng-CAM IgG (lanes 1-5) or anti-N-CAM IgG (lanes 6-10). In both A and B, bound antibodies were then detected by incubation with [125 I]-protein A followed by autoradiography.

cross-reaction of anti-N-CAM antibodies with Ng-CAM was detectable. The original anti-TCS N-CAM, however, was found to cross-react with purified Ng-CAM to an eightfold higher level.

Each antibody preparation was then preincubated with purified N-CAM or Ng-CAM, and added to a culture to determine its ability to inhibit fasciculation. The ability of each antibody to inhibit fasciculation was strongly neutralized by 1.5 μ g or less of Ng-CAM, but not by 30 μ g of N-CAM (Table III). These results indicate that the ability of polyclonal anti-N-CAM and anti-TCS N-CAM antibodies to inhibit fasciculation is, in fact, primarily due to anti-Ng-CAM antibodies present at low levels.

The striking contrast between the relative potencies of anti-N-CAM and anti-Ng-CAM antibodies in inhibiting brain membrane vesicle aggregation and ganglion neurite fasciculation indicated that these two adhesion molecules make different relative contributions to adhesion in these two systems. To determine whether these results might be due to

Table II. Quantitative Evaluation of the Specificity of Anti-CAM Antibodies

CAM	Ng of [125 I]-protein A bound per μ g of CAM		
	Anti-Ng-CAM	Anti-N-CAM	Anti-TCS N-CAM
Ng-CAM	9.83	0.07	0.57
N-CAM	<0.01	3.47	2.83

0.3- μ g aliquots of Ng-CAM and N-CAM were resolved by SDS PAGE and immunoblotted using the indicated polyclonal antibodies. Immunoblots were prepared and quantitated as described in Materials and Methods.

differential expression of the two molecules in the different tissues, extracts of brain membranes and separate samples of neurite outgrowths and cell bodies from dorsal root ganglia were immunoblotted with antibodies to N-CAM and Ng-CAM (Fig. 4 B). A quantitation of these immunoblots (Table IV) indicated that freshly dissected dorsal root ganglia had an Ng-CAM/N-CAM ratio that was 2.5-fold greater than that of brain membrane vesicles. Moreover, during tissue culture in the presence of nerve growth factor the ratio of Ng-CAM to N-CAM rose further still, particularly in neurite outgrowths. This dramatic increase in Ng-CAM expression relative to N-CAM expression may be indicative of the normal response of dorsal root ganglia to nerve growth factor, a trophic factor which has recently been demonstrated (18) to have a similar effect on the relative expression of Ng-CAM and N-CAM in PC12 cells.

The observation that anti-Ng-CAM monoclonal antibodies (10F6 and 16F5) which inhibited neuron-glia adhesion (22) also inhibited fasciculation (Table I), made it necessary to rule out the possibility that neuron-glia interactions are involved in the fasciculation assay. To evaluate this possibility, the distribution of glial cells in neurite outgrowths from dorsal root ganglia was determined using a monoclonal antibody, GA3 (glial antigen 3), that recognizes glia but not neurons in dissociated chicken brain cell culture (Fig. 5 b). In dorsal root ganglia cultures, GA3 staining was observed in the vicinity of the ganglion (Fig. 5 e), but no GA3 staining was observed in the regions of the neurite outgrowth where fasciculation was scored (see Fig. 3). As expected, neurites were strongly Ng-CAM positive (Fig. 5 f). These results suggest that although Ng-CAM was involved in fasciculation in this system, neuron-glia interactions were not involved.

Cell Migration

We next explored a case in which neuron-glia interactions are known to be involved. During development of the chicken cerebellum (around day 15), the external granule cell layer divides into two layers, an outer proliferative zone and an inner premigratory zone (19). Granule cells in the premigratory zone then differentiate morphologically by sending out parallel fibers, while their cell bodies descend through the molecular layer along the Bergmann glia fibers into the internal granule cell layer (34). The migration of these cells can be traced by pulse-labeling with [3 H]thymidine either in vivo (19) or in vitro (32, 33), provided that the labeled thymidine is present when the cells undergo their final cell division in the proliferative zone.

Immediately after incubation with [3 H]thymidine, almost all labeled cells were located in the external granule cell layer (Fig. 6 a, Table V). During 3 d in culture in the presence

Table III. Neutralization of Inhibition of Fasciculation by Purified CAMs

Antibody ($\mu\text{g/ml}$)*	N-CAM ($\mu\text{g/ml}$)	Ng-CAM ($\mu\text{g/ml}$)	% Inhibition \ddagger	Neutralization (%) \S
Anti-Ng-CAM (10)	0	0	60 \pm 6	—
Anti-Ng-CAM (10)	30	0	50 \pm 3	16 \pm 5
Anti-Ng-CAM (10)	0	3	-5 \pm 10	108 \pm 16
Anti-Ng-CAM (10)	0	0.5	11 \pm 7	82 \pm 11
Anti-N-CAM (400)	0	0	37 \pm 3	—
Anti-N-CAM (400)	30	0	37 \pm 5	0 \pm 14
Anti-N-CAM (400)	0	15	-4 \pm 8	110 \pm 22
Anti-N-CAM (400)	0	1.5	10 \pm 4	73 \pm 12
Anti-(TCS N-CAM) (50)	0	0	58 \pm 5	—
Anti-(TCS N-CAM) (50)	30	0	55 \pm 3	6 \pm 5
Anti-(TCS N-CAM) (50)	0	1	3 \pm 7	96 \pm 12

* Polyclonal rabbit antibodies were used as Fab' fragments. Antibodies were preincubated with the indicated CAMs for 30 min at 25°C before addition to cultures.

$$\ddagger \% \text{ Inhibition} = \frac{\text{diam (non-immune)} - \text{diam (antibody + CAM)} \pm \text{SEM}}{\text{diam (non-immune)}} \times 100$$

$$\S \% \text{ Neutralization} = \frac{\text{diam (antibody + CAM)} \pm \text{SEM} - \text{diameter (antibody only)}}{\text{diam (CAM only)} - \text{diam (antibody only)}} \times 100$$

of non-immune antisera, most labeled cells migrated to the internal granule cell layer (Table V). When cultured in the presence of anti-Ng-CAM Fab', the great majority of labeled cells remained in the external granule cell layer (Fig. 6 b, Table V), suggesting that cell migration was inhibited by this antibody. In contrast, anti-N-CAM antibodies did not significantly inhibit cell migration (Fig. 6 c, Table V), having no greater effect than control antibodies to L-CAM (liver CAM [20], a molecule which is not present in the cerebellum). When a mixture of anti-Ng-CAM Fab' and anti-N-CAM Fab' was used, no increase of inhibition was observed (Table V) over anti-Ng-CAM Fab' alone. These results are consistent with a role for Ng-CAM in cell migration on glia, but none, or only a minor one, for N-CAM in the cerebellum at this stage of development.

Several control experiments were performed to determine the viability of cells, the distribution of N-CAM and Ng-CAM, and the degree of penetration of added antibodies in the explants. After 3 d in culture, the cells in explants were loosely packed and contained ~15% pycnotic nuclei resulting from central necrosis. The general cytoarchitecture, however, was quite well preserved (Fig. 6, b and c). Less than 1% of the labeled cells contained pycnotic nuclei, making it unlikely that dead cells were being counted as nonmigratory cells. The distribution of Ng-CAM in the explants was similar (Fig. 6 d) to that in cerebellar tissue sections (Fig. 6 e): no staining in the proliferative zone of the external granule cell layer, intense staining in the pre-migratory zone and molecular layer where cell migration is initiated, and very weak staining in the internal granule cell layer. The distribution of N-CAM in the explants was also indistinguishable from its distribution in tissue sections (data not shown). The fact that N-CAM was present on cell surfaces throughout the cerebellum (Fig. 6 f) while anti-N-CAM antibodies did not inhibit cell migration suggests that the effect of anti-Ng-CAM on cell migration is specific and not due merely to a general coating of cell surfaces with antibodies. Furthermore, in at least some regions of the cerebellum, N-CAM molecules are functional in cell adhesion as evidenced by the

observation that both polyclonal and monoclonal anti-N-CAM antibodies inhibited the aggregation of plasma membrane vesicles prepared from 17-d embryonic cerebella (data not shown). Finally, when explants cultured in the presence of anti-CAM Fab' were later sectioned and stained with anti-Fab' antibodies, the staining patterns seen were similar to normal tissue distributions of the corresponding CAMs, confirming that adequate penetration of the tissue by the Fab' fragments had occurred.

To study the differential involvement of Ng-CAM in cell migration further, the effects of several different antibodies were tested (Table V). Monoclonal antibodies 3G2 and 16F5, which recognize only the molecule of M_r 200,000 and the derivative M_r 135,000 polypeptide, inhibited migration. Moreover, separate antibodies raised against the M_r 200,000 molecule and the purified M_r 135,000 and 80,000 polypeptides (which are structurally related to independent regions of the M_r 200,000 species [22]) all inhibited cell migration significantly. In contrast, neither monoclonal antibody anti-N-CAM No. 1, which inhibits N-CAM-mediated cell aggregation, nor monoclonal antibody 15G8, which recognizes the polysialic acid in the embryonic form of N-CAM (8), inhibited cell migration. However, anti-N-CAM No. 5, which recognizes a common carbohydrate epitope present on Ng-

Table IV. Relative Concentrations of Ng-CAM and N-CAM in Various Neural Tissues

Tissue	Ng-CAM/N-CAM Ratio*
14-d Brain	0.32 \pm 0.05
10-d DRG \ddagger	0.81 \pm 0.13
10-d DRG Cell Bodies (Cultured)	1.99 \pm 0.13
10-d DRG Neurites (Cultured)	4.95 \pm 1.69
6-d Retina	0.04 \pm 0.01

Duplicate aliquots of Nonidet P-40 extracts of the indicated tissue fractions were prepared, immunoblotted using polyclonal anti-N-CAM and anti-Ng-CAM antibodies, and the immunoblots quantitated as described in Materials and Methods.

* Mean \pm SEM in four independent experiments (two for retina).

\ddagger DRG, dorsal root ganglia.

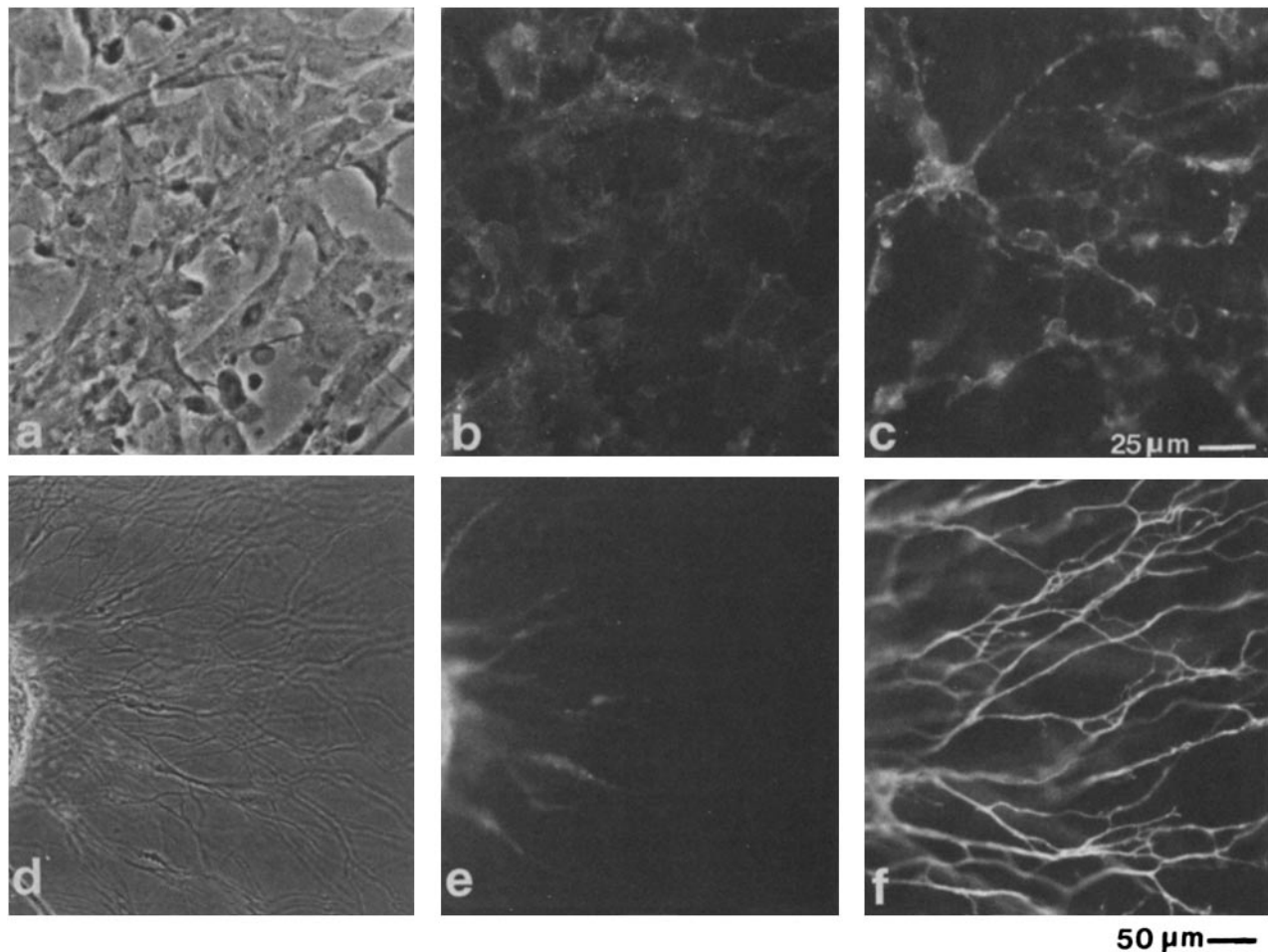


Figure 5. Immunofluorescent staining of cultured brain cells and dorsal root ganglia. (a–c) Primary cultures of 12-d brain cells containing both neurons and glia were prepared as previously described (24). (d–f) Dorsal root ganglia were cultured for 2 d as described in Materials and Methods. (a and d) Phase-contrast images. Immunofluorescent localization of antigens recognized by glia-specific monoclonal antibody GA3 (b and e) and rabbit anti-Ng-CAM antibody (c and f) was performed as previously described (24). a and b are photographs of the same field as are d and e. The edge of the ganglia is at the left margin of d and e. Note that glial cells (e) are found near the edge of the ganglia but not in the region of neurite outgrowths. Part of f is out of focus because the neurites form a three-dimensional array in the collagen gel.

CAM, N-CAM, and cytotactin (23, 24), caused significant inhibition of cell migration. These findings strongly support the interpretation that the inhibition of migration was correlated with anti-Ng-CAM specificity.

Formation of Histological Layers

Cells of the developing retina appear in a relatively uniform distribution at 6 d of embryonic development. By 9 d of development, the neurite-rich inner and outer plexiform layers have segregated from cell body-rich layers. This morphological development also occurs *in vitro* where it can be perturbed by added antibodies. In previous studies (5), antibodies to TCS N-CAM were found to inhibit the formation of these histological layers. The results described above indicating that anti-TCS N-CAM cross-reacted with Ng-CAM prompted us to re-examine the ability of the different highly specific anti-CAM antibodies to inhibit development in this system.

Formation of histological layers in the developing retina was inhibited by both polyclonal (Fig. 7, b and e) and

monoclonal (data not shown) anti-N-CAM antibodies, but not by polyclonal anti-Ng-CAM antibodies (Fig. 7 c). Retinas cultured in the presence of anti-N-CAM antibodies consistently showed disorder in the pattern of histological layers: they lacked a defined outer plexiform layer in many locations, cells were frequently scattered into the inner plexiform layer, and the cell bodies in the nuclear layers were less compactly packed than in control or anti-Ng-CAM-treated retinas. In contrast, retinas cultured in the presence of similar concentrations of anti-Ng-CAM antibodies or antibodies from unimmunized rabbits showed patterns resembling retinas developing *in vivo*. The fact that anti-N-CAM antibodies inhibited the morphological development of the retina while anti-Ng-CAM antibodies had no apparent effect is consistent with the relatively low Ng-CAM/N-CAM ratio in the 6-d retina (Table IV). These results were clearly in sharp contrast to those seen for fasciculation in explanted ganglia and for granule cell migration in cerebellar explants.

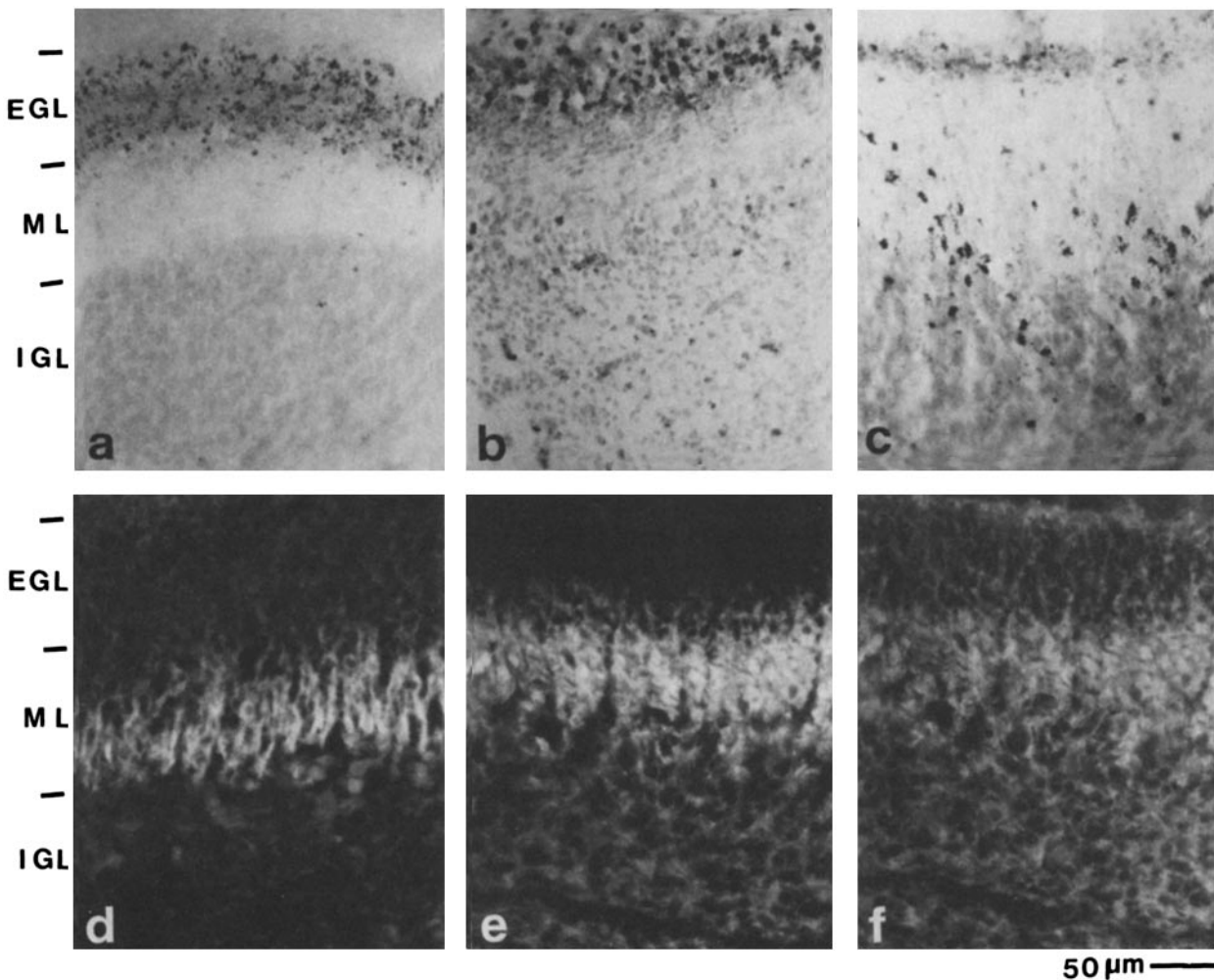


Figure 6. Effects of anti-Ng-CAM and anti-N-CAM on the migration of cerebellar granule cells. (a-c) Autoradiography of [^3H]thymidine-labeled cells. A cerebellar explant was fixed immediately after pulse labeling with [^3H]thymidine (a). Explants were also cultured for 3 d in the presence of 2.5 mg/ml anti-Ng-CAM Fab' (b) or 2.5 mg/ml anti-N-CAM Fab' (c). After fixation, explants were prepared for autoradiography as described in Materials and Methods. Cells in a-c are slightly out of focus because they are on a different focal plane than the silver grains. (d-f) Immunofluorescent staining of cerebellar sections was performed as previously described (7). (d) A section from an explant cultured for 3 d was stained with anti-Ng-CAM. (e and f) A cerebellar section from a 17-d chicken embryo was double-stained using polyclonal rabbit anti-Ng-CAM (e) and monoclonal anti-N-CAM (f). EGL, external granule layer; IGL, internal granule layer.

Discussion

The present studies provide evidence that N-CAM and Ng-CAM molecules on the same neuronal membranes differ in molecular specificity and binding mechanism. Furthermore, the relative amounts of Ng-CAM and N-CAM in different neural regions is correlated with their respective contributions to adhesion as measured in several assays by antibody perturbation. The membrane vesicle aggregation experiments confirm the homophilic nature of N-CAM binding, and indicate that if an adhesive ligand for Ng-CAM exists, it is neither Ng-CAM nor N-CAM. Perturbation experiments with carefully characterized antibodies were consistent with the conclusion that Ng-CAM plays a more predominant role than N-CAM in mediating the fasciculation of neurite outgrowths from cultured dorsal root ganglia. This was also the case in the migration of granule cells from the external to the internal granule cell layer in cerebellar explants. In contrast, the data suggested that N-CAM plays the predominant role in

the formation of histological layers in the developing retina.

Quantitation of the amounts of N-CAM and Ng-CAM showed a correlation between the relative expression of CAMs and the predominant adhesive mechanism operating in tissue of a given neural region. Consistent with this correlation, it was found that during culture of dorsal root ganglia in the presence of nerve growth factor, the Ng-CAM to N-CAM ratio in neurites rises at least sixfold to a level 15 times that found in total brain membranes. Similarly, immunohistological examination of cerebellar tissue sections has shown a large increase in the relative Ng-CAM staining in regions in which active granule cell migration was occurring (22, 43).

Because Ng-CAM appears to mediate neuron-neuron as well as neuron-glia interactions, it was important to determine its binding mechanism on neurons using immunological methods. The results of such antibody perturbation experiments must be interpreted with caution: the fact that an

Table V. Effects of Anti-N-CAM and Anti-Ng-CAM on Cerebellar Granule Cell Migration

Antibody*	% [³ H]Thymidine-labeled cells in			% Inhibition [§]
	EGL [‡]	ML [‡]	IGL [‡]	
Day 0				
Medium only	88.8 ± 3.0	2.2 ± 0.3	9.0 ± 5.3	—
Day 3				
Medium only	19.7 ± 3.7	17.0 ± 1.6	63.7 ± 5.3	8
Non-immune	20.3 ± 3.6	10.8 ± 1.8	68.8 ± 2.2	0
Anti-L-CAM	32.2 ± 1.0	6.0 ± 2.0	62.0 ± 1.0	12
Anti-Ng-CAM				
Anti-Ng-CAM	67.3 ± 5.8	20.7 ± 6.9	12.3 ± 0.9	95
Anti-135K component	60.3 ± 2.6	14.7 ± 5.7	25.0 ± 4.2	73
Anti-200K component	49.0 ± 11.8	9.0 ± 1.4	42.0 ± 10.8	45
Anti-80K component	60.0 ± 3.6	12.6 ± 4.1	27.3 ± 4.8	70
MAb 3G2	63.0 ± 6.0	11.0 ± 1.0	26.0 ± 5.0	72
MAb 16F5	63.7 ± 3.7	7.0 ± 1.6	29.3 ± 2.1	67
Anti-N-CAM				
Anti-N-CAM	33.7 ± 10.1	4.3 ± 1.9	61.7 ± 8.4	14
MAb Anti-N-CAM No. 1	29.0 ± 0.5	14.5 ± 6.5	56.5 ± 6.5	22
MAb 15G8	22.7 ± 3.1	10.0 ± 0.0	67.0 ± 3.3	7
MAb Anti-N-CAM No. 5	44.5 ± 4.3	12.0 ± 2.5	43.8 ± 2.2	42
Anti-Ng-CAM + anti-N-CAM	59.5 ± 1.5	7.5 ± 2.5	32.5 ± 1.5	66

* Antibodies used were polyclonal rabbit antibodies unless monoclonal antibody (MAb) is indicated. Polyclonal antibodies were used as Fab' fragments at a concentration of 2.5 mg/ml. Monoclonal antibodies were used as IgG (3G2, 16F5, anti-N-CAM No. 1) or IgM (15G8, anti-N-CAM No. 5) at 5 mg/ml.

‡ The distribution of [³H]thymidine-labeled cells (mean ± SD) was calculated as described in Materials and Methods. EGL, external granular layer; ML, molecular layer; IGL, internal granular layer.

§ The percent of the [³H]thymidine-labeled cells in the IGL was used to estimate the degree of inhibition of cell migration:

$$\% \text{ inhibition} = \frac{\% (\text{non-immune, day 3}) - \% (\text{antibody, day 3})}{\% (\text{non-immune, day 3}) - \% (\text{medium, day 0})} \times 100$$

antibody binds to a protein does not guarantee that the protein's function will be inhibited and the fact that an antibody blocks adhesion does not guarantee that the molecule it recognized is actually an adhesive ligand. It must also be kept in mind that in an in vitro adhesion assay, observed rates of aggregation reflect the summation of positive contributions from specific adhesion and negative contributions from parameters such as membrane charge that are intrinsic to cells and parameters such as shear rate that are dependent on the design of the assay. It is not surprising, therefore, that although N-CAM and Ng-CAM participate in independent adhesive mechanisms, antibodies to either CAM can strongly inhibit aggregation. These observations simply indicate that, in the presence of either anti-CAM antibody, the positive contributions of unblocked adhesion molecules are insufficient to overcome the threshold provided by the negative forces. Despite these caveats, appropriately controlled membrane vesicle aggregation experiments suggest that Ng-CAM-mediated adhesion is heterophilic, i.e., Ng-CAM binds *trans* to a ligand on other cells that is not Ng-CAM (see Fig. 8 A). In the most striking of these experiments, coating vesicles with anti-Ng-CAM antibodies inhibited their aggregation but had little effect on their co-aggregation with uncoated vesicles. This result might be expected if Ng-CAM binding were heterophilic because only one-half of the possible (Ng-CAM)-X bonds (Fig. 8 A) are blocked in such a coating experiment. In contrast, coating vesicles with anti-N-CAM antibodies completely inhibited their ability to aggregate as well as their ability to co-aggregate with uncoated vesicles. This result corroborates further the direct evidence

that N-CAM binding is homophilic (27); in a homophilic mechanism, coating of a membrane with anti-N-CAM would block all N-CAM-N-CAM bonds in which that membrane might participate.

Because Ng-CAM is not expressed on glia in the central nervous system (24), Ng-CAM-mediated neuron-glia adhesion must also be heterophilic. The present conclusion that Ng-CAM-mediated neuron-neuron adhesion is heterophilic must be considered tentative, however, until the ligand for Ng-CAM is definitively identified, in view of the indirect nature of the evidence and the problems inherent in analyzing the adhesive properties of a heterogeneous population of membrane vesicles. The ligand for Ng-CAM does not appear to be N-CAM as shown by the experiments with reconstituted vesicles. Moreover, the possibility that Ng-CAM is involved in adhesion only by modulating the adhesivity of N-CAM molecules on the same cell (*cis* interaction, Fig. 8 B) is not consistent with the small effect of antibodies to N-CAM in the fasciculation and migration assays. Nonetheless, the data do not rule out the possibility of a *cis* interaction between the two molecules that could occur in addition to *trans* effects.

With an understanding of the comparative molecular binding mechanisms of N-CAM and Ng-CAM in hand, we were better able to evaluate the independent functions of these molecules in a variety of developmental processes. That Ng-CAM plays a more central role than N-CAM in mediating the fasciculation of neurite outgrowths in cultured dorsal root ganglia was suggested by the observations that Ng-CAM antibodies were effective in inhibiting fasciculation at 50-fold

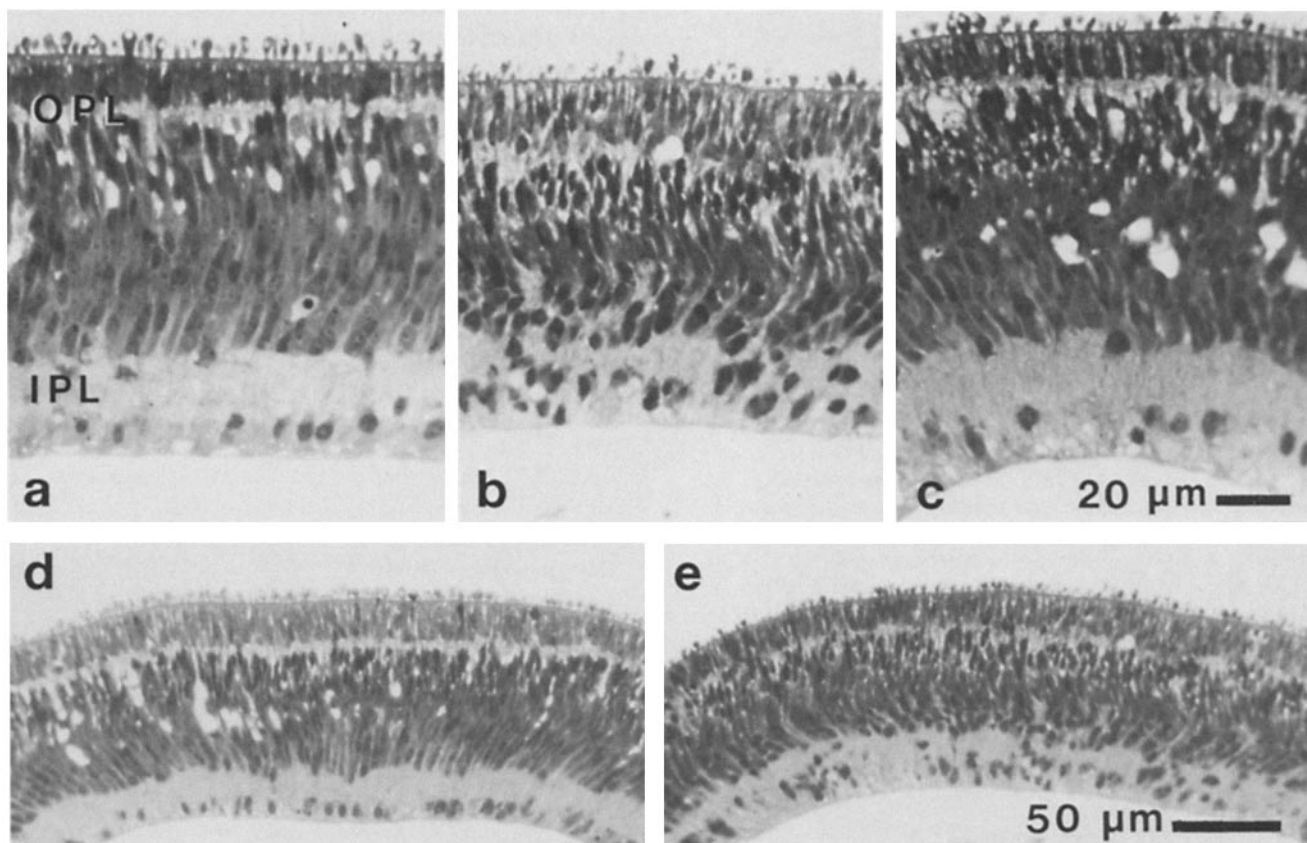


Figure 7. Formation of histological layers in the developing retina. Explants from neural retinas were cultured as described in Materials and Methods in the presence of Fab' fragments (1 mg/ml) from unimmunized rabbits (*a* and *d*) or rabbits immunized with N-CAM (*b* and *e*) or Ng-CAM (*c*). After 4 d *in vitro*, the explants were fixed and the formation of retinal layers was analyzed in histological sections. Note the narrowing, mosaic disruption, or obliteration of the outer plexiform layer and the presence of displaced cells in the inner plexiform layer. *d* and *e* are low magnification views of the sections shown in *a* and *b*, respectively. OPL, outer plexiform layer; IPL, inner plexiform layer.

lower doses than anti-N-CAM antibodies, and that monoclonal anti-Ng-CAM antibodies inhibited fasciculation while monoclonal anti-N-CAM antibodies did not. Furthermore, the ability of polyclonal anti-N-CAM antibodies to inhibit fasciculation was found to be primarily due to the presence of antibodies cross-reactive with Ng-CAM (Table II). The ability (37) of anti-TCS N-CAM (an antiserum prepared against partially purified N-CAM before Ng-CAM had been identified) to inhibit fasciculation was definitely shown to be due to the presence of anti-Ng-CAM reactivity in the polyvalent antibody preparation.

Although N-CAM does not appear to play a major role in the fasciculation of neurite outgrowths in cultured 10-d dorsal root ganglia, we cannot rule out the possibility that it is involved in fasciculation in systems where N-CAM-mediated adhesion is enhanced by parameters such as increased expression relative to Ng-CAM or decreased sialylation (27). The present results thus may not be inconsistent with the recent observations that (*a*) antibodies to the NILE glycoprotein (which is equivalent to rodent Ng-CAM [18]) preferentially inhibit fasciculation in cultures of 14-d embryo rat brain cells while antibodies to N-CAM preferentially inhibit fasciculation in cultures of postnatal 5-d cerebellum cells (41), and (*b*) anti-N-CAM antibodies inhibit the fasciculation of neurite outgrowths from cultured 7-d lumbar dorsal

root ganglia (38). Nevertheless, in preliminary experiments, we find that Ng-CAM is also the predominant adhesion molecule involved in the fasciculation of both 7-d lumbar dorsal root ganglia (containing the embryonic form of N-CAM) and 18-d lumbar dorsal root ganglia (containing the adult form of N-CAM) *in vitro*. Therefore, even when N-CAM was present in the highly adhesive adult form (27), Ng-CAM was the predominant adhesion molecule as determined by antibody perturbation.

Given the markedly non-linear dependence of known CAM binding on surface concentration (27), it is not surprising that differential prevalence modulation and polarity modulation of Ng-CAM and N-CAM in various neural regions leads to differential contributions of these CAMs to various neuronal interactions. Our studies indicate that differential prevalence modulation as well as polarity modulation in response to nerve growth factor may both be occurring in cultured dorsal root ganglia. It was recently observed that culturing PC12 cells in the presence of nerve growth factor enhanced their expression of Ng-CAM but not N-CAM (18). During culture of dorsal root ganglia in the presence of nerve growth factor, the Ng-CAM/N-CAM ratio increased 2.5-fold in the region of the original explant and sixfold in neurite outgrowths. Nevertheless, this polarity modulation of Ng-CAM was not as great as has previously been observed

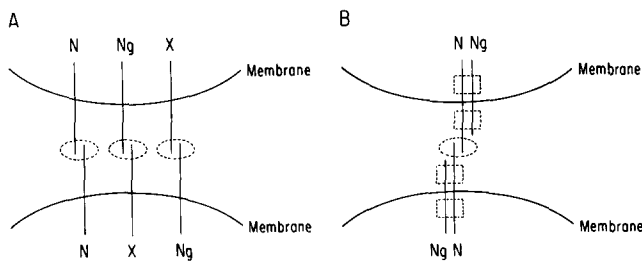


Figure 8. *Trans* and *cis* binding mechanisms. (A) In a strict *trans* binding model, N-CAM (N) on one membrane binds to N-CAM on a second membrane (homophilic binding). Ng-CAM (Ng) binds to its ligand, X (heterophilic binding). Note that coating only one of two membranes with antibodies to a homophilic ligand nevertheless blocks all bonds involving that molecule, while coating only one of two membranes with antibodies to a heterophilic ligand blocks only one-half the bonds involving that molecule. Dashed ovals indicate *trans* bonds. (B) In a *cis* binding model, Ng-CAM has no *trans* ligand, but interacts with N-CAM on the same membrane and thereby affects its *trans* binding. Antibodies to Ng-CAM would affect adhesion either by steric interference with the N-CAM binding region or by perturbing the *cis* interaction between Ng-CAM and N-CAM. Dashed ovals indicate *trans* bonds, dashed rectangles indicate *cis* interactions between Ng-CAM and N-CAM which might occur either in intercellular or extracellular portions of the molecules. Models in which Ng-CAM participates in both *trans* bonds and *cis* interactions are also possible.

by immunohistological methods in the central nervous system where only very faint staining was seen on somata of non-migrating neurons (14). Whether this reflects a real difference between the central nervous system and dorsal root ganglia, or is an artifact resulting from the failure of some Ng-CAM-rich growing neurites to leave the cell body region of the original explant *in vitro* remains to be determined. It is pertinent, however, that we have observed in preliminary experiments that the Ng-CAM/N-CAM ratio directly determined in dorsal root ganglia dissected from older embryos is actually lower than in 10-d dorsal root ganglia, raising the possibility that the contribution of N-CAM to fasciculation *in vivo* is greater than *in vitro* where the presence of a high concentration of nerve growth factor may have artificially increased the Ng-CAM/N-CAM ratio. Alternatively, the low Ng-CAM/N-CAM ratio in ganglia from older animals may reflect the fact that Ng-CAM-rich neurite outgrowths would not have been dissected along with the ganglia. In any case, the present results and those on PC12 cells suggest that the expression of CAMs *in vivo* may be controlled by growth factors and they raise the possibility that the ability of nerve growth factor to promote neurite outgrowth may itself depend in part on neurite-neurite interactions mediated by Ng-CAM.

Effects related to differential modulation also appear to apply to morphogenetic processes dependent upon neuron-glia interactions. In the present studies, cerebellar granule cell migration, which depends strongly on interactions of the leading edge of the neuronal cell body with Bergmann glia, was preferentially inhibited by anti-Ng-CAM antibodies (Table V) despite the fact that the aggregation of cerebellar neuronal membrane vesicles was inhibited by both anti-N-CAM and anti-Ng-CAM. The binding of neurons to mature central nervous system glia is inhibited by anti-Ng-CAM but not by anti-N-CAM antibodies (22). The composite results are

consistent with the conclusion (34), based on histological data, that granule cell migration in the cerebellum is mediated mainly by an interaction between these neurons and radial glial fibers and not directly by neuron-neuron interactions.

The present and previous experiments indicate how a relatively small number of adhesive molecules of unique molecular specificity could cause cells to form highly complex histological patterns provided that the function of the molecules was appropriately modulated. In previous studies, it was observed, for example, that during the maturation of the brain, a chemical modulation of N-CAM occurs to different extents in different regions: in the perinatal period, the amount of polysialic acid in the N-CAM molecule decreases to one-third that seen in the embryonic period (6, 36). This change, called E to A conversion, is correlated with an increase in N-CAM binding efficacy (27) which appears to be independent from the changes in binding efficacy that reflect alterations in the local surface concentration of N-CAM. The various combinations of modulation of CAMs of different specificities by changes in relative prevalence, by their polarized distribution on cells, by chemical modification, and by their differential expression induced by growth factors could together geometrically increase the number of patterns that CAMs could specify in contributing to neural histogenesis.

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