Structure of a New Nervous System Glycoprotein, Nr-CAM, and Its Relationship to Subgroups of Neural Cell Adhesion Molecules

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Abstract. We have identified and characterized a new glycoprotein in the chicken nervous system using immunological and molecular biological methods and we have examined its tissue distribution. Analysis revealed that this protein is very similar in structure to the chicken neuron-glia cell adhesion molecule, Ng-CAM, and to mouse L1. cDNA clones encompassing the entire coding sequence of this Ng-CAM related molecule, called Nr-CAM, have been isolated and sequenced. A glycoprotein containing one major component of M_r 145,000 on SDS-PAGE was purified from brain by lentil lectin affinity chromatography and FPLC, and its amino-terminal sequence was identical to that predicted from the Nr-CAM cDNA. The complete cDNA sequence encodes six Ig-like domains, five fibronectin type III repeats, a predicted transmembrane domain, and a short cytoplasmic domain. On Northern blots, nucleic acid probes for Nr-CAM recognized one major RNA species of ~7 kb and much lesser amounts of larger RNAs. Most of the same probes hybridized to single bands on genomic Southern blots, suggesting that Nr-CAM is encoded by a single gene that may be alternatively processed to yield several mRNAs. In support of this notion, two Nr-CAM cDNA clones had a 57-bp sequence located between the second and third Ig-like domains that was not found in two other Nr-CAM cDNA clones, and two other clones were isolated that lacked the 279-bp segment encoding the fifth fibronectin-like type III repeat.

Antibodies against the purified protein and synthetic peptides in Nr-CAM both recognized a predominant M_r 145,000 species and a much less prevalent species of M_r 170,000 in neural tissues. Levels of Nr-CAM expression increased in the brain until approximately embryonic day (E) 12, followed by slightly lower levels of expression at E18 and after hatching. Immunofluorescent staining with anti-Nr-CAM antibodies showed that most neurons in the retina were positive at E7 and the pattern of expression became restricted to several layers on neuronal cell bodies and fibers during development. Anti-Nr-CAM antibodies labeled specifically cell surfaces on neurons in culture.

Although the structure of Nr-CAM resembles that of chicken Ng-CAM and mouse L1, the identity with each of these neural CAMs does not exceed 40%. The differences indicate that Nr-CAM is distinct from Ng-CAM and L1, but there are sufficient similarities to suggest that all of these molecules are members of a subgroup of neural CAMs in the N-CAM superfamily. The structure of Nr-CAM, its close relationship to this subgroup of neural CAMs, and its pattern of expression during development are all consistent with the hypothesis that it functions in cell adhesion during neural development.

THE development of the intricate networks of the nervous system involves complex and coordinated interactions between different cells. Cells interact directly with other cells via cell adhesion molecules (CAMs) that are anchored in the plasma membrane as well as with extracellular substrate adhesion molecules (SAMs) via membrane proteins such as the integrins (see reviews in 23). There is a significant amount of overlap in CAM and SAM expression across different tissues, suggesting that redundant adhesive mechanisms may provide for plasticity during development and regeneration. Indeed, it is likely that combinations of adhesion molecules will be necessary for particular functions (50, 76). Furthermore, while cell adhesion and migration require CAMs and SAMs, cells also express a variety of molecules, such as cytotactin, that can act as repulsive agents to cells and thereby restrict migration pathways of neurons and their axons (8, 17, 22, 72).

The expression of CAMs in the nervous system such as the neural CAM, N-CAM, and the neuron-glia CAM, Ng-CAM, varies temporally and spatially in development and the levels of CAM expression can be correlated with cell adhesive behavior (16, 50). For example, the ability of specific antibodies against N-CAM and Ng-CAM to inhibit neuron aggregation depends on the relative prevalence of these two CAMs on cells isolated from different regions (40). Moreover, the

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molecules appear to contribute differently during histogenesis of different neural tissues. For example, the migration of granule cells in developing cerebella was inhibited by anti-Ng-CAM but not by anti-N-CAM antibodies, and, conversely, histogenetic layering in the retina was inhibited by anti-N-CAM but not by anti-Ng-CAM antibodies (50). These data are consistent with the suggestion (20) that up to dozens of neural CAMs may be necessary for the establishment of functional neuroanatomy.

Neural CAMs can be grouped into two major structural families, one resembling N-CAM (14) and the other resembling the liver CAM, L-CAM (31) and its mammalian homologue uvomorulin or E-cadherin (88). Most N-CAM-like molecules are calcium independent and contain various numbers of Ig-like domains whereas members of the cadherin family are calcium dependent and contain a structurally different type of repeated domain. Many of the Ig-like molecules also contain fibronectin type III (FnIII)[1]–like domains. Various vertebrate molecules in the Ig family including N-CAM (14, 47), Ng-CAM (6, 39)/G4 (10)/8D9 antigen (60), L1 (52, 64), contactin (71)/F11 (5, 9)/F3 (33), TAG-1 (30), myelin-associated glycoprotein (1, 59, 79), and Po (15, 27) have been shown to be involved in cell adhesion. Invertebrate proteins that resemble certain vertebrate Ig-like molecules in their overall organization, including fasciclin II (45), fasciclin III (67), and neuroglian (3) also can function as homophilic ligands when transfected into cell lines (36, 84). Like N-CAM and Ng-CAM, all these CAMs show restricted patterns of expression during development. Subgroups of these molecules share certain amino acid sequences, nucleic acid sequences, and carbohydrate epitopes (3, 6, 14, 21, 30, 33, 37, 81).

Close analysis of the number of domains and the amino acid identities among all these molecules suggest that they can be grouped into at least three subfamilies (6, 37). Members of each subfamily have the same number of Ig and FnIII repeats and they show significantly greater amino acid similarities to each other than to members of other subfamilies. For example, N-CAM has five Ig and two FnIII domains, whereas chicken Ng-CAM, mouse L1, and *Drosophila* neuroglian each have six Ig and five FnIII domains. Furthermore, the amino acid sequences of Ng-CAM, L1, and neuroglian resemble each other more closely than they resemble any other known molecule (3, 6, 64).

In the course of using antibodies against Ng-CAM to isolate cDNA clones (6), we detected a new molecule that shares at least one epitope with the cytoplasmic region of Ng-CAM but is the product of a different gene. Analysis of the full-length coding sequence of this molecule, which we have called Nr-CAM (Ng-CAM related), indicates that, like Ng-CAM, it contains six Ig domains, five FnIII repeats, a transmembrane region, and a short cytoplasmic domain. A glycoprotein of M_r ~145,000 that contains an amino-terminal sequence identical to that predicted from the cDNA sequence was isolated, and antibodies against the protein were found to stain the surfaces of certain neurons in culture and in tissue sections. The structure and expression pattern of Nr-CAM suggest that it is closely related to Ng-CAM and that it may be involved in related aspects of cell-cell adhesion during neural development. A preliminary report of this work has previously appeared (Grumet, M., M. P. Burgoon, V. Mauro, G. M. Edelman, and B. A. Cunningham. 1989. *J. Neurosci.* 15:568a [Abstr.]).

1. *Abbreviation used in this paper*: FnIII, fibronectin type III.

Materials and Methods

DNA

cDNA libraries were constructed in λgt11 from total RNA or poly $(A)^+$ RNA isolated from 9- to 14-d embryonic chicken brains by the RNAse H method using oligo (dT) or using synthetic oligonucleotides as primers as described (43, 61). Three chicken λgt11 cDNA libraries prepared from adult brain, embryonic cerebella, and total embryo RNA were purchased from Clontech (Palo Alto, CA). A λgt10 cDNA library constructed from chicken embryo brain RNA was kindly provided by Drs. Joan Levi and Hidesabura Hanafusa. Antibody screening of the libraries was performed (95), using polyclonal antibodies against denatured Ng-CAM protein which recognize all the components of the Ng-CAM (6). Positive clones were isolated to homogeneity, and the inserts were excised from λgt11 arms by restriction with EcoRI endonuclease (61).

cDNA inserts were labeled with ^{32}P-dCTP using random oligonucleotide primers (26) and used to screen λgt11 libraries to obtain overlapping cDNA clones. For sequence analysis, cDNA inserts were subcloned into the pBluescript (KS) vector (Stratagene, La Jolla, CA) and sequenced by the dideoxynucleotide chain-termination method using Sequenase (U.S. Biochemical, Cleveland, OH) (80). The sequence of larger inserts was obtained either by the use of synthetic internal oligonucleotides or by deletion cloning in pBluescript vectors. Two deletion cloning methods were used: clones were digested with restriction endonucleases which recognized sites in the polylinker and within the insert, or they were deletion cloned using Exonuclease III and mung bean nuclease. Oligonucleotides used for priming cDNA synthesis and sequencing reactions were synthesized at the Rockefeller University Protein Sequencing Facility.

RNA, Northern Blots, and Southern Blots

Total RNA was prepared from embryonic chicken brains by rapid extraction of freshly dissected tissue in LiCl/urea using a Polytron homogenizer (32). Poly $(A)^+$ RNA was isolated on oligo (dT) columns (Pharmacia, Piscataway, NJ). RNA transfer blots and DNA transfer blots were performed using standard techniques (61). Restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and Boehringer Mannheim Diagnostics (Indianapolis, IN). For genomic Southern analyses, chicken liver genomic DNA (Clontech, Palo Alto, CA) was digested to completion with appropriate restriction endonucleases and resolved on individual lanes of 0.8% agarose gels (61). Nucleic acids were transferred to Immobilon-N (Millipore Continental Water Systems, Bedford, MA), the membranes were fixed and hybridized with ^{32}P-labeled cDNA probes (61). Some hybridizations were done using Rapid Hybridization Buffer (Amersham Corp., Arlington Heights, IL).

Analysis of Protein Sequence

Sequence data were compiled using the Staden ANALYSEQ programs (85). The Dayhoff protein sequence database (National Biomedical Research Foundation, Washington, DC) and the translated Genbank database (release 63) were searched using the rapid homology search program FASTA (ktup = 1; 68). The FASTA program was also used in the pairwise alignments of the internal tandem repeats in Nr-CAM, Ng-CAM, and L1 and in comparisons to other neural CAMs. Hydrophobicity analyses of the sequence were performed by means of an automated hydrophobicity program (25) using parameters given by Kyte and Doolittle with a sliding window of 19 residues (57).

Protein

Nr-CAM was purified from 14-d chicken embryo brain membranes (49) which were extracted in 50 mM Tris (pH 7.5)/300 mM NaCl/1% NP-40/200 U/ml trasylol/1 mM PMSF, passed over a column of DE-52 (Whatman Inc., Clifton, NJ) equilibrated in the same buffer, and depleted of Ng-CAM, N-CAM, and cytotactin by affinity chromatography using immobilized monoclonal antibodies 10F6, anti-N-CAM No. 1, and 1D8, respectively (38, 41, 42, 48, 49). Then the extracts were passed slowly over a column of lentil

lectin Sepharose 4B (Pharmacia, Piscataway, NJ), the column was washed, and fractions were eluted by incubation for 4 h in the same buffer containing 0.2 M α-methyl-D-glucoside (Sigma Chemical Co., St. Louis, MO). The sugar was removed from the eluate by dialysis in 50 mM Tris (pH 7.5)/150 mM NaCl and the material was passed slowly over another column of lentil lectin Sepharose 4B. The column was washed extensively with 50 mM Tris (pH 7.5)/150 mM NaCl and eluted in the same buffer containing 0.2 M α-methyl-D-glucoside. The eluate was dialyzed in 50 mM Tris (pH 7.5)/75 mM NaCl and fractionated on a Mono Q column (Pharmacia) by FPLC using an NaCl gradient from 75 to 300 mM; Nr-CAM eluted between 130 and 180 mM NaCl. For analytical analysis, proteins were resolved in SDS-PAGE (58) and stained with Coomassie blue or with silver (65).

For amino-terminal sequence determinations, intact Nr-CAM and V8-protease digests (11) of the molecule were resolved on SDS-PAGE, transferred to Immobilon (Millipore Continental Water Systems), stained with Ponceau S and sequenced (63). Amino-terminal sequencing of intact and fragmented forms of Nr-CAM was performed at the Rockefeller University Protein Sequencing Facility by automated Edman degradation.

Immunoblotting

To identify Nr-CAM protein, antibodies were generated against two peptides predicted from the cDNA sequence. Peptides representing amino acids 153–174 and 837–856 (Fig. 2) were synthesized with a cysteine residue at the carboxyl terminus at the Rockefeller University Protein Sequencing Facility. The peptides were purified by HPLC and covalently coupled to keyhole limpet hemocyanin (KLH; Calbiochem-Behring Corp., San Diego, CA) using pyridyldithioproprionic acid *N*-hydrosuccinimide ester (Sigma Chemical Co.). For preparation of antisera, the peptides coupled to KLH and the FPLC-purified Nr-CAM were injected into rabbits. Monoclonal and polyclonal antibodies including anti-Ng-CAMs, anti-N-CAMs, HNK-1, and anti-cytotactin were prepared as described (13, 38, 42, 49). For protein extracts, tissues were homogenized in 10 vol of 50 mM Tris (7.5)/150 mM NaCl/1% NP-40/200 U/ml trasylol/1 mM PMSF, and centrifuged at 100,000 *g* for 1 h. Protein in the supernatant fraction was determined by the method of Bradford (4). Immunoblots were performed after resolution on SDS-PAGE and transfer to nitrocellulose (90). Treatments of Nr-CAM with endoglycosidase F (Boehringer Mannheim Diagnostics) were as described (93) and the proteins were detected after resolution on SDS/PAGE and immunoblotting with polyclonal antibodies to Nr-CAM.

Immunofluorescence

Unfractionated cultures prepared from embryonic day-9 chick brain and retina containing neurons and glial cells were grown on coverslips for 2 d, fixed for 10 min with 3.7% formaldehyde, and stained with antibodies (41). Neurons and glial cells were identified by morphology and by staining with antibodies as described (39).

Tissues from White Leghorn chicken embryos were fixed overnight in 4% (wt/vol) paraformaldehyde/240 mM phosphate buffer, pH 7.6, and then cryoprotected successively in sucrose solutions (12, 16, and 18% in water) for 2-h each. Cryostat sections of 10–16 μm were collected on gelatin-coated slides and stored desiccated at 4°C. For immunofluorescence, sections were stained with rabbit anti-Nr-CAM, anti-Ng-CAM, and anti-N-CAM sera or Ig, and monoclonal antibodies against Ng-CAM (10F6 and 16F5) as described (89). Slides were mounted in 90% glycerol/PBS and sections were photographed on Tri-X film with a Nikon UFX camera on a Zeiss Universal microscope equipped with IIIRS epifluorescence optics.

Results

Isolation and Sequencing of cDNA Clones

While characterizing chicken Ng-CAM cDNA clones using polyclonal antibodies against denatured Ng-CAM protein, we isolated an antibody-positive cDNA clone 701 (Fig. 1) from an embryonic chicken brain library. The insert in this clone encoded a peptide sequence very similar to but distinct from the sequence of chicken Ng-CAM (6). This insert was used as a probe to isolate larger cDNA clones from several different chicken cDNA libraries. Clone 730 (1.6 kb) extended in the 3′ direction, and contained a translation termination codon and clone 704 (1.4 kb) extended in the 5′ direction (Fig. 1). Additional cDNA clones extending 5′ from clone 704 to a potential signal sequence were obtained by walking, using cDNA fragments as probes. Eight overlapping cDNA clones were isolated and sequenced in both directions providing a DNA sequence with a single open reading frame (Fig. 2).

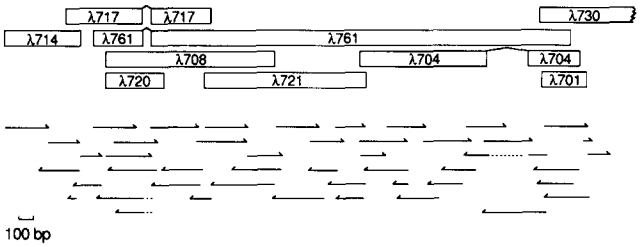

Figure 1. Schematic representation of Nr-CAM cDNA clones and the sequencing strategy. The nine cDNA clones used to determine the sequence of Nr-CAM are indicated (to scale) in a 5′ (*left*) to 3′ (*right*) direction. The λgt11 clones 704, 717, and 761 do not have sequences found in other clones and the gaps in these clones are indicated by single angled lines. Direction and extent of sequencing are indicated by arrows and gaps are indicated by dashed lines.

The deduced protein sequence (Fig. 2) has a potential translation start site at nucleotide position 33. The first 24 amino acids (−24 to −1) have the features of a signal sequence for cotranslational insertion into the ER membrane, and the most likely cleavage site for a signal peptide (69, 91) is at the end of this segment. The termination codon at nucleotide position 3837 delineates an open reading frame of 1268 amino acids. Other reading frames contain multiple stop codons.

Hydrophobicity analysis (Fig. 3) indicated the presence of one major hydrophobic domain in Nr-CAM (amino acids 1,108–1,130) that is rich in nonpolar amino acids that are characteristic of transmembrane segments. Its location suggests that Nr-CAM has an extracellular region of 1,107 amino acids and a cytoplasmic domain of 114 amino acids. In the cytoplasmic domain there are four potential casein kinase II phosphorylation sites (S/T-X-X-D/E; 56) at serines 1,166 and 1,194, and threonines 1,161 and 1,180, three potential protein kinase C phosphorylation sites (S/T-X-R/K; 94) at serines 1,182 and 1,217, and threonine 1,185, and one potential cAMP phosphorylation site (R/K-R/K-X-S; 34) at serine 1,178. In the extracellular domain there are 18 potential N-linked glycosylation sites (N-X-S/T; 62). In addition to the putative transmembrane region, there are three minor hydrophobic peaks (Fig. 3). The first one corresponds to the signal sequence, as expected (91). The others are short segments at amino acids 219–231 and 576–597 (Fig. 2). They do not have the characteristic features of typical transmembrane domains and their possible significance is unknown.

The extracellular region of Nr-CAM includes the two major motifs of repeated domains that are also found in several neural CAMs. Beginning at the amino terminus are six similar domains (Fig. 2) that resemble those found in Igs and a number of neural CAMs as exemplified by N-CAM (14, 46). Each domain contains ~100 residues and all have highly conserved pairs of cysteines and surrounding amino acids characteristic of the C-2 subtype of Ig domains (21, 92) found in neural CAMs.

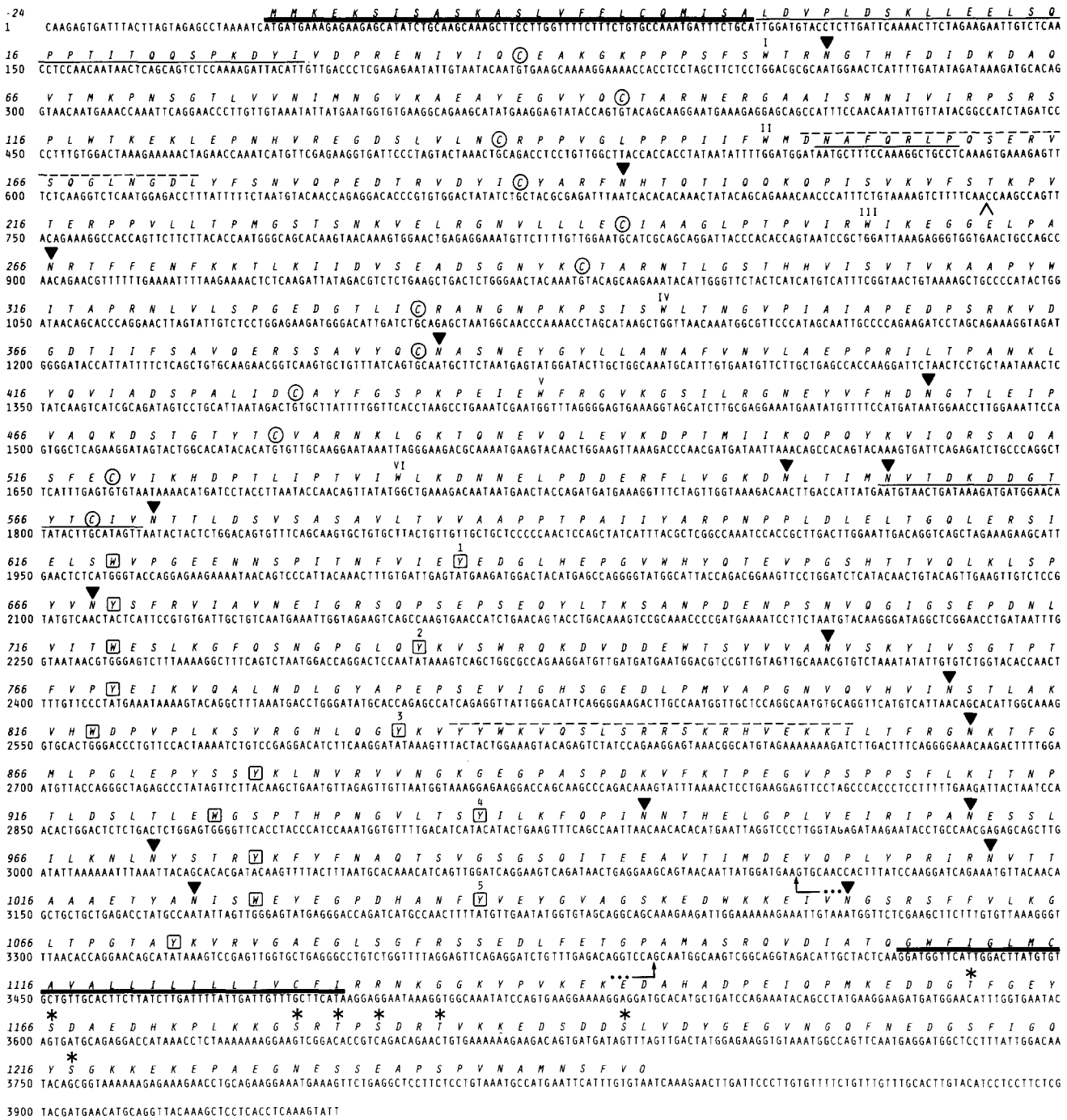

Figure 2. Nucleotide sequence and deduced amino acid sequence of Nr-CAM. The longest open reading frame contains 1,268 amino acids terminating at an ocher termination codon (*O*). Two hydrophobic regions representing the signal peptide (−24 to −1) and the transmembrane region (1,108–1,130) are underlined by a thick bar. Thinner underlining denotes amino acid sequences determined by protein chemical analyses of the M_r 145,000 form of Nr-CAM and peptides obtained from it after treatment with V8 protease. Dashed overlining indicates amino acid sequences that were synthesized and used to generate antibodies in rabbits. Potential sites of asparagine-linked glycosylation are marked with inverted triangles. Potential phosphorylation sites are indicated by asterisks. The Ig domains are numbered from I to VI over the conserved tryptophans and the characteristic cysteines are circled. The FnIII repeating units are numbered 1 to 5 over conserved tyrosines and the conserved tryptophans and tyrosines are boxed. The position of the additional sequence encoded by clones 708 and 720 between Ig domains two and three is indicated by a caret, and the 279-bp sequence representing the fifth FnIII repeat that is not found in clone 704 is bounded by angled arrows. These sequence data are available from EMBL/Genbank/DDBJ under accession number X58482.

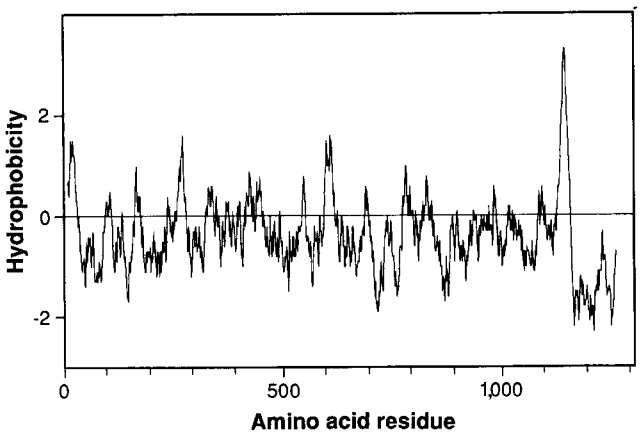

Figure 3. Hydrophobicity plot of the predicted amino acid sequence of Nr-CAM. Amino acid residues are numbered from the translation start site (amino acid −24 in Fig. 2). Positive values indicate hydrophobicity.

Between the Ig-like regions and the membrane spanning region are five domains that resemble each other and the type III repeats found in fibronectin (53, 83). Each of these domains contains ~100 amino acids including highly conserved tryptophan and tyrosine residues in the amino and carboxy terminal regions, respectively. The fourth and fifth FnIII domains are less well conserved.

Between the second and third Ig-like domains (between nucleotides 738 and 739) there is a 20–amino acid sequence (MDSLNDTIAANLSDTDIYGA) encoded by cDNA clones 708 and 720, whereas these amino acids are replaced by a single threonine residue in the sequence encoded by cDNA clones 717 and 761. The results indicate that this segment may be an alternatively spliced exon in Nr-CAM mRNAs. In addition, the fifth FnIII repeat, (nucleotide positions 3,111–3,390), is encoded by a 279-bp sequence found in clone 761. This segment is not present in clone 704 suggesting that this may be another alternatively spliced region of the gene. Both potential mRNA splices do not interrupt structural domains;

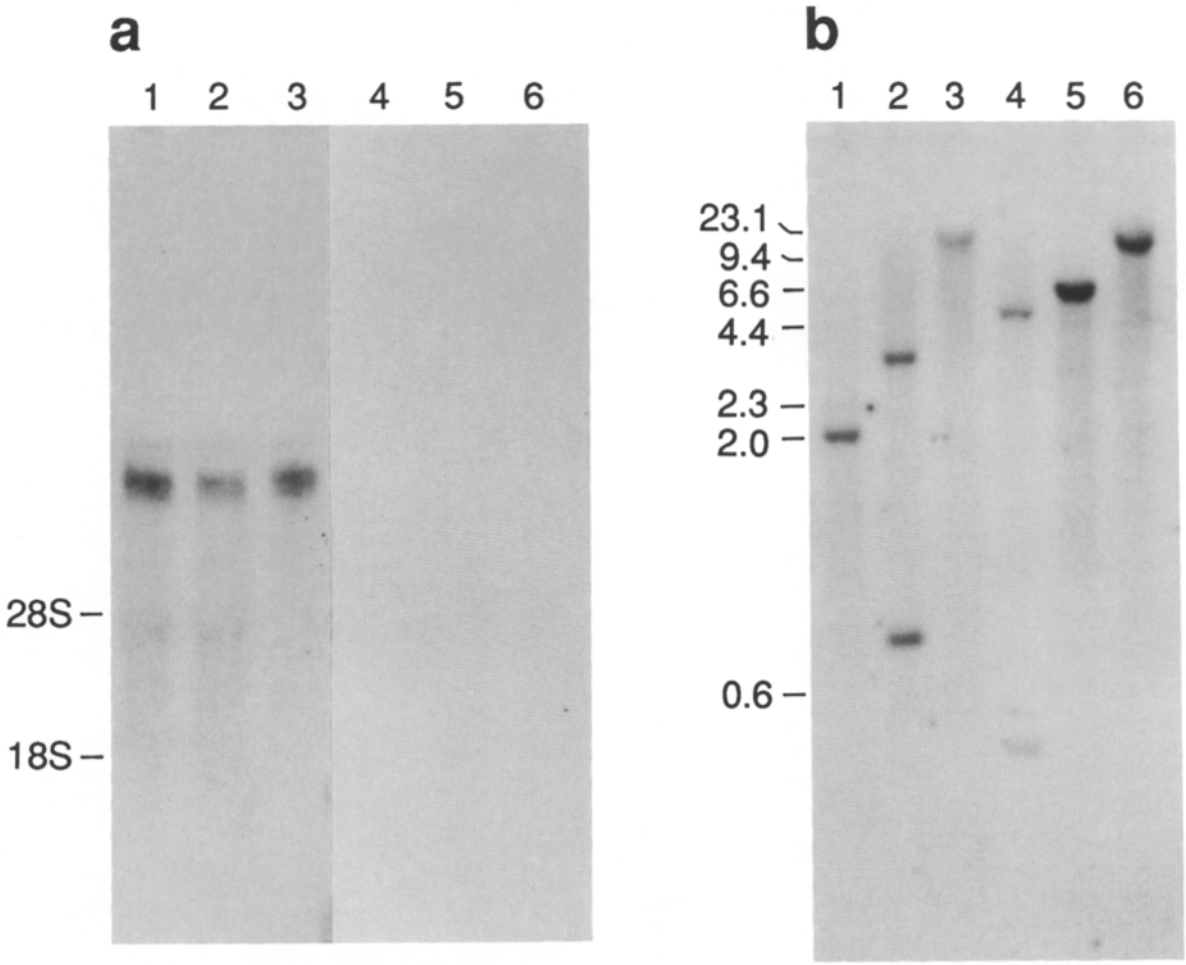

Figure 4. Northern and Southern blots using Nr-CAM cDNA probes. (*a*) Total brain RNA (15 μg) from 12- (lane *1*) and 18-d (lane *2*) embryos, poly-(A)$^+$ RNA (1 μg) from 12-d chick embryo brain (lane *3*), and total RNA from 14-d chick embryo heart (lane *4*), gizzard (lane *5*), and liver (lane *6*) were electrophoresed on 0.8% formaldehyde gels transferred to Immobilon-N and hybridized with an Nr-CAM probe from clone 721. Positions of 28S and 18S ribosomal RNA are indicated. (*b*) Recognition of genomic DNA by Nr-CAM cDNA clones. 8 μg of adult chicken genomic DNA was restricted with PstI (lane *1*), EcoRI (lane *2*), BamHI (lane *3*), XbaI (lane *4*), PvuII (lane *5*), and ApaI (lane *6*), electrophoresed on 0.8% agarose gels, transferred to Immobilon-N, and probed with Nr-CAM cDNA probe made from clone 717. Relative migration of molecular weight standards is indicated at the left in kilobases.

one is located between the second and third Ig domains and the other encompasses the entire fifth FnIII repeat that is located amino terminal to the transmembrane domain. If both segments can be involved in alternative RNA splicing, then at least four Nr-CAM polypeptides ranging in size from 127,346 to 139,605 D could be produced.

Nr-CAM mRNA and Its Gene

In Northern analyses, various Nr-CAM cDNA probes hybridized with a predominant mRNA of approximately 7 kb (Fig. 4 *a*, lanes *1–3*) which is significantly larger than the ~6 kb mRNA detected with all Ng-CAM probes (6). The 7-kb RNA species was detected in neural tissues, but it was not detected in chick liver, gizzard, heart (Fig. 4 *a*, lanes *4–6*), and skin (data not shown). Levels of Nr-CAM mRNA in brain increased until approximately embryonic day 12 (Fig. 4 *a*, lane *2*); slightly lower levels were detected at later embryonic stages and after hatching. Both cDNA probes from clones 721 (Fig. 4) and 704 (data not shown) hybridized to one major species in total brain (lanes *1* and *2*) and poly(A)$^+$ RNA (lane *3*) of ~7 kb and to at least one larger species that is much less prevalent. The detection of more than one mRNA species is consistent with the notion that there are alternatively spliced forms of Nr-CAM mRNAs.

Northern blot analysis indicated that mRNAs for Nr-CAM were relatively stable when compared to mRNAs for Ng-CAM which were particularly sensitive to degradation (6). This difference may be related to the large difference in GC content. The GC content for Nr-CAM is low (41%) whereas that for Ng-CAM is high (68%) (6). In addition, the third nucleotide in each codon, that is not important for encoding most amino acids, is 41% GC for Nr-CAM and 82% GC for Ng-CAM.

Southern blots of chicken liver genomic DNA digested with many restriction enzymes were hybridized with a cDNA subfragment of clone 717 and yielded single bands (Fig. 4 *b*). Several other restriction enzymes resulted in Southern blots with two bands. Preliminary Southern blots on genomic clones encoding part of the Nr-CAM gene using the same restriction enzymes and cDNA probes gave identical results (unpublished observations). These findings suggest that a single gene encodes Nr-CAM and that certain cDNA probes span introns containing particular restriction sites. The combined data from Northern and Southern analysis are consistent with the notion that Nr-CAM is transcribed from a single gene and that the mRNA may be alternatively processed into multiple species.

Similarity of Nr-CAM to Other Neural CAMs

A comparison of the amino acid sequence of Nr-CAM to the Dayhoff protein sequence database, the translated Genbank database, and specifically to the sequences of chicken Ng-CAM (6), mouse L1 (64), N-CAM (14), chicken contactin (71) and F11 (5), mouse F3 (33), and other neural CAMs, showed that it was most similar to that of chicken Ng-CAM and mouse L1 (Table I). The FASTA rapid homology search program (68) showed that the six Ig domains in Nr-CAM are similar to each other, and that the individual Ig domains of chicken Nr-CAM are most similar to the corresponding domains in chicken Ng-CAM and mouse L1 with a one-to-one correspondence of highest FASTA scores between each domain in Nr-CAM and the corresponding domains in Ng-CAM and L1. The pairwise FASTA sequence comparisons were used to align Ng-CAM and L1 with Nr-CAM, and residues that are the same in all three molecules are shown in bold face (Fig. 5). The pairwise similarities between Nr-CAM and Ng-CAM, and between Nr-CAM and L1 are indicated by vertical lines in Fig. 6. In general, the amino acid sequences in Nr-CAM are slightly more similar to L1 (40% overall identity) than to Ng-CAM (36% overall identity) except in the sixth Ig domain and the fifth FnIII repeat (Fig. 6).

Of the Ig domains, the second and the third are the most

Table I. Relationship between Nr-CAM and Other Neural CAMs

	Nr-CAM	Ng-CAM	L1	Neuroglian	Contactin/F11	F3	TAG-1	Chick N-CAM	Mouse N-CAM	Fasciclin II
Nr-CAM (C)	–									
Ng-CAM (C)	**2,112** (1,291)	–								
L1 (M)	**2,570** (1,260)	**2,467** (1,286)	–							
Neuroglian (I)	1,620 (1,227)	1,023 (1,267)	1,416 (1,224)	–						
Contactin/F11 (C)	817 (875)	792 (815)	962 (883)	929 (778)	–					
F3 (M)	841 (976)	840 (873)	994 (989)	878 (781)	**4,027** (1,010)	–				
TAG-1 (R)	999 (934)	915 (865)	1072 (993)	1066 (975)	**2,644** (1,033)	**2,584** (1,009)	–			
N-CAM (C)	420 (685)	269 (443)	362 (697)	355 (685)	226 (427)	214 (514)	312 (652)	–		
N-CAM (M)	417 (688)	274 (581)	322 (712)	318 (559)	279 (551)	209 (246)	322 (665)	**4,200** (1,121)	–	
Fasciclin II (I)	175 (132)	140 (254)	230 (331)	197 (240)	282 (572)	243 (586)	279 (328)	463 (457)	610 (620)	–

Protein sequences for each of the neural CAMs listed were compared in a pairwise fashion using the FASTA program of Pearson and Lipman (1988) (ktup = 1). The FASTA scores are shown for the number of amino acid residues indicated in parentheses over which the maximum identity extended. Numbers in boldface highlight the highest similarities between more closely related proteins. Letters in parentheses indicate species origin of protein; *C*, chicken; *M*, mouse; *I*, insect; *R*, rat.

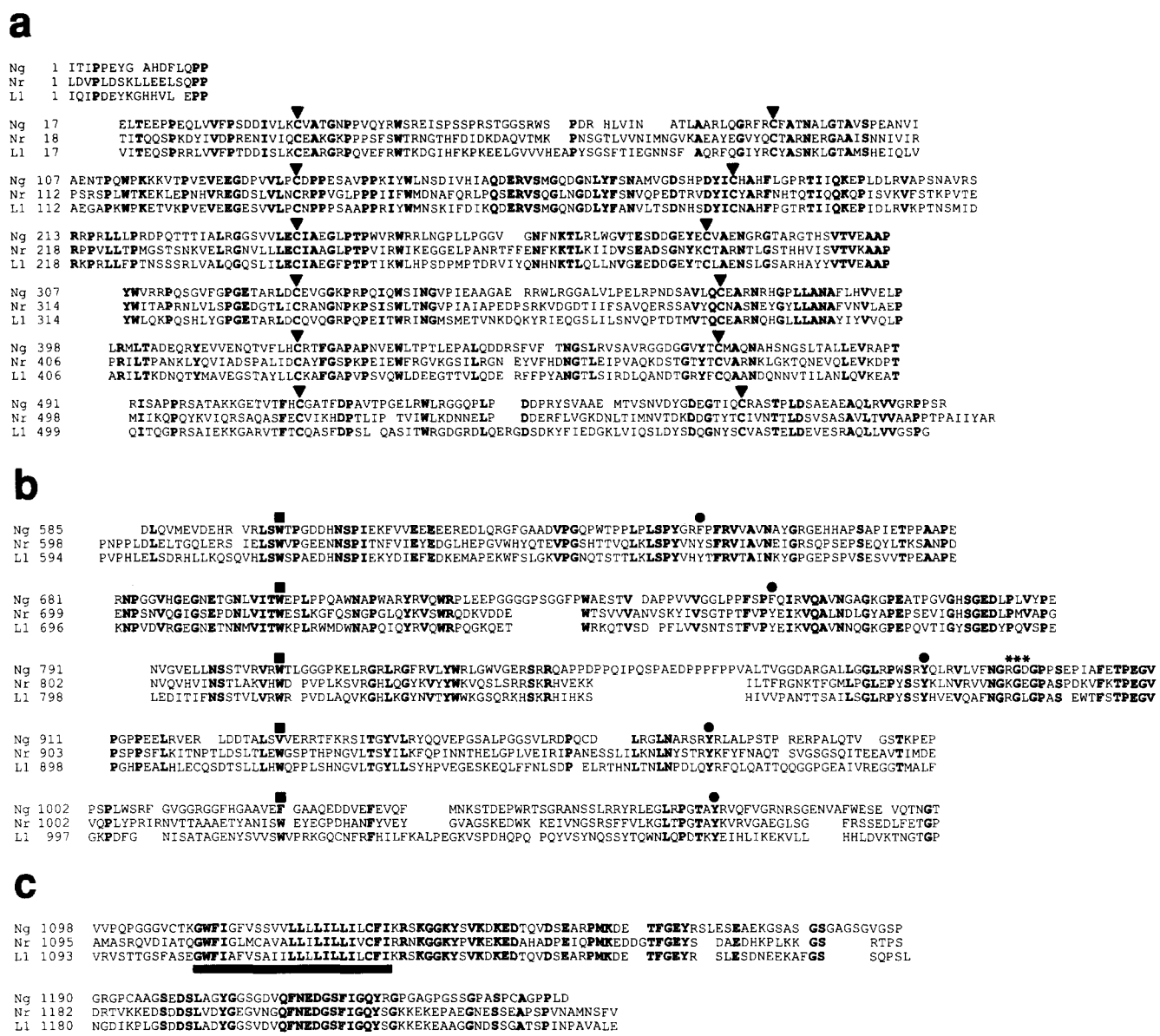

Figure 5. Comparison of the complete amino acid sequences among chicken Nr-CAM, Ng-CAM, and mouse L1. The position of the first amino acid residue of each line is given for Ng-CAM (*Ng*), Nr-CAM (*Nr*), and L1 (*L1*). Sequences were initially aligned pairwise using the FASTA program (ktup = 1). Gaps were introduced to maximize the identities between the sequences. Identical residues among the three sequences are indicated in bold. (*a*) Alignment of six consecutive Ig domains in Nr-CAM to corresponding domains in Ng-CAM and L1. Characteristic cysteine residues likely to be involved in intrachain disulfide bonds are highlighted by inverted triangles, and the six comparisons are aligned through the first cysteine in each pair. (*b*) Alignment of five consecutive FnIII repeating units in Nr-CAM to corresponding repeats in Ng-CAM and L1. Characteristic tryptophans and tyrosines are highlighted by filled squares and circles, respectively. The five pairs of domains are aligned through the first tryptophans in each. The RGD sequence in Ng-CAM is highlighted by three asterisks. (*c*) Alignment of the transmembrane (*underlined*) and cytoplasmic segments in Nr-CAM to corresponding segments in Ng-CAM and L1.

similar (38% identity) among the three molecules, followed by the third (36% identity), with the lowest level of identity (19%) in the sixth. Among all of the Ig domains of Nr-CAM, Ng-CAM, and L1, the most highly conserved regions appear as patterns of alternating amino acids surrounding the highly-conserved cysteines (Figs. 5 *a* and 6), which may reflect conserved patterns that are important for folding of the β-pleated sheets found in the Ig domains.

In pairwise comparisons between Nr-CAM and Ng-CAM, and between Nr-CAM and L1 (Fig. 6), the second and third Ig domains are still the most highly conserved (42–48% similarity). The comparisons also show that some regions in Nr-CAM are more similar to those in Ng-CAM, while others are more similar to those in L1. For example, in the fourth Ig domain the two cysteines are located in a region containing many residues in common to the three molecules and the intervening region contains alternative segments that are more similar either to Ng-CAM or to L1.

A highly conserved segment is found in the middle of the second Ig domain which also shows some similarity to the

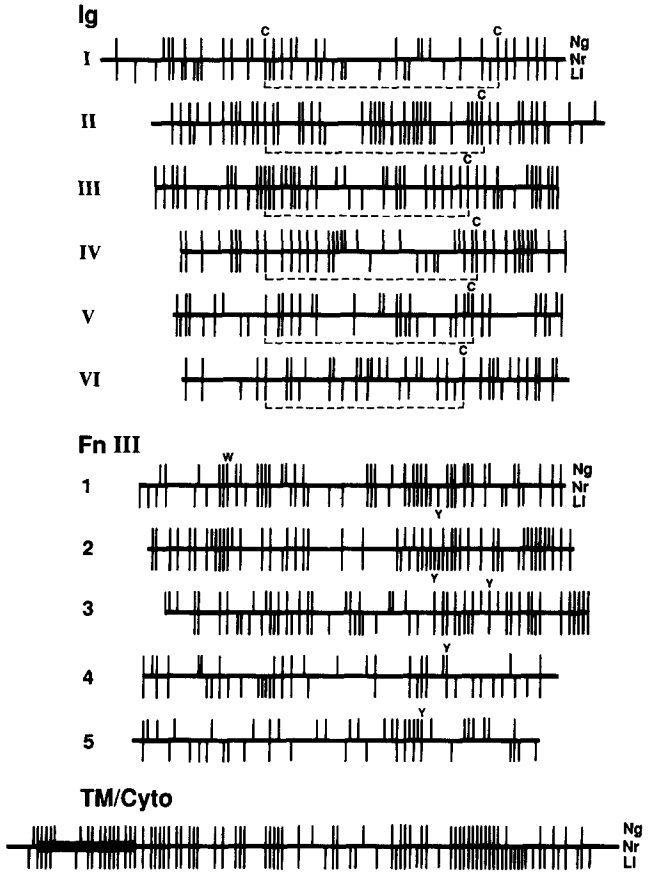

Figure 6. Linear representation of amino acid identities among domains of chicken Nr-CAM, Ng-CAM, and mouse L1. Each of the Ig domains (*I–VI*) and the FnIII repeats (*1–5*), and the transmembrane and cytoplasmic region (*TM/Cyto*) are represented by horizontal lines corresponding to domains shown in Fig. 5. Vertical lines above indicate amino acid identities between Nr-CAM (*Nr*) and Ng-CAM (*Ng*), and below indicate amino acid identities between Nr-CAM and L1. The paired cysteines (*C*) in each Ig domain are connected by dashed lines, and the positions of the tryptophans (*W*) and tyrosines (*Y*) in the FnIII repeats are indicated. The transmembrane domain is indicated by a thicker horizontal line.

second Ig domain in N-CAM. In N-CAM, this region is believed to be important for homophilic binding (14) and for heparin binding (12, 77). This region of Nr-CAM, Ng-CAM, and L1 does not contain the putative consensus sequence for heparin binding found in N-CAM, but it may play a role in cell binding mediated by these molecules.

The similarity among the first three FnIII repeats of Nr-CAM, Ng-CAM and L1 range from 38% in the second domain to 29% in the first domain (Figs. 5 *b* and 6). The lowest levels of similarity among these molecules are in the last two repeats that are only 15 and 9% identical, respectively. The pairwise identities in the last two FnIII repeats are significantly greater inasmuch as there are small regions in Nr-CAM that are similar to either Ng-CAM or L1. This region is probably closest to the plasma membrane in the extracellular portion of these molecules and may have been weakly conserved because it is not important for cell binding.

There are significant differences between Nr-CAM, Ng-CAM and L1 in the third FnIII repeat (Fig. 5 *b*). Ng-CAM contains an 18-amino acid stretch not found in Nr-CAM and L1 that is located very close to the amino terminus of the M_r 80,000 component of Ng-CAM (its amino terminal sequence is APPDPP . . .) and may be important for its cleavage from the M_r 200,000 species (6). Moreover, Ng-CAM contains an RGD sequence near the end of the repeat; an RGD sequence in a comparable position in fibronectin is involved in binding to integrins (51). Nr-CAM and L1 do not contain RGD sequences in their FnIII repeats (Fig. 5 *b*).

The amino acid sequences of Nr-CAM, Ng-CAM, and L1 are more similar to each other in the predicted transmembrane domains and portions of the cytoplasmic regions than in any other region (~50% identical). In these regions, several continuous segments of as many as 12 amino acids are identical in the three proteins (Figs. 5 *c* and 6). The carboxyl half of the cytoplasmic domain of Nr-CAM is much more similar to L1 (66% identity) than to Ng-CAM (43% identity).

Identification and Characterization of Nr-CAM Protein

To identify the Nr-CAM protein and also compare its localization in the nervous system to that of neural CAMs, specific anti-Nr-CAM antibodies were generated. Two peptides were synthesized (Fig. 2) representing regions of the second Ig domain and the third FnIII repeat. Antibodies were prepared against the peptides coupled to KLH. Both specifically bound to an M_r 145,000 species in extracts prepared from chicken brain that was not detected in extracts of other tissues (Fig. 7). The anti-peptide antibodies were therefore used to detect the protein during purification from detergent extracts of brain membranes.

Ng-CAM, N-CAM, and cytotactin were first removed from the extracts by affinity absorption and Nr-CAM was then isolated on lentil lectin columns that bind all these molecules. The eluate was fractionated by FPLC on a Mono Q column to yield fractions containing one major component of M_r 145,000 which was recognized specifically by the anti-peptide antibodies (Fig. 7, *a* and *b*). Polyclonal antibodies were then generated against the purified protein, and they specifically recognized the same M_r 145,000 protein detected by the antipeptide antibodies (Fig. 7, *b* and *c*) and an additional minor species of M_r 170,000 (Fig. 7 *d*).

To verify that the M_r 145,000 species represents the protein specified by the Nr-CAM cDNA clones, it was resolved on SDS-PAGE, transferred to Immobilon, and the amino-terminal sequence was determined. This sequence was identical to that beginning at the amino terminus predicted from the cDNA sequence (Fig. 2). In addition, the M_r 145,000 species was digested with *Staphylococcal aureus* V8 protease and resolved on SDS-PAGE. Two amino acid sequences were obtained that matched the predicted sequence at amino acid positions 154 and 836 (Fig. 2). These results indicate that the M_r 145,000 species represents all or a major part of the molecule encoded by Nr-CAM mRNA. In addition, the observation that detergents were required to release the M_r 145,000 protein from membranes whereas treatments that usually remove peripherally associated proteins including high pH and salt did not remove Nr-CAM (unpublished observations) indicated that the protein extends through the transmembrane region.

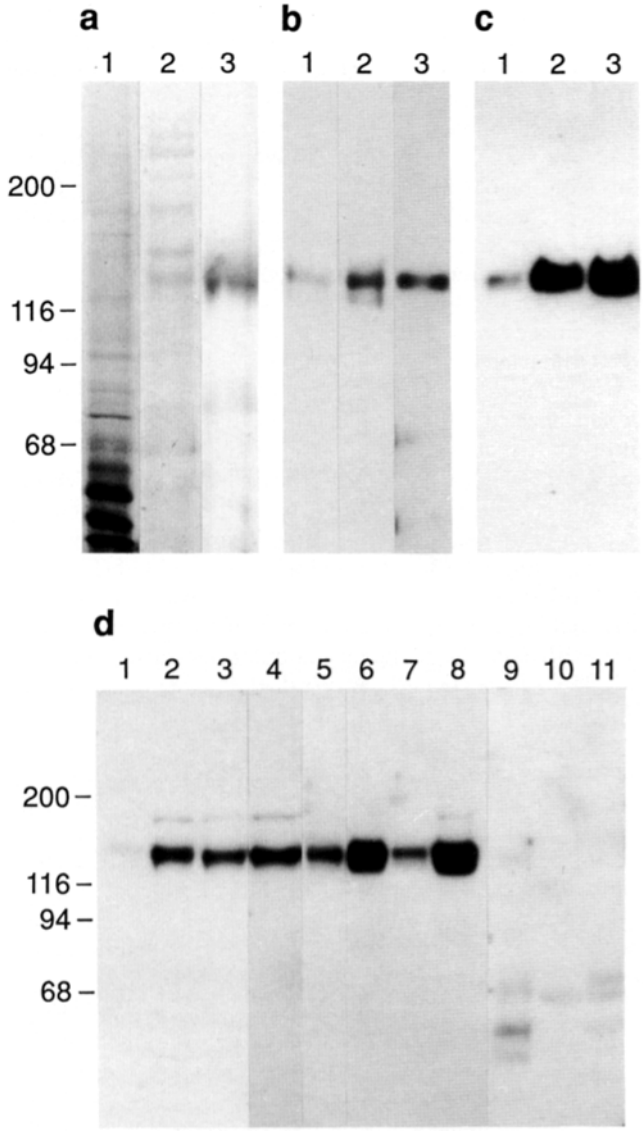

Figure 7. Detection and isolation of Nr-CAM protein. Extracts of 14-d embryonic chick brain membranes (lane *1*), extracts purified on lentil lectin columns (lane *2*), and FPLC-purified Nr-CAM (lane *3*) were resolved on SDS-PAGE. The gels were either (*a*) stained with Coomassie blue (lanes *1* and *2*) and silver (lane *3*), or transferred to nitrocellulose and immunoblotted with (*b*) rabbit antibodies against a peptide corresponding to amino acids 153–173 (Fig. 2) and (*c*) rabbit antibodies against FPLC-purified Nr-CAM. (*d*) Extracts of chicken tissues containing 100 μg protein prepared from E6, E9, E12, and E15 retina (lanes *1–4*, respectively), and from 10-d posthatch chicken retina, cerebellum, spinal cord, brain, intestine, liver, and lung (lanes *5–11*, respectively) were immunoblotted with rabbit antibodies against Nr-CAM. Molecular mass standards are indicated as $M_r \times 10^{-3}$.

The nature of the M_r 170,000 component is less clear. It is immunologically related to the M_r 145,000 component but it was only detected in immunoblots so we could not determine whether it had the same amino terminal sequence as the M_r 145,000 component. The M_r 170,000 species could be a larger component that gives rise to the M_r 145,000 form by proteolysis or it could be an alternatively spliced form of Nr-CAM.

Both the M_r 145,000 and 170,000 species are larger than the polypeptides predicted from the cDNA sequence (M_r 127,000–140,000, see Figs. 1 and 2). The fact that Nr-CAM bound to lentil lectin columns suggest that this difference could be due at least in part to glycosylation. In accord with this notion, treatment of the M_r 145,000 species with endoglycosidase F decreased its relative molecular mass ~15,000. In addition, it was detected on immunoblots by monoclonal antibody HNK-1 (data not shown) which recognizes certain N-linked carbohydrates found in several CAMs and SAMs (33, 40, 42, 48, 54, 55, 75, 78), particularly those of nervous system origin.

Localization of Nr-CAM Protein

The tissue distribution of Nr-CAM mRNA and protein indicated that it was present in neural tissues but not in non-neural tissues (Figs. 4 and 7). The expression of Nr-CAM in different tissues during development was examined by immunoblotting with antibodies against the Nr-CAM peptides and against the purified M_r 145,000 species. No reactivity was found in extracts of 3-d chick embryos, a time before differentiation of neurons (89). At later stages and progressively until approximately embryonic day 10–14, the levels of Nr-CAM increased in the brain and retina (Fig. 7 *d*, lanes *1–6*). In addition to the predominant M_r 145,000 species, the M_r 170,000 species was found in lesser amounts in neural tissues both during development and after hatching. These tissues included brain, retina, cerebellum and spinal cord (Fig. 7 *d*). Little or none of the two species were detected in non-neural tissues including intestine, liver, and lung (Fig. 7 *d*, lanes *9–11*); the nature of the lower molecular mass bands in these samples is unknown.

To determine which neural cells express the protein in vitro, cultures of brain and retina from 9-d chicken embryos were analyzed by immunofluorescence with antibodies against Nr-CAM (Fig. 8) and compared to the staining patterns of antibodies against N-CAM, Ng-CAM, and other antigens that distinguish neurons from glia in culture (39). In such cultures, the neurons and their processes are more apparent than the underlying flat glial cells which can be best discerned by their nuclei. Because antibodies against denatured Ng-CAM had detected a fusion protein produced by the original Nr-CAM cDNA clone encoding the cytoplasmic region of Nr-CAM, it was necessary to use antibodies that had no such cross reactivity. The antibodies used here were generated against native Ng-CAM and native Nr-CAM and did not cross react with Nr-CAM and Ng-CAM, respectively, in immunoblotting experiments (data not shown).

In cultures of unfractionated neural cells, stellate neurons and their fasciculated processes stained with antibodies against N-CAM (Fig. 8, *a* and *b*) and Ng-CAM (Fig. 8, *c* and *d*) whereas the underlying nonneuronal astroblasts were very weakly positive for N-CAM but were negative for Ng-CAM as described previously (39, 41). Antibodies against Nr-CAM specifically labeled the surface of some, but not all, neurons and neurites in brain cultures; they bound very weakly or not at all to the flat astroblasts (Fig. 8, *e* and *f*). Staining of cultures prepared from retina (Fig. 8, *g* and *h*) showed the presence of Nr-CAM on most neuronal cell bodies (*arrowhead*) and neurites but not on the underlying glial cells. The fact that the neurites in the retinal cultures were shorter

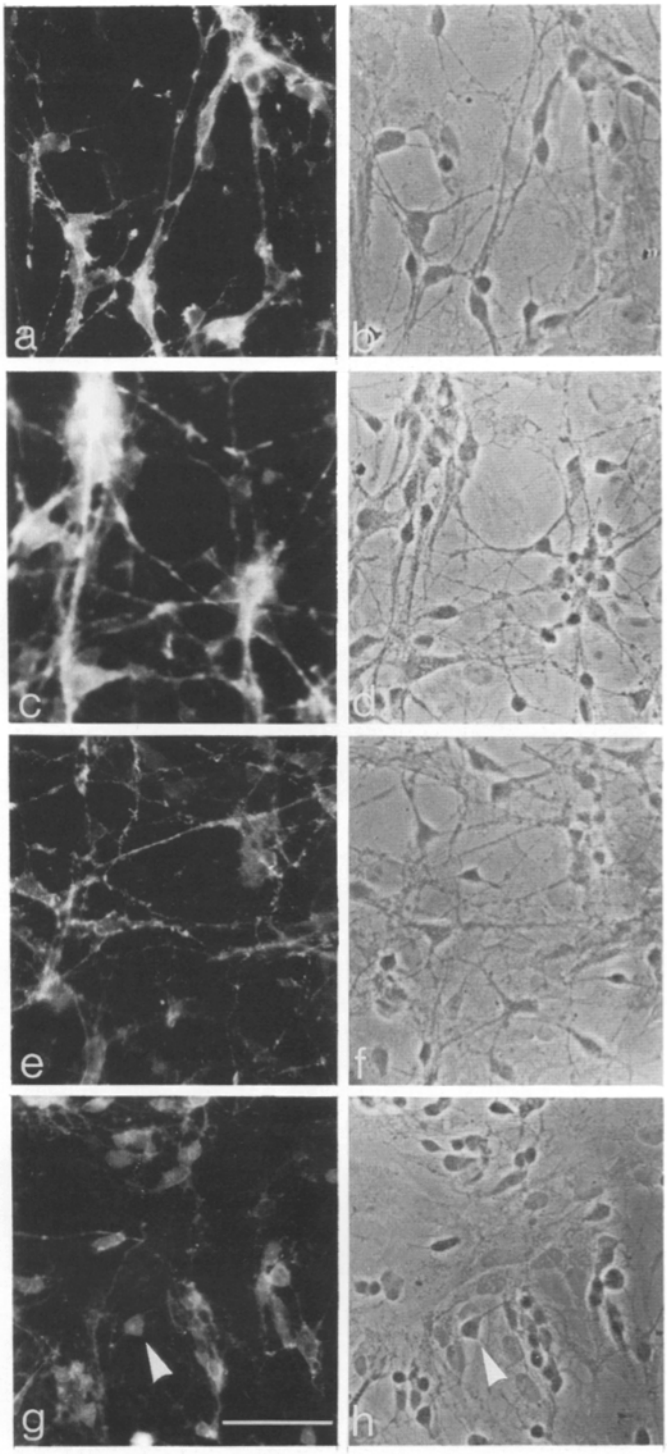

Figure 8. Immunofluorescence staining of chicken brain and retina cultures containing both neurons and astroglial cells. Unfractionated cultures prepared from 9-d embryo brain (*a–f*) and retina (*g* and *h*) were stained with rabbit antibodies against N-CAM (*a* and *b*), Ng-CAM (*c* and *d*), and Nr-CAM (*e–h*). Fluorescence (*a*, *c*, *e*, and *g*) and phase-contrast (*b*, *d*, *f*, and *h*) micrographs of the same respective fields are shown. In *g*, for example, the stellate process bearing neurons (*arrowhead*) overlay the flat glial cells. Bar, 50 μm.

and less fasciculated made them less prominent than those in brain cultures. Antibodies against Ng-CAM also labeled some but not all of the retinal neurons and also failed to label glial cells (data not shown). It was consistently observed that while most retinal neurons were Nr-CAM positive, only some were Ng-CAM positive. Conversely, most brain neurons were Ng-CAM positive but only some were Nr-CAM positive (compare Fig. 8, *e* and *f* with *g* and *h*). These results suggest that Nr-CAM and Ng-CAM are differentially expressed on different types of neurons at different stages and locations.

Differences in the localization of Nr-CAM, Ng-CAM, and N-CAM during development were also found when sections of tissues were labeled by immunofluorescence. For example, at E7, Nr-CAM (Fig. 9 *a*) and N-CAM (16) were uniformly distributed throughout the retina, while Ng-CAM (Fig. 9 *b*) was localized primarily to the ganglion cells and their fibers in the optic fiber layer (OFL). At E17, antibodies to all three molecules labeled this layer intensely, but the labeling pattern in the other retinal layers differed (Fig. 9, *d* and *e*; 16). Nr-CAM was also found on cell bodies in the ganglion cell layer (GCL), and on fibers in the outer plexiform layer (OPL) and in the inner and outer nuclear layers (INL and ONL, respectively). At 10 d after hatching, anti-Nr-CAM antibodies labeled fibers in the OFL, inner plexiform layer (IPL), and OPL, and cell bodies in several layers including the INL and ONL. In contrast, double labeling of the same sections with monoclonal antibodies against Ng-CAM showed staining almost exclusively in the OFL (Fig. 9 *h*). Although anti-N-CAM antibodies also stained the OFL and the IPL, the intensity of the staining in the outer layers of the retina was much weaker than the Nr-CAM stain and the detailed staining patterns for the two molecules differed in most layers (Fig. 9, *g* and *i*).

In the developing cerebellum, Nr-CAM, like N-CAM (40), was found on cell bodies and fibers in many layers including those on the external granular layer. Like Ng-CAM (40), the highest level of Nr-CAM staining in the cerebellum was found on fibers in the molecular layer, but Ng-CAM was not found on the external granule cells. Anti-Nr-CAM antibodies also stained the surfaces of cells in the grey matter of the spinal cord, in dorsal root ganglia, in the lens, and in nerves and ganglia in various tissues (data not shown).

All of these results indicate that, during retinal development, Nr-CAM is more widely and differently distributed than Ng-CAM and that, with time, its localization changes dramatically.

Discussion

We have isolated and initially characterized a new cell surface glycoprotein in the nervous system, Nr-CAM, that is structurally related to chicken Ng-CAM and mouse L1 in that all these proteins contain six Ig domains, five FnIII repeats, a single transmembrane domain, and a cytoplasmic domain. These structural features are summarized for Nr-CAM in Fig. 10. Because we obtained two different cDNA clones spanning each of two segments, the model incorporates the notion that there may be several variant forms of the molecule that arise by alternative RNA splicing. In support of this idea at least two mRNAs were detected on North-

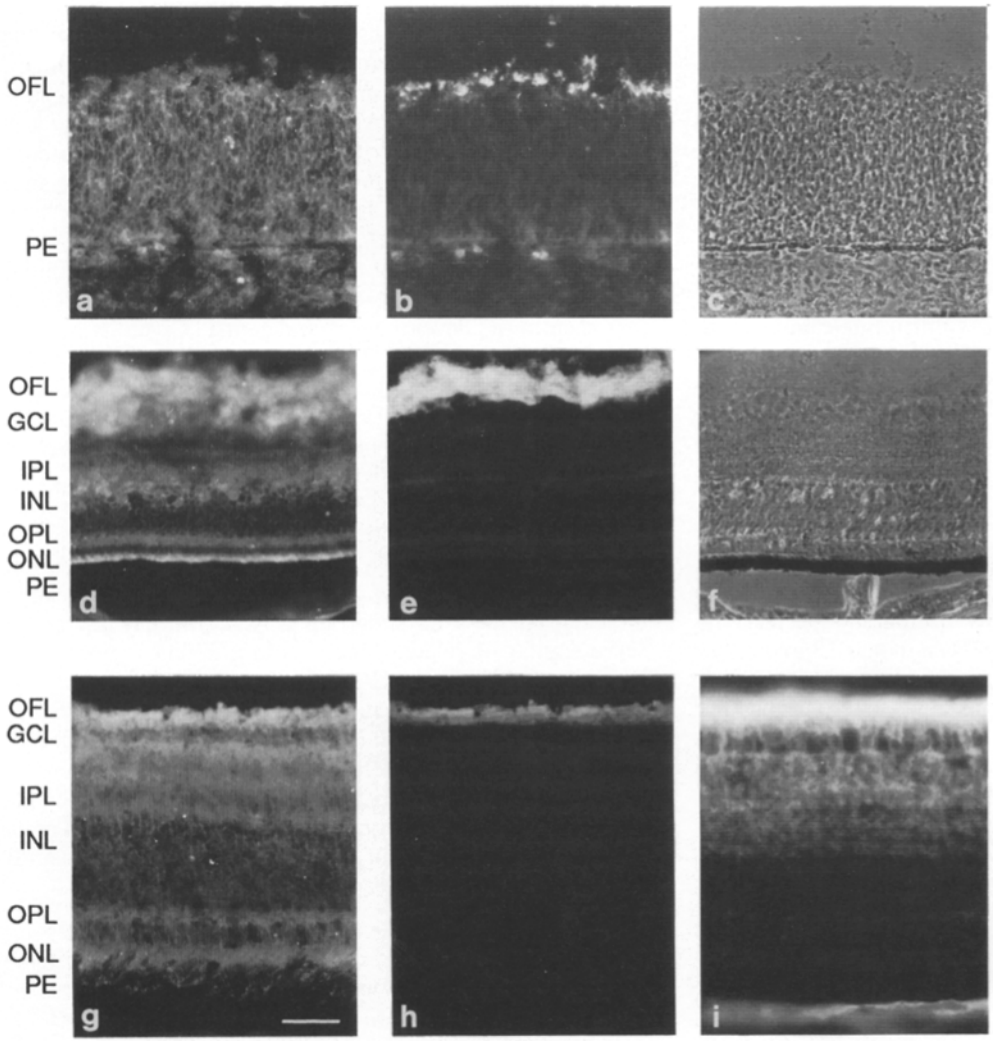

Figure 9. Immunofluorescence staining of transverse retinal sections with antibodies against Nr-CAM. Retinal sections from E7 (*a–c*), E17 (*d–f*), and 10-d posthatch (*g–i*) chickens were double labeled with rabbit antibodies against Nr-CAM and fluorescein-labeled goat anti-rabbit antibodies (*a*, *d*, and *g*), and monoclonal antibodies against Ng-CAM and rhodamine-labeled goat anti-mouse antibodies (*b*, *e*, and *h*). Micrographs of the same respective fields are shown using filters for fluorescein (*a*, *d*, and *g*), rhodamine (*b*, *e*, and *h*), and phase contrast (*c* and *f*). (*i*) Section was labeled with rabbit antibodies against N-CAM. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *OFL*, optic fiber layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *PE*, pigment epithelium. Bar, 50 μm.

ern blots (Fig. 4 *a*), and two different sized proteins were detected on immunoblots (Fig. 7 *d*).

Detailed pairwise comparisons of the amino acid sequences of Nr-CAM with other molecules confirmed that it is a member of the N-CAM family of neural CAMs (21) and indicated that throughout its sequence it is most closely related to chicken Ng-CAM, mouse L1, rat NILE, and human L1 (44, 70; detailed comparison to NILE and human L1 must await completion of their structures). FASTA scores for comparisons between pairs of these molecules were >2,000, whereas scores for other pairs of molecules were much lower; for example, the FASTA score for Nr-CAM/N-CAM was only 420 (Table I). Interestingly, *Drosophila* neuroglian (3) has the same organization of Ig domains and FnIII repeats, and its sequence gave the highest FASTA score when compared to Nr-CAM (Table I). This similarity is due in part to the carboxyl-terminal 111 amino acids in neuroglian which are 38% identical to Nr-CAM; this stretch includes a sequence of nine amino acids that is identical in Nr-CAM, Ng-CAM, L1, NILE, and human L1 (Fig. 5) (6, 44, 64, 70).

Figure 10. Model of the domain structure of Nr-CAM. Ig domains are shown as loops in the amino-terminal portion of the polypeptide and FNIII repeats are shown as boxes. The two open boxes indicated by arrows represent potential mRNA splice variants (see text). The predicted transmembrane region is indicated by a vertical bar and the general location of potential phosphorylated sites (*P*) is indicated.

The combined data indicate that Nr-CAM, Ng-CAM, L1 (and probably NILE) and neuroglian are members of one subgroup in the N-CAM superfamily of neural CAMs (Table I). Using similar criteria, chick contactin/F11, rat TAG-1, and mouse F3 are most similar to each other and have been suggested to be members of a different subgroup; each of these molecules contains six Ig domains, four FnIII repeats, and has a phosphatidylinositol membrane anchor (6, 37). It also has been suggested (33) that mouse F3 is equivalent to chick contactin/F11 (this is reflected in the very high FASTA score of 4027), and that TAG-1 is a different, but related molecule (30).

FASTA scores between N-CAMs from chick (14) and mouse (2) are very high (Table I). N-CAMs are encoded by various spliced mRNAs (14, 35) and they may be considered to comprise a third subgroup which contain five Ig domains, two FnIII repeats, a transmembrane domain and different sized cytoplasmic domains. Fasciclin II has been suggested to be a member of the N-CAM subgroup (45) because it shares this organization of domains and shows the highest FASTA score when compared to N-CAMs (Table I). So far, therefore, three distinct subgroups of N-CAM-related molecules can be discerned.

When one compares molecules within a single animal species, the structure of Nr-CAM is found to be most like that of Ng-CAM. A slightly higher level of identity, however, exists between Nr-CAM and L1, which is marginally better than the identity between L1 and Ng-CAM; it is not clear whether this difference is significant. It is unlikely that either chicken Nr-CAM or Ng-CAM is equivalent to mouse L1 given that they are at best only 40% identical and that the level of identity is much greater (~80% identity, see Table I) between other members of the N-CAM superfamily believed to be equivalent molecules in different species. The relatively low level of identity between mouse L1 and either chicken Nr-CAM or Ng-CAM, is consistent with the possibility that there is a chicken equivalent of L1 that has not been detected as yet. Alternatively, certain molecules with related structures and probably with related functions may have evolved differently in chickens and mice.

Other molecules whose structures have not been determined in detail could not be included in the computer comparisons, but they nonetheless may be related to Nr-CAM. While this manuscript was being prepared, for example, it was reported that retinal chick proteins of 140- and 130-kD, called Bravo (18), which are detected on retinal fibers in the retina but not in the tectum, have amino-terminal sequences similar to mouse L1. The amino-terminal sequence of Bravo is much more similar (74% identity) to the amino-terminal sequence of Nr-CAM; however, there are ambiguities in the amino-terminal sequence of Bravo which make it difficult to distinguish whether it is very closely related or identical to Nr-CAM. Neurofascin (74) also shares certain properties with Nr-CAM including the general size of some of its components and its localization in the nervous system, but it is known to be immunologically distinct from Bravo (18). Definitive comparisons among these molecules will only be possible when the structures of Bravo and neurofascin have been more completely determined.

In addition to their structural similarities, many members of the Ig superfamily have been implicated in functions related to cell adhesion. For example, in the subgroup of molecules that are structurally related to Nr-CAM, it is known that Ng-CAM, L1, and NILE are important for axonal fasciculation (28, 29, 50, 86) and neuroglian has been demonstrated to mediate cell-cell adhesion after transfection into non-neuronal cell lines (36). All these molecules are found on neurons and are particularly prevalent on axons (40, 73, 87, 89). Nr-CAM, like these neural CAMs, is expressed on the surface of developing neurons and is localized at cell–cell junctions (Figs. 8 and 9). The dynamic changes in Nr-CAM localization and its restriction to certain neuronal cell bodies and fibers during development, are consistent with the hypothesis that it may be important for neural cell adhesion and axonal fasciculation. In preliminary experiments, Fab' fragments of antibodies against Nr-CAM partially inhibited the aggregation of retinal neurons (Grumet, M., G. M. Edelman, and B. A. Cunningham, unpublished observations) suggesting that Nr-CAM is a CAM. Given the present work, it is also possible to study Nr-CAM function by preparing constructs that encode the protein and transfecting them into cells which normally do not express Nr-CAM, as has been done for other CAMs (24, 66, 84).

It is noteworthy that, of the tissues examined in this initial study, the most dynamic distribution pattern for Nr-CAM was seen in the developing neural retina. Given its relative abundance in retina (Fig. 7 *d*), Nr-CAM may be particularly important for cell layering and fiber interactions in this tissue. Previous studies (7, 50) of CAM function during retinal layering implicated N-CAM but not Ng-CAM, and it is therefore important to test the differential involvement of Nr-CAM. The prevalence of Nr-CAM along with Ng-CAM in the optic fiber layer and in the optic nerve (data not shown) suggest that, like Ng-CAM, it may be important in nerve formation and axon guidance. It is possible that structurally related molecules in the same subgroup as Nr-CAM may interact to serve similar or related functions in different regions. The localization of different CAMs or their isoforms on individual cells can differ dramatically in different regions (16, 19, 40, 82) and this may result in part from the ability of different CAMs or isoforms to interact with each other (47). Further analyses of the function of Nr-CAM and a more detailed investigation of its expression should allow determination of its functional contribution in particular regions during neural development and regeneration.

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References

1. Arquint, M., J. Roder, L. S. Chia, J. Down, D. Wilkinson, H. Bayley, P. Braun, and R. Dunn. 1987. Molecular cloning and primary structure of myelin-associated glycoprotein. *Proc. Natl. Acad. Sci. USA.* 84:600–604.
2. Barthels, D., M.-J. Santoni, W. Wille, C. Ruppert, J.-C. Chaix, C. Hirsch, J. C. Fontecilla-Camps, and C. Goridis. 1987. Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a Mr 79,000 polypeptide without a membrane-spanning region. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:907–914.
3. Bieber, A. J., P. M. Snow, M. Hortsch, N. H. Patel, J. R. Jacobs, Z. R. Traquina, J. Schilling, and C. S. Goodman. 1989. Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell.* 59:447–460.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
5. Brummendorf, T., J. M. Wolff, R. Frank, and F. G. Rathjen. 1989. Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. *Neuron.* 2:1351–1361.
6. Burgoon, M. P., M. Grumet, V. Mauro, G. M. Edelman, and B. A. Cunningham. 1991. Structure of the chicken neuron-glia cell adhesion molecule, Ng-CAM: origin of polypeptides and relation to Ig superfamily. *J. Cell Biol.* 112:1017–1029.
7. Buskirk, D. R., J.-P. Thiery, U. Rutishauser, and G. M. Edelman. 1980. Antibodies to a neural cell adhesion molecule disrupt histogenesis in cul-

tured chick retinae. *Nature (Lond.).* 285:488–489.
8. Caron, P., and M. E. Schwab. 1988. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.* 106:1281–1287.
9. Chang, S., F. G. Rathjen, and J. A. Raper. 1987. Extension of neurites on axons is impaired by antibodies against specific cell surface glycoproteins. *J. Cell Biol.* 104:355–362.
10. Chang, S., F. G. Rathjen, and J. A. Raper. 1990. Neurite outgrowth promoting activity of G4 and its inhibition by monoclonal antibodies. *J. Neurosci. Res.* 25:180–186.
11. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.
12. Cole, G. J., and R. Akeson. 1989. Identification of a heparin binding domain of the neural cell adhesion molecule N-CAM using synthetic peptides. *Neuron.* 2:1157–1165.
13. Crossin, K. L., S. Hoffman, M. Grumet, J.-P. Thiery, and G. M. Edelman. 1986. Site-restricted expression of cytotactin during development of the chicken embryo. *J. Cell Biol.* 102:1917–1930.
14. Cunningham, B. A., J. J. Hemperly, B. A. Murray, E. A. Prediger, R. Brackenbury, and G. M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science (Wash. DC).* 236:799–806.
15. D'Urso, D., P. J. Brophy, S. M. Staugaitis, C. S. Gillespie, A. B. Frey, J. G. Stempak, and D. R. Colman. 1990. Protein zero of peripheral nerve myelin: biosynthesis, membrane insertion, and evidence for homotypic interaction. *Neuron.* 2:449–460.
16. Daniloff, J., C.-M. Chuong, G. Levi, and G. M. Edelman. 1986. Differential distribution of cell adhesion molecules during histogenesis of the chicken nervous system. *J. Neurosci.* 6:739–758.
17. Davies, J. A., G. M. W. Cook, C. D. Stern, and R. J. Keynes. 1990. Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones. *Neuron.* 2:11–20.
18. de la Rosa, E. J., J. F. Kayyem, J. M. Roman, Y. D. Stierhof, W. J. Dreyer, and U. Schwarz. 1990. Topologically restricted appearance in the developing chick retinotectal system of Bravo, a neural surface protein: experimental modulation by environmental cues. *J. Cell Biol.* 111:3087–3096.
19. Dodd, J., S. B. Morton, D. Karagogeos, M. Yamamoto, and T. M. Jessell. 1988. Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron.* 1:105–116.
20. Edelman, G. M. 1984. Cell surface modulation and marker multiplicity in neural patterning. *Trends Neurosci.* 7:78–84.
21. Edelman, G. M. 1987. CAMs and Igs: cell adhesion and the evolutionary origins of immunity. *Immun. Rev.* 100:11–45.
22. Edelman, G. M., and K. L. Crossin. 1991. Cell adhesion molecules: implications for a molecular histology. *Annu. Rev. Biochem.* In press.
23. Edelman, G. M., B. A. Cunningham, and J. P. Thiery. 1990. Morphoregulatory Molecules. Neurosciences Institute. John Wiley & Sons, Inc., New York.
24. Edelman, G. M., B. A. Murray, R.-M. Mege, B. A. Cunningham, and W. J. Gallin. 1987. Cellular expression of liver and neural cell adhesion molecules after transfection with their cDNAs results in specific cell-cell binding. *Proc. Natl. Acad. Sci. USA.* 84:8502–8506.
25. Eisenberg, D. 1984. Three-dimensional structures of membrane and surface proteins. *Annu. Rev. Biochem.* 53:595–623.
26. Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266–267.
27. Filbin, M. T., F. S. Walsh, B. D. Trapp, J. A. Pizzey, and G. I. Tennekoon. 1990. Role of myelin Po protein as a homophilic adhesion molecule. *Nature (Lond.).* 344:871–872.
28. Fischer, G., J. Kunemund, and M. Schachner. 1986. Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell surface glycoprotein L1. *J. Neurosci.* 6:605–612.
29. Friedlander, D. R., M. Grumet, and G. M. Edelman. 1986. Nerve growth factor enhances expression of neuron-glia cell adhesion molecule in PC12 cells. *J. Cell Biol.* 102:413–419.
30. Furley, A. J., S. B. Morton, D. Manalo, D. Karagogeos, J. Dodd, and T. M. Jessel. 1990. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite-promoting activity. *Cell.* 61:157–170.
31. Gallin, W. J., B. C. Sorkin, G. M. Edelman, and B. A. Cunningham. 1987. Sequence analysis of a cDNA clone encoding the liver cell adhesion molecule, L-CAM. *Proc. Natl. Acad. Sci. USA.* 84:2808–2812.
32. Geliebter, J. 1987. Dideoxynucleotide sequencing of RNA and uncloned cDNA. *BRL Focus* 9:5–8.
33. Gennarini, G., G. Cibelli, G. Rougon, M.-G. Mattei, and C. Goridis. 1989. The mouse neuronal cell surface protein F3: a phosphatidylinositol-anchored member of the immunoglobulin superfamily related to chicken contactin. *J. Cell Biol.* 109:775–788.
34. Glass, D. B., M. R. El-Maghrabi, and S. J. Pilkis. 1986. Synthetic peptides corresponding to the site phosphorylated in 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as substrates of cyclic nucleotide-dependent protein kinases. *J. Biol. Chem.* 261:2987–2993.
35. Goridis, C., and W. Wille. 1988. The three size classes of mouse NCAM proteins arise from a single gene by a combination of alternative splicing and use of different polyadenylation sites. *Neurochem. Int.* 12:269–272.
36. Grenningloh, G., A. Bieber, J. Rehm, P. Snow, Z. Traquina, M. Hortsch, N. Patel, and C. S. Goodman. 1990. Molecular genetics of neuronal recognition in Drosophila: evolution and function of immunoglobulin superfamily cell adhesion molecules. *Cold Spring Harbor Symp. Quant. Biol.* In press.
37. Grumet, M. 1991. Structure, expression and function of Ng-CAM, a member of the immunoglobulin superfamily involved in neuron-neuron and neuron-glia adhesion. *J. Neurosci. Res.* In press.
38. Grumet, M., and G. M. Edelman. 1984. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J. Cell Biol.* 98:1746–1756.
39. Grumet, M., and G. M. Edelman. 1988. Neuron-glia cell adhesion molecule interacts with neurons and astroglia via different binding mechanisms. *J. Cell Biol.* 106:487–503.
40. Grumet, M., S. Hoffman, C.-M. Chuong, and G. M. Edelman. 1984. Polypeptide components and binding functions of neuron-glia cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 81:7989–7993.
41. Grumet, M., S. Hoffman, and G. M. Edelman. 1984. Two antigenically related neuronal CAMs of different specificities mediate neuron-neuron and neuron-glia adhesion. *Proc. Natl. Acad. Sci. USA.* 81:267–271.
42. Grumet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA.* 82:8075–8079.
43. Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene (Amst.).* 25:263–269.
44. Harper, J. R., J. T. Prince, P. A. Healy, J. K. Stuart, S. J. Nauman, and W. B. Stallcup. 1991. Isolation and sequence of partial cDNA clones of human L1: homology of human and rodent L1 in the cytoplasmic region. *J. Neurochem.* 56:797–804.
45. Harrelson, A. L., and C. S. Goodman. 1988. Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. *Science (Wash. DC).* 242:700–707.
46. Hemperly, J. J., B. A. Murray, G. M. Edelman, and B. A. Cunningham. 1986. Sequence of a cDNA clone encoding the polysialic acid-rich and cytoplasmic domains of the neural cell adhesion molecule N-CAM. *Proc. Natl. Acad. Sci. USA.* 83:3037–3041.
47. Hoffman, S., and G. M. Edelman. 1983. Kinetics of homophilic binding by E and A forms of the neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 80:5762–5766.
48. Hoffman, S., and G. M. Edelman. 1987. A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. *Proc. Natl. Acad. Sci. USA.* 84:2523–2527.
49. Hoffman, S., B. C. Sorkin, P. C. White, R. Brackenbury, R. Mailhammer, U. Rutishauser, B. A. Cunningham, and G. M. Edelman. 1982. Chemical characterization of a neural cell adhesion molecules purified from embryonic brain membranes. *J. Biol. Chem.* 257:7720–7729.
50. Hoffman, S., D. R. Friedlander, C.-M. Chuong, M. Grumet, and G. M. Edelman. 1986. Differential contributions of Ng-CAM and N-CAM to cell adhesion in different neural regions. *J. Cell Biol.* 103:145–158.
51. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48: 549–554.
52. Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990. The neural cell adhesion molecule N-CAM enhances L1-dependent cell–cell interactions. *J. Cell Biol.* 110:193–208.
53. Kornblihtt, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755–1759.
54. Kruse, J., G. Keilhauer, A. Faissner, R. Timpl, and M. Schachner. 1985. The J1 glycoprotein: a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature (Lond.).* 316:146–148.
55. Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner. 1984. Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature (Lond.).* 311:151–153.
56. Kuenzel, E. A., J. A. Mulligan, J. Sommercorn, and E. G. Krebs. 1987. Substrate specificity determinants for Casein Kinase II as deduced from studies with synthetic peptides. *J. Biol. Chem.* 262:9136–9140.
57. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.
58. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
59. Lai, C., M. A. Brow, K.-A. Nave, A. B. Noronha, R. H. Quarles, F. E. Bloom, R. J. Milner, and J. G. Sutcliffe. 1987. Two forms of 1B236/myelin-associated glycoprotein, a cell adhesion molecule for postnatal neural development, are produced by alternative splicing. *Proc. Natl. Acad. Sci. USA.* 84:4337–4341.
60. Lemmon, V., K. L. Farr, and C. Lagenaur. 1989. L1-mediated axon outgrowth occurs via a homophilic binding mechanism. *Neuron.* 2:1597–1603.
61. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning:

A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
62. Marshal, R. D. 1972. Glycoproteins. *Annu. Rev. Biochem.* 41:673–702.
63. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262:10035–10038.
64. Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Fruh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature (Lond.).* 334:701–703.
65. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307–310.
66. Nagafuchi, A., Y. Shirayoshi, K. Okazaki, K. Yasuda, and M. Takeichi. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature (Lond.).* 329:341–343.
67. Patel, N. H., P. M. Snow, and C. S. Goodman. 1987. Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. *Cell.* 48:975–988.
68. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA.* 85:2444–2448.
69. Popowicz, A. M., and P. F. Dash. 1988. SIGSEQ: a computer program for predicting signal sequence cleavage sites. *Computer Appl. Biosci.* 4:405–406.
70. Prince, J. T., N. Milona, and W. B. Stallcup. 1989. Characterization of a partial cDNA clone for the NILE glycoprotein and identification of the encoded polypeptide domain. *J. Neurosci.* 9:876–883.
71. Ranscht, B. 1988. Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. *J. Cell Biol.* 107:1561–1573.
72. Raper, J. A., and J. P. Kapfhammer. 1990. The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. *Neuron.* 2:21–29.
73. Rathjen, F. G., and M. Schachner. 1984. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen), which is involved in cell adhesion. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1–10.
74. Rathjen, F. G., J. M. Wolff, S. Chang, F. Bonhoeffer, and J. A. Raper. 1987. Neurofascin: a novel chick cell-surface glycoprotein involved in neurite-neurite interactions. *Cell.* 51:841–849.
75. Rathjen, F. G., J. M. Wolff, R. Frank, F. Bonhoeffer, and U. Rutishauser. 1987. Membrane glycoproteins involved in neurite fasciculation. *J. Cell Biol.* 104:343–353.
76. Reichardt, L. F., J. L. Bixby, D. E. Hall, M. J. Ignatius, K. M. Neugebauer, and K. J. Tomaselli. 1989. Integrins and cell adhesion molecules: neuronal receptors that regulate axon growth on extracellular matrices and cell surfaces. *Dev. Neurosci.* 11:332–347.
77. Reyes, A. A., R. Akeson, L. Brezina, and G. J. Cole. 1990. Structural requirements for neural cell adhesion molecule-heparin interaction. *Cell Reg.* 1:567–576.
78. Ruegg, M. A., E. T. Stoeckli, R. B. Lanz, P. Streit, and P. Sonderegger. 1989. A homologue of the axonally secreted protein axonin-1 is an integral membrane protein of nerve fiber tracts involved in neurite fasciculation. *J. Cell Biol.* 109:2363–2378.
79. Salzer, J. G., W. P. Holmes, and D. R. Colman. 1987. The amino acid sequences of the myelin-associated glycoproteins: homology to the immunoglobulin gene superfamily. *J. Cell Biol.* 104:957–965.
80. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
81. Schachner, M., H. Antonicek, T. Fahrig, A. Faissner, G. Fischer, V. Kunemund, R. Martini, A. Meyer, E. Persohn, E. Pollerberg, R. Probstmeier, and K. Sadoul. 1989. Families of neural cell adhesion molecules. *In* Morphoregulatory Molecules. G. M. Edelman, B. A. Cunningham, and J. P. Thiery, editors. 443–468.
82. Schlosshauer, B., U. Schwarz, and U. Rutishauser. 1984. Topological distribution of different forms of neural cell adhesion molecule in the developing chick visual system. *Nature (Lond.).* 310:141–143.
83. Schwarzbauer, J. E., R. S. Patel, D. Fonda, and R. O. Hynes. 1987. Multiple sites of alternative splicing of the rat fibronectin gene transcript. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2573–2580.
84. Snow, P. M., A. J. Bieber, and C. S. Goodman. 1989. Fasciclin III: a novel homophilic adhesion molecule in Drosophila. *Cell.* 89:313–323.
85. Staden, R. 1984. Graphic methods to determine the function of nucleic acid sequences. *Nucleic Acids Res.* 12:521–538.
86. Stallcup, W. B., and L. L. Beasley. 1985. Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. *Proc. Natl. Acad. Sci. USA.* 82: 1276–1280.
87. Stallcup, W. B., L. L. Beasley, and J. M. Levine. 1985. Antibody against nerve growth factor-inducible large external (NILE) glycoprotein labels nerve fiber tracts in the developing rat nervous system. *J. Neurosci.* 5:1090–1101.
88. Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development (Camb.).* 102:639–655.
89. Thiery, J.-P., A. Delouvée, M. Grumet, and G. M. Edelman. 1985. Initial appearance and regional distribution of the neuron-glia cell adhesion molecule, in the chick embryo. *J. Cell Biol.* 100:442–456.
90. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
91. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683–4690.
92. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily: domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381–405.
93. Wolff, J. M., F. G. Rathjen, R. Frank, and S. Roth. 1987. Biochemical characterization of polypeptide components involved in neurite fasciculation and elongation. *Eur. J. Biochem.* 168:551–561.
94. Woodgett, J. R., K. L. Gould, and T. Hunter. 1986. Substrate specificity of protein kinase C. *Eur. J. Biochem.* 161:177–184.
95. Young, R. A., and R. W. Davis. 1983. Yeast RNA polymerase III genes: isolation with antibody probes. *Science (Wash. DC).* 222:778–782.