Neuron, Vol. 4, 697–709, May, 1990, Copyright © 1990 by Cell Press

Differential Splicing Generates a Nervous System–Specific Form of Drosophila Neuroglian

Michael Hortsch, Allan J. Bieber, Nipam H. Patel, and Corey S. Goodman
Howard Hughes Medical Institute
and Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

We recently described the characterization and cloning of Drosophila neuroglian, a member of the immunoglobulin superfamily. Neuroglian contains six immunoglobulin-like domains and five fibronectin type III domains and shows strong sequence homology to the mouse neural cell adhesion molecule L1. Here we show that the neuroglian gene generates at least two different protein products by tissue-specific alternative splicing. The two protein forms differ in their cytoplasmic domains. The long form is restricted to the surface of neurons in the CNS and neurons and some support cells in the PNS; in contrast, the short form is expressed on a wide range of other cells and tissues. Thus, whereas the mouse L1 gene appears to encode only one protein that functions largely as a neural cell adhesion molecule, its Drosophila homolog, the neuroglian gene, encodes at least two protein forms that may play two different roles, one as a neural cell adhesion molecule and the other as a more general cell adhesion molecule involved in other tissues and imaginal disc morphogenesis.

Introduction

Several vertebrate neural cell adhesion molecules are members of the immunoglobulin superfamily, including N-CAM (Cunningham et al., 1987; Barthels et al., 1987), MAG (Arquint et al., 1987; Salzer et al., 1987), L1 (Moos et al., 1988), and contactin/F11 (Ranscht, 1988; Brümmendorf et al., 1989). These molecules have been implicated in such events as neurite outgrowth, growth cone guidance, axon fasciculation, and myelination. Some of the genes encoding these immunoglobulin superfamily cell adhesion molecules, such as N-CAM and MAG, generate multiple protein forms as a result of differential splicing (Murray et al., 1986; Cunningham et al., 1987; Owens et al., 1987; Barbas et al., 1988; Small et al., 1988; Santoni et al., 1989; Frail and Braun, 1984; Lai et al., 1987). These protein isotypes can differ from each other by their mechanism of membrane attachment, by the size of their cytoplasmic domains, or by the inclusion or exclusion of relatively small amino acid inserts in their extracellular domains. Some of these protein isoforms are expressed in a tissue-specific fashion. For example, a muscle-specific form of N-CAM is generated by the inclusion of a muscle-specific exon encoding 37 amino acids not found in other tissues (Dickson et al., 1987; Thompson et al., 1989). The unique biochemical and functional properties of some of these different protein isoforms are not well understood.

Homologs or close relatives of two of the vertebrate immunoglobulin superfamily cell adhesion molecules have recently been identified in insects (Harrelson and Goodman, 1988; Bieber et al., 1989). Grasshopper fasciclin II is related to N-CAM (Harrelson and Goodman, 1988), whereas Drosophila neuroglian is highly related on the structural and sequence level to L1 (Bieber et al., 1989). Both fasciclin II and neuroglian are integral membrane glycoproteins with multiple immunoglobulin domains followed by multiple fibronectin type III domains, and within the developing nervous system, both are expressed on subsets of axon pathways. Drosophila neuroglian is expressed in a wide variety of tissues in the developing embryo, including the central and peripheral nervous systems (CNS and PNS), salivary glands, trachea, hindgut, muscle, and ectodermal tissues (Bieber et al., 1989).

This wide expression pattern of Drosophila neuroglian differs from the more restricted appearance of its vertebrate homolog, L1. Within the mouse, L1 is restricted largely to the developing nervous system, and within the nervous system, L1 is confined to certain regions and cell types. For example, only certain layers and cells of the mouse neocortex and the cerebellum express L1 (Fushiki and Schachner, 1986; Rathjen and Schachner, 1984; Persohn and Schachner, 1987). In the developing chick nervous system, the L1 homolog Ng-CAM is restricted to extending neurites and migrating neurons (Daniloff et al., 1986). Very few cells outside of the nervous system express L1, although it has been reported on certain proliferating epithelial progenitor cells in the mouse intestine (Thor et al., 1987). Molecular and biochemical analyses of the L1 protein and cDNAs have so far yielded evidence for only a single type of L1 protein in vertebrates (Rathjen and Schachner, 1984; Moos et al., 1988; Prince et al., 1989).

Here we report on the discovery of two different protein forms of the Drosophila L1 homolog, neuroglian; these two neuroglian isoforms arise by tissue-specific, developmentally regulated alternative splicing. The two forms of neuroglian have identical extracellular domains, but differ in the size of their cytoplasmic domains. Using a monoclonal antibody (MAb) that is specific for the long form of the protein, we show that the long form is entirely restricted to the surface of neurons in the CNS and PNS of both embryos and larvae. The long form–specific MAb stains neither CNS glia nor other nonneuronal tissues labeled by a MAb that recognizes an epitope shared by the long and short forms of neuroglian. Thus, it appears as if the neuronal expression and function of L1/neuroglian have remained relatively conserved in both the arthropods and the chordates; whereas in at least some

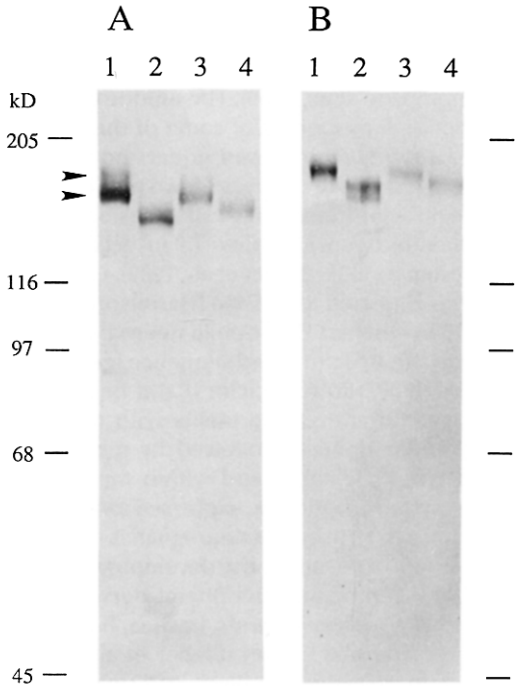


Figure 1. Deglycosylation of Neuroglian Protein

Membranes were prepared from 10–14 hr embryos and treated with TFMS (lanes 2) or endoglycosidase H (lanes 4). Samples shown in lanes 1 and 3 were incubated in deglycosylation buffer without TFMS or endoglycosidase H. Aliquots of the deglycosylation reactions were separated on a 7.5% SDS-polyacrylamide gel and electroblotted to nitrocellulose. Blot A was incubated with a 1/1000 dilution of rat anti-neuroglian serum followed by horseradish peroxidase-conjugated secondary antibody and was developed by diaminobenzidine. Blot B was processed in parallel using a 1/1000 dilution of BP-104 monoclonal ascites fluid. Arrows indicate the two different forms of the neuroglian protein.

arthropods, but not the chordates, the gene has evolved a second protein form and a second pattern of expression (and presumably a second function as a more general cell adhesion molecule) in a variety of nonneuronal tissues.

Results

Anti-Neuroglian Antibodies Detect Two Different Forms of Neuroglian Protein

We recently described the cloning and sequence of a Drosophila neuroglian cDNA encoding a mature protein of 1216 amino acids with an apparent molecular mass on SDS-polyacrylamide gels of 167 kd (Bieber et al., 1989). Using a rat anti-neuroglian polyclonal antiserum, a second minor 180 kd form of neuroglian can be detected in embryonic membranes on Western blots (Figure 1A, lane 1; see Figure 9A); this second form was also seen in the previously published immunoprecipitation experiments (see Figure 3A in Bieber et al., 1989).

We generated and characterized two different MAbs that recognize Drosophila neuroglian and that help to distinguish between the two forms of the protein. MAb 1B7 (Bieber et al., 1989) recognizes both the 167 and the 180 kd form of the protein; it stains the outside of living cells, suggesting that it recognizes a common epitope in the extracellular domain of the two forms of the protein. MAb BP-104 specifically recognizes the larger protein (Figure 1B; see Figure 9B); it requires detergent permeabilization of tissue to bind to the protein, suggesting that it recognizes an epitope specific to the cytoplasmic domain of this form of the protein. MAb BP-104 fails to bind to the 167 kd neuroglian species on Western blots or in any other assay system. Using the MAb BP-104 coupled to protein G-Sepharose beads as an affinity matrix, the 180 kd neuroglian form was purified to homogeneity and subjected to an amino-terminal sequence determination (with the kind help of Arie Admon). The derived amino acid sequence was identical to the previously published amino terminus of the main 167 kd neuroglian form (Bieber et al., 1989; data not shown).

Complete deglycosylation of embryonic membrane proteins with trifluoromethanesulfonic acid (TFMS) results in a downshift of approximately 12 kd for both neuroglian forms, whereas removal of N-linked high-mannose-type oligosaccharides with endoglycosidase H causes a decrease in their apparent molecular mass of about 5 kd (Figure 1). The glycosylation pattern of both neuroglian proteins forms appears to be very similar if not identical. Since the size differences between the fully glycosylated and the TFMS-treated, deglycosylated proteins are the same for both neuroglian species, a structural variation in their polypeptide backbones appears to be the reason for their different electrophoretic mobilities.

The Two Neuroglian Protein Forms Differ in Their Cytoplasmic Domains

The antibody specific for the long form of the protein (MAb BP-104) was used to screen the Zinn 9–12 hr Drosophila embryonic λgt11 cDNA expression library. From this screen, a cDNA clone encoding the 180 kd form of neuroglian was isolated. Using MAb BP-104, 2 positive recombinant phage (containing identical 4.3 kb inserts) out of a total of 5×10^5 phage screened were isolated from this expression library. Sequence analysis and S1 nuclease protection experiments (data not shown) indicated that the first 5′ 3699 bp are identical to the previously described neuroglian sequence (Bieber et al., 1989). The next 38 nucleotides just 3′ to the point where the two cDNAs diverge show a high degree of homology (76% identity on the nucleotide level; Figure 2A). Farther 3′, beyond nucleotide 3737, the two neuroglian cDNA sequences diverge completely and show no significant homology (Figure 2A).

The deduced amino acid sequences of the two open reading frames show that the extracellular and transmembrane domains of both forms are identical. However, after the transmembrane domain, the two neuroglian protein forms share only the first 68 amino acids of their cytoplasmic domains. The short, more

Figure 2. Nucleotide and Protein Sequences of the 3′ Ends of the Neuroglian mRNAs

(A) 3′ end sequences of neuroglian cDNAs coding for the two forms of the neuroglian protein. Both sequences are colinear up to base pair 3699, where they diverge. In frame stop codons and sites for restriction endonucleases used for the subcloning of specific DNA fragments are indicated. (B) C-terminal sequences of the short and long forms of Drosophila neuroglian compared with mouse L1. The first 68 amino acids of the cytoplasmic domains of both neuroglian forms are identical.

abundant form of the protein continues for another 17 amino acid residues before being terminated by an in frame stop codon (Figure 2B). The high degree of homology on the nucleotide level between the two neuroglian cDNAs in this region is also reflected in their amino acid sequences. Eleven out of the 17 C-terminal amino acids of the short form are found in the protein sequence of the longer form. However, in contrast to the short form, the long form of the protein extends for another 62 amino acids. It is this extra cytoplasmic domain that is recognized by MAb BP-104. The entire cytoplasmic domain of the long form of the neuroglian protein form encompasses 148 amino acid residues, compared with 85 amino acids in the cytoplasmic domain of the short form (Figure 2B).

The long form–specific cytoplasmic extension has a unique amino acid composition (Figure 2B). Only 4 different amino acids account for 77% of the 62 amino acids in this extra domain: alanine (23/62), glycine (12/62), serine (8/62), and proline (5/62). Using several different secondary structure prediction programs, no prevalent secondary protein structure was projected. Furthermore, no repeated pattern of amino acids could be recognized. However, a high probability for turn structures was predicted by these computer programs at most of the glycine–glycine sequence sites within this domain.

Comparison of the amino acid sequence of mouse L1 reveals that the size of the L1 cytoplasmic domain (114 amino acids) falls between the two Drosophila neuroglian forms (Figure 2B). Although the C-terminal 25 amino acids of the mouse L1 protein also have a high alanine/glycine/serine/proline content of 52%, there is no apparent sequence homology to the long form–specific extension of neuroglian. Interestingly, the cytoplasmic domain specific for the 180 kd (long) form of N-CAM contains 47% alanine/glycine/serine/proline residues (Cunningham et al., 1987). It is this domain of the 180 kd N-CAM molecule that has been implicated in the binding to spectrin and the specific anchoring of this N-CAM species to the cellular cytoskeleton (Pollerberg et al., 1986, 1987).

The Two Forms of Neuroglian Protein Are Encoded by Different mRNAs

The long and short forms of the neuroglian protein are translated from two different mRNAs. RNA protection experiments were performed to demonstrate the existence of different mRNA species for Drosophila neuroglian. Fragments from both cDNAs starting at the ScaI site at nucleotide 3583 to the 3′ EcoRI cloning site were subcloned in both Bluescript KS/+ and Bluescript SK/+ vectors. [α-^{32}P]UTP-labeled RNA probes were transcribed using T7 RNA polymerase and hy-

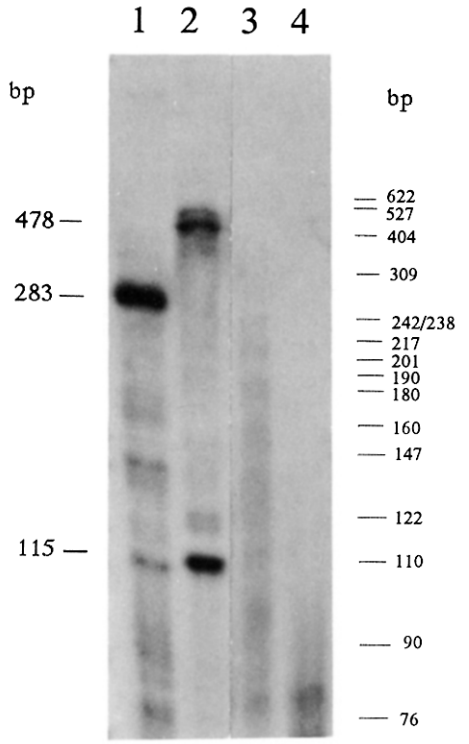


Figure 3. Two Different Neuroglian mRNAs Are Detected by RNA Protection Experiments Using Probes from the Two Neuroglian cDNAs

Sense (lanes 3 and 4) and antisense (lanes 1 and 2) ^{32}P-labeled RNA probes were generated from subcloned 3′ end ScaI–EcoRI fragments of the short (lanes 1 and 3) and long (lanes 2 and 4) neuroglian cDNAs, using T7 RNA polymerase. Probes were hybridized to 30 μg of total Drosophila 12–24 hr embryonic RNA, treated with 10 μg/ml RNAase T1, and separated on an 8% urea–polyacrylamide gel. Protected fragments were detected by autoradiography for 12 hr at −80°C. ^{32}P-labeled, HpaII-cut pBR-322 fragments were used as size markers.

bridized to 30 μg of total RNA isolated from 12–24 hr Drosophila embryos. Single-stranded RNA molecules were digested using RNAase T1, and protected RNA fragments were analyzed on 8% urea–polyacrylamide gels. Only antisense RNA probes yielded protected RNA–RNA hybrid fragments (Figure 3, lanes 1 and 2). A fragment of 115 nucleotides representing the shared sequence between the two cDNAs (starting at the ScaI restriction site to the point where the two sequences diverge) was protected by both antisense RNA probes. In addition, the RNA probe derived from the short neuroglian cDNA protected a homologous 283 nucleotide fragment, whereas the long form–specific probe hybridized over 478 nucleotides to its homologous mRNA species.

The intensities of the protected RNA bands indicate that the mRNA encoding the smaller neuroglian protein form is several times more abundant than the mRNA coding for the longer polypeptide. The relative abundances of the different mRNA species therefore correspond to the relative amounts of the two neuroglian protein forms found in embryonic membranes (Figure 1; see Figure 9).

The Long Form of Neuroglian Is Specifically Expressed on the Surface of CNS and PNS Neurons and on a Few PNS Support Cells

Neuroglian protein expression is widespread in the Drosophila embryo (Bieber et al., 1989). MAb 1B7 recognizes both forms of the neuroglian protein and stains hindgut, muscle, trachea, and salivary glands, in addition to neurons and glia in both the CNS and the PNS (Figure 4A). MAb BP-104 is specific for the long form of the protein, and its staining is entirely restricted to the nervous system (Figure 4B). Within the CNS, it is expressed on neurons, but not on glia (Figure 5); in the PNS, it is expressed on both neurons and a small number of support cells (Figures 6A and 6B). No other tissues or cells outside the nervous system are ever stained by MAb BP-104 during embryonic development. Even within the nervous system, the number of BP-104-positive cells is considerably smaller than the number of 1B7-positive cells. Certain glial cells in the CNS, such as those along the peripheral nerve roots, are stained by MAb 1B7, but are not recognized by MAb BP-104 (Figures 4C and 4D), suggesting that these glia express the short form but not the long form of the protein. Axon tracts, such as the segmental and intersegmental nerves, are heavily stained by MAb BP-104, showing that axons do express the long form.

Figure 5 shows an example of the restricted expression of the long form of the protein in the CNS. In a more dorsal focal plane, certain cells at the surface of the ventral ganglia, including a pair of mesodermal cells (the muscle pioneers) and the longitudinal glial cells, are stained by MAb 1B7 but not by MAb BP-104 (Figures 5A and 5B). In contrast, the RP1 and RP2 neurons are recognized by both antibodies. In a deeper plane of focus, both antibodies stain many of the longitudinal and commissural axon tracts and certain identified neurons (e.g., the aCC, pCC, and RP3 neurons; Figures 5C and 5D). In the PNS, the long form of the protein is expressed on the cell bodies of all PNS neurons (arrowhead in Figure 6A), on a small number of PNS support cells (including the innermost sheath cell; data not shown), and on all major PNS sensory pathways (arrowhead in Figure 6B); however, it is not expressed on most of the side branches of these PNS pathways that contain the axons of motoneurons.

Expression of the long form begins at around 6 hr of embryogenesis as a single neuron in each CNS hemisegment, located about one-third the length of a segment anterior from the segment boundary, expresses this form of the protein. Expression then begins on the surface of adjacent CNS cells. The pattern of expression extends both rostrally and caudally along the length of the CNS in a stripe about 4–5 cells

wide (Figure 6C). By hour 7, the longitudinal stripe of expression in the CNS is continuous, with no expression at the midline or at the lateral margin of the CNS; expression in the PNS has begun. Interestingly, this pattern of expression of the long form of neuroglian does not appear to match simply the order of neuronal differentiation as seen with a variety of antibodies that reveal the order of differentiation and axon outgrowth. Rather, this longitudinal stripe of expression of the long form of neuroglian within the CNS prefigures the location where the longitudinal axon tracts will form and appears even before the longitudinal glia have migrated into this same position (Jacobs et al., 1989).

This restricted expression of the long form of neuroglian by neurons was also observed at later stages of development. As represented by a wing disc in Figure 7A, the epidermal cells in all imaginal discs are stained rather uniformly by MAb 1B7. In contrast, the epidermal cells in the discs do not stain with MAb BP-104, indicating that these cells express the short but not the long form of the protein. Only neurons and axon tracts in the discs express the neuron-specific long form of the protein (Figure 7B). Staining of eye imaginal discs with MAb BP-104 results in a strong labeling of the newly generated photoreceptor cells behind the morphogenetic furrow, as well as strong staining of the axons in the optic stalk connecting the eye disc with the lamina of the optic lobe (Figures 7C and 7D). Note in Figure 7C, that although the photoreceptor axons entering the optic lobes express the long form of neuroglian, most of the cells within the optic lobes have not yet begun to express the long form (in contrast, at this stage, most neurons in the ventral nerve cord do already express the long form). As can be seen in Figure 7D, the surfaces of all photoreceptor cells are stained rather evenly by MAb BP-104, indicating that they too express the neuron-specific long form of the neuroglian protein.

The Expression of the Two Forms of Neuroglian Is Developmentally Regulated

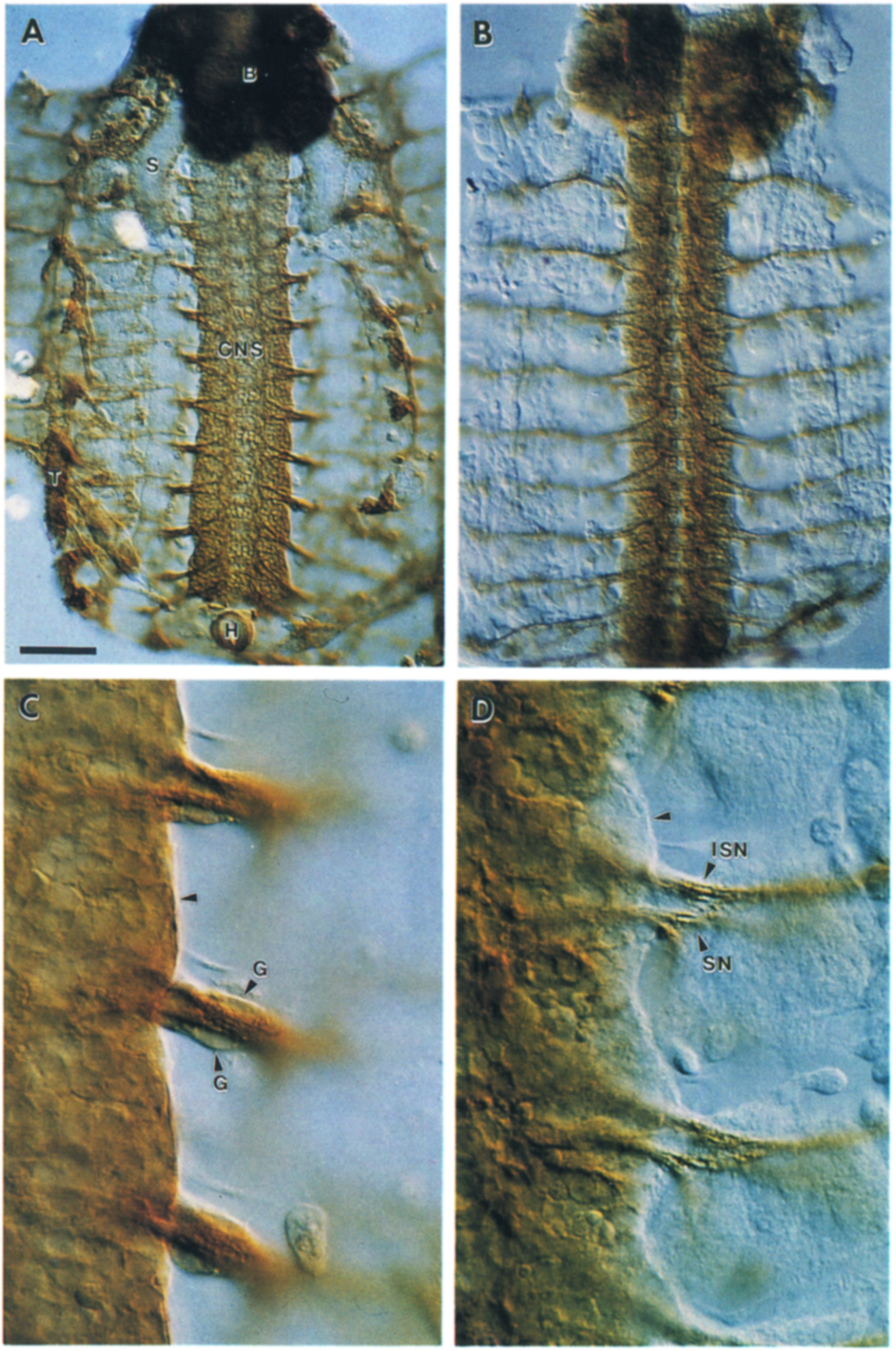
The appearance of mRNAs coding for the two different forms of neuroglian, as well as their corresponding protein products, was examined during Drosophila embryogenesis using Northern and Western blot analyses. Using the restriction endonuclease sites (BamHI for the short form and Cfr10I for the long neuroglian form) indicated in Figure 2A, cDNA fragments specific for the two different transcripts were isolated and subcloned into Bluescript SK/+. [α-^{32}P]UTP-labeled antisense RNA probes were transcribed from the T7 promoter and used for probing Northern blots with total RNA from staged Drosophila embryos. The RNA probe specific for the short form of neuroglian detected a single mRNA species of 5.5 kb, whereas the probe specific for the long neuroglian species hybridized to two mRNAs of 5.7 and 7.6 kb (Figure 8). Significant quantities of mRNA encoding the more abundant, short neuroglian protein are detectable from 9 hr of development onward. The two mRNA species for the larger neuroglian polypeptide begin to be expressed slightly later in development and reach a peak at 12–15 hr of development, during the period of axon outgrowth. It is unclear in which region the 7.6 kb mRNA species differs from the 5.7 kb transcript.

The expression pattern of the different mRNA species for neuroglian reflects the appearance of the corresponding protein products (Figure 9). Very little neuroglian protein is detected before 8 hr of embryonic development. The shorter form of the neuroglian protein becomes strongly expressed during the 8–12 hr window; the neuron-specific long form of the protein increases in abundance just slightly after the short form. Thus, the expression of neuroglian protein on the cell surface seems to be primarily regulated at the transcriptional level using tissue-specific alternative splicing to determine when and where each form of the protein is generated.

Discussion

We previously described the characterization and cloning of Drosophila neuroglian, a member of the immunoglobulin superfamily that appears to be the arthropod homolog of the vertebrate neural cell adhesion protein L1 (Bieber et al., 1989). In this paper, we report on the expression and sequence of two different forms of the neuroglian protein. Two monoclonal antibodies, 1B7 and BP-104, were used as specific probes to study the temporal and spatial expression of the two different forms of the neuroglian protein. MAb 1B7 recognizes both the long and short forms of the protein, whereas MAb BP-104 recognizes the unique cytoplasmic extension of the long form of the protein. The longer, less abundant form has an extra 62 amino acids at the C-terminus of its cytoplasmic domain as compared with the small, major form of the protein; the amino acid sequences of the extracellular and transmembrane domains of both forms are identical.

Both forms of neuroglian are encoded by a single gene and generated by tissue-specific, alternative splicing. During embryogenesis and larval development, neuroglian is expressed in a wide variety of tissues, including many of the neurons and glia of the CNS and PNS, the trachea, the hindgut, muscle, ectodermal tissues, and the imaginal discs. The expression of the long form is entirely restricted to the surface of neurons in the CNS and neurons and a few support cells in the PNS and is completely absent from nonneuronal tissues during embryonic and larval stages of development. Within the developing CNS, the differences in the expression of the short and long forms is striking: several different classes of glial cells, which prefigure the longitudinal tracts and peripheral nerve roots (Jacobs and Goodman, 1989), specifically express the short form of the protein but not the neuron-spe-


A
B
S
CNS
T
H
B
C
G
G
D
ISN
SN

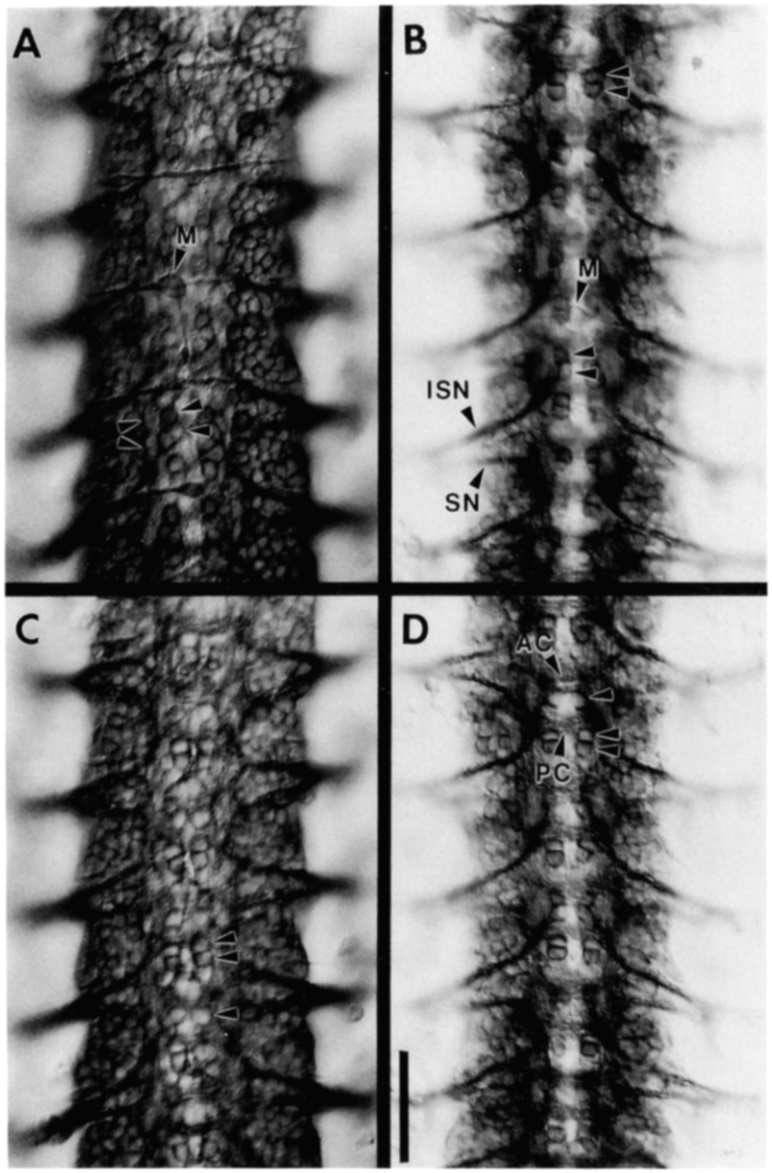


Figure 5. The Long Form of Neuroglian Shows a More Restricted Pattern of CNS Expression Than the Short Form

Staining of the ventral nerve cord with MAb 1B7 (A and C), which recognizes both forms of neuroglian, or MAb BP-104 (B and D), which recognizes only the long form of the protein. (A) and (B) show neuroglian-positive cells in a most dorsal plane of focus in the CNS. Neurons RP2 and RP1 (right pair of arrowheads in [A]) are stained with both antibodies, whereas the longitudinal glia (left pair of arrowheads in [A]) as well as the muscle pioneers (M) stain with MAb 1B7 but not with the long form-specific MAb BP-104. (C) and (D) present a deeper plane of focus. Many axon tracts in the anterior (AC) and the posterior (PC) commissures and in the segmental (SN) and intersegmental (ISN) nerves, as well as certain identified neurons such as aCC, pCC (pairs of arrowheads in [C] and [D]), and RP3 (single arrowhead in [C] and [D]), are stained by both MAbs. Arrowheads: (A) Left pair, longitudinal glia; right pair, RP2 and RP1. (B) Top right pair, aCC and pCC; bottom right pair, RP2 and RP1. (C) Top right pair, aCC and pCC; bottom right single, RP3. (D) Top right single, RP3; bottom right pair, aCC and pCC. Bar, 30 μm.

cific long form. Lacking an antibody specific for the short form of neuroglian, we cannot exclude at this time the possibility that some neuronal cells coexpress both forms of the neuroglian protein on their surfaces.

The expression of the long, but not the short, form of neuroglian thus appears in line with the expression of its homolog, the neural cell adhesion molecule L1, in mouse and other vertebrates. In the developing vertebrate CNS, L1 is expressed on specific regions and

Figure 4. Expression of the Long Form of Neuroglian Protein Is Restricted to the Surface of Neurons

(A) and (B) show 12 hr Drosophila embryos dissected onto glass slides and stained with MAb 1B7 (A) or MAb BP-104 (B). MAb BP-104 specifically recognizes the long form of the neuroglian protein, whereas MAb 1B7 recognizes an epitope common to both forms. Neuroglian protein is detected by MAb 1B7 in the brain (B), the central and peripheral nervous systems (CNS), salivary gland (S), trachea (T), and the hindgut (H). In contrast, MAb BP-104 (B) stains only the CNS and PNS and none of these other tissues. (C) and (D) depict a close-up of the peripheral nerve roots as stained with either MAb 1B7 (C) or MAb BP-104 (D). Both antibodies stain the axons of the segmental (SN) and the intersegmental (ISN) nerve roots. Note: The arrows mark where these two nerves come together just outside the CNS, at a distinctive set of glia. At this location, their axons mix and diverge into several different pathways. The glial cells (G), which are associated with the peripheral nerve roots, are recognized only by MAb 1B7. The single arrowheads mark the peripheral edge of the ventral nerve cord. Bar, 50 μm (A); 38 μm (B); 10 μm (C and D).

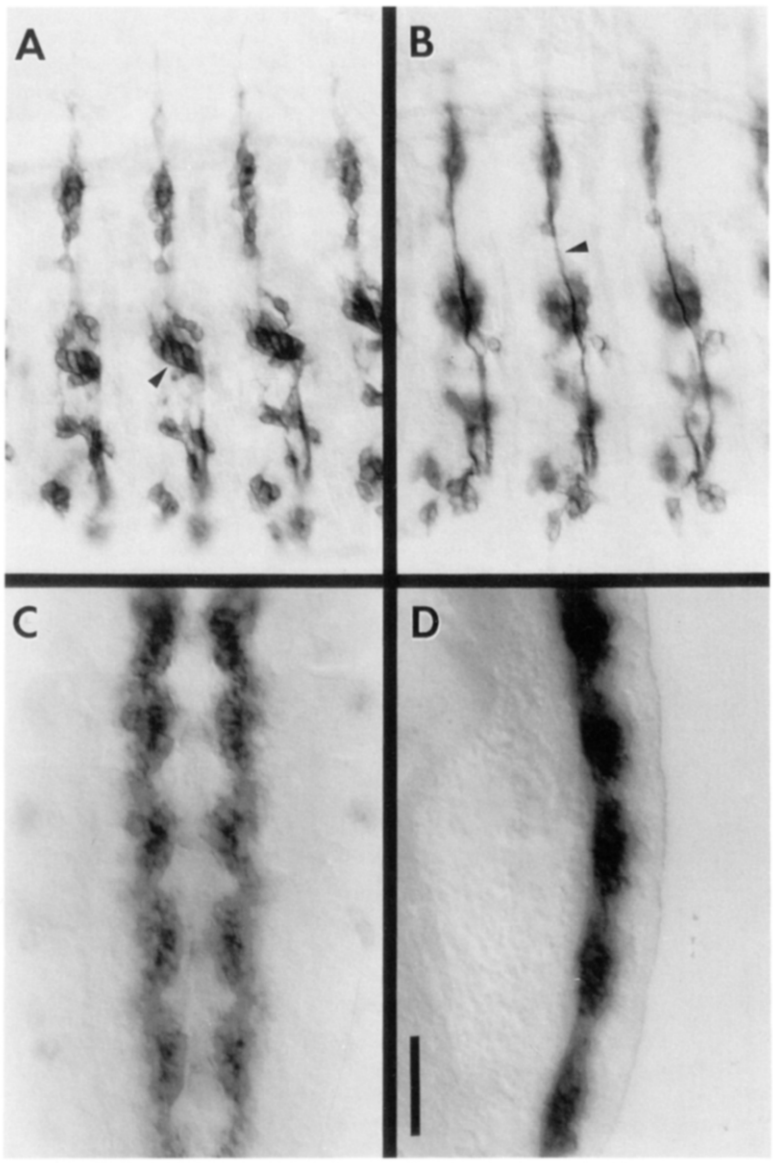


Figure 6. Early Embryonic CNS and PNS Expression of the Neuron-Specific Long Form of Neuroglian Protein

MAb BP-104 strongly stains all neurons (arrowhead in [A]) and axon tracts (arrowhead in [B]) in the PNS of the Drosophila embryo. It also stains a few PNS support cells, including the innermost sheath cells (data not shown). Staining for the long form of the protein is first detectable at around 6 hr of development in a small repeated cluster of cells along the midline of the CNS; the embryo shown here is 7 hr old and shows the continuous stripe of longitudinal staining ([C]), ventral view; [D], lateral view of the same embryo). See text for details. Bar, 30 μm.

types of neurons at times of cell migration and neurite outgrowth and appears at points of neuron–neuron contact, but not at contact sites of neurons and glia (Grumet and Edelman, 1984; Persohn and Schachner, 1987). Astrocytes and oligodendrocytes in the CNS do not express L1 (Rathjen and Schachner, 1987), although some experimental evidence has led to the hypothesis that L1 may be involved in a heterotypic interaction in the vertebrate CNS between neurons, which express the protein, and glial cells, which do not (Grumet et al., 1984; Grumet and Edelman, 1988). In the PNS of mice and rats, however, most Schwann cells express L1 at some point in development (Salton et al., 1983a; Faissner et al., 1984; Seilheimer et al., 1989), and L1 appears to be involved in mediating neuronal growth on Schwann cells in culture (Stallcup and Beasly, 1985; Bixby et al., 1988; Seilheimer and Schachner, 1988). Antibody perturbation experiments indicate that the L1 protein is also involved in neurite outgrowth, and axon fasciculation and appears to function in a homophilic manner (Fischer et al., 1986; Lagenaur and Lemmon, 1987; Landmesser et al., 1988).

The strong expression of the long form of neuroglian on axon tracts in both the CNS and the PNS of Drosophila embryos and larvae suggests that neuroglian may serve a function in axon fasciculation in arthropods similar to that of its homolog, L1, in chordates. Although the expression of the neuron-specific long form of Drosophila neuroglian shows many parallels with the appearance of L1 in vertebrates, the overall pattern of neuroglian versus L1 expression shows some significant differences. Evidently, sometime in the evolution of the arthropods, but not the chordates, the ancestral L1/neuroglian gene evolved a

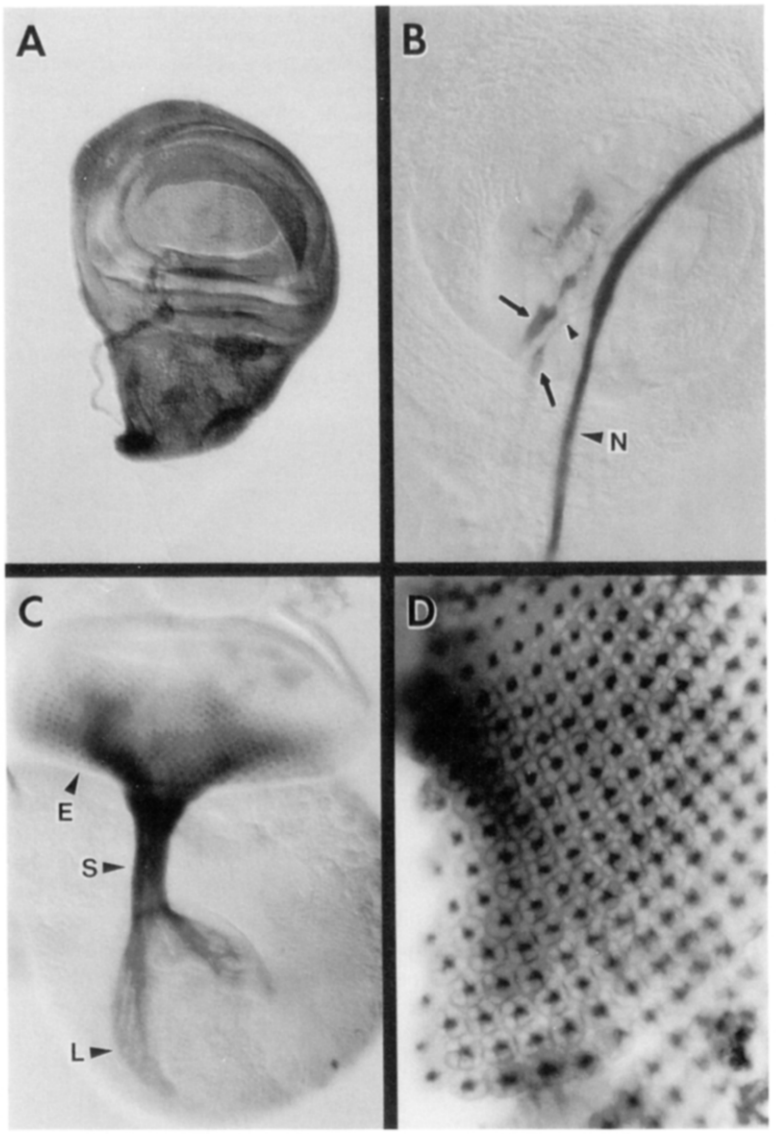


Figure 7. Epidermal Cells of the Imaginal Discs Express the Short Form of Neuroglian, whereas the Neurons and Axon Tracts in the Discs Express the Long Form

(A) and (B) show a comparison of MAb 1B7 (A; wing disc) versus MAb BP-104 (B; leg disc) staining of imaginal discs. MAb BP-104 recognizes only the long form of neuroglian. Whereas MAb 1B7 stains the disc tissue uniformly, MAb BP-104 stains only the embryonic nerves (N) and the newly formed neurons (arrows) and axons (arrowhead) within the discs at this stage. In (C) and (D), MAb BP-104 staining in the eye disc (E) and the optic stalk (S) is shown. As seen at the lower magnification (C), the axon tracts of the optic stalk (S) connecting the photoreceptor cells with the lamina (L) of the optic lobe express high levels of the long form of the protein. At higher magnification (D), the staining of the photoreceptor cells with MAb BP-104 can be seen. MAb 1B7 stains most cells throughout the eye disc (not shown).

second protein form and a second pattern of expression (and presumably a second role as a more general cell adhesion molecule) in a variety of nonneuronal tissues. Given that the tissue specificity of certain cell adhesion molecules, such as P-cadherin, differs significantly even between phylogenetically closely related species (Shimoyama et al., 1989), it is perhaps not too surprising to see differences in the tissue specificity of Drosophila neuroglian versus vertebrate L1. Thus, whereas L1 in vertebrates appears to come in only one protein form and to function primarily as a neural cell adhesion molecule, neuroglian in Drosophila appears to have two forms, two different patterns of expression, and presumably two different developmental roles: one as a neural cell adhesion molecule and the other as a more general cell adhesion molecule involved in the events of tissue and imaginal disc morphogenesis.

Nothing is known about the functional significance of the two different forms of the neuroglian protein with different cytoplasmic domains. Since both forms have identical extracellular domains, a difference in binding specificity between the two seems unlikely. However, as was shown for E-cadherin, the structure of the cytoplasmic domain can have a significant influence on the binding affinity to its extracellular domain (Nagafuchi and Takeichi, 1988). Nevertheless, a differential involvement of the two neuroglian polypeptides in some cytoplasmic event appears more likely. One alternative is that the two forms interact differently with cytoskeletal components. Supporting this possibility are the findings on the 180 kd (long) form of N-CAM. It has a unique, 261 amino acid cytoplasmic insert (Cunningham et al., 1987) and interacts specifically with cellular spectrin molecules (Pollerberg et al., 1987). This interaction results in a lower

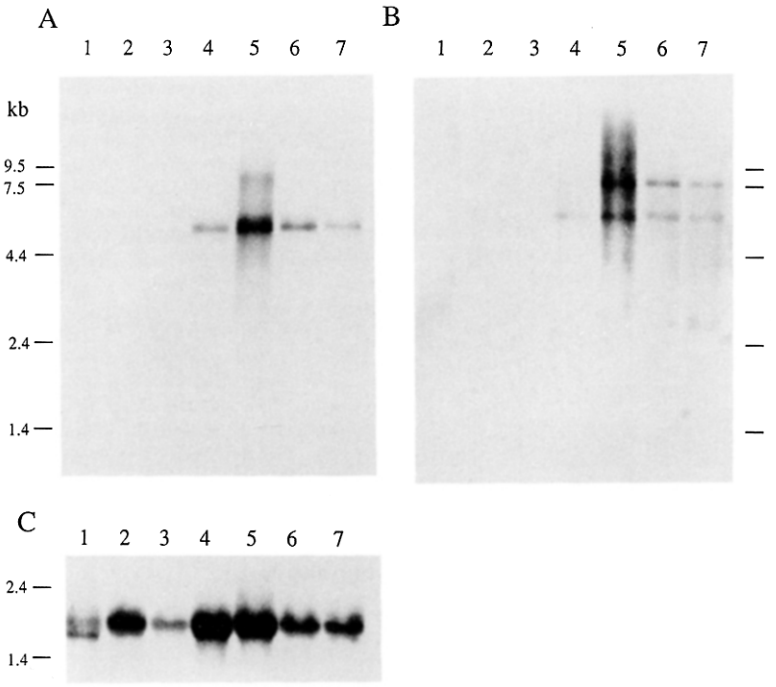


Figure 8. Developmental Northern Blots for the Two Neuroglian mRNAs

Total RNA was prepared from staged Drosophila embryos. Aliquots (30 μg) were separated on a 1% agarose gel (lanes 1, 0–3 hr; lanes 2, 3–6 hr; lanes 3, 6–9 hr; lanes 4, 9–12 hr; lanes 5, 12–15 hr; lanes 6, 15–18 hr; lanes 7, 18–21 hr) and transferred to nylon filters. These filters were hybridized to T7 RNA polymerase transcribed, ^{32}P-labeled single-stranded RNA probes specific for the short (A) or the long (B) form of neuroglian. (C) The Northern blot shown in (A) was subsequently probed with a ^{32}P-labeled cDNA fragment for Drosophila α tubulin 4 (Natzle and McCarthy, 1984).

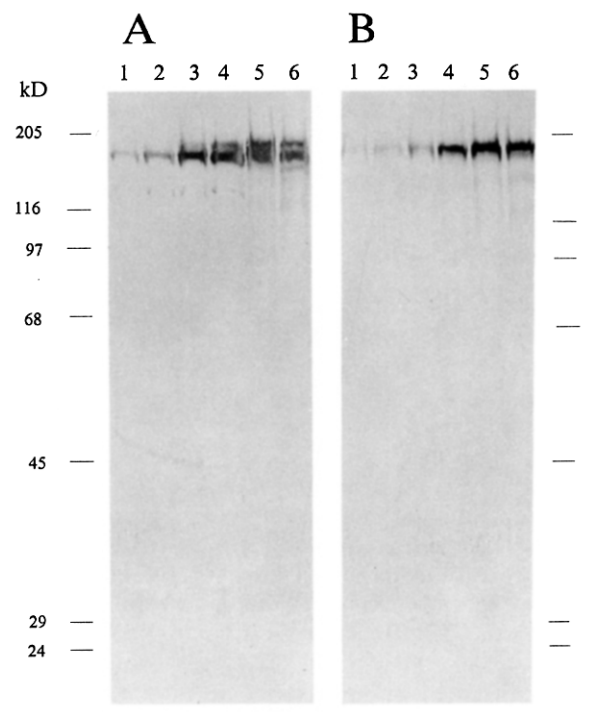


Figure 9. Embryonic Developmental Western Blots Stained with Anti-Neuroglian Antibodies

Membranes from staged Drosophila embryos were prepared, and 20 μg aliquots were separated on a 7.5% SDS-polyacrylamide gel before being electroblotted to nitrocellulose filters (lanes 1, 0–4 hr; lanes 2, 4–8 hr; lanes 3, 8–12 hr; lanes 4, 12–16 hr; lanes 5, 16–20 hr; lanes 6, 20–24 hr). Blot A was incubated with a 1/1000 dilution of rat anti-neuroglian serum followed by a horseradish peroxidase–conjugated secondary antibody and developed with diaminobenzidine. Blot B was probed with a 1/1000 dilution of BP-104 monoclonal mouse ascites fluid.

diffusion coefficient of the 180 kd N-CAM form in the plane of the membrane as compared with the 120 kd and 140 kd forms (Pollerberg et al., 1986). The unusual amino acid composition of the long form–specific cytoplasmic domain of neuroglian could be indicative of an interaction with some cytoskeletal protein such as spectrin. Although the 180 kd–specific domain of N-CAM shares no primary sequence homology with the long form–specific domain of neuroglian, both have an unusual high content of the same 4 amino acids: alanine, glycine, proline, and serine. Whether this unusual amino acid content is involved in spectrin binding is unknown.

Both N-CAM and L1 are phosphorylated at one or several serine and threonine residues (Sorkin et al., 1984; Mackie et al., 1989; Salton et al., 1983b; Faissner et al., 1985). Although the 8 additional serine residues in the long neuroglian form are not present in the context of a known phosphorylation signal, they could be targets of a so far uncharacterized, serine-specific protein kinase. It was recently postulated by Schuch et al. (1989) that cell adhesion molecules can influence cellular secondary messenger systems. The different cytoplasmic domains of neuroglian described here could have very different functions and activities in such a process.

Different isoforms with different C-terminal cytoplasmic domains have been observed among several vertebrates cell adhesion molecules (Cunningham et al., 1987; Owens et al., 1987; Lai et al., 1987). Thus far, however, biochemical, immunological, and cDNA analyses have not revealed a second form of the L1 protein in vertebrates (Salton et al., 1983b; Rathjen et al., 1987; Moos et al., 1988; Prince et al., 1989). In mouse, rat,

and chicken, the mature L1 protein appears as a single polypeptide with an apparent molecular mass of approximately 200 kd. The existence of two different polypeptide forms of neuroglian therefore appears unique to Drosophila. The cDNA sequences downstream from the point where the two transcripts diverge are coded for by two different exons (M. Hortsch et al., unpublished data). The exon encoding the short form-specific cytoplasmic domain resides 5′ to the exon encoding the long form-specific domain. Since the first 5′ 38 nucleotides of both exons are highly conserved, we interpret this to represent a fairly recent exon duplication event that now results in the generation of two different neuroglian gene products in flies. The identification of neuroglian homologs in other more primitive insects such as grasshopper might help to elucidate how widespread the multiple neuroglian/L1 protein forms are and when in the phylogeny of the arthropods the exon duplication occurred.

Experimental Procedures

Generation and Screening of Monoclonal Antibodies and Antisera

The generation of MAb 1B7 and of rat anti-neuroglian serum antibodies has been previously described by Bieber et al. (1989). MAb BP-104 was generated using membrane proteins from cultured embryonic Drosophila neurons. The procedures for neuron culture and membrane isolation have been previously described by Patel et al. (1987). BALB/c mice were immunized with intraperitoneal injections containing approximately 1 mg of protein. The primary injection consisted of membrane protein suspended in a 1:1 mixture of PBS (2 mM $NaH_2PO_4 \cdot H_2O$, 8 mM Na_2HPO_4, 170 mM NaCl [pH 7.4]) and Freund's complete adjuvant. Mice were given three boosting injections at 3 week intervals. Boosting injections were identical to the primary injection except that incomplete Freund's adjuvant was used. Three days prior to fusion, a final injection of protein in PBS alone was administered. Mouse spleen cells were fused to NS-1 myeloma cells (Oi and Herzenberg, 1980), and hybridoma supernatants were screened by immunofluorescence on 10–15 hr whole-mount Drosophila embryos as previously described (Patel et al., 1987).

Immunocytochemistry

Staged embryos were dissected onto glass slides and stained with MAbs as described by Bieber et al. (1989). Imaginal discs were dissected from late third instar larvae or collected in a mass disc isolation. Staining of whole mounts and imaginal discs was performed using horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) as outlined by Patel et al. (1989). For some preparations, staining was enhanced by the addition of $NiCl_2$ to a concentration of 0.05% to the diaminobenzidine staining solution. A Zeiss Axiophot microscope with Nomarski optics was used to view and photograph mounted embryos, dissected embryonic nervous systems, and imaginal discs.

SDS-PAGE and Western Blot Analysis

Total membrane proteins from 10–14 hr Drosophila embryos were prepared as previously described (Patel et al., 1987). Proteins were separated on 7.5% SDS-polyacrylamide gels, and immunoblots were performed according to Burnette (1981), with the modifications described by Hortsch et al. (1985).

Deglycosylation of Proteins

Total membrane proteins from 10–14 hr embryos were deglycosylated with TFMS as described by Edge et al. (1981). Deglycosylation with endoglycosidase H was carried out according to the manufacturers specifications (Boehringer Mannheim Biochemica). Deglycosylated protein was analyzed by immunoblotting as described above.

cDNA Expression Cloning

A 1/1000 dilution of MAb BP-104 mouse ascites fluid was used to screen the Kai Zinn 9–12 hr Drosophila embryo λgt11 cDNA expression library (Zinn et al., 1988). Nitrocellulose filters were processed as described for Western blots using a horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody and diaminobenzidine for the detection of positive phage.

DNA Sequencing

DNA sequencing was performed according to the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemical Corp.), [^{35}S]dATP, and 60 cm buffer gradient gels (Biggin et al., 1983). Subcloning into the Bluescript KS/+ vector (Stratagene) and M13mp10 has been described previously (Bieber et al., 1989).

RNA Protection Experiments

RNA protection experiments were carried out using a modification of the method described by Zinn et al. (1983). ScaI–EcoRI fragments isolated from the 3′ ends of both neuroglian cDNA probes were subcloned into SmaI- and EcoRI-cut, phosphatized Bluescript KS/+ and SK/+ vectors and linearized plasmid DNA was transcribed in the presence of [α-^{32}P]UTP by T7 RNA polymerase. Probe (10^6 cpm) was hybridized to 30 μg of total Drosophila 12–24 hr embryonic RNA overnight at 45°C. The mixture of RNAase A and RNAase T1 used by Zinn et al. (1983) resulted in the degradation of some of the RNA–RNA hybrid fragments. Presumably this was due to base slippage in the AT-rich 5′ parts of the probes. RNAase treatment was therefore performed for 30 min at 37°C using 10 μg/ml RNAase T1, resulting in a slightly higher background. Protected RNA fragments were separated on 8% urea–polyacrylamide gels and visualized by autoradiography at −80°C.

Northern Blot Analysis

Total RNA was prepared from staged Drosophila embryos, separated by formaldehyde-containing 1% agarose gels (Lehrach et al., 1977), and blotted onto GeneScreen Plus membranes. Hybridization was carried out according to the method of Church and Gilbert (1984), using [α-^{32}P]UTP-labeled RNA probes transcribed by T7 polymerase or DNA probes radiolabeled by the extension of sequences randomly primed with oligonucleotides as described by Feinberg and Vogelstein (1983).

Acknowledgments

We thank Z. Traquina for excellent technical assistance, P. Snow for sharing his initial observations of multiple forms of the neuroglian protein, B. Altenberg (EMBL, Heidelberg) for her help with the sequence analysis and the secondary protein structure prediction programs, U. Gaul for the eye disc shown in Figure 7D, and the Fristrom lab for the mass isolated discs. Special thanks to A. Admon (HHMI, UC Berkeley) for his advice and help in determining the amino-terminal amino acid sequence of the long neuroglian form and to R. Bodmer (UCSF) for help with the identification of PNS cells. This work was supported by NATO and DFG postdoctoral fellowships to M. H., National Institutes of Health and HHMI postdoctoral fellowships to A. J. B., and NIH grant HD21294 to C. S. G.

Received December 26, 1989; revised February 2, 1990.

References

Arquint, M., Roder, J., Chia, L.-S., Down, J., Wilkinson, D., Bayley, H., Braun, P., and Dunn, R. (1987). Molecular cloning and primary structure of myelin-associated glycoprotein. Proc. Natl. Acad. Sci. USA *84*, 600–604.

Barbas, J. A., Chaix, J.-C., Steinmetz, M., and Goridis, C. (1988).

Differential splicing and alternative polyadenylation generates distinct NCAM transcripts and proteins in the mouse. EMBO J. *7*, 625–632.

Barthels, D., Santoni, M. J., Wille, W., Ruppert, C., Chaix, J.-C., Hirsch, M. R., Fontecilla, J. C., and Goridis, C. (1987). Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a M_r 79000 polypeptide without a membrane-spanning region. EMBO J. *6*, 907–914.

Bieber, A. J., Snow, P. M., Hortsch, M., Patel, N. H., Jacobs, J. R., Traquina, Z. R., Schilling, J., and Goodman, C. S. (1989). Drosophila neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. Cell *59*, 447–460.

Biggin, M. D., Gibson, T. J., and Hong, G. F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA *80*, 3963–3965.

Bixby, J. L., Lilien, J., and Reichardt, L. F. (1988). Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro. J. Cell Biol. *107*, 353–361.

Brümmendorf, T., Wolff, J. M., Frank, R., and Rathjen, F. G. (1989). Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. Neuron *2*, 1351–1361.

Burnette, W. N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. *112*, 195–203.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R., and Edelman, G. M. (1987). Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation and alternative splicing. Science *236*, 799–806.

Daniloff, J. K., Chuong, C.-M., Levi, G., and Edelman, G. M. (1986). Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system. J. Neurosci. *6*, 739–758.

Dickson, G., Gower, H. J., Barton, C. H., Prentice, H. M., Elsom, V. L., Moore, S. E., Cox, R. D., Quinn, C., Putt, W., and Walsh, F. S. (1987). Human muscle neural cell adhesion molecule (N-CAM): identification of a muscle-specific sequence in the extracellular domain. Cell *50*, 1119–1130.

Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., Jr., and Weber, P. (1981). Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal. Biochem. *118*, 131–137.

Faissner, A., Kruse, J., Nicke, J., and Schachner, M. (1984). Expression of neural cell adhesion molecule L1 during development, in neurological mutants and in the peripheral nervous system. Dev. Brain Res. *15*, 69–82.

Faissner, A., Teplow, B. D., Kübler, D., Keilhauer, G., Kinzel, V., and Schachner, M. (1985). Biosynthesis and membrane topology of the neural cell adhesion molecule L1. EMBO J. *4*, 3105–3113.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6–13.

Fischer, G., Künemund, V., and Schachner, M. (1986). Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell surface glycoprotein L1. J. Neurosci. *6*, 605–612.

Frail, D. E., and Braun, P. E. (1984). Two developmentally regulated messenger RNAs differing in their coding region may exist for the myelin-associated glycoprotein. J. Biol. Chem. *259*, 14857–14862.

Fushiki, S., and Schachner, M. (1986). Immunological localization of cell adhesion molecules L1 and NCAM and the shared carbohydrate epitope L2 during development of the mouse neocortex. Dev. Brain Res. *24*, 153–167.

Grumet, M., and Edelman, G. M. (1984). Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. J. Cell Biol. *98*, 1746–1756.

Grumet, M., and Edelman, G. M. (1988). Neuron–glia cell adhesion molecule interacts with neurons and astroglia via different binding mechanisms. J. Cell Biol. *106*, 487–503.

Grumet, M., Hoffman, S., Chuong, C.-M., and Edelman, G. M. (1984). Polypeptide components and binding functions of neuron–glia cell adhesion molecule. Proc. Natl. Acad. Sci. USA *81*, 7989–7993.

Harrelson, A. L., and Goodman, C. S. (1988). Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. Science *242*, 700–708.

Hortsch, M., Avossa, D., and Meyer, D. I. (1985). A structural and functional analysis of the docking protein: characterization of active domains by proteolysis and specific antibodies. J. Biol. Chem. *260*, 9137–9145.

Jacobs, J. R., and Goodman, C. S. (1989). Embryonic development of axon pathways in the *Drosophila* CNS. I. A glial scaffold appears before the first growth cones. J. Neurosci. *9*, 2402–2411.

Jacobs, J. R., Hiromi, Y., Patel, N. H., and Goodman, C. S. (1989). Lineage, migration, and morphogenesis of longitudinal glia in the Drosophila CNS as revealed by a molecular lineage marker. Neuron *2*, 1625–1631.

Lagenaur, C., and Lemmon, V. (1987). An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci. USA *84*, 7753–7757.

Lai, C., Brow, M. A., Nave, K.-A., Noronha, A. B., Quarles, R. H., Bloom, F. E., Milner, R. J., and Sutcliffe, J. G. (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.

Landmesser, L., Dahm, L., Schultz, K., and Rutishauser, U. (1988). Distinct roles for adhesion molecules during innervation of embryonic chick muscle. Dev. Biol. *130*, 645–670.

Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977). RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry *16*, 4743–4751.

Mackie, K., Sorkin, B. C., Nairn, A. C., Greengard, P., Edelman, G. M., and Cunningham, B. A. (1989). Identification of two protein kinases that phosphorylate the neural cell-adhesion molecule, NCAM. J. Neurosci. *9*, 1883–1896.

Moos, M., Tacke, R., Scherer, H., Teplow, D., Früh, K., and Schachner, M. (1988). Neural adhesion molecule L1 is a member of the immunoglobulin superfamily with binding domains similar to fibronectin. Nature *334*, 701–703.

Murray, B. A., Hemperly, J. J., Prediger, E. A., Edelman, G. M., and Cunningham, B. A. (1986). Alternatively spliced mRNAs code for different polypeptide chains of the chicken neural cell adhesion molecule (NCAM). J. Cell Biol. *102*, 189–193.

Nagafuchi, A., and Takeichi, M. (1988). Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J. *7*, 3679–3684.

Natzle, J. E., and McCarthy, B. J. (1984). Regulation of Drosophila α- and β-tubulin genes during development. Dev. Biol. *104*, 187–198.

Oi, V. T., and Herzenberg, L. A. (1980). Immunoglobulin producing hybrid cell lines. In Selected Methods in Cellular Immunology, B. B. Mishell and S. M. Shiigi, eds. (New York: Freeman), pp. 351–357.

Owens, G. C., Edelman, G. M., and Cunningham, B. A. (1987). Organization of the neural cell adhesion molecule (NCAM) gene: alternative exon usage as the basis for different membrane-associated domains. Proc. Natl. Acad. Sci. USA *84*, 294–298.

Patel, N. H., Snow, P. M., and Goodman, C. S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. Cell *48*, 975–988.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., and Goodman, C. S. (1989). Expression of *engrailed* proteins in arthropods, annelids, and chordates. Cell *58*, 955–968.

Persohn, E., and Schachner, M. (1987). Immunoelectron microscopic localization of the neural cell adhesion molecules L1 and NCAM during postnatal development of the mouse cerebellum. J. Cell Biol. *105*, 569–576.

Pollerberg, E., Schachner, M., and Davoust, J. (1986). Differentiation-state dependent surface mobilities of two forms of the neural cell adhesion molecule. Nature *324*, 462–465.

Pollerberg, E. G., Burridge, K., Krebs, K. E., Goodman, S. R., and Schachner, M. (1987). The 180-kd component of the neural cell adhesion molecule NCAM is involved in cell–cell contacts and cytoskeleton–membrane interactions. Cell Tissue Res. *250*, 227–236.

Prince, J. T., Milona, N., and Stallcup, W. B. (1989). Characterization of a partial cDNA clone for the NILE glycoprotein and identification of the encoded polypeptide domain. J. Neurosci. *9*, 876–883.

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.

Rathjen, F. G., and Schachner, M. (1984). Immunological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J. *3*, 1–10.

Rathjen, F. G., Wolff, J. M., Frank, R., Bonhoeffer, F., and Rutishauser, U. (1987). Membrane glycoproteins involved in neurite fasciculation. J. Cell Biol. *104*, 343–353.

Salton, S. R. J., Richter-Landsberg, C., Greene, L. A., and Shelanski, M. L. (1983a). Nerve growth factor-inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. J. Neurosci. *3*, 441–454.

Salton, S. R. J., Shelanski, M. L., and Greene, L. A. (1983b). Biochemical properties of the nerve growth factor-inducible large external (NILE) glycoprotein. J. Neurosci. *3*, 2420–2430.

Salzer, J. L., Holmes, W. P., and Colman, D. R. (1987). The amino acid sequences of the myelin-associated glycoproteins: homology to the immunoglobulin gene superfamily. J. Cell Biol. *104*, 957–965.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Santoni, M. J., Barthels, D., Vopper, G., Boned, A., Goridis, C., and Wille, W. (1989). Differential exon usage involving an unusual splicing mechanism generates at least eight types of NCAM cDNA in mouse brain. EMBO J. *8*, 385–392.

Schuch, U., Lohse, M. J., and Schachner, M. (1989). Neural cell adhesion molecules influence second messenger systems. Neuron *3*, 13–20.

Seilheimer, B., and Schachner, M. (1988). Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neuron growth on Schwann cells in culture. J. Cell Biol. *107*, 341–351.

Seilheimer, B., Persohn, E., and Schachner, M. (1989). Neural cell adhesion molecule expression is regulated by Schwann cell–neuron interaction in culture. J. Cell Biol. *108*, 1909–1915.

Shimoyama, Y., Yoshida, T., Terada, M., Shimosato, Y., Abe, O., and Hirohashi, S. (1989). Molecular cloning of a human Ca^{2+}-dependent cell–cell adhesion molecule homologous to mouse placental cadherin: its low expression in human placental tissues. J. Cell Biol. *109*, 1787–1794.

Small, S. J., Haines, S. L., and Akeson, R. A. (1988). Polypeptide variation in an N-CAM extracellular immunoglobulin-like fold is developmentally regulated through alternative splicing. Neuron *1*, 1007–1017.

Sorkin, B. C., Hoffman, S., Edelman, G. M., and Cunningham, B. A. (1984). Sulfation and phosphorylation of the neural cell adhesion molecule, NCAM. Science *225*, 1476–1478.

Stallcup, W. B., and Beasly, L. L. (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.

Thompson, J., Dickson, G., Moore, S. E., Gower, H. J., Putt, W., Kenimer, J. G., Barton, C. H., and Walsh, F. S. (1989). Alternative splicing of the neural cell adhesion molecule gene generates variant extracellular domain structure in skeletal muscle and brain. Genes Dev. *3*, 348–357.

Thor, G., Probstmeier, R., and Schachner, M. (1987). Characterization of the cell adhesion molecules L1, NCAM and J1 in the mouse intestine. EMBO J. *6*, 2581–2586.

Zinn, K., DiMaio, D., and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell *34*, 865–879.

Zinn, K., McAllister, L., and Goodman, C. S. (1988). Sequence analysis and neuronal expression of fasciclin I in grasshopper and Drosophila. Cell *53*, 577–587.