N-cadherin, NCAM, and Integrins Promote Retinal Neurite Outgrowth on Astrocytes In Vitro

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Abstract. Retinal ganglion neurons extend axons that grow along astroglial cell surfaces in the developing optic pathway. To identify the molecules that may mediate axon extension in vivo, antibodies to neuronal cell surface proteins were tested for their effects on neurite outgrowth by embryonic chick retinal neurons cultured on astrocyte monolayers. Neurite outgrowth by retinal neurons from embryonic day 7 (E7) and E11 chick embryos depended on the function of a calcium-dependent cell adhesion molecule (N-cadherin) and β_1-class integrin extracellular matrix receptors. The in-

hibitory effects of either antibody on process extension could not be accounted for by a reduction in the attachment of neurons to astrocytes. The role of a third cell adhesion molecule, NCAM, changed during development. Anti-NCAM had no detectable inhibitory effects on neurite outgrowth by E7 retinal neurons. In contrast, E11 retinal neurite outgrowth was strongly dependent on NCAM function. Thus, N-cadherin, integrins, and NCAM are likely to regulate axon extension in the optic pathway, and their relative importance varies with developmental age.

IN the developing primary visual system of vertebrates, the endfeet of neuroepithelial astroglial precursors are prominent components of the routes taken by retinal ganglion cell axons within the eye, optic nerve, and optic tectum (Rager, 1980; Easter et al., 1984; Silver and Rutis-hauser, 1984; Lemmon, 1985, 1986; Bork et al., 1987). In the retina, growth cones contact the endfeet of radially oriented glia as well as the inner limiting basement membrane as they grow toward the optic stalk (Rager, 1980; Easter et al., 1984). In the optic nerve, small fascicles of axons are separated by glial processes, and their growth cones frequently contact glial surfaces (Rager, 1980; Silver and Rutishauser, 1984; Bovolenta and Mason, 1987). Although the role of glial surfaces in the ordered growth of optic fibers into the tectum is unknown, the optic nerve fiber layer of the embryonic tectum is penetrated by numerous radial glial endfeet (Lemmon, 1985, 1986; Bork et al., 1987). Thus, observations of optic pathway development in vivo suggest that astroglial precursors stimulate and guide the elongation of retinal ganglion cell axons.

Studies of central nervous system development and regeneration have demonstrated that astrocytes provide an effective cellular substrate for axonal elongation (Bohn et al., 1982; Smith et al., 1986). Astrocytes isolated from embryonic or early postnatal brain are good substrates for rapid neurite extension by both central and peripheral neurons in vitro (Noble et al., 1984; Fallon, 1985; Tomaselli et al., 1986). In particular, embryonic chick retinal neurons extend profuse, unfasciculated neurites on astrocyte surfaces while they do not respond as well to fibroblast monolayers (McCaffery et

al., 1984; Fallon, 1985). Therefore, astrocytes are distinct from other cell types in that they express highly active neurite outgrowth-promoting factors. Retinal neurons also attach and extend neurites on substrates coated with the purified extracellular matrix (ECM)[1] proteins, laminin, fibronectin, and collagen types I and IV (Akers et al., 1981; Adler et al., 1985; Cohen et al., 1986; Hall et all., 1987; Neugebauer, K., unpublished observations). Laminin, which is the most effective of these ECM proteins in promoting neurite outgrowth by retinal neurons (Hall et al., 1987), is expressed on the surfaces of cultured astrocytes (Liesi et al., 1983). Recent evidence indicates, however, that laminin is not the only neurite outgrowth-promoting factor expressed by astrocytes. First, retinal neurons isolated from embryonic day-11 (E11) chick embryos no longer respond to purified laminin, yet they continue to extend neurites rapidly on astrocyte surfaces (Cohen et al., 1986; Hall et al., 1987). Second, neurite outgrowth by peripheral neurons on astrocytes is only mildly inhibited by a monoclonal antibody (CSAT) that completely inhibits neurite outgrowth on these ECM-derived substrates (Tomaselli et al., 1988). This antibody recognizes the integrin β_1 subunit shared by a family of ECM receptor heterodimers (see Hynes, 1987, for nomenclature) and blocks neuronal attachment and process outgrowth on laminin, fibronectin, collagens, and native ECMs (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986, 1987; Cohen et al.,

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1. *Abbreviations used in this paper:* E, embryonic day; ECM, extracellular matrix.

1987; Hall et al., 1987). Thus, cultured astrocytes express neurite-promoting factors that are distinct from these ECM proteins.

Recently, attention has been focused on the role of cell adhesion molecules (CAMs) as cell surface–associated neurite-promoting factors. N-cadherin is a 130-kD cell surface glycoprotein that mediates calcium-dependent adhesion between neural cells (Grunwald et al., 1982; Hatta et al., 1985) and is expressed by both central and peripheral neurons (Hatta et al., 1986, 1987; Crittenden et al., 1988). Neurite outgrowth by peripheral neurons grown on astrocytes depends strongly on the function of N-cadherin (Tomaselli et al., 1988). N-cadherin also functions in peripheral neurite outgrowth on skeletal myotubes and Schwann cells (Bixby et al., 1987, 1988). The calcium-independent CAM, NCAM, is also widely distributed in the nervous system (cf. Daniloff et al., 1986). Although it promotes neuronal adhesion and process outgrowth on myotubes in vitro (Rutishauser et al., 1983; Bixby and Reichardt, 1987; Bixby et al., 1987), NCAM does not appear to be involved in peripheral neurite outgrowth on astrocytes or Schwann cells (Tomaselli et al., 1988; Bixby et al., 1988) or on the surfaces of other peripheral axons (Chang et al., 1987). However, as NCAM is expressed on astrocytes in culture (Noble et al., 1985; Keilhauer et al., 1985), it remains a candidate for mediating interactions that lead to neurite outgrowth by some types of neurons on astrocytes.

As a first step towards defining the molecular mechanisms underlying axon extension in the retinotectal pathway, we have examined embryonic retinal neurite outgrowth on astrocytes in vitro, using antibodies that inhibit the function of N-cadherin, NCAM, and the integrin β_1-class ECM receptor heterodimers. Our results implicate all three of these neuronal receptors in neurite outgrowth by retinal neurons on astrocytes and suggest changes in the relative importance of NCAM function as development proceeds.

Materials and Methods

Animals

Fertile White Leghorn chicken eggs were purchased from Feather Hill Farm (Petaluma, CA) and were maintained at 38°C and 95% humidity until use. Mice were from Simonson Laboratories (Gilroy, CA), and newborn Sprague-Dawley rats were from Bantin and Kingman (Fremont, CA).

Chemicals and Reagents

Murine laminin was purified from the Engelbreth-Holm-Swarm sarcoma by Dr. D. E. Hall (University of California, San Francisco) using published procedures (Timpl et al., 1979). L-[^{35}S]methionine was from Amersham Corp. (Arlington Heights, IL). Aquasol was from New England Nuclear (Boston, MA). Protein A-Sepharose CL-4B was obtained from Pharmacia, Inc., (Piscataway, NJ). DEAE cellulose (DE-52) was from Whatman Inc. (Clifton, NJ). Pepsin was from Worthington Biochemical Corp. (Freehold, NJ), and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Antibodies

Hybridoma cells secreting the CSAT monoclonal antibody were the generous gift of Dr. A. F. Horwitz (University of Illinois, Urbana, IL) and were grown as described (Neff et al., 1982). CSAT recognizes an epitope on the avian integrin β_1 subunit and will henceforth be referred to as anti–integrin β_1 (Buck et al., 1986; see Hynes, 1987, for integrin nomenclature). The 224-1A6-A1 (1A6) hybridoma cell line (Lemmon et al., 1982) was kindly provided by Dr. David Gottlieb (Washington University, St. Louis, MO).

1A6 is identical to monoclonal antibody 105 which has been shown to bind an extracellular epitope of NCAM but not to inhibit NCAM function as assayed by brain vesicle aggregation (Watanabe et al., 1986). Hybridoma cells secreting the monoclonal antibody A2B5 which recognizes gangliosides on the surfaces of neurons and type II astrocytes (Eisenbarth et al., 1979; Raff et al., 1983) were purchased from the American Type Culture Collection (Rockville, MD). For the preparation of ascites fluid, hybridoma cells were injected intraperitoneally into 10-wk-old BALB/c female mice that had been primed 10 d previously with tetramethylpentadecane (pristane). CSAT IgG was purified from ascites fluid by protein A-Sepharose CL-4B chromatography as described in Neff et al. (1982), and 1A6 IgG was purified by ammonium sulfate precipitation followed by ion exchange chromatography on DEAE cellulose as described in Hudson and Hay (1980). Fab fragments of CSAT and 1A6 IgG were prepared by papain digestion followed by ion exchange chromatography on DEAE cellulose as described in Hudson and Hay (1980).

A rabbit antiserum to chick brain NCAM was generated as described in Bixby and Reichardt (1987); the antiserum recognizes all three forms of NCAM (ld, sd, and ssd) in immunoblots of brain membranes and inhibits adhesion and neurite outgrowth by chick peripheral neurons on skeletal myotubes in vitro (Bixby and Reichardt, 1987; Bixby et al., 1987). The anti-N-cadherin serum was generated in New Zealand White rabbits against a purified 90-kD proteolytic fragment of a 130-kD Ca^{2+}-dependent CAM expressed by avian neural retinal cells (Crittenden et al., 1988). The anti-N-cadherin serum recognizes a single 130-kD protein in immunoblots of chick retinal membrane proteins separated by two-dimensional gel electrophoresis (Crittenden et al., 1988). This polypeptide has been referred to in the literature as NcalCAM (Bixby et al., 1987; Crittenden et al., 1988) but is now known to cross react with monoclonal antibodies to N-cadherin. Anti-NCAM and anti-N-cadherin IgG were prepared by ammonium sulfate precipitation followed by chromatography on DEAE (Hudson and Hay, 1980). Fab' fragments of these antibodies were prepared by pepsin digestion followed by reduction and alkylation as described in Hudson and Hay (1980).

Cell Culture

Since the integrin β_1 monoclonal antibody is highly species-specific, rat astrocytes were chosen as a substrate for neurite outgrowth by chick retinal neurons to minimize the effects of this antibody on the astrocytes themselves. Similarly, the N-cadherin and NCAM antisera were generated against avian antigens, but do cross react to some extent with rat antigens. Astrocytes were isolated from neonatal rat cortices as described (Fallon, 1985) and >90% expressed the astrocyte marker, glial fibrillary acidic protein (GFAP). Of these GFAP-positive cells, 90–95% were flat, polygonal cells that resembled type I astrocytes isolated from rat optic nerve; the remaining 5–10% were process-bearing and, like type II astrocytes, expressed the cell surface gangliosides recognized by the A2B5 monoclonal antibody (Raff et al., 1983). Enzymatically dissociated E7 and E11 chick retinal neurons were separated from non-neuronal cells by differential adhesion to tissue culture plastic and grown in defined medium as described in Hall et al. (1987). When the neurons were cocultured with astrocytes, the defined medium was supplemented with 0.5% FCS as in Cohen et al. (1986).

Substrate Preparation and Coculture

In some experiments, astrocytes were cultured as small "island" monolayers (5 mm in diameter) centered on 13-mm glass coverslips that had been previously coated with laminin (10 μg/ml in Ca^{2+}, Mg^{2+}-free PBS) as described in Tomaselli et al. (1986). For other experiments, coverslips were coated with 1 mg/ml poly-D-lysine in water, washed with Ca^{2+}, Mg^{2+}-free PBS, and seeded with ∼10^5 astrocytes, such that after 24–48 h of culture, a confluent monolayer of astrocytes extended to the coverslip edge. In coculture experiments, ∼10^5 retinal neurons were plated per coverslip and cultured for 16–20 h at 37°C in the absence or presence of antibodies. Antibodies were diluted in medium and sterile filtered through 0.45 μm nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA) before use.

Attachment Assays

Astrocytes (∼20,000 cells/0.28 cm^2 well) were plated into 96-well tissue culture plates (Flow Laboratories, Inc., McLean, VA) that had been previously coated with poly-D-lysine. After 1–2 d of culture, retinal cell attachment to confluent monolayers of astrocytes was tested as follows. Retinal neurons were labeled for 3–5 h in methionine-free growth medium containing [^{35}S]methionine (100 μCi/ml). The labeled cells were pelleted by centrifugation at 1,000 g for 5 min and resuspended in normal growth medium.
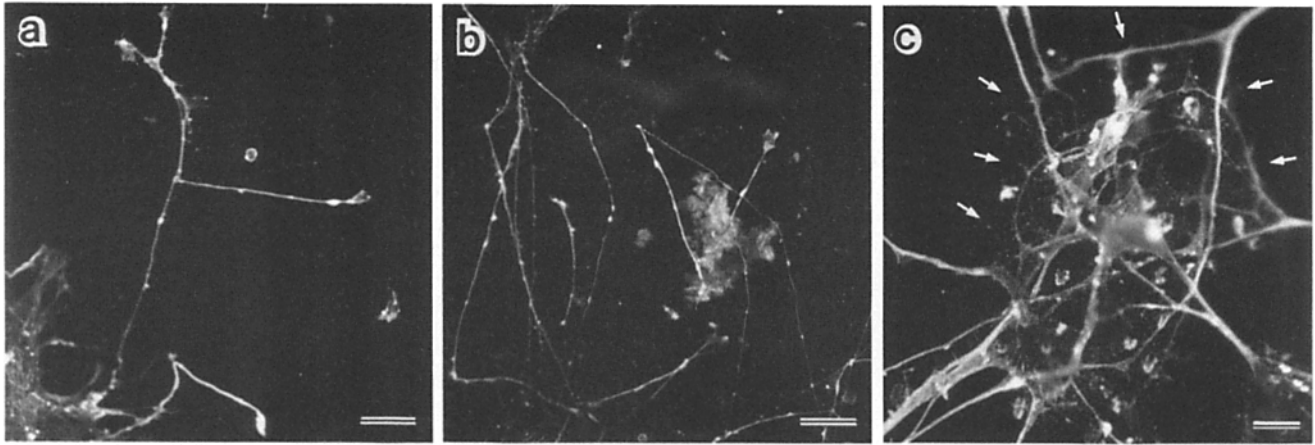
Figure 1. E7 retinal neurite outgrowth on astrocytes and laminin. Neurons were cultured for 16 h, fixed, and labeled with the A2B5 monoclonal antibody followed by a rhodamine-conjugated second antibody. (*a*) Retinal neurites on a laminin substrate; (*b*) neurites on a confluent astrocyte monolayer; and (*c*) a higher magnification view of neurite outgrowth at the border of a single astrocyte (outlined by arrows) surrounded by laminin. Note that the neurites tend to follow the edge of the astrocyte before fasciculating and growing onto the laminin. Bars: (*a* and *b*) 50 μm; (*c*) 10 μm.

Approximately 50,000 cells (∼500,000 cpm) were added to each well, and the plates were spun for 3 min at ∼600 g to ensure an even distribution of retinal neurons on the astrocyte monolayer. Cells were allowed to attach for 1 h at 37°C. Nonadherent retinal neurons were removed by adding 0.1 ml of medium to each side of the well and aspirating the supernatants. This wash procedure was sufficient to completely remove neurons from wells coated with the nonadhesive protein, BSA. A more stringent wash was achieved by performing this wash procedure three times instead of once. Wells were then examined through a microscope to confirm that the astrocytes themselves were not being lost from the substrate. Retinal cells that adhered to the astrocyte monolayer were then extracted in 0.1 ml 1% SDS in PBS overnight, mixed with 3 ml Aquasol, and counted in a scintillation counter. In each experiment, all determinations were made in triplicate.

Analysis of Retinal Neurite Outgrowth on Laminin and Astrocyte Substrates

Retinal neuron–astrocyte cocultures were fixed in 3.7% formaldehyde and stained with the A2B5 monoclonal antibody as described in Tomaselli et al. (1986). Briefly, fixed cultures were blocked and permeabilized in 1% normal goat serum/0.05% saponin/PBS, and then incubated for 1 h in a 1:500 dilution of A2B5 ascites fluid. After washing five times in PBS, cultures were incubated for 1 h in a 1:200 dilution of rhodamine isothiocyanate-coupled goat anti-mouse second antibody (Cappel Laboratories, Inc., Malvern, PA). Coverslips were mounted with gelvatol and viewed with a 63× planopochromat oil immersion lens on a Zeiss inverted IM microscope equipped with rhodamine optics. Since the A2B5 monoclonal antibody vividly stains both the cell bodies and processes of all avian

neural retinal cells (Eisenbarth et al., 1979), the number of neurons bearing processes and the length of these processes could be determined. Type II astrocytes were also labeled by A2B5, but were easily distinguished from neurons on the basis of their fibrous morphology (see Raff et al., 1983). The percentage of A2B5 positive neurons with a neurite greater than two cell body diameters in length was counted. Between 100 and 200 neurons per coverslip were tabulated in this manner. To determine neurite lengths, processes that were visible from cell soma to growth cone were drawn by camera lucida. Drawings were traced onto a computerized digitizing pad (GTCO, Inc., Rockville, MD) which calculated their lengths. Retinal neurons usually extended a single, unbranched neurite. When a neuron had more than one process, the lengths of individual neurites were summed to give the total neuritic output per neuron. All values presented in the tables and figures, therefore, refer to the total neuritic output per neuron.

Results

E7 Retinal Neurite Outgrowth on Laminin Substrates and Astrocyte Monolayers

Neurite outgrowth by embryonic chick retinal neurons grown in vitro was examined by quantifying the percent of neurons bearing neurites and the total length of neuronal processes extended by each neuron. Retinal neurons from E7 embryos were grown on substrates consisting of small islands of rat cortical astrocyte monolayers centered on glass coverslips that were coated with a concentration of laminin (10 μg/ml) that is optimal for attachment and neurite outgrowth by E7 retinal neurons (Hall et al., 1987). After 16–20 h of culture, retinal neurons extended a profuse array of neurites on both the astrocyte monolayers and the surrounding laminin substrate (Fig. 1, *a* and *b*), and neurites frequently crossed the border between the two substrates (Fig. 1 *c*). Of the neuronal cell bodies in contact with the astrocyte monolayer, 41% extended neurites, compared with 32% of those in contact with the laminin substrate (Table I). Comparison of the average lengths of retinal neurites extended on these substrates showed that those on astrocyte monolayers were ∼2.2-fold longer than those on laminin (Table I). Moreover, neurites at the astrocyte–laminin border appeared to prefer the astrocyte substrate, often following the edge of the astrocyte island before growing onto the laminin surround

Table I. Neurite Outgrowth by E7 Retinal Neurons on Laminin and Astrocytes

Substrate	Percent with neurites	Average neurite length (μm)
Laminin (10 μg/ml)	32 ± 5 (6)	110 ± 13 (33)
Astrocytes	41 ± 6 (4)	243 ± 18 (76)

E7 retinal neurons were grown on astrocyte islands centered on laminin-coated coverslips for 16–20 h. Neurons were stained with A2B5 antibody followed by a rhodamine-conjugated second antibody, and the percentage of labeled neurons with a neurite greater than 2-cell diameters was determined for the number of coverslips indicated in parentheses. Fluorescent neurites were drawn by camera lucida, and the total neurite length per neuron was determined using a computerized digitizing pad. The number of neurites measured is indicated in parentheses. All values represent the mean ± SEM. The difference between percent of cells with neurites is not significant ($P > 0.05$, Student's *t* test); the difference between average neurite length is significant ($P < 0.0005$).

Table II. Effects of Antibodies on E7 Neurite Outgrowth

Antibody	Astrocytes		Laminin	
	Percent with neurites	Percent average length*	Percent with neurites	Percent average length*
None	41 ± 6 (4)	119 ± 11 (60)‡	32 ± 5 (6)	109 ± 11 (33)
1A6				
1 mg/ml IgG	42 ± 5 (4)	100 ± 3 (72)	27 ± 3 (5)	100 ± 10 (42)
1 mg/ml Fab	42 ± 4 (5)	100 ± 5 (128)		
Integrin β₁				
0.10 mg/ml IgG	20 ± 1 (3)	71 ± 6 (40)§	1 ± 1 (3)	–
0.25 mg/ml Fab	32 ± 6 (3)	81 ± 5 (91)§	8 ± 4 (3)	ND
0.50 mg/ml Fab	22 (1)	85 ± 9 (37)§		
N-cadherin				
1 mg/ml IgG	9 ± 2 (4)	40 ± 4 (51)§	24 ± 3 (8)	100 ± 10 (29)
1 mg/ml Fab'	22 ± 5 (4)	61 ± 4 (93)§		
2 mg/ml Fab'	19 (1)	52 ± 6 (42)§		
NCAM				
1 mg/ml IgG	25 ± 3 (4)	56 ± 5 (67)§	27 ± 5 (8)	103 ± 11 (25)
1 mg/ml Fab'	38 ± 5 (4)	93 ± 6 (95)‡		
2 mg/ml Fab'	32 (1)	109 ± 11 (33)‡		

E7 retinal neurons were cultured for 16–20 h on either confluent astrocyte monolayers or on laminin in the absence or presence of antibodies. After fixation and immunostaining with A2B5, the percent of neurons bearing neurites was determined as described in Materials and Methods; the number of cultures examined is indicated in parentheses. Fluorescently labeled neurites were traced and their lengths determined as described in Materials and Methods; the number of neurites measured is indicated in parentheses. All values represent the mean ± SEM; note that neurite lengths are expressed as percent of 1A6 control. The average neurite length on laminin in the presence of 1A6 IgG is 101 ± 10 μm, and on astrocytes in the presence of 1A6 Fab is 191 ± 9 μm. The Student's t test was used to assign significance levels to differences in average neurite length from 1A6 controls as indicated.
* Relative to 1A6 control.
‡ Not significantly different from 1A6 control ($P > 0.05$).
§ Significantly different from 1A6 control ($P < 0.005$).

(Fig. 1 c); neurites were rarely seen skirting the astrocytes in favor of the laminin substrate. These observations suggest that astrocyte cell surfaces are a more favorable substrate for process extension than laminin alone.
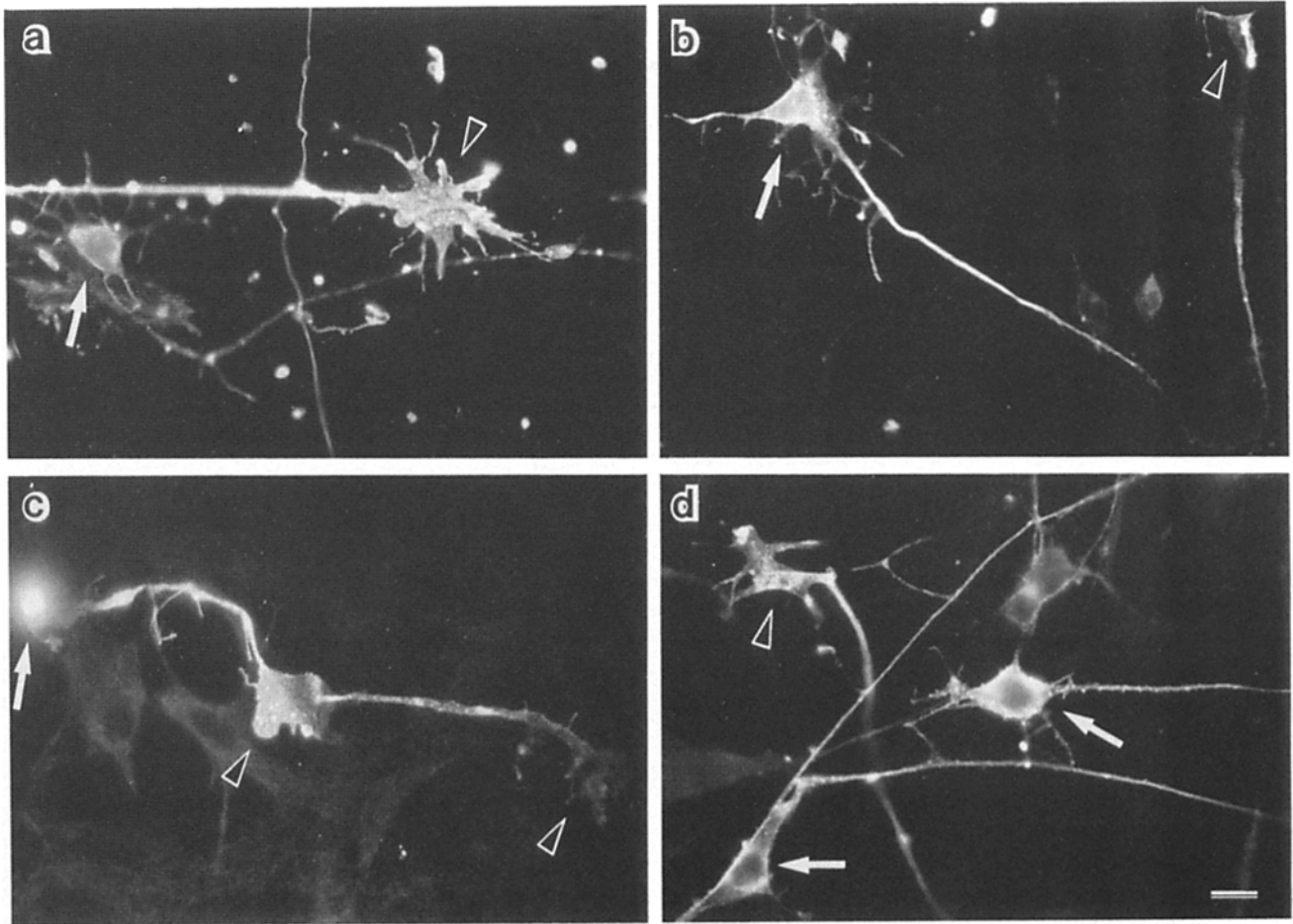
Neurite Outgrowth by E7 Retinal Neurons on Astrocyte Monolayers: Inhibition by N-Cadherin and Integrin β₁ Antibodies

To define the molecular interactions underlying E7 retinal neurite outgrowth on astrocyte surfaces, antibodies that recognize and inhibit the activity of avian neuronal cell surface proteins were tested for their effects on astrocyte-stimulated neurite outgrowth. The monoclonal antibody 1A6, which recognizes an extracellular epitope of avian NCAM but does not inhibit any detectable NCAM function (Watanabe et al., 1986; Bixby and Reichardt, 1987; Bixby et al., 1987), was used to control for possible nonspecific effects of antibody binding to cell surfaces. Compared with cultures grown in the absence of antibodies, neither 1A6 IgG nor 1A6 Fab significantly affected the percent of process-bearing neurons or the average length of neurites extended on either laminin or astrocytes (Table II).

A monoclonal antibody (CSAT) that recognizes the avian β₁ subunit of the integrin family of ECM receptor heterodimers (Horwitz et al., 1985; Buck et al., 1986; reviewed in Hynes, 1987, and Ruoslahti and Pierschbacher, 1987), has previously been shown to eliminate retinal neuron attachment and process outgrowth on laminin, fibronectin, and collagen types I and IV (Hall et al., 1987; Neugebauer, K., unpublished observations). When E7 retinal neurons were grown on astrocyte monolayers in the presence of high concentrations of either anti–integrin β₁ IgG (0.1 mg/ml) or

Fab fragments (0.25 mg/ml), the percent of neurons bearing processes was reduced by 25–50% as compared with 1A6 Fab–containing control cultures (Table II). The average lengths of the remaining processes were significantly reduced by 15–30% (Table II; Fig. 3). These effects on astrocyte-stimulated neurite outgrowth were relatively weak compared with the virtual elimination of neurite outgrowth by integrin β₁ antibodies on laminin (see Table II). Since doubling the concentration of anti–integrin β₁ Fab to 0.5 mg/ml had only a slightly stronger effect on neurite outgrowth on astrocytes, the 0.25 mg/ml concentration of anti–integrin β₁ Fab used in these and subsequent experiments appears to have been saturating. As illustrated in Fig. 2, the overall morphology of neurons cultured in the presence of integrin β₁ antibodies resembled that of neurons in 1A6-containing cultures despite the inhibitory effects of integrin β₁ antibodies on neurite outgrowth.

Antibodies that recognize and inhibit the function of N-cadherin, a Ca^{2+}-dependent cell adhesion molecule expressed on avian retinal neurons (Crittenden et al., 1988), had dramatic effects on several aspects of retinal neurite outgrowth on astrocyte monolayers. In the presence of 1 mg/ml anti–N-cadherin Fab's, the percent of process-bearing E7 retinal neurons was reduced by ~50% (Table II). Neurites that did grow in the presence of anti–N-cadherin Fab's were visibly shorter than those seen in control cultures, reflecting a ~40% reduction in average neurite length (Table II; Fig. 2 c, Fig. 3). Often these neurites had enlarged growth cones (Fig. 2 c). Doubling the concentration of anti–N-cadherin Fab' did not significantly enhance these effects, indicating that the effects of 1 mg/ml anti–N-cadherin Fab' were saturating (Table II). Interestingly, bivalent anti–N-cadherin IgG had

Figure 2. E7 retinal neurite outgrowth on astrocyte monolayers in the presence of antibodies to neuronal cell surface proteins. Neurons were cultured on astrocytes for 16 h in the presence of antibodies, fixed, and stained with A2B5 antibody. (*a*) Neurite outgrowth in the presence of 1 mg/ml 1A6 Fab fragments is extensive: portions of three long neurites traverse the field. (*b*) In the presence of 0.25 mg/ml anti–integrin β_1 Fab, neurites do grow but are shorter on average. (*c*) This single neuron grown in the presence of 1 mg/ml anti–N-cadherin IgG is quite short ($\sim$120 μm) and has two growth cones (*arrowheads*). (*d*) Neurite outgrowth in the presence of 2 mg/ml anti–NCAM Fab' is not visibly affected; portions of four long neurites crisscross the field. Examples of retinal cell bodies (*arrows*) and growth cones (*arrowheads*) are visible in each field. Bar, 10 μm.
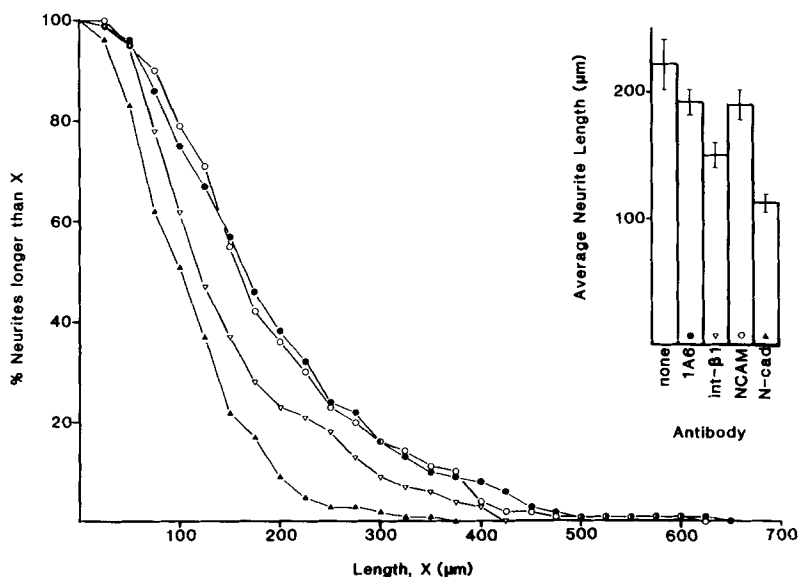
Figure 3. Effects of antibodies on E7 retinal neurite length on astrocytes after 16–20 h of culture. Distribution of neurite lengths in the presence of the following Fab fragments: (●) 1 mg/ml 1A6 Fab (*n* = 128 neurites); (▽) 0.1 mg/ml integrin β_1 Fab (*n* = 91); (○) 1 mg/ml NCAM Fab' (*n* = 95); (▲) 1 mg/ml N-cadherin Fab' (*n* = 93). Neurite lengths were binned by 25-μm intervals and plotted versus the percentage of neurites measured that were longer than the length (×) indicated. The distribution of neurite lengths in the presence of 1A6 Fab was identical to that in the absence of antibodies (omitted for clarity). (*Inset*) Histogram of average neurite lengths (mean ± SEM) in the absence or presence of Fab fragments of antibodies. 60 neurites were measured for the "no antibody" condition. Data from four separate experiments were pooled to yield the final distribution curve and histogram.

Table III. Effects of Combined Antibodies on E7 Retinal Neurite Outgrowth on Astrocytes

Antibody	Percent with neurites	Percent average length*
1A6 IgG	40 ± 8 (2)	100 ± 10 (39)
Integrin IgG	19 ± 1 (2)	63 ± 8 (40)‡
N-cadherin IgG	9 ± 3 (2)	37 ± 5 (50)‡
Integrin + N-cadherin	5 ± 2 (1)	33 ± 6 (14)‡§
1A6 Fab	48 (2)	100 ± 9 (30)
NCAM Fab'	44 ± 1 (2)	92 ± 13 (32)
N-cadherin Fab'	29 ± 1 (2)	61 ± 9 (30)‡
NCAM + N-cadherin	26 (1)	64 ± 9 (25)‡§

E7 retinal neurons were grown for 16 h on confluent astrocyte monolayers in the presence of the indicated antibodies. All antibodies were added to a final concentration of 1 mg/ml, except anti-integrin β_1 IgG was used at 0.1 mg/ml. After the cultures were fixed and stained, the percent of neurons bearing neurites was determined (n = the number of cultures examined) and the neurite length was measured as described (n = the number of neurites traced). All values represent the mean ± SEM. The Student's t test was used to assign significance levels to differences in average neurite lengths from 1A6 controls as indicated.
* Relative to 1A6 control.
‡ Significantly different from 1A6 control ($P < 0.0005$).
§ Not significantly different from anti-N-cadherin IgG alone ($P > 0.05$).

stronger inhibitory effects than did monovalent Fab' fragments (Table II). Inhibition of retinal neurite outgrowth by N-cadherin antibodies was substrate specific, since neurite outgrowth on laminin substrates was not significantly affected by anti-N-cadherin IgG (Table II).

To determine whether the inhibitory effects of integrin β_1 and N-cadherin antibodies were additive, E7 retinal neurons were cultured on astrocytes in the presence of both antibodies. A representative experiment shown in Table III demonstrates that the combined presence of integrin β_1 and N-cadherin bivalent antibodies resulted in a small but statistically insignificant increase in the inhibition observed with anti-N-cadherin alone.

When E7 retinal neurons were cultured on astrocytes in the presence of anti-NCAM Fab' fragments at concentrations as high as 2 mg/ml, no parameter of neurite outgrowth examined was significantly affected. Instead, neurons treated with anti-NCAM Fab' were indistinguishable from control on the basis of morphology (Fig. 2 d), neurite length distribution (Fig. 3), percent of neurons with neurites or average neurite length (Table II). Even when added with anti-N-cadherin Fab', anti-NCAM Fab's did not produce a decrease in neurite outgrowth beyond the effects of anti-N-cadherin alone (Table III). Thus, we were unable to detect any effects of anti-NCAM Fab' on E7 retinal neurite outgrowth on astrocytes. However, in cultures treated with 1 mg/ml anti-NCAM IgG, a ∿50% decrease in the average neurite length was observed (Table II). As our preparation of anti-NCAM Fab's is known to block the function of chick NCAM (Bixby and Reichardt, 1987; Bixby et al., 1987), the effects of bivalent NCAM antibodies in the present experiments seem most likely to be due to cross-linking of their target antigen rather than a specific inhibition of NCAM function. Interestingly, NCAM IgG binding only inhibited process outgrowth on astrocytes, as it had no significant effects on process outgrowth on laminin substrates (Table II).
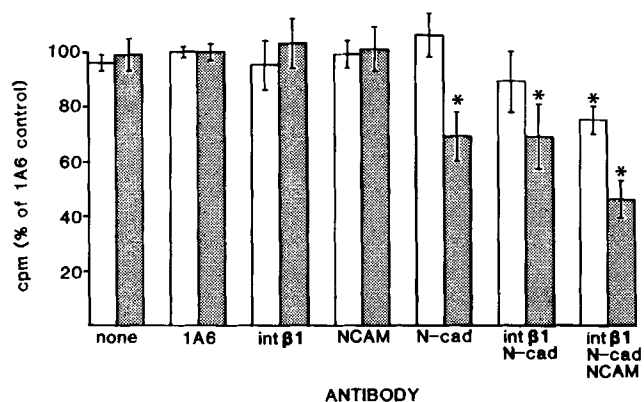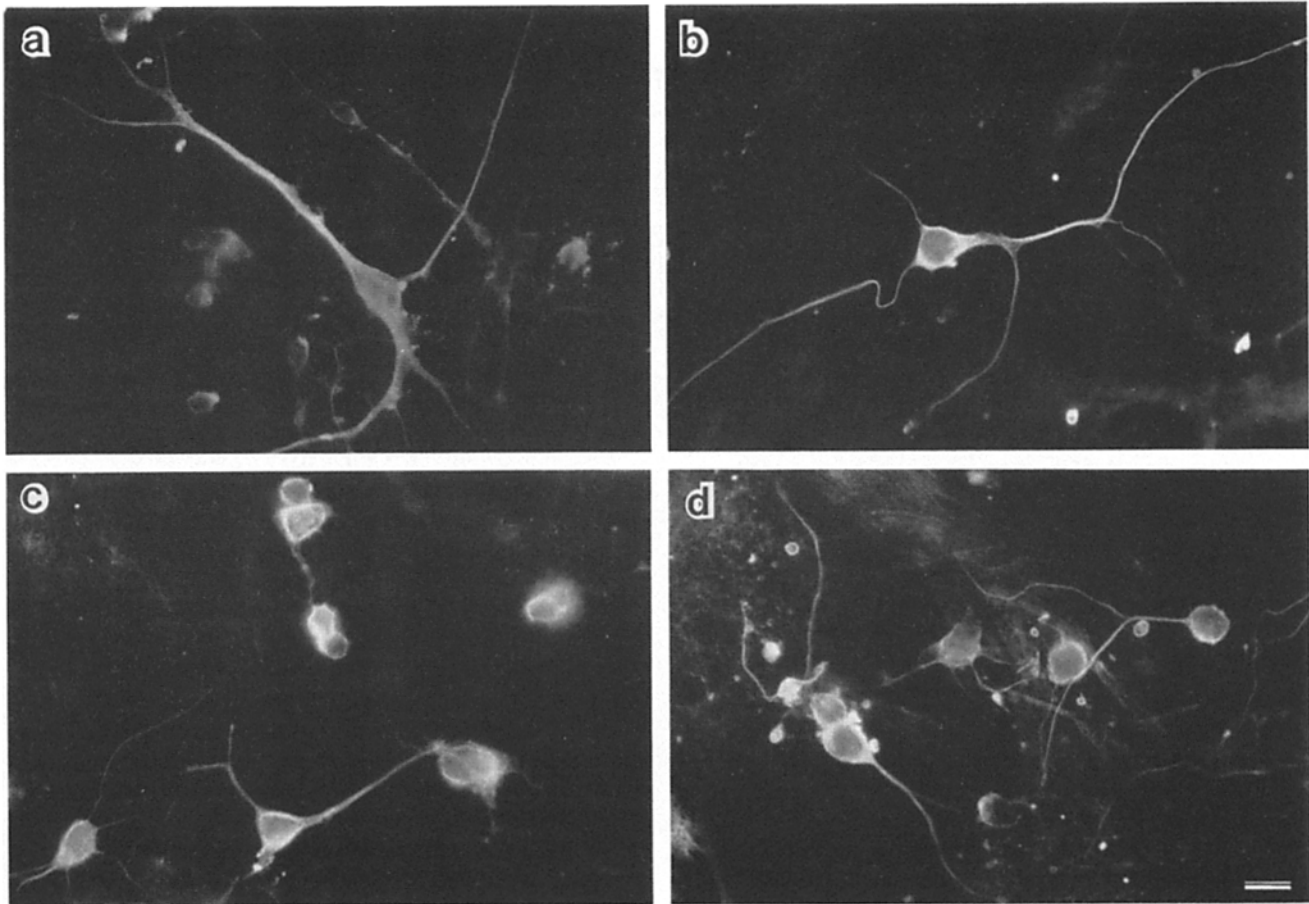

ANTIBODY

Figure 4. Attachment of ^{35}S-labeled E7 retinal neurons to astrocyte monolayers in the absence or presence of antibodies. All antibodies indicated are IgG, except anti-NCAM which is Fab'. Antibodies were added to a final concentration of 1 mg/ml, except that anti-integrin β_1 was used at 0.1 mg/ml. Neurons were allowed to attach to astrocyte monolayers for 1 h. Wells were subjected to either one (open bars) or three (stippled bars) washes. Radioactivity associated with adherent neurons was quantitated by extracting the cells with 1% SDS and counting the extracts in a scintillation counter. Under both assay conditions, 80–100% of the counts added to each well was associated with the monolayer in absence of antibody, although fewer counts were usually detected after the additional washing procedure. Triplicate determinations were made in each experiment, and the results of three to six experiments per variable were averaged. Each value is expressed as a percent of cpm remaining with the astrocytes in the presence of control 1A6 IgG (mean ± SEM). Significant differences from 1A6 control were determined by the Student's t test (*, $P < 0.005$); bars without asterisks are not significantly different ($P > 0.05$). Note that under extensive wash conditions, NCAM antibodies significantly decreased attachment beyond the effects of N-cadherin plus integrin β_1 antibodies ($P < 0.05$).

Attachment of E7 Retinal Neurons to Astrocyte Monolayers

To determine whether the inhibition of neurite outgrowth by either integrin β_1 or N-cadherin antibodies was correlated with a reduction in retinal neuron attachment to astrocytes, the attachment of [^{35}S]methionine-labeled E7 retinal neurons to confluent monolayers of astrocytes was measured in the absence or presence of antibodies. In each experiment, neurons contacting the astrocyte monolayers were subjected to two wash conditions differing in stringency (see Materials and Methods). The results in Fig. 4 show that after one wash of the monolayer (open bars), neither anti-integrin β_1 IgG, anti-NCAM Fab', nor anti-N-cadherin IgG significantly reduced retinal neuron attachment to astrocytes when applied individually. The combination of all three antibodies decreased attachment by ∿25% compared with control under these conditions (Fig. 4).

After a more extensive washing procedure (three washes), only anti-N-cadherin IgG produced effects when applied alone, reducing attachment by 30% (Fig. 4, stippled bars). While integrin β_1 antibodies did not enhance the inhibitory effects of N-cadherin antibodies, the combination of integrin β_1, N-cadherin, and NCAM antibodies did reduce attachment by an additional 30% (Fig. 4). Thus, anti-NCAM ap-

Figure 5. E11 retinal neurite outgrowth on astrocytes after 16 h of culture in the absence and presence of antibodies. Neurons are fluorescently stained with A2B5 antibody. (*a* and *b*) Two distinct neuronal morphologies in the absence of antibodies. Note that the neurites in *b* extend out of the field. (*c*) In the presence of 1 mg/ml anti-N-cadherin Fab', many neurons lack neurites, and the neurites present are short and often wispy. (*d*) In the presence of 1 mg/ml anti-NCAM Fab', neurites are obviously shortened. Bar, 10 μm.

pears to potentiate the inhibition of attachment by N-cadherin antibodies. This result, in combination with the observed insensitivity of retinal neurite outgrowth to NCAM antibodies, suggests that NCAM function may be more important for adhesion than for neurite outgrowth by E7 retinal neurons.

Process Outgrowth by E11 Retinal Neurons on Astrocytes: Inhibition by N-cadherin, Integrin β_1, and NCAM Antibodies

The possibility that retinal neurons of different developmental ages use different mechanisms for neurite outgrowth on astrocytes was examined using E11 retinal neurons in the coculture paradigm used for E7 neurons. Unlike E7 retinal neurons, E11 retinal neurons do not attach or extend processes on either laminin or fibronectin, although they continue to do so on collagens and astrocytes (Cohen et al., 1986; Hall et al., 1987). E11 retinal neurons grown on astrocytes for 16–20 h exhibited diverse morphologies, ranging from cells with large somata and many bifurcating processes to cells with small somata and one or two straight neurites (Fig. 5, *a* and *b*). Spatulate growth cones with multiple filopodia were not characteristic of E11 retinal neurons as

they were for E7 retinal neurons (Fig. 5). Thus, older retinal neurons grown on astrocytes regenerate growth cones that resemble those observed in the optic nerve and tract in situ (Bovolenta and Mason, 1987). Although fewer E11 than E7 neurons extended processes (29 vs. 41%; Tables I and IV), E11 neurites were comparable to E7 neurites in average length (~200 μm; see Figs. 3 and 6). E11 neurite lengths were more heterogeneous in that approximately twice as many E11 as E7 neurons had either very short (<100 μm) or very long (>500 μm) neuritic arbors (compare Figs. 3 and 6). However, we were unable to correlate neurite length with neurons grouped by morphological characteristics. Similarly, the effects of antibodies on E11 neurite outgrowth (see below) could not be attributed to differential effects on neurons with distinct morphologies (data not shown).

Anti-N-cadherin Fab' fragments inhibited E11 neurite outgrowth on astrocytes to a similar extent as on E7 retinal neurons. Compared with 1A6 Fab, anti-N-cadherin Fab's consistently reduced the percent of process-bearing E11 neurons by ~50%, and the average length of remaining neurites by ~35% (Fig. 6; Table IV). Fig. 6 shows that anti-N-cadherin increases the proportion of neurites shorter than 100 μm from 35 to 65%. In the presence of anti-N-cadherin, E11
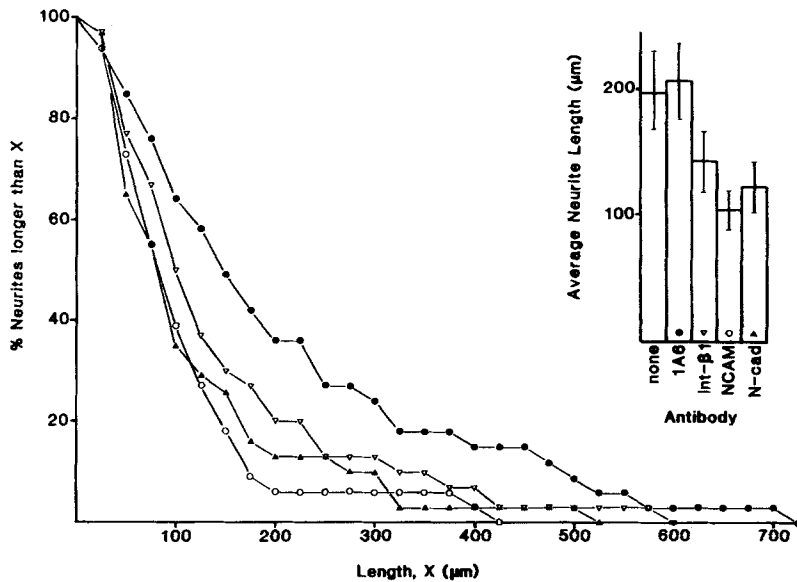
Figure 6. Effects of antibodies on E11 retinal neurite length on astrocytes after 16 h of culture. Distribution of E11 neurite lengths in the presence of the following Fab fragments: (●) 1 mg/ml 1A6 Fab (n = 33); (▽) 0.25 mg/ml anti-integrin β₁ Fab (n = 30); (○) 1 mg/ml anti-NCAM Fab' (n = 33); (▲) 1 mg/ml anti-N-cadherin Fab' (n = 31). (Inset) Histogram of average neurite lengths (mean ± SEM) in the absence or presence of Fab fragments. 33 neurites were measured for the "no antibody" condition. Data from a representative experiment was used to generate the distribution curve and histogram.

retinal neurons appeared well attached to the astrocytes, although their neurites were visibly shorter (Fig. 5 c).

When anti-integrin β₁ Fab fragments were tested, both the average neurite length and the percent of process-bearing neurons were reduced by ~30% (Table IV; Fig. 6). About 50% of the neurites in integrin β₁-containing cultures were shorter than 100 μm (Fig. 6). These effects of anti-integrin β₁ Fabs were not anticipated, as E11 retinal neurons have lost their integrin-dependent responsiveness to purified laminin and fibronectin (Hall et al., 1987).

In contrast to that of E7 retinal neurons, E11 retinal neurite outgrowth on astrocytes was sensitive to anti-NCAM Fab' fragments in the medium. E11 retinal neurons extended neurites in the presence of 1 mg/ml anti-NCAM Fab's (Fig. 5 d), but the percent of process-bearing neurons was reduced by ~50% and the average length of neurites was reduced by ~25% (Table IV). Fig. 6 shows that anti-NCAM Fab' fragments virtually eliminated long neurites in the cultures, as ~90% of the neurites were shorter than 175 μm. Thus, these experiments show that inhibitory effects of anti-NCAM on retinal neurite outgrowth on astrocytes depends on the developmental age of the retinal neurons.

Table IV. Antibody Effects on E11 Retinal Neurite
Outgrowth on Astrocytes

Antibody	Percent with neurites	Percent average length*
None	29 ± 3 (4)	117 ± 10 (109)
1A6 Fab	32 ± 6 (4)	100 ± 8 (110)‡
Integrin Fab	20 ± 1 (2)	67 ± 8 (65)§
NCAM Fab'	16 ± 3 (4)	76 ± 7 (117)§
N-cadherin Fab'	15 ± 1 (2)	64 ± 8 (48)§

E11 retinal neurons were cultured on confluent astrocyte monolayers for 16–20 h in the absence or presence of Fab' fragments. All Fab fragments were added to a final concentration of 1 mg/ml, except anti-integrin β₁ was used at 0.25 mg/ml. A2B5-stained neurons were scored for the percent with neurites (n = the number of cultures examined). The lengths of fluorescent neurites were determined as described in Materials and Methods (n = the number of neurites measured). All values represent the mean ± SEM. The Student's t test was used to compare differences in average neurite length to 1A6 control.
* Relative to 1A6 control.
‡ Not significantly different from 1A6 control (P > 0.05).
§ Significantly different from 1A6 control (P < 0.05).

Discussion

Our observations of chick retinal neurite outgrowth on astrocytes in the presence of antibodies to neuronal cell surface proteins support the following major conclusions. (a) The ability of embryonic chick retinal neurons to extend neurites rapidly when cultured on astrocyte cell surfaces depends strongly on the function of the Ca²⁺-dependent cell adhesion molecule, N-cadherin, and to a lesser extent, on members of the integrin family of ECM protein receptor heterodimers. (b) NCAM's role in mediating retinal neurite outgrowth on astrocytes depends on the developmental age of the retinal neurons, such that E11 but not E7 neurons require NCAM function for maximal neurite extension. (c) Retinal neurons use additional receptors for attachment and neurite outgrowth on astrocytes, since neither was completely inhibited by any individual antibody or combination of antibodies tested. (d) The molecules used by retinal neurons for process outgrowth on astrocytes are similar to those used by peripheral ciliary ganglion neurons but differ in two important respects: retinal neurite outgrowth relies on (i) at least one additional CAM (NCAM) and (ii) integrin β₁-class ECM receptors even at developmental ages at which they have lost their responsiveness to purified laminin.

N-cadherin is a 130-kD neuronal cell surface protein that is important in the calcium-dependent aggregation of avian retinal neurons (Grunwald et al., 1982). N-cadherin mediates peripheral neurite outgrowth on cultured myotubes, Schwann cells, and astrocytes (Bixby et al., 1987, 1988; Tomaselli et al., 1988). N-cadherin is likely to act by a homophilic binding mechanism (i.e., neuronal N-cadherin binding to astrocyte N-cadherin), since a closely related epithelial cell CAM, E-cadherin, has been shown to function, at least in part, in this way (Nagafuchi et al., 1987). It is unclear whether any of the effects of N-cadherin antibodies observed in these experiments can be attributed to the binding of antibodies to astrocyte N-cadherin; however, since we have shown that saturating levels of anti-N-cadherin were used (see Table II), we are confident that N-cadherin on the neuronal surface was maximally inactivated. Here we have shown that N-cadherin antibodies strongly reduced both E7

and E11 retinal neurite outgrowth on astrocytes. The extent of this inhibition was similar for neurons of both ages; in the presence of anti–N-cadherin Fab' fragments, ~50% fewer E7 and E11 neurons grew neurites, and neurites in both cultures were 35–40% shorter than those that grew in control cultures. The growth cones of E7 retinal neurons often appeared enlarged in the presence of N-cadherin but not other antibodies. E11 retinal neurites did not bear spatulate growth cones under any of the conditions tested. N-cadherin is also involved in E7 retinal neuron adhesion to astrocytes, as determined by quantitative attachment assays. However, even under the most stringent conditions of our assay, N-cadherin antibodies reduced attachment by only ~30%, an amount insufficient to account for the potency of their effects on process outgrowth. The importance of N-cadherin in retinal neuron process outgrowth on astrocytes and its presence in the developing chick neural retina and optic nerve (Hatta et al., 1986, 1987) suggest that N-cadherin also promotes axonal extension on glial surfaces within the primary visual pathway.

Members of the integrin family of ECM receptors also mediate process outgrowth by neurons on astrocytes. The integrin β_1 subunit (Hynes, 1987; Ruoslahti and Pierschbacher, 1987) is shared by cell surface glycoprotein heterodimers that bind laminin and fibronectin directly (Horwitz et al., 1985; Akiyama et al., 1986; Buck et al., 1986; Tomaselli et al., 1988). Anti–integrin β_1 monoclonal antibodies inhibit attachment and neurite outgrowth by a variety of avian neurons on substrates coated with purified laminin, fibronectin, and collagen types I and IV (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986; Cohen et al., 1987; Hall et al., 1987; Neugebauer, K., unpublished observations). Since integrin β_1 antibodies also inhibit neurite outgrowth on substrates coated with a laminin–heparan sulfate proteoglycan complex secreted by astrocytes as well as on intact ECMs (Tomaselli et al., 1986, 1988; Cohen et al., 1987), they were expected to prevent retinal neuron interactions with the fibronectin and laminin that rat cortical astrocytes are known to express in vitro (Liesi et al., 1983; Price and Hynes, 1985).

Anti–integrin β_1 Fab fragments reduced the percent of process-bearing E7 retinal neurons and the average neurite length by ~30% each. These effects are comparable to those of integrin β_1 antibodies on ciliary ganglion neurite outgrowth on astrocytes (Tomaselli et al., 1988). Integrin β_1 antibodies did not significantly inhibit adhesion of retinal neurons to astrocytes in our assays, even though these antibodies virtually eliminate the attachment of retinal neurons to laminin, fibronectin, and collagen I and IV substrates (Hall et al., 1987; Neugebauer, K., unpublished observations). Thus, integrin receptor interactions with ECM components expressed on astrocyte surfaces do not appear to play a major role in neuron-astrocyte attachment.

Anti–integrin β_1 Fab fragments inhibited E7 and E11 retinal neurite outgrowth on astrocytes to the same extent. E11 retinal neurons have previously been shown to lose their ability to attach to and extend neurites on laminin and fibronectin substrates (Cohen et al., 1986, 1987; Hall et al., 1987). However, they retain integrins on their surface and their ability to attach to collagen types I and IV in an integrin-dependent manner (Cohen et al., 1987; Hall et al., 1987; Neugebauer, K., unpublished observations). Thus, the present

results suggest that astrocytes express ECM proteins in addition to laminin and fibronectin (e.g., collagen types I and IV) whose neuronal receptor(s) includes the integrin β_1 subunit. Alternatively, E11 retinal neurons may recover their response to laminin and/or fibronectin when grown in contact with astrocyte cell surfaces. In this respect, the behavior of retinal neurons differs from that of ciliary ganglion neurons: neurite outgrowth by these peripheral neurons on astrocytes is not inhibited by integrin β_1 antibodies at a developmental age (E14) when they, like E11 retinal neurons, no longer attach or extend neurites on purified laminin (Tomaselli et al., 1988).

Anti–NCAM Fab' fragments did not affect any parameter of E7 neurite outgrowth on astrocytes even when added in combination with N-cadherin antibodies (cf. Table III). Anti–NCAM Fab' alone did not detectably inhibit E7 retinal neuron attachment to astrocyte monolayers. Anti–NCAM Fab' did, however, have significant effects on attachment when added in combination with N-cadherin and integrin β_1 antibodies. Together, these results suggest that NCAM plays a relatively minor role in E7 retinal neuron interactions with astrocytes. In contrast, the same anti–NCAM Fab's dramatically inhibited neurite outgrowth by developmentally older (E11) retinal neurons: the percent of process-bearing E11 neurons was decreased by 50% and the average length of E11 neurites was decreased by 25%. Since homophilic NCAM binding in liposome assays is highly dependent on its concentration in the membrane (Hoffman and Edelman, 1983), the striking developmental change in the sensitivity of retinal neurite outgrowth to NCAM antibodies may reflect the twofold increase in NCAM levels observed in the retina between E5 and E10 (Daniloff et al., 1986). Alternatively, changes in the molecular form of NCAM on the surface of the neurites may explain these findings: although sialic acid-poor 180,000- and 140,000-M_r forms of NCAM predominate in both E5 and E10 retina, there is an apparent transition from sialic acid-rich to poor forms in the optic nerve between these ages (Schlosshauer et al., 1984).

The inhibition of E7 retinal neurite outgrowth on astrocytes by anti–NCAM IgG is the only instance in our experiments in which an IgG had effects not seen with Fab' fragments. Specifically, both IgG and Fab's generated from the same NCAM antiserum used here reduce ciliary ganglion neurite elongation on skeletal myotubes (Bixby et al., 1987), while neither is inhibitory on astrocytes or Schwann cells (Tomaselli et al., 1988; Bixby et al., 1988). One possible explanation for these results is that in the process of cross-linking NCAM on the surface of retinal neurons, NCAM IgG redistributes other relevant proteins (e.g., N-cadherin) in the plane of the membrane. The cell adhesion molecule L1/NgCAM has been shown to co-redistribute with the 180-kD form of NCAM on neural membranes after treatment with bivalent anti–NCAM IgG (Thor et al., 1986), establishing a precedent for this possibility.

Since neither attachment nor neurite outgrowth by retinal neurons on astrocytes was completely inhibited by the antibodies used in this study (see Fig. 4 and Table III), adhesive molecules in addition to N-cadherin, integrin β_1 heterodimers, and NCAM must be active. It is possible that this activity represents a novel adhesive mechanism. However, three previously identified molecules that mediate neuronal interactions with astrocytes are also good candidates: (a)

cytotactin, an ECM glycoprotein complex present in the optic fiber and inner plexiform layers of the embryonic chick retina (Crossin et al., 1986); (b) AMOG (adhesion molecule on glia), an integral membrane protein that promotes cerebellar granule cell adhesion to astrocytes (Antonicek et al., 1987); and (c) L1/NgCAM (Grumet and Edelman, 1988), a cell adhesion molecule present in the developing optic pathway (Daniloff et al., 1986; Lemmon and McLoon, 1986). L1 has been implicated in neurite outgrowth on other neurites and on Schwann cells (Chang et al., 1987; Bixby et al., 1988), and purified L1 promotes neurite extension when used as a culture substrate (Lagenaur and Lemmon, 1987). It is unlikely that all neurons use such additional mechanisms, since ciliary ganglion neurite outgrowth on astrocytes can be nearly completely accounted for by the combined activity of N-cadherin and the integrin β_1 heterodimers (Tomaselli et al., 1988).

The above results suggest roles for integrins, N-cadherin, and NCAM in process outgrowth and adhesion in the developing retinotectal pathway. The early chick neural retina consists of a sheet of neuroepithelial cells that gradually differentiate into the various retinal cell types, beginning with the appearance of the first postmitotic retinal ganglion cells at embryonic day 2.5-3 (Kahn, 1974). These cells migrate to the most vitreal portion of the retina and extend axons toward the optic stalk, using a laminin-rich basal lamina and Müller glial endfeet as growth substrates (Rager, 1980; Easter et al., 1984; Cohen et al., 1987). At E7, ganglion cell axon initiation peaks (Halfter et al., 1983), and other postmitotic retinal cell types are migrating to appropriate layers of the neural retina (Kahn, 1974). Although the ordered growth of axons to the optic stalk relies on the integrity of the basal lamina (Halfter and Deiss, 1984, 1986), directional cues for growth are not provided by the intact, isolated basal laminae (Halfter et al., 1987). Our observations of E7 retinal neurite outgrowth on astrocyte surfaces implicate N-cadherin as well as integrin-class ECM receptors in axonal extension at this early developmental stage, raising the possibility that N-cadherin on glial endfeet may guide advancing axons to the optic stalk.

Axonal growth within the optic nerve peaks between E7 and E11 and occurs primarily along the endfeet of astroglial precursors and preexisting axonal surfaces (Rager, 1980; Silver and Rutishauser, 1984). Laminin may be involved in the elongation of the first ganglion cell axons to enter the optic pathway (E3), but it disappears from the optic nerve by E7 (Cohen et al., 1987). NCAM is localized at the surface of glial endfeet in the nerve where it may play a role in axon extension (Silver and Rutishauser, 1984; Thanos et al., 1984). The observed developmental change in NCAM's role in retinal neurite outgrowth on astrocytes is consistent with the hypothesis that axonal growth in the optic pathway becomes progressively more dependent on cell surfaces.

At the optic tectum, retinal ganglion cell axons synapse with a topological specificity that generates a tectal map of the visual field. Neuronal recognition via differential adhesion (chemoaffinity) has long been thought to account for this targeting phenomenon (Sperry, 1963). Since glial endfeet line the developing optic nerve fiber layer of the tectum (Lemmon, 1985, 1986; Bork et al., 1987). They may provide cues for axon growth and guidance. The sensitivity of astrocyte-stimulated E11 retinal neurite outgrowth to NCAM

antibodies is consistent with evidence that NCAM is involved in accurately targeting axons at the tectum (Fraser et al., 1984). As N-cadherin and integrins remain active on the surface of E11 retinal neurons, it will be of interest to determine whether these receptor systems also regulate synaptogenic events at the optic tectum.

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