Neural Cell Adhesion Molecule Regulates Cell Contact–mediated Changes in Choline Acetyltransferase Activity of Embryonic Chick Sympathetic Neurons

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Abstract. E10 chick sympathetic ganglion cells display a cell contact–dependent rise in choline acetyltransferase (ChAT) specific activity over the first several days in culture. This effect can be mimicked by addition of crude membrane fractions prepared from E10 retina and adult chicken brain, but not by those from E10 brain. The effects of both cell–cell and membrane–cell contact are inhibited by the addition of anti-NCAM Fab fragments. The membranes capable of increasing ChAT and those which are ineffective all contain NCAM, however their relative levels of NCAM poly-sialic acid differ. Whereas membranes with high poly-sialic acid NCAM are ineffective, selective enzymatic removal of polysialic acid renders them capable of producing an increase in ChAT. The inhibition of NCAM-mediated adhesion produced by Fab fragments can be compensated for by addition of wheat germ ag-glutinin, but only with membranes whose NCAM has low levels of polysialic acid. Taken together, these data suggest that NCAM can regulate cell contact–mediated increases in ChAT activity. We propose that NCAM-mediated adhesion promotes contact between cell membranes to allow the transmission of an otherwise NCAM-independent signal. In addition, NCAM's poly-sialic acid moiety appears to influence the ability of cells to transmit this signal, even in the presence of an alternative adhesion mechanism.

C ELL–CELL recognition via adhesion between plasma membranes contributes to both the formation of specific patterns within a neural tissue and the establishment of neuronal pathways and connections (see reference 13 for general review). In some cases the adhesion appears to be largely a mechanical phenomenon; that is, cell surface adhesion molecules physically hold two membranes together. However, there is evidence that cell–cell adhesion can also generate biochemical signals which lead to alterations in gene expression and/or functional properties of neural cells. For example, direct contact between glial cells and neurons in vitro promotes biochemical changes in several different types of glial cells. Such changes include the ability to express mature biochemical properties (cortisol-mediated glutamine synthetase induction in chick retinal and tectal glial cells; 27, 28), stimulation of cell division (rat Schwann cells early in development; 18, 45–47), and morphological differentiation accompanied by cessation of proliferation (mouse cerebellar astrocytes; 19). Neuron–neuron contact

has also been shown to produce changes in cellular bio-chemistry in vitro. Levels of neurotransmitter biosynthetic enzymes (tyrosine hydroxylase and/or choline acetyltrans-ferase [ChAT])[1] and of neuropeptides (substance P) are reg-ulated by cell–cell contact in rat and chick sympathetic neu-rons, as well as in two other related neural crest derivatives, rat pheochromocytoma (PC12) cells and bovine adrenal chromaffin cells (1–3, 25, 29, 57).

In most of these systems, the effects of cell–cell contact have been studied in three ways: (*a*) increased cell density or co-culture of glial cells with neurons has been used to pro-duce increased amounts of cell contact (1, 2, 29, 36, 46); (*b*) addition of crude plasma membrane fractions to cultured cells has been shown to mimic cell contact (3, 18, 25, 36, 44, 47); whereas (*c*) addition of conditioned medium fails to mimic cell contact (1, 3, 19, 46). As has been suggested in studies of neuron–Schwann cell contact (35), there are two general biochemical mechanisms by which cell contact could cause alterations in cell biochemistry: (*a*) cell adhesion mol-ecules could serve to hold the appropriate cells together, whereas different molecules actually generate signals be-tween cells; or (*b*) a single molecule or ligand-receptor com-plex could function simultaneously in both capacities. Whereas studies with crude membranes indicate the require-

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1. *Abbreviations used in this paper*: ChAT, choline acetyltransferase; endo N, endoneuraminidase N; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; WGA, wheat germ agglutinin.

ment for cell contact, they are not able to distinguish between these two mechanisms.

To investigate the role of cell adhesion in cell contact–dependent changes in neuronal neurotransmitter enzymes, we have focussed on a particular molecule, the neural cell adhesion molecule (NCAM). NCAM is a well-characterized cell adhesion ligand (for recent reviews see references 9, 38, 39) which is expressed on many types of neural cells, including sympathetic ganglion cells (51). Our experimental approach has been to use NCAM-containing cell membranes, namely those derived from chick brain or retina, to stimulate contact-dependent regulation of ChAT in chick sympathetic neurons. The role of NCAM has been analyzed by specific perturbation of the molecule's function in two ways: inhibition of NCAM-mediated adhesion by anti-NCAM Fab fragments, and enhancement of adhesion by desialylation of the molecule using a bacteriophage endoneuraminidase N (endo N; 43). In addition, we have used a lectin to agglutinate membranes to cells, thereby bypassing NCAM's adhesive function and allowing us to evaluate NCAM's importance in signal generation.

Materials and Methods

Embryonic Chick Sympathetic Ganglion Cell Culture

Paravertebral sympathetic ganglia from the lumbar and sacral levels of 10-d-old chick embryos (stage 36) were used for primary cultures, which were prepared using previously described methods (4). Briefly, upon dissection, ganglia were collected in PBS at room temperature. The ganglia were then incubated with 0.01% trypsin in Ca^{2+}/Mg^{2+}-free PBS for 20 min at 37°C. After trypsinization, ganglia were treated for ~2 min with 0.02 mg/ml DNase and then washed with an excess first of PBS and then of F14 medium (54) containing 10% heat-inactivated horse serum (F14/HS). The ganglia were then dissociated into single cells by passing them through the tip of a Pasteur pipette 10–20 times with moderate force. The resulting cell suspension contained both neurons and nonneuronal cells. In some experiments, these two populations were separated from one another by a preplating step (3 h at 37°C on uncoated tissue culture plastic), because nonneuronal cells adhere preferentially to tissue culture plastic in the presence of horse serum (55). The number of cells per milliliter was approximated using a hemocytometer, and cells were plated at a given density onto polyornithine and laminin-coated 35-mm tissue culture plastic dishes (11) in a total volume of 1.5 ml F14/HS containing 20 ng/ml nerve growth factor (NGF). Under these conditions, ~90% of the neurons initially plated survived for up to 5 d in culture (11). In addition, as previously described, when the total cell suspension was plated, the presence of horse serum in the medium prevented the nonneuronal cells from overgrowing the dish over a 2–4-d culture period (55).

An accurate value for the number of cells per dish at the start of culture was determined by counting the total number of phase-bright cells attached to the dish 1–3 h after plating. Cell counts were carried out by scanning along a strip with an area of 1.7% of the total surface area of the dish using a phase-contrast microscope with 125× magnification. The number of surviving neurons was counted at various times after plating as well. Neurons were defined as those cells having well-defined phase-bright cell bodies with neurites at least 5 cell diameters long after 48 h in culture. Nonneuronal cells were flat and phase dark after several hours in culture, and could be counted when cell density was low.

ChAT Activity

ChAT activity was determined according to the method of Fonnum (12) in extracts of cultured cells prepared by adding the homogenization buffer directly to culture dishes. All activity measured in both mixed cultures and pure neuronal cultures could be blocked by the specific ChAT inhibitor naphthylvinyl pyridine (50 µM; 56).

Specific enzymatic activity is expressed as activity per cell, with number of total cells or neurons determined by counting, as described above.
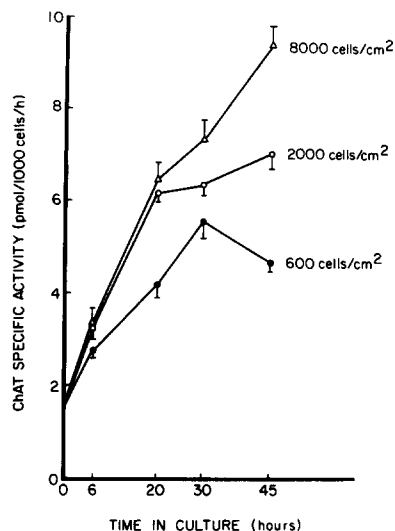

Figure 1. ChAT specific activity as a function of time in culture at low, intermediate, and high density. Sympathetic ganglion cells (both neurons and nonneuronal cells) were plated at densities of ~600, 2,000, or 8,000 cells/cm², and ChAT specific activity was determined 6, 20, 30, or 45 h later. The zero time point represents the value obtained from dissociated cells which were not plated. Values are means ± SEM of nine values from four independent experiments.

Preparation of Crude Membrane Vesicles

Membrane vesicles were prepared from various tissues using a modification of previously described methods (22). Briefly, tissues were dissected, rinsed with Hepes-buffered saline (HBS; 10 mM Hepes, 150 mM NaCl, pH 7.4, containing 100 KIU aprotonin, 50 µg/ml DNAase, and 2 mM $CaCl_2$), then homogenized in the same buffer. Homogenates were first centrifuged at low speed (120 g) for 5 min at 4°C to remove unhomogenized tissue and nuclei. The resulting supernatant was then centrifuged at 17,000 g for 20 min at 4°C. The pellet was resuspended in HBS containing 0.6 M sucrose, transferred to an ultracentrifuge tube, and a cushion of 1.2 M sucrose in HBS was layered underneath. Tubes were centrifuged in a Ti60 rotor at 35,000 rpm for 45 min at 4°C. The crude membrane vesicle fraction was collected from the interface of the two sucrose concentrations. Vesicles were washed twice with HBS. The final pellet was diluted 1:20 in F14/HS. This dilution resulted in an average protein concentration of 0.46 ± 0.02 mg/ml (average of values determined for E10 retina, E10 brain, and adult brain membranes, n = 8), as determined using the Peterson protein assay (32) with BSA as a standard.

Addition of Vesicles to Cells in Culture. 100 µl of diluted membrane vesicles was added to each 35-mm culture dish, which already contained cells that had been allowed to attach to the dish for 5 h. The dishes were then centrifuged at 1,600 g in the flat bottom of swinging bucket rotors for 15 min at 4°C, which resulted in a rapid and homogeneous adhesion of the membrane fractions to the entire surface of the dish. Careful examination at high magnification revealed that the cell bodies themselves were also densely coated with vesicles. The amount of membrane added (46 µg/dish or 30.7 µg/ml) was comparable with that used in previous studies of related cell culture systems (rat sympathetic neurons, 36–72 µg/35-mm dish (3) or three doses of 100 µg/ml (25); bovine adrenal chromaffin cells, 30 µg/ml [47]). Neuronal counts were made immediately before membranes were added and at the end of the culture period. Values for ChAT activity obtained from membrane-coated dishes without cells were subtracted from those obtained with cells.

Treatment of Vesicles with Endoneuraminidase N. The final vesicle pellet was resuspended in HBS at a dilution of 1:20. To 25 µl of this vesicle suspension, 32 U of purified endo N (43) was added in a total volume of 600 µl HBS. After incubation at 4°C for 30 min, vesicles were washed once with a large excess of HBS and the final pellet was resuspended at a dilution of 1:100 in ice-cold F14/HS. 500 µl of this suspension was added to cells in culture. To monitor removal of NCAM polysialic acid, samples of untreated and treated vesicles were analyzed by immunoblot (see following).

Immunological Reagents

NCAM. Anti–NCAM IgG was purified from polyclonal antisera raised in rabbits against chicken NCAM. Purity of IgG fractions was monitored by SDS-PAGE using 15% separating gels and silver stain. Fab fragments were prepared from the IgGs by digestion with pepsin (43). No heavy chain was detectable on gels of the Fab preparations.

G4. G4 antigen was affinity immunopurified from adult chicken brain using G4 mAb (34), and was generously provided to us by Dr. Fritz Rathjen, Max-Planck Institute for Developmental Biology, Tuebingen, FRG. Polyclonal anti-G4 antiserum was raised in rabbits, and IgG fractions and Fabs were prepared as described for NCAM. Chicken G4 antigen is biochemically and functionally related to the mouse L1 cell adhesion molecule. G4 and L1 have 50% identical NH$_2$-terminal amino acid sequences, behave similarly in two-dimensional IEF/SDS-PAGE gels, and are both associated with fiber tracts and involved in neurite fasciculation in vitro (34).

Gel Electrophoresis and Immunoblot Techniques

Gel electrophoresis was carried out using 7.5% polyacrylamide separating gels containing SDS (26). Separated proteins were then electrophoretically transferred to nitrocellulose for immunoblotting (53). Nonspecific binding was blocked by incubating the nitrocellulose sheets with Blotto (23) for 1 h at 37°C. Primary antibody was diluted in Blotto and incubated with the nitrocellulose for 2 h at room temperature. Unreacted antibody was washed away using a series of 4 5-min washes in phosphate buffer (55). Bound antibody was then reacted with a peroxidase-conjugated second antibody (anti-IgG of the appropriate species, also diluted in Blotto), and visualized by color development of the enzyme reaction using 0.05% 4-chloro-1-naphthol and 0.01% H$_2$O$_2$ as substrates (20).

Results

Characterization of the Effect of Cell–Cell Contact on Neurotransmitter Biosynthetic Enzyme Activity in Chick Sympathetic Neurons

To study the role of NCAM in cell contact–mediated changes in neurotransmitter enzyme levels in vitro, a model system was required in which NCAM function could be reliably perturbed. The chick embryo was used because Fab fragments prepared from polyclonal antisera against chicken NCAM have been shown to be effective and specific in blocking NCAM function in a variety of different developmental events (17, 24, 31, 40, 42, 48, 50). We chose to examine the effect of cell contact on ChAT specific activity in sympathetic neurons, because previous studies using rat sympathetic neurons indicated that this enzyme is regulated by cell–cell contact (2, 3, 25).

To produce different degrees of cell–cell contact, sympathetic ganglion cells from embryonic day 10 (including both neurons and nonneuronal cells) were cultured at different densities, ranging from 6,000–80,000 cells/dish (600–8,000 cells/cm²). This procedure resulted in an initial homogeneous distribution of >85% single cells at each of the different densities. ChAT specific activity remained low at all densities for the first 4 h in culture (Fig. 1). However, by
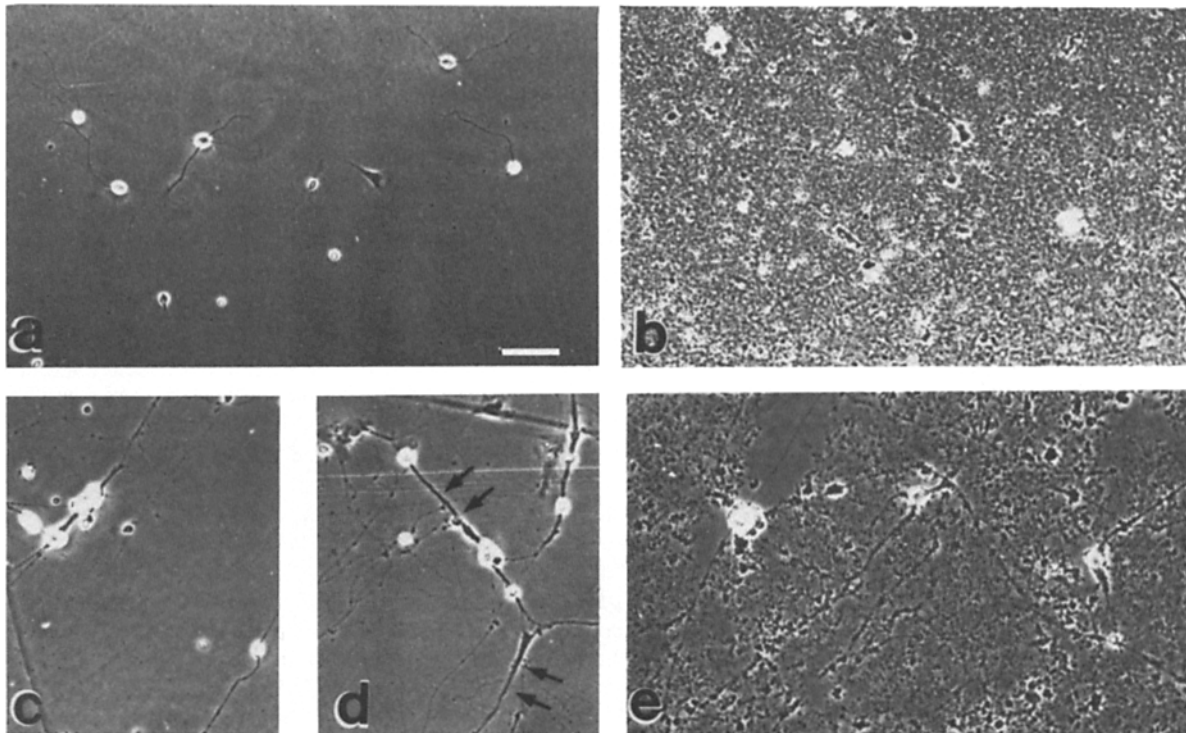

Figure 2. Appearance of sympathetic ganglion cell cultures after membrane treatment. Sympathetic ganglion cells were cultured at a density of ∼1,500 cells/cm². 5 h after the initial plating, E10 retinal membranes were added to the dishes as described (see Methods). Photographs were taken after 1 d in culture (*a* and *c*, untreated controls; *b*, E10 retinal membranes added) and after 3 d in culture (*d*, untreated controls; *e*, E10 retinal membranes added). (*e*) The cultures develop normally in the presence of membranes. Note the increased neuronal size and the extensive neuritic network that has developed (compare to *a*). (*c*) Even at low density, some clusters of neurons are present after 1 d in culture. (*d*) Nonneuronal cells present in the cultures tend to align themselves along the neurites. Arrows point to the flattened, phase-dark nonneuronal cells, easily seen after several days in culture. Bar, 50 μm.

8 h after plating, ChAT activity began to increase, even at the lowest density (Fig. 1). As plating density was increased, the plateau of increased activity was reached at later times, i.e., at low density the plateau was reached after only 1 d in culture, whereas at intermediate and high densities, the plateau was reached after 2 d or longer (Fig. 1).

Although the extent of cell contact was difficult to quantify, it was qualitatively clear that even after only 24 h at low cell density, some of the single cells had migrated together to form small clusters (Fig. 2 *C*). At intermediate and high densities, larger clusters were formed. Previous studies using rat sympathetic neurons suggested that this normally occurring migration of the neurons in culture is responsible for the effect on ChAT activity (3). In the present cultures, the non-neuronal cells tended to align themselves along the neurites (Fig. 2 *D*). Thus, there were at least three different types of contact occurring in these cultures: cell body–cell body, neurite–neurite, and neurite–glial cell. However, an effect on ChAT activity similar to that seen in mixed cultures was also present in >95% pure neuronal cultures (see Methods). Neurons maintained at intermediate density (1,500 neurons/cm^2) for 30 h had higher ChAT specific activity (13.8 $\pm$ 1.10 pmol/h per 1,000 cells) than those maintained at low density (650 neurons/cm^2; 6.0 $\pm$ 0.53 pmol/h per 1,000 cells), suggesting that the presence of neurons alone was sufficient to produce the effect. A significant and variable degree of neuron–neuron aggregation occurred during the preplating step used to prepare pure neuronal cultures which, in turn, affected the baseline of ChAT activity at any given density. Thus, pure neuronal cultures were not used for studies of membrane–cell contact described below.

To further assess the importance of cell–cell contact, conditioned medium from high-density cultures was added to cells plated at low density. After 17 h in culture, there was no effect of conditioned medium on ChAT activity (Table I).

We next examined the ability of crude membrane fractions to mimic the effect of increased cell density. Membranes from embryonic day 10 chick brain or retina were added to intermediate density cultures 5 h after the initial plating. Neither brain nor retinal membranes altered the long-term survival of the neurons (data not shown), or the development of characteristic long neurites (Fig. 2 *E*). However, addition of either type of membrane substantially inhibited the formation of clusters of neuronal cell bodies (Fig. 2 *E*). Thus,

TIME AFTER ADDITION OF MEMBRANE (hours)

Figure 3. Effect of retinal and brain membranes on ChAT activity. Sympathetic ganglion cells were plated at a density of ~2,000 cells/cm^2. 5 h after the initial plating, membranes prepared from E10 retina (*RT*) or E10 brain (*BR*) were added to the dishes as described (see Methods). 8, 18, or 30 h later, ChAT specific activity was determined. Addition of both types of membrane decreased cell–cell contact, probably reflecting a reduction in cell migration. Thus, although values for cells alone are shown for reference (*control*), the enhancement of ChAT activity by retinal membranes is best represented by comparison with the levels obtained with E10 brain membranes. The zero time point represents the values obtained from dishes immediately after the addition of membranes. Values are means $\pm$ SEM of 11 values obtained in four independent experiments.

when E10 brain membranes were added to the cells, ChAT specific activity increased only slightly from the low level seen in freshly dissociated cells (Fig. 3). This inhibitory effect relative to untreated control cultures probably reflects the reduction in cell migration caused by the presence of membrane vesicles on the substrate. Despite the decrease in cell–cell contact, the addition of retinal membranes brought about an increase in ChAT activity to levels about three times those obtained in cultures treated with E10 brain membranes (Fig. 3).

Role of NCAM: Effect of Anti-NCAM Fab Fragments

Cell–Cell Contact. As a first step in determining whether NCAM was involved in cell contact–mediated biochemical events, the effect of anti-NCAM Fab fragments on ChAT activity was assessed in cultures of sympathetic neurons. Sympathetic ganglion cells were kept in suspension culture in uncoated tissue culture plastic dishes for 8 h to allow cell surface proteins, including NCAM, to recover after trypsinization (41). During the initial 3 h, the nonneuronal cells were allowed to attach to the plastic surface. The neuron-enriched suspension was then carefully removed and placed in a second uncoated dish in NGF-containing medium for the remainder of the 8 h period. Cells were then preincubated for 30 min with either anti-NCAM Fab or preimmune Fab plated at high density (6,000 cells/cm^2), and ChAT activity was determined 1 d later. In two independent experiments, ChAT specific activity was decreased nearly threefold by anti-NCAM Fab fragments (preimmune Fab, ChAT sp act 12.5 $\pm$ 1.2; anti-NCAM Fab, ChAT sp act 4.54 $\pm$ 0.33; means $\pm$ SEM of six dishes).

Cell–Membrane Contact. We next examined the ability of anti-NCAM Fab fragments to inhibit the effect of retinal

Table I. Effect of High Density Conditioned Medium on ChAT Activity in Low Density Cultures

	ChAT specific activity
	*pmol/h per 1,000 cells**
Low density‡	2.53 $\pm$ 0.09
Low density + 50% CM§	2.78 $\pm$ 0.08
High density‖	6.56 $\pm$ 0.34
High density + 50% CM	6.87 $\pm$ 0.27

* ChAT specific activity was determined after 20 h in culture. Values are means $\pm$ SEM of six values obtained in two independent experiments.
‡ Low density was 530 cells/cm^2.
§ Conditioned medium (CM) was obtained from high density cultures as follows. Sympathetic ganglion cells were plated at a density of 9,200 cells/cm^2 and were cultured for 4 d without medium change. The conditioned medium was then removed and stored frozen at −20°C. Cultures treated with CM received 50% CM and 50% fresh F14/HS, together with 30 ng/ml NGF.
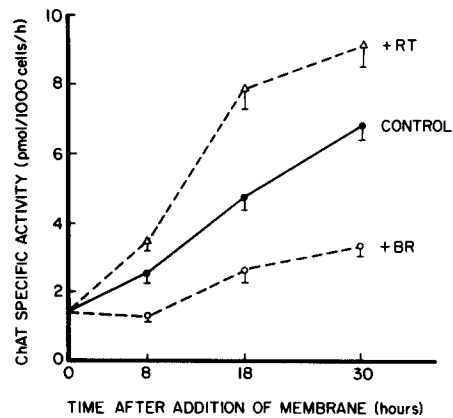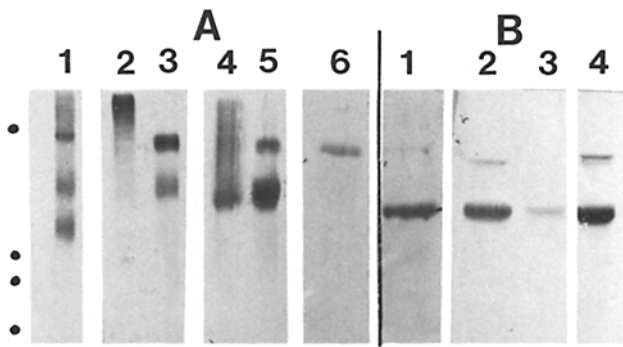‖ High density was 7,250 cells/cm^2.

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Figure 4. NCAM and G4 present in retinal and brain membranes and sympathetic neurons. The NCAM and G4 forms present on membranes obtained from E10 retina and brain, adult chicken brain, and sympathetic neuronal cultures were monitored by immunoblots after transfer of proteins separated on SDS-PAGE to nitrocellulose. (A) NCAM was detected using a polyclonal anti–chicken NCAM antiserum. (Lane 1) Adult chicken brain membranes; (lane 2) E10 brain membranes; (lane 3) E10 brain membranes treated with endo N as described (see Methods); (lane 4) E10 retinal membranes; (lane 5) E10 retinal membranes treated with endo N; (lane 6) total protein from sympathetic ganglion cell cultures after 1 d in vitro. The diffuse staining in the high molecular mass range reflects NCAM molecules with high polysialic acid content (lanes 2 and 4). Treatment with endo N removes most of this diffuse staining, yielding two more discrete bands at ∿ mol wt 180 and 140 kD (lanes 3 and 5). Because NCAM forms present in adult chicken brain membranes (lane 1) and sympathetic neurons (lane 6) contain little polysialic acid, more discrete bands are seen. (B) G4 was detected using a polyclonal anti–chicken G4 antiserum, and appears as two discrete bands of ∿ mol wt 180 and 130 kD. (lane 1) Adult chicken brain membranes; (lane 2) E10 brain membranes; (lane 3) E10 retinal membranes; (lane 4) total protein from sympathetic ganglion cell cultures after 1 d in vitro. Molecular mass standards, indicated by dots, are: myosin (200,000), phosphorylase b (92,500), BSA (69,000), and ovalbumin (46,000).

Importance of NCAM Polysialic Acid Content

NCAM is present on retinal membranes, which were effective in stimulating ChAT activity, as well as on E10 brain membranes, which were ineffective. However, E10 retinal NCAM has a relatively low polysialic acid content compared with E10 brain NCAM (Fig. 4 A; see also references 8, 22). The possible role of NCAM polysialic acid in the membrane-mediated increase in ChAT activity was thus examined in two ways: (a) selective removal of polysialic acid from NCAM using the bacteriophage enzyme endo N, and (b) addition of membranes from adult chicken brain, in which the naturally occurring NCAM is similar to E10 retina in its content of polysialic acid (Fig. 4 A).

In the first protocol, retinal and brain membranes were each incubated with endo N, and aliquots of treated and untreated vesicles were analyzed by SDS-PAGE to determine the effectiveness of the treatment (Fig. 4 A). Retinal membranes that had been treated with endo N were not altered in their ability to mediate an increase in ChAT. In contrast, E10 brain membranes, which when untreated did not produce an increase in ChAT, became effective in increasing enzyme activity after endo N treatment (Table III). In the second protocol, exposure of cells to adult chicken brain membranes resulted in an increase in ChAT activity (Table III). In all cases, the enhancement of ChAT activity produced by the membranes was blocked by anti-NCAM Fab fragments (Table III), and anti-G4 Fab fragments were ineffective (Table II).

membranes on ChAT activity. The Fab fragments were added to the cells together with the membranes ∿5 h after the initial plating. 17 h later, ChAT specific activity was determined. As a control for the specificity of the effects of anti-NCAM Fab fragments, membranes were also added together with Fab fragments of a polyclonal antiserum directed against the G4 antigen, another cell adhesion molecule (related to L1, NILE, and NgCAM; 6, 16, 33, 34, 49) which is present on both retinal and brain membranes, as well as on sympathetic neurons (Fig. 4). Both immunoblot analysis (Fig. 4; compare panel A, lane 6 with panel B, lane 4) and immunostaining (7) indicate that the relative amounts of NCAM and G4 present on sympathetic neurons are comparable. G4 is present on both E10 and adult chicken brain membranes in relatively high amounts (Fig. 4 B, lanes 1 and 2), but on E10 retinal membranes to a lesser extent (Fig. 4 B, lane 3).

In these experiments, anti-NCAM Fab fragments inhibited the effect of retinal membranes on ChAT activity, whereas anti-G4 Fab fragments and those prepared from nonimmune serum had no effect (Table II). Neither the decrease in ChAT activity seen after the addition of brain membranes (Table II) nor cell viability (data not shown) was altered by any of the Fab fragments.

Table II. Effect of Fab Fragments on Membrane-mediated Alterations in ChAT Activity

Membranes added*	Fab‡	ChAT specific activity§
		pmol/h per 1,000 cells
None	—	5.00 ± 0.08
	Nonimmune	4.83 ± 0.25
	Anti-NCAM	4.91 ± 0.21
	Anti-G4	5.22 ± 0.19
E10 Retina	—	12.2 ± 0.52
	Nonimmune	13.3 ± 0.47
	Anti-NCAM	3.51 ± 0.09
	Anti-G4	12.7 ± 0.33
E10 Brain	—	3.33 ± 0.11
	Nonimmune	2.95 ± 0.12
	Anti-NCAM	2.74 ± 0.14
	Anti-G4	3.57 ± 0.10
E10 Brain + endo N	—	6.56 ± 0.01
	Anti-G4	6.66 ± 0.02
Adult brain	—	13.7 ± 0.2
	Nonimmune	14.1 ± 0.3
	Anti-G4	13.2 ± 0.3

* Sympathetic ganglion cells were cultured at a density of ∿2,000 cells/cm². 5 h later, membranes prepared from the tissues indicated were added to the dishes as described (see Methods). In some experiments, E10 brain membranes were treated with endo N as described (see Methods).
‡ Membranes were preincubated with 50 μg of the appropriate Fab fragment/μl original pellet volume for 30 min on ice, before membranes plus Fab fragments were added to the cells.
§ 18 h after the addition of membranes to cells, ChAT specific activity was determined in extracts from each dish. Data are means ± SEM of five to nine values obtained in five independent experiments.

Table III. Role of NCAM's Polysialic Acid Content in the Effectiveness of Membranes to Mediate an Increase in ChAT Activity

Membranes added*	Anti-NCAM‡	ChAT activity§
None	−	5.1 ± 0.2
	+	4.8 ± 0.3
E10 Brain	−	3.3 ± 0.1
	+	2.7 ± 0.1
E10 Brain + endo N	−	6.7 ± 0.2
	+	2.6 ± 0.1
E10 Retina	−	14.4 ± 0.6
	+	3.9 ± 0.3
E10 Retina + endo N	−	15.2 ± 0.8
	+	3.2 ± 0.3
Adult brain	−	13.6 ± 0.4
	+	3.3 ± 0.2

* Sympathetic ganglion cells were cultured at a density of ∼2,000 cells/cm². 5 h later, membranes were added to the dishes as described (see Methods). The relative content of NCAM polysialic acid (PSA) for each of the membrane preparations can be estimated from the mobility of NCAM in SDS-PAGE as visualized on immunoblots (see Fig. 4).
‡ Membrane vesicles were preincubated with 50 μg anti-NCAM Fab fragment/μl original pellet volume for 30 min on ice, before membranes plus Fab fragments were added to the cells.
§ 18 h after the addition of membranes to cells, ChAT specific activity was determined (pmoles/h per 1,000 cells). Data are means ± SEM of seven to nine values obtained from three independent experiments.

Is the Role of NCAM Direct or Indirect?

Whereas the data presented above suggested a role for NCAM in the cell contact–mediated change in ChAT activity, they are not able to distinguish between a direct effect of NCAM as a signal generator and the indirect effect of agglutination of membranes to cells, i.e., a purely adhesive function. To address this issue, we have examined the ability of an alternative adhesion mechanism, agglutination by a plant lectin (wheat germ agglutinin; WGA), to substitute for NCAM-mediated adhesion.

This series of experiments was carried out using E10 retinal and brain membranes whose NCAM-mediated adhesion was blocked by incubation with anti-NCAM Fab fragments. The membranes were then added to cells that had been pretreated with WGA. Whereas retinal membranes added together with anti-NCAM Fab fragments had no effect on ChAT activity, their ability to increase ChAT activity was restored when the membranes were agglutinated to the cells with WGA (Fig. 5). However, when E10 brain membranes were added to the cells in the presence of anti-NCAM, ChAT activity remained low, even in the presence of WGA (Fig. 5). Only after treatment of E10 brain membranes with endo N to remove NCAM polysialic acid did WGA-mediated agglutination result in an increase in ChAT activity (Fig. 5). This effect does not reflect a difference in the number of ^{125}I-WGA binding sites present on endo N–treated brain membranes (1.25 μg/ml ^{125}I-WGA plus 400 μg/ml cold WGA incubated with control or endo N–treated brain vesicles for 10 min at 4°C; −endo N = 49,479 ± 3,589 cpm bound, +endo N = 52,944 ± 4,641 cpm bound; heat-denatured ligand ^{125}I-WGA = 1,805 ± 125 cpm bound).
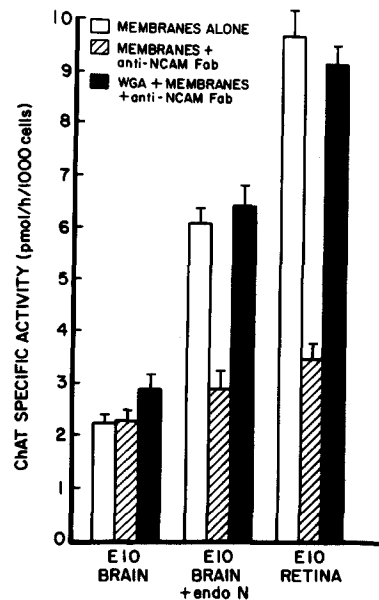

Figure 5. Lectin-mediated agglutination of membranes to cells: role of NCAM polysialic acid in the effectiveness of membranes to increase ChAT activity. Sympathetic ganglion cells were plated at a density of ∼2,000 cells/cm². 5 h later, some of the cells were incubated with 10 μg/ml WGA at 37°C for 30 min. Membrane vesicles were prepared from E10 brain or retina and treated with endo N as described (see Methods). Aliquots of E10 brain membranes, E10 brain membranes treated with endo N, and E10 retinal membranes were then preincubated with anti-NCAM Fab fragments (50 μg anti-NCAM Fab/μl original pellet vol for 15 min at 4°C). Membranes alone (*open bars*) or membranes + anti-NCAM Fab (*hatched bars*) were then added to control cells, and membranes + anti-NCAM Fab were also added to the cells which had been precoated with WGA (*solid bars*). ChAT specific activity was determined 16 h later. Values are the means ± SEM of seven values obtained in three independent experiments. Control values were as follows (expressed as pmol/h per 1,000 cells): untreated cells, 5.03 ± 0.11; cells + WGA, 5.61 ± 0.22; cells + WGA + anti-NCAM Fab, 5.50 ± 0.08; cells + brain membranes + WGA, 2.73 ± 0.07; cells + retinal membranes + WGA, 10.1 ± 0.10.

Discussion

In E10 chick sympathetic neurons, there is a cell contact–dependent increase in the specific activity of ChAT over the first several days in culture. This effect is similar to that which has been previously described using rat sympathetic neurons, where contact between neurons results in an increase in both ChAT specific activity and substance P concentration (2). Our data suggest that NCAM is important for this effect in two ways. First, NCAM-mediated binding appears to be necessary to hold cell membranes together to allow the transmission of an independent signal. This requirement can be replaced by an artificial lectin-mediated adhesion system. Second, NCAM's polysialic acid moiety appears to influence the ability of cells to transmit this signal, even in the presence of the alternative adhesion mechanism.

The molecular mechanism of the signal that directly increases ChAT activity is not known, although both soluble and membrane-bound cholinergic neuronal differentiation factors have been described (5, 14, 15, 21, 25, 30). Moreover, the nature of the increase itself is not well defined. Previous

studies using chick sympathetic neurons have suggested the existence of two discrete neuronal subpopulations which can be selected by culture conditions, one of which exhibits primarily cholinergic characteristics and the other adrenergic characteristics after several days in culture (10, 37). However, those experiments could not distinguish between selection of a subpopulation versus culture-induced loss of the ability to respond to a cholinergic inducing factor. Nonetheless, under culture conditions where essentially all of the neurons survived (similar to the high density cultures in the present study), both ChAT and tyrosine hydroxylase were expressed in a ratio of ~3:1, i.e., the cholinergic enzyme predominated, even though two-thirds of the cells exhibited another adrenergic marker (37).

Zurn and Mudry (57) have studied chick superior cervical ganglion neurons from E8-12 in long-term culture under conditions where only a subpopulation of cells survive (50% of those originally plated). Their data indicate that these neurons are capable of synthesizing both acetylcholine and catecholamines. As culture density was increased from ~10,000 to 50,000 cells/cm^2, cholinergic properties diminished and adrenergic properties dominated. Nonetheless, at the lowest density (10,000 cells/cm^2), corresponding to high density in the present study, synthesis of acetylcholine was greater than that of catecholamines (57). These studies are consistent with the interpretation that tyrosine hydroxylase and ChAT can be coexpressed, with their ratio regulated by cell–cell contact. Because our cultures contain the total cell population, the data in the present study cannot distinguish between an induction of ChAT in adrenergic cells versus an increase of ChAT in an already cholinergic subpopulation.

Our results demonstrate the ability of NCAM to indirectly influence the transmission of a signal which leads to changes in ChAT levels. This influence includes two components, one that correlates with the presence or absence of NCAM-mediated adhesion, and a second that reflects the molecule's polysialic acid content. Regulatory effects of NCAM-mediated adhesion have been observed in other cell–cell interactions, namely junctional communication among neural plate cells and the innervation of muscles (17, 40, 52).[2] Neither gap junctions nor neuromuscular junctions are believed to contain NCAM as an integral component, yet the perturbation of NCAM-mediated adhesion by antibody in culture has been shown to block or delay their formation (17, 40).[2]

NCAM's polysialic acid content also appears to regulate the efficiency of NCAM–NCAM binding (22, 43). However, recent studies suggest that this is only one of the consequences of a more global influence of the polysialic acid moiety, which is very large and abundant, on the overall degree of membrane–membrane apposition.[3] In the present studies, the ability of NCAM polysialic acid to block the increase in ChAT activity, even when NCAM binding function is replaced by lectin-mediated agglutination, is consistent with the idea that polysialic acid may serve as a selective screen around the cells which can prevent some ligands from interacting with their receptors.[3]

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Do the observations made in this study have implications for the development of sympathetic neurons in vivo? As indicated above, temporal changes in NCAM expression have been proposed to regulate the initial formation of gap junctions in the neural plate and neuromuscular junctions in the chick hindlimb (52).[2] Chick neural crest cells also display temporal regulation of NCAM expression. In particular, they lose NCAM during the process of migration, and then reexpress NCAM in the developing ganglia (51). Taken together, data from these three systems are consistent with the hypothesis that the expression of the NCAM polypeptide in developing tissues, together with variations in its polysialic acid content, may serve as permissive "gates" which control contact-dependent cell–cell interactions.[3] Future studies will be directed towards a closer examination of both the expression of the NCAM polypeptide and its polysialic acid content in the course of development of the sympathetic ganglion.

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