Initial Appearance and Regional Distribution of the Neuron–Glia Cell Adhesion Molecule in the Chick Embryo

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ABSTRACT This study represents a global survey of the times of the first appearance of the neuron–glia cell adhesion molecule (Ng-CAM) in various regions and on particular cells of the chick embryonic nervous system. Ng-CAM, originally characterized by means of an in vitro binding assay between glial cells and brain membrane vesicles, first appears in development at the surface of early postmitotic neurons. By 3 d in the chick embryo, the first neurons detected by antibodies to Ng-CAM are located in the ventral neural tube; these precursors of motor neurons emit well-stained fibers to the periphery. To identify locations of appearance of Ng-CAM in the peripheral nervous system (PNS), we used a monoclonal antibody called NC-1 that is specific for neural crest cells in early embryos to show the presence of numerous crest cells in the neuritic outgrowth from the neural tube; neither these crest cells nor those in ganglion rudiments bound anti-Ng-CAM antibodies. The earliest neurons in the PNS stained by anti-Ng-CAM appeared by 4 d of development in the cranial ganglia. At later stages and progressively, all the neurons and neurites of the PNS were found to contain Ng-CAM both in vitro and in vivo. Many central nervous system (CNS) neurons also showed Ng-CAM at these later stages, but in the CNS, the molecule was mostly associated with neuronal processes (mainly axons) rather than with cell bodies; this regional distribution at the neuronal cell surface is an example of polarity modulation.

In contrast to the neural cell adhesion molecule and the liver cell adhesion molecule, both of which are found very early in derivatives of more than one germ layer, Ng-CAM is expressed only on neurons of the CNS and the PNS during the later epoch of development concerned with neural histogenesis. Ng-CAM is thus a specific differentiation product of neuroectoderm. Ng-CAM was found on developing neurons at approximately the same time that neurofilaments first appear, times at which glial cells are still undergoing differentiation from neuroepithelial precursors. The present findings and those of previous studies suggest that together the neural cell adhesion molecule and Ng-CAM mediate specific cellular interactions during the formation of neuronal networks by means of modulation events that govern their prevalence and polarity on neuronal cell surfaces.

Various reciprocal interactions between neurons and glia have been described (1–4) as underlying key events in the development and the maintenance of both the architecture and the function of the nervous system. The studies suggest that transient or permanent adhesions between neurons and glia and their precursors are critical in the ontogeny and the stabilization of the neuronal network. Specific cell adhesion molecules (CAMs) have been identified and characterized in terms of their cellular distribution in developmental and adult life (5, 6). Neurons contain the cell surface glycoprotein, the neural cell adhesion molecule (N-CAM), which mediates

1 Abbreviations used in this paper: CAM, cell adhesion molecule; CNS, central nervous system; FITC, fluorescein isothiocyanate; NC-1, monoclonal antibody specific for neural crest cells; N-CAM, neural cell adhesion molecule; Ng-CAM, neuron–glia cell adhesion molecule; PEG, polyethylene glycol; PNS, peripheral nervous system.
binding to other neurons or to precursors of embryonic striated muscle (7–9). Perturbation experiments suggest that N-CAM is involved in neural fasciculation (10–12) and in the establishment and maintenance of mapped projections (13). N-CAM on one surface has been shown (6, 14–16) to bind to N-CAM on another (homophilic binding); the efficacy of this binding is dramatically altered by changes in the surface density of the molecule (16).

Despite the existence of such mechanisms of modulation, for neurons to adhere specifically to glial cells in the presence of other neurons, adhesion molecules must exist that differ in specificity from N-CAM and that operate by means of a heterophilic mechanism of binding (5, 17, 18). A quantitative in vitro cell adhesion assay has recently been designed to identify some of these molecules (17). In the presence of monovalent Fab’ fragments of antibodies to N-CAM, brain membrane vesicles from 14-d chick embryos were able to adhere specifically to glial cells derived from the same tissue. An immunologically based indirect approach that was fundamental to the identification of N-CAM (19) was used to partially purify a neuron cell surface molecule involved in the adhesion to glial cells. Specific antibodies to this molecule were shown to inhibit the adhesion between brain membrane vesicles and glial cells (17) and more recently between neuronal and glial cells (20). The binding assays suggested that the neuron–glia cell adhesion molecule (Ng-CAM) and N-CAM had different functions in the nervous system inasmuch as Fab’ fragments of antibodies to N-CAM did not prevent the binding of neurons to glial cells (21). In vitro, Ng-CAM was located at the surface of neurites and neuron cell bodies and was present on individual cells along with N-CAM; Ng-CAM was not found on glial cells under the same conditions. This finding and the data from the binding assay suggest that Ng-CAM binding is heterophilic, i.e., to another kind of CAM on the glial surface (17, 20, 22).

Biochemical analyses of the two neuronal CAMs revealed major structural differences in their molecular weight, carbohydrate contents, and peptide produced by Staphylococcus aureus V8 protease (20, 22). It is notable, however, that these two neuronal surface molecules share at least one or two antigenic determinants (22); recent evidence suggests (20) that one of these antigenic determinants involves carbohydrate moieties. During maturation of the nervous system, N-CAM shows a decrement of two-thirds of its sialic acid content, a process that results in an increase in the efficacy of homophilic binding (16, 23). In contrast, there is no evidence as yet that Ng-CAM is grossly modified in its structure during development. The structural analyses underline the need to determine the differences in cellular functions of N-CAM and Ng-CAM during neural histogenesis.

Global surveys (24–27) of CAM distribution, particularly in early development, have been very important in interpreting their functional relation to morphogenesis. As shown by immunohistochemistry, N-CAM first appears early during development at the blastoderm stage; another cell adhesion molecule originally purified from liver (L-CAM) (24) is also widespread at this early stage of development. During gastrulation and early neurogenesis, N-CAM and L-CAM are segregated into different tissues (25). N-CAM becomes increasingly abundant in the neural primordium and also at least transiently in many mesodermal tissues (26). L-CAM becomes restricted to the non-neural ectoderm, endoderm, and to mesodermal derivatives such as the urogenital system (27).

Because of their early appearance in more than one germ layer, these two molecules have been proposed to be important in the formation of most early embryonic structures and have been designated (6) as primary CAMs.

The discovery and partial characterization of Ng-CAM have prompted us to examine its distribution during early development by means similar to those used for other CAMs; our expectation was that a comparison of distribution patterns of Ng-CAM and N-CAM would provide strong clues concerning their respective roles in neural morphogenesis. The original distribution of Ng-CAM in the chick throughout embryogenesis was therefore investigated using immunofluorescence labeling, with particular emphasis on the times at which the molecule first appeared in various regions. Antibodies to a surface marker (NC-1) recently identified on precursor cells of the nervous system (28, 29) were used in addition, both to characterize these cells and to sharpen the description of Ng-CAM localization particularly in the periphery. The earliest appearance of Ng-CAM on the first postmitotic neurons at 3 d of incubation and its subsequent restricted distribution only on neurons of the central nervous system (CNS) and the peripheral nervous system (PNS) suggest that Ng-CAM is a secondary CAM. This is consistent with its appearance during differentiation exclusively in derivatives of neuroectoderm as well as with its postulated heterophilic binding mechanism.

**MATERIALS AND METHODS**

**Preparation of Anti-Ng-CAM Antibodies:** Ng-CAM was released from embryonic chick membranes by treatment with trypsin and partially purified from the extract by chromatography on DEAE cellulose, Sepharose S-300, and lentil lectin-Sepharose columns using an immunologically based in vitro binding assay to detect the molecule (17). Monoclonal antibodies (16F5, 11D7, 10F6, 9D10) were prepared after immunization of BALB/c mice with the partially purified Ng-CAM. Monoclonal antibody 16F5 obtained from ascitic fluid was coupled to Sepharose CL-2B by the cyanogen bromide method and was used for affinity purification of Ng-CAM from detergent extracts of chick embryo brain membranes (17, 20). Rabbits were immunized with 50 μg of affinity-purified Ng-CAM at 2-wk intervals (first injection in complete Freund’s adjuvant, second injection in incomplete Freund’s adjuvant, subse- quent injections in PBS) and were first bled after the third injection. The immunoglobulin fraction was isolated by ammonium sulfate precipitation and ion-exchange chromatography (19). All the monoclonal antibodies used in this study (16F5, 10F6, 11D7, 9D10) were IgGs and recognized the major M, 135,000 component of chicken Ng-CAM (see Fig. 1).

**NC-1:** The monoclonal antibody NC-1 was produced after immunization of BALB/c mice with 8-d-old quail ciliary ganglia (28). NC-1 has been shown to be an IgM that reacts with surface components of migrating neural crest cells and later with neurons and glia of the avian PNS and CNS (29). The IgM was purified from ascites fluid; proteins precipitated between 30% and 50% ammonium sulfate were resuspended in 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0, and dialyzed against the same buffer. The fraction containing IgM was purified by gel filtration on Sephacryl S-300 and identified by SDS PAGE. After a fourfold concentration in dialysis tubing covered with crystalline sucrose, the IgM was dialyzed in 0.05 M NaH2BO3/0.15 M NaCl, pH 9.2. 1 ml of a 1% solution of NC-1 IgM in phosphate-buffered saline was incubated with 8 mg of fluorescein isothiocyanate (FITC)-celite (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. The reaction was stopped with 200 μl of 1 M NH4Cl, pH 6, the celite was removed by centrifugation, and the FITC-coupled IgM was dialyzed against large volumes of PBS, with four buffer changes in 4 d. FITC–NC-1 was routinely used at 1:1,000 dilution for direct immunolabeling.

**Immunoblotting:** SDS gel electrophoresis was carried out on 6% acrylamide gels (30) and proteins were transferred to nitrocellulose paper (31). For monoclonal antibodies, the nitrocellulose was reacted sequentially with 50 μg of monoclonal antibody, 50 μg of rabbit anti-mouse immunoglobulin, and 1 x 10^6 cpm of 125I-protein A. For immunoblots with rabbit anti-Ng-CAM, 50 μg of the IgF fraction was used followed by 1 x 10^6 cpm of 125I-protein A. Labeled material was detected by autoradiography.

**Cell Cultures of Dorsal Root Ganglia:** 10-d-old chick ganglia
were trypsinized with 0.1% crude trypsin (Difco Laboratories, Detroit, MI; 1:250) in PBS at 37°C for 20 min, dissociated with fire-polished pasteur pipettes in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) containing 10% newborn calf serum, and washed in the same medium. Cells were plated on fibronectin-coated coverslips and cultured for 24 h in Dulbecco's modified Eagle's medium/10% newborn calf serum/10% quail embryo yolk sac extract/5% nerve growth factor (5 U/ml). The yolk sac extract was prepared by homogenization in a Dounce homogenizer of 9-day-old quail yolk sac in Dulbecco's modified Eagle's medium at 4°C, and after centrifugation at 40,000 rpm for 1 h the supernatant fraction was collected and used (one yolk sac per 5 ml of medium). Under these conditions, neurites appear in the culture as soon as 2 h; the conditions facilitate the survival of many more neurons than in culture in the presence of nerve growth factor alone.

**Preparation of Embryos for Immunohistology:** While leghorn chick embryos were used throughout these studies, embryos were fixed in 3.7% formaldehyde in PBS for 2 h at 4°C and staged according to the number of somites and according to Hamburger and Hamilton (32).

**Polyethylene Glycol Sections and Frozen Sections:** Embryos were washed in PBS and dehydrated with a graded series of ethanol solutions. They were then impregnated with polyethylene glycol (PEG) 1000 (Serva, Heidelberg, Federal Republic of Germany) for 2 h at 45°C. After embedding in PEG 1500 with 10–20% PEG 1000, depending on the ambient humidity, 7-μm sections were prepared and then attached to glass slides with rubber cement (for details, see references 33, 34). For frozen sections, embryos were impregnated with 12–18% sucrose in PBS and processed as described (27).

**Immunolabeling:** For immunolabeling of cells, living or fixed cultures in 3.7% formaldehyde/PBS were incubated with FITC-NC-1 and with rabbit Igg anti-Ng-CAM antibodies (10 μg/ml) in PBS containing 0.5% BSA, followed by goat IgG anti-rabbit antibodies coupled to rhodamine (100 μg/ml, Nordic, Tilburg, The Netherlands).

Double immunolabeling with NC-1 or with the various antibodies to Ng-CAM gave results identical to those obtained with control cultures stained separately with each antibody.

Frozen sections were rehydrated in PBS for 10 min and incubated at room temperature with antibodies. All double-immunolabeling experiments were carried out with a rabbit IgG against Ng-CAM and FITC-NC-1 following the same procedure used for the cell cultures. When labeling with Ng-CAM monoclonal antibodies, either ascites fluid (dilution 1:100 in PBS) or hybridoma culture supernatants were used. After washing, sections were incubated with rabbit IgG against mouse Ig (Nordic, 20 tag/ml) and with FITC-coupled sheep anti-rabbit IgG (Pasteur Institute, dilution 1:150). In all cases, sections were mounted in 90% glycerol, 10% PBS containing 0.1% para-phenylenediamine, and were photographed with a Leitz Vario Orthomat camera mounted on a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N J) (26).

**RESULTS**

The specificity of the reagents used in this study was determined by immunoblotting (Fig. 1). Both the polyclonal and monoclonal antibodies specifically recognized the major M, 135,000 component of Ng-CAM. No N-CAM or other polypeptides present in detergent extracts of brain membranes were discerned by the anti-Ng-CAM antibodies and specific antibodies to N-CAM did not bind to Ng-CAM (20, 22). In addition to the M, 135,000 and 200,000 components of Ng-CAM recognized by the monoclonal antibodies, the polyclonal rabbit antibodies to Ng-CAM also recognized a component of M, 80,000 that is immunologically related to the M, 200,000 component of Ng-CAM (20). Despite these detailed immunological relationships revealed by chemical means, the overall patterns of histochemical staining with the different monoclonal and polyclonal antibodies to Ng-CAM were found to be indistinguishable. It is important to note that by a combination of criteria including polypeptide composition and structure, binding of specific antibodies, involvement in cell-cell adhesion, and modulation by prevalence, position and chemical alteration, Ng-CAM and N-CAM are clearly different (5, 6, 17, 18, 20–22).

To define further the cellular environment of those neurons expressing Ng-CAM and to serve as a reference, we also immunolabeled all sections examined with NC-1. While Ng-CAM was found to be restricted to neurons (17, 20, 22), antigenic determinants for NC-1 are found on both neuronal and glial surfaces and, in addition, provide a particularly useful marker for migrating neural crest cells (28, 29). An illustration of the difference in the staining patterns is seen in Fig. 2, which shows cells isolated from dorsal root ganglia of the PNS cultured in vitro. Consistent with previous findings (22), Ng-CAM was present on neurons exclusively (Fig. 2 A) whereas NC-1 stained both stellate neurons and flat glial cells (Fig. 2 B). This is in accord with the observation that the determinants for the latter antibody can be seen on the surfaces of precursor cells of the PNS during their migration (28, 29). The identities of the neurons and some of the glia were confirmed by immunofluorescence staining of cells in culture using specific antibodies to neurofilament proteins and S-100 protein, respectively (data not shown). These distinctive properties of NC-1 were useful in providing a background reference for the more localized staining patterns of anti-Ng-CAM, particularly in analyzing the PNS.

Following this approach, the distribution of immunoreactivity with the various immune reagents was determined in a temporal series of embryonic sections. In this series, Ng-CAM was first detected at 3 d in the CNS of the chick embryo and slightly later in the PNS. Subsequently, Ng-CAM was seen in many regions of the brain and spinal cord as the cells of these regions left the neuroepithelial stage. In the PNS, initial appearances of Ng-CAM were concomitant with the onset of the differentiation of ganglia and plexuses.
3 d (30 Somites)

At the level of the last somites in the trunk, a very limited area of the ventrolateral aspect of the neural tube was stained (Fig. 3A). In a slightly more rostral section, neuronal cell bodies and neurites were also stained (Fig. 3B); stained fibers traversed the sclerotome heading towards the myotome. In the dorsal region of the neural tube, other cells were also stained but with lower frequency. When the same section was labeled with NC-1, it became clear that numerous crest cells had accumulated between the neural tube, the myotome, and the sclerotome. Some of these crest cells entirely covered the early neural fibers (Fig. 3C). At the 10th somite level of the same embryo, even more intense staining for Ng-CAM was observed in the ventral aspect of the neural tube (Fig. 3D), and pioneer fibers that were already in contact with the myotome were also stained for Ng-CAM. This slightly oblique section clearly showed the neural crest pathways in the trunk; on the right side of the neural tube in Fig. 3E, neural crest cells recognized by NC-1 formed a continuum from the dorsal aspect of the neural tube to the gut. On the left side of the neural tube in Fig. 3E, those crest cells unable to penetrate the sclerotome remained at the dorsal border of the neural tube. Other crest cells accumulated lateral to the aorta and within the gut by means of different routes including those defined by the basement membrane of the ventral aspect of the myotome and the intersomitic pathways (34). Double-immunolabeling experiments showed that, at this stage, Ng-CAM did not appear on any of the crest cells or on precursors to sensory and autonomic neurons. In Fig. 4A and B are shown views at higher magnification of the ventral part of the neural tube of Fig. 3D; Ng-CAM is present on neurites and growth cones and, as revealed by NC-1 staining, these early fibers were completely adjacent to and occasionally surrounded by crest cells or Schwann cell precursors. In Fig. 4B and E show examples of highly branched pioneer fibers; where crest cells were also extremely abundant, NC-1 staining did not appear on these fibers which were completely hidden by the crest cells. In contrast to the NC-1-staining pattern, which included both cell bodies and neuronal cell processes, Ng-CAM was found in greatest abundance on neuronal fibers and only in lesser amounts on cell bodies.

4 d (Stage 23 in Hamburger and Hamilton[32])

In the trunk, precursors of motor neuron fibers that stained for Ng-CAM were found to be grouped into bundles; in the neural tube itself, Ng-CAM distribution was still restricted to the ventral aspect. Comparison of the data in Fig. 5A and B clearly shows that at this stage Ng-CAM was not found in the dorsal root and sympathetic ganglion rudiments.

At the cephalic level, the first fibers accumulating at the outer aspect of the mesencephalic and rhombencephalic vesicles were stained for Ng-CAM (Fig. 5D). In addition to staining these fibers, NC-1 also recognized cell bodies and processes within the neuroepithelium (Fig. 5E).

4.5–5 d (Stage 25)

In the trunk, motor nerves were organized into bundles that were uniformly stained by anti-Ng-CAM (Fig. 6A, D, and E). NC-1-positive crest cells associated with these nerves, as well as other such cells localized around the mesonephric rudiment (Fig. 6B) or along the aorta, were not stained for Ng-CAM; these cells later give rise respectively to the adrenal medulla and to the aortic plexuses. The first sensory neurons in the dorsal root ganglia to express Ng-CAM were detected at this stage (Fig. 6D).

During this period, the number of fibers stained in the brain rapidly increased. In the telencephalon (Fig. 7A–C), Ng-CAM appeared to be restricted to the fiber network whereas NC-1 also stained many cells in the neuroepithelium. In many locations in the CNS, Ng-CAM staining was distributed in a polar fashion, being weakly positive on neuronal cell bodies and strongly positive on neuronal fibers.

6–6.5 d (Stage 29)

In the neural retina, by 4 d the first axons accumulating at the inner limiting membrane were stained (not shown); by 6½ d, the optic nerve (Fig. 7, D and E) was strongly stained and could be easily followed at the level of the choroid fissure next to the pecten. Neuroepithelial cells including the pecten (35) and the ganglion cell bodies remained unstained.

Most, if not all, of the nerve fibers arising from the vestibulocochlear ganglion complex were labeled for Ng-CAM; as shown in Fig. 7, G and H, the acoustic ganglion nerve fibers inserted into the inner ear sensory epithelium were visualized. In addition to the acoustic and vestibular ganglia, the inner ear is also derived from the otic placodes; nonetheless, Ng-CAM was never found in any of the other structures of the inner ear. All of the cranial sensory ganglia were positive for Ng-CAM including neurons of the nodose ganglion, which are derived entirely from an epibranchial placode (not shown),
FIGURE 3 Immunofluorescence labeling for Ng-CAM and NC-1 epitope at early stages. Tranverse PEG sections in 31-somite stage chick embryo. Sections in A, B, and D are stained with rabbit anti-Ng-CAM IgG, in C and E are stained with NC-1, and in F the same section in D and E is shown by phase contrast. (A) At the last somite level, only a very faint Ng-CAM immunoreactivity can be detected in the ventral region of the neural tube. (B) At the 15th somite level, Ng-CAM is found at the surface of the first postmitotic precursors of motoneurons; both cell bodies and neurites are stained; (C) same section as B, NC-1 monoclonal antibody heavily stained migrating neural crest cells localized between the neural tube and the myotome; the more ventral crest cells cover the first motoneuron fibers that already have reached the myotome. Note that NC-1 also stains the periphery of the notochord. (D) 10th somite level: Ng-CAM is found mostly at the ventral side of the neural tube and on all motor neuron fibers. (E) On the same section stained with NC-1, many neural crest cells can be identified lateral to the neural tube, further ventrally under the myotome, next to the aorta, and within the gut. ao, aorta; dm, dermomyotome; e, ectoderm; en, endoderm; m, mesoderm; n, notochord; nt, neural tube; sc, sclerotome; sg, sympathetic ganglion; sp, splanchnopleural mesoderm. Bar, 40 μm. (A–C) × 200; (D–F) × 150.

and the trigeminal ganglion (Fig. 8 C) in the development of which both crest and placodal cells participate (36).

In the trunk, sensory and sympathetic ganglia were positive for Ng-CAM staining (Fig. 8 A and B). The spinal cord was also intensely stained both in the ventral horn and in the dorsal white columns.

By using NC-1 and antibodies to Ng-CAM, it was possible to follow the ontogeny of the enteric plexuses. Crest cells that penetrated into the pharynx as early as 2 d of incubation (34) migrated for several days while progressively colonizing the developing gut (37). At 5 d, pioneer crest cells at the level of the umbilicus were not stained for Ng-CAM (not shown); however, in the stomach, several of the crest cells recognized by NC-1 (Fig. 8 E) had already differentiated into neurons bearing Ng-CAM (Fig. 8 D). In the duodenum, many cells became positive for Ng-CAM at 6½ days, but these cells had not yet become organized as a concentric plexus (Fig. 8 F).

The studies of the distribution of Ng-CAM up to this stage in chick development demonstrated that it is present throughout the embryo only on neurons and particularly on neurites at times when neurons became postmitotic. To provide a framework for understanding the function of Ng-CAM in later histogenesis, we studied several regions; examples are provided below.

Later Embryonic Development

By 9–10 d (stage 35), when crest cells had reached their targets and had stopped migration, all the PNS ganglia and nerves were positively stained for Ng-CAM. At this stage, cells in the ganglia of the rectum were positive for Ng-CAM, and by 13 d both Meissner and Auerbach plexuses were heavily labeled (Fig. 9 A). Individual fibers could be detected within
FIGURE 4 Ng-CAM and NC-1 epitope distribution at the ventral aspect of the neural tube. Transverse PEG sections in 31-somite stage chick embryo, 10th somite level. Sections in A and C are stained with rabbit anti-Ng-CAM IgG; in B and D sections are stained with NC-1. (A) Ng-CAM is found in the ventrolateral part of the neural tube and on the neurites and growth cones. (B) Immunostaining with NC-1 allowed visualization of neural crest cells forming a string along all the neurites. (C) At a similar level, Ng-CAM stained fibers are highly branched within the sclerotome. (D) Same section, neural crest cells are very numerous both dorsally along the neural tube and in the fibers in which individual neurites could not be distinguished. Note that some of the fibers are in contact with the myotome. my, myotome; n, notochord. Bar, 20 μm. x 560.

the mucosa reaching the epithelium while bundles of interconnecting ganglia were visible in the smooth muscle (Fig. 9 B). The Remak ganglion, a mixed sympathetic and parasympathetic ganglion was also heavily stained (not shown). In birds, adrenal medullary cells are arranged around cords of the cortex; these cells could be easily recognized with NC-1 (Fig. 9 E). Interestingly, the adrenal medulla (Fig. 9,D-F), which is derived entirely from crest cells, was also stained by antibodies to Ng-CAM, although its chromaffin cells do not harbor processes. Fibers corresponding to the CNS innervation of the medulla also contained Ng-CAM (not shown).

In the CNS at stage 35, nerve fibers were stained in many different areas including the inner plexiform layer of the retina. By 14 d (stage 40) both the optic nerve and the inner plexiform layer were heavily labeled, although not uniformly (Fig. 10 A and B). Neuronal cell bodies and the outer plexiform layer showed barely detectable levels of staining. In the cerebellum, the molecular layer and the inner granular cell layers were strongly stained (Fig. 10 C). In the external granular layer, neuron cell bodies and their long processes running parallel to the pial surface could occasionally be seen (Fig. 10 D); these fibers probably corresponded to those seen on a tangential section (Fig. 10 E). Later, at 18 d of incubation, the most intensely stained regions of the cerebellum corre-
sponded to the fiber tracts in the white matter region and to the molecular layer (Fig. 11A). Proliferating external granule cells adjacent to the pial surface were not stained by antibodies for Ng-CAM. In contrast, external granule cells in the premigratory zones of the external granule layer were labeled by antibodies to Ng-CAM (20). In the tectum, several fiber-rich laminae including layers 6–10 as described by Ramon y Cajal (38) were heavily stained by antibodies to Ng-CAM and the deep fiber layers were also stained (Fig. 11B). More recent detailed studies suggest a dynamic pattern of staining across the layers of each of these regions as cell and fiber tract movements occur (Rickmann, M., C.-M. Chuong, M. Grumet, W. M. Cowan, and G. M. Edelman, unpublished observations). The overall pattern of Ng-CAM distribution on the CNS neurons was similar: in general, the staining was intense on fibers and faint on cell bodies. Exceptions could be found, however, as seen in migrating granule cells.

A summary of the temporal course and distribution of Ng-CAM appearance as detected by this global survey is presented in Table 1.
DISCUSSION

A high degree of order is seen in the successive steps of neurogenesis including neural induction, neurulation, segregation of precursors for the CNS and PNS, and the establishment of neural connections. One of the major issues is whether this order is generated by successive gene expressions of very large amounts of cell surface molecules of different binding specificities or whether a smaller number of CAMs undergoing controlled cell surface modulation can regulate the establishment of neural patterns (18). At present, the evidence, although not conclusive, favors the latter interpretation (5, 6, 18).

Our main goal in the present experiments was to provide a description of the sequence of initial appearances and distributions of Ng-CAM in various neural regions, to relate this distribution to that found for N-CAM, and to provide additional bases for a functional interpretation of the role of Ng-CAM in neurogenesis. It is important to stress that the emphasis was on obtaining an initial description at the global level rather than focusing upon an exhaustive description of each neural region; more extensive studies of the details of each region will obviously be required.

The major conclusions of this survey are: (a) Ng-CAM appears much later than N-CAM in embryogenesis and is first seen on postmitotic neurons. (b) Both in the CNS and PNS, Ng-CAM appears prior to glial cell differentiation. (c) Ng-CAM staining patterns, although they appear different in detail, form a subset of the NC-1 distribution patterns, which include the neural crest cells and their derivatives (28, 29). (d) In the CNS, Ng-CAM staining is faint on cell bodies, and much more intense on neurites, axons, and growth cones. In the periphery, it appears to be distributed more evenly be-
tween cell bodies and processes. In cultured CNS neurons, it is also more evenly distributed (20). (e) The sequential patterns of Ng-CAM staining, particularly in the CNS, are very different from those observed for N-CAM (25, 26). While N-CAM staining is pervasive from the earliest stages of embryogenesis and undergoes prevalence modulation early (25) and embryo to adult conversion late (16, 23), the staining for Ng-CAM, even within a given region of the CNS, is more sharply
FIGURE 8 Spinal cord and peripheral nervous system. (A and B) Transverse PEG section at trunk level; stage 29 (6–6½ d) stained with rabbit anti-Ng-CAM IgG; anti-Ng-CAM staining was found in the spinal cord white matter, motor roots, sensory and sympathetic ganglion cells, and fibers. (C) At the mesencephalic level, both cell bodies and fibers were stained in the trigeminal ganglion by monoclonal antibody 11D7. (D–F) Frozen sections from stage 25 (4½–5 d). A number of crest cells accumulating in the gizzard were stained by NC-1 (E) and expressed Ng-CAM (D) while exhibiting small neurites. (F) In the duodenum from a stage 29 embryo, many crest cells organizing into one plexus were stained by anti-Ng-CAM antibodies. *drg*, dorsal root ganglion; *dwc*, dorsal white column; *en*, endoderm; *n*, notochord; *ne*, nerve; *se*, serosa; *sg*, sympathetic ganglion; *vh*, ventral horn. Bar, 50 μm. (A) x 60; (B and F) x 160; (C–E) x 150.

distributed in layers and is often associated with migrating cells and axons. So far, no evidence has been found for unusual carbohydrates or embryo to adult conversion in Ng-CAM (20, 22). Unlike N-CAM (25, 26) and L-CAM (25, 27), Ng-CAM is the first CAM found to be restricted in distribution to derivatives of only one germ layer (i.e., neuroectodermal derivatives of ectoderm). For this reason it is termed a secondary CAM, in that it appears later in development than N-CAM and L-CAM and is apparently involved in histogenetic events rather than in early embryogenetic processes.

The first Ng-CAM-positive cells appear in the neural tube at about the 30-somite stage, at the time that some neuron precursors begin to withdraw from the cell cycle (39, 40). Very rapidly, these cells send out Ng-CAM-positive processes in the somitic region. Ng-CAM remained predominantly limited to the ventral aspect of the neural tube up to 5 d, at which time the dorsal white column also appeared to be stained. In the head, the first appearance of Ng-CAM was noted in the optic nerve by 4 d, at a time when the first postmitotic ganglion cells in the retina are found (41). The sequence of appearance is consistent with that of neuronal maturation and neurite extension in these areas (38, 42, 43).

Direct comparison of the distributions of the NC-1 epitope and of Ng-CAM showed that the latter does not appear on the surface of placodal and crest cell until 4 d of incubation during gangliogenesis. The earliest PNS neurons to be stained are found in the head, particularly in the ciliary ganglion rudiment; ciliary neuron birth dates have been determined to be between 2½ and 5 d in the chick embryo (44). In the dorsal
FIGURE 9 Peripheral nervous system in 14-d-old chick embryos; frozen sections. (A–C) Rectum. Antibodies to Ng-CAM labeled (A) both Auerbach and Meissner plexuses and interconnecting fibers and (B) very fine neurites reaching the epithelium; (C) section in B shown by phase contrast. (D–F) In the adrenal gland, differentiated crest cells that were stained by NC-1 (E) surround each rod of the cortex; many of these cells also expressed Ng-CAM (D) at their surface. (F) Same field as D and E shown by phase contrast. au, Auerbach plexus; en, endoderm; mei, Meissner plexus; mu, smooth muscle; sbm, submucosa. Bar, 50 μm. (A) × 50; (B and C) × 180; (D–F) × 170.

root ganglia, Ng-CAM is detectable on bipolar neurons by 4½ d, possibly corresponding to the first postmitotic neurons belonging to the lateralventral compartment (45, 46).

It is clear that in both the CNS and in the PNS, Ng-CAM is expressed early after the birth date of neurons. A striking feature in the CNS was that the molecule appeared most obviously on nascent processes. In the chick embryo, the first cells to be decorated by antibodies to neurofilaments are found during the same time and position in the neural tube as those expressing Ng-CAM (47). Neurofilament proteins provide some of the earliest reliable differentiation markers for neurons and have been shown to accumulate in cell bodies and processes either at the last round of division or just after mitosis. In the dorsal root ganglion rudiments, neurofilament-containing cells have been visualized by 4½ d in situ or when grown in vitro (48, 49). This distribution in birds and mammals (50, 51) is in excellent agreement with the classical observations of the appearance of neurons by use of silver impregnation techniques (38, 42, 43).

In view of its proposed function (neuron–glia binding), it is significant that Ng-CAM appeared on neurons and neurites at just those stages which precede the appearance of differentiated glia. In the dorsal root ganglion, S-100 used as a marker for satellite cells can be detected after 5 d but increases substantially only by day 10 of incubation (52), whereas Ng-CAM is present on all the neurons by day 7. Other markers such as glial fibrillary acidic protein also appear late; in the PNS, only a subpopulation of glia found in the enteric plexuses contains glial fibrillary acidic protein (53). Vimentin, which is also present in glial cells, is known to be expressed in the neural tube and in the neural crest before glial cell differentiation (48, 49, 54), and cannot be considered as a strict glial marker (54).

Ng-CAM is clearly neither the first nor the last marker to appear on differentiating neurons. The data indicate, for example, that cells expressing other definitive neuronal mark-
FIGURE 10  Central nervous system in 14-d-old chick embryo; frozen sections. Sections in A and C–E show staining with rabbit IgG anti-Ng-CAM, and in B is shown the same field in A by phase contrast. (A) In the neural retina, the optic nerve and regions of the inner plexiform layer are heavily stained for Ng-CAM. Most cell bodies including ganglion cells remain unstained. (C) In the cerebellum, the molecular layer, the inner granular layers, and cells in deeper layers are recognized by Ng-CAM antibodies. In the external granular layer, some cells bearing long processes parallel to the molecular layer were also stained for Ng-CAM (D). (E) Tangential section of cerebellum. egl, external granular layer; gc, ganglion cell layer; ip, inner plexiform layer; ml, molecular layer; on, optic nerve. Bar, 20 μm. (A and B) X 225; (C–E) X 250.

FIGURE 11  Ng-CAM staining in the central nervous system at later stages. Frozen sections in 18-d-old chick embryo. In the cerebellum (A), both the molecular layer and the fiber tracts in the white matter zone are intensely stained whereas most other regions are either weakly stained or not stained. In the tectum (B) laminae 6–10 of Ramon y Cajal (38) and the deep fiber layer are stained. df1, deep fiber layer; gl, granular layer; ml, molecular layer; wm, white matter. Bar, 50 μm. X 180.

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ers do not necessarily express Ng-CAM: neural crest cells synthesizing acetylcholine during migration (55) and early sympathetic aggregates containing catecholamines and tyrosine hydroxylase (56-58) were not recognized by antibodies to Ng-CAM. In contrast, many neurons with Ng-CAM at their surface are fully differentiated. In the duodenum at 6 d, choline acetyltransferase is not yet detectable (59) and neuropeptides such as vasoactive intestinal polypeptide and substance P are not found before days 10 and 14 of incubation, respectively (60); Ng-CAM is nevertheless present.

These combined correlations sharply pose the problem of whether Ng-CAM is expressed in newly born neurons as an early autonomously programmed function or whether Ng-CAM synthesis is induced by contact with glial cell precursors. Using NC-1 as a marker for crest cells and their neural derivatives and anti-Ng-CAM staining, it was found that pioneer fibers always exit the ventral neural tube in regions already occupied by crest cells and become closely associated with these cells. Thus, in vivo, neuron precursors are very likely to be in close contact with non-neuronal cells, a fact making the choice between the two hypotheses of autonomous expression or of glial induction extremely difficult. A decision on this issue by in vitro experiments seems more promising. For example, the expression of S-100 (61) can be modulated in vitro; in the absence of neurons, glial cells synthesize only a low level of S-100, whereas in the presence of living or paraformaldehyde fixed neurons, a substantial increase in S-100 is observed in the glial cells in direct contact with the neurons. A similar approach may shed light on whether crest cells can induce or greatly enhance the synthesis of Ng-CAM by neurons.

One of the most intriguing observations in the current study is the polar or asymmetric distribution of Ng-CAM on neurons, particularly in the CNS, in vivo. Ng-CAM was mostly found on neurites and axons but was difficult to discern at the surface of individual cell bodies in many different areas of the CNS except in cell bodies of migrating cells such as pioneer fibers always exit the ventral neural tube in regions already occupied by crest cells and become closely associated with these cells. Thus, in vivo, neuron precursors are very likely to be in close contact with non-neuronal cells, a fact making the choice between the two hypotheses of autonomous expression or of glial induction extremely difficult. A decision on this issue by in vitro experiments seems more promising. For example, the expression of S-100 (61) can be modulated in vitro; in the absence of neurons, glial cells synthesize only a low level of S-100, whereas in the presence of living or paraformaldehyde fixed neurons, a substantial increase in S-100 is observed in the glial cells in direct contact with the neurons. A similar approach may shed light on whether crest cells can induce or greatly enhance the synthesis of Ng-CAM by neurons.

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granule cells of the cerebellum. The molecule thus undergoes polarity modulation, a form of local cell surface modulation that is also seen clearly for L-CAM in several globular epithelia (24, 27). This kind of modulation is less apparent for N-CAM at similar stages. Although both the mechanism of the polarity modulation of Ng-CAM and its relationship to the biosynthesis of the molecule remain to be explored, our knowledge of the existence of this form of modulation may have a bearing upon our understanding of the mutually integrated functions of Ng-CAM and N-CAM during the development of maps of fiber tracts, particularly in the CNS.

In considering such functions, the overall sequences of very early ontogenetic appearances of the various CAMs may be contrasted with the later distributions of the two neuronal molecules. It is significant that N-CAM and L-CAM appear at the earliest stages of chick embryogenesis (25) defining them as primary CAMs. Ng-CAM, in contrast, must be considered a secondary CAM inasmuch as it appears later only in the derivatives of the neuroectoderm. The topological relationships among these known CAMs in the embryo may be made clearer by updating the previously described composite fate map (25) of the blastoderm (Fig. 12). As shown in

**TABLE I**

| Incubation period | Peripheral nervous system | Central nervous system |
|-------------------|--------------------------|-----------------------|
| 3 d (30 somites, stage 18) | Ciliary ganglion | Precursors of motoneurons (ventral neural tube) |
| 4 d (stage 23) | Fibers in mesencephalon | Optic nerve |
| 4½ d (stage 25) | Cranial sensory ganglia | Ventral horn and dorsal white matter of spinal cord |
| 6–6½ d (stage 29) | Dorsal root ganglion | Fiber networks in telencephalon and rhombencephalon |
| 9–10 d (stage 35) | Sympathetic ganglia | Ventral horn and dorsal white matter of spinal cord |
| 13 d | Enteric ganglia (rectum) | Inner plexiform layer in retina |
| | All ganglia | Fiber layers in tectum |
| | | Molecular layer of cerebellum |

**FIGURE 12** Fate map of the distribution of the three well-characterized CAMs in the chick blastodisk (Stage O, Vakaet, reference 62). (A) The presumptive territories of the different tissues have been established by marking techniques including quail grafts into chick hosts (see reference 62 for a review and new data on the position of the definitive endoderm). (B) The distributions of the three adhesion molecules (N-CAM, *[]*; L-CAM, [//]; and Ng-CAM [[]) determined during the embryonic and organogenetic periods (1–13 d) are superimposed on the fate map of tissues shown in A. Initially, only N-CAM and L-CAM are present uniformly in the blastodisk (25). Ng-CAM emerges later and is restricted to the nervous tissue including presumptive crest cells and neurogenic placodes lying at the border of the top inner half circle. The presumptive territories for Ng-CAM are thus a distinct subset of those mapped for N-CAM. N-CAM is also transiently found in many mesodermal tissues (lower inner circle); L-CAM surrounds the N-CAM and Ng-CAM areas in a simply connected ring including the non-neural ectoderm (upper part) and the definitive endoderm (lower part). The region not yet occupied by a known CAM corresponds to the hemangioblastic tissue (blank regions). Note that both N-CAM and L-CAM appear on cells derived from more than one germ layer, but that Ng-CAM is restricted to cells derived from neuroectoderm. Because of their very early and widespread distributions, N-CAM and L-CAM are considered to be primary CAMs. In contrast, Ng-CAM, which appears later and in a more restricted manner, is considered to be a secondary CAM. The vertical bar represents the primitive streak (PS); Ec, extraembryonic and extraembryonic ectoderm; En, endoderm; H, heart; IP, lateral plate (splanchno- and somatopleural mesoderm); N, nervous system; No, prechordal and chordamesoderm; S, somite. (Data on N-CAM and L-CAM were derived from reference 25.)
earlier studies, L-CAM and N-CAM are first found uniformly distributed in the blastoderm. In the fate map, however, the presumptive territory corresponding to the nervous system and transiently to mesodermal tissues containing N-CAM is surrounded by a territory containing calcium-dependent L-CAM corresponding to the non-neural ectoderm, the endoderm, and to a minor extent, the mesoderm. This map makes it clear that both of these primary CAMs are present on derivatives of more than one germ layer. Neural induction and other subsequent interactions leading to the formation and compartmentalization of the nervous system are associated with surface modulation of the prevalence of these two adhesion molecules in different regions. The neural primordium and the neural crest lose L-CAM, but the neurogenic placodes retain it until their internalization (27). N-CAM increases in amount in the neural tube, is transiently lost from crest cells, but is permanently retained in neurogenic placodes (26).

As shown in the present study, Ng-CAM is restricted to the rostral part of the N-CAM zone (Fig. 12), i.e., the nervous system including presumptive crest cells and neurogenic placodes (25). In continuing development, both N-CAM and Ng-CAM can both appear at the surface of individual neurons. The above description of early events indicates that there are major differences in appearance times, distribution, and modulation of the two neuronal CAMs.

In addition to the selective regional appearance of Ng-CAM, the subsequent maturation of the nervous system includes the conversion of N-CAM from the embryonic to the adult forms (5, 63, 64); this transition occurs at different times in both the PNS and the CNS (63, 64). The present observations make it possible to compare some of the detailed differences that occur in distributions of Ng-CAM and N-CAM during later histogenetic events that are nonetheless prior to embryonic to adult conversion in N-CAM. The major difference that emerges from the comparison appears to be that whereas N-CAM is more globally distributed over neurons at all stages, increasing in amount on neural tissues that are active in neural morphogenesis and then decreasing to adult levels (64), Ng-CAM shows much more sharply localized dynamic changes in prevalence. Thus, it is not only found mainly on axon bundles and on migrating cell bodies, but it shows sharp differential changes in distribution in different layers of developing neural laminae such as those of the cerebellum. Although these changes in prevalence modulation of Ng-CAM have not yet been quantitated as they have for N-CAM (64), they are so extreme as to leave little doubt that there are very strong differences in the amounts of Ng-CAM at the cell surface in highly localized regions. This is exemplified by the distributions in the cerebellum and also in the optic tectum (Fig. 11). This important generalization that Ng-CAM distributions show dynamic changes over more localized portions of developing fiber tracts than do N-CAM distributions remains to be confirmed both by electron microscope observations and by more quantitative studies of the kind already applied to N-CAM (64).

It may be useful to correlate these observations on comparative distribution during histogenesis with what is known of the diversity in structure and binding specificities of Ng-CAM and N-CAM as well as to suggest some hypothetical activities of these CAMs in vivo that can be verified or falsified by future experiments. Whatever other functions Ng-CAM may have on neuronal surfaces, it is clear that it plays a key role in the heterotypic binding of neurons to glia (probably astroglia) by a heterophilic mechanism (17, 20, 22). In vitro, neurons do not bind to glia by an N-CAM-mediated mechanism (20, 22). In view of the demonstrated role of gial guide fibers in neuronal migration, particularly in the cerebellum and cerebral cortex (1), it is reasonable to suggest that modulation of Ng-CAM at the cell surface is related to movements of this type. A pertinent example is provided by the external granule cells during their descent along the Bergmann glia. The axons of each external granule cell fasciculate with other axons to form the parallel fibers of the molecular layer whereas the cell body and its extending process adhere transiently to the radial glia. The implied role of Ng-CAM in this migration is consistent with the order of its appearance, with its rapidly changing distribution in structures such as the cerebellum, and with the fact that it is not as ubiquitously distributed in all parts of the CNS as in the N-CAM during development. The correlations implied by this proposal, along with some of the similar properties of Ng-CAM and of a recently described antigen in the mouse (65) suggests that this so-called L1-antigen may in fact be Ng-CAM. A detailed description of these similarities appears elsewhere (22). One discrepancy, worthy of note, is that Ng-CAM has not been found so far on Schwann cells, whereas L1-antigen has been definitely identified on these cells. Further anatomical studies combined with additional biochemical and in vitro assays should help to determine whether the functions proposed here can definitely be attributed to Ng-CAM.

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