Distinct Molecular Interactions Mediate Neuronal Process Outgrowth on Non-neuronal Cell Surfaces and Extracellular Matrices

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Abstract. We have compared neurite outgrowth on extracellular matrix (ECM) constituents to outgrowth on glial and muscle cell surfaces. Embryonic chick ciliary ganglion (CG) neurons regenerate neurites rapidly on surfaces coated with laminin (LN), fibronectin (FN), conditioned media (CM) from several non-neuronal cell types that secrete LN, and on intact extracellular matrices. Neurite outgrowth on all of these substrates is blocked by two monoclonal antibodies, CSAT and JG22, that prevent the adhesion of many cells, including neurons, to the ECM constituents LN, FN, and collagen. Neurite outgrowth is inhibited even on mixed LN/poly-D-lysine substrates where neuronal attachment is independent of LN. Therefore, neuronal process outgrowth on extracellular matrices requires the function of neuronal cell surface molecules recognized by these antibodies.

The surfaces of cultured astrocytes, Schwann cells, and skeletal myotubes also promote rapid process outgrowth from CG neurons. Neurite outgrowth on these surfaces, though, is not prevented by CSAT or JG22 antibodies. In addition, antibodies to a LN/proteoglycan complex that block neurite outgrowth on several LN-containing CM factors and on an ECM extract failed to inhibit cell surface–stimulated neurite outgrowth. After extraction with a nonionic detergent, Schwann cells and myotubes continue to support rapid neurite outgrowth. However, the activity associated with the detergent insoluble residue is blocked by CSAT and JG22 antibodies. Detergent extraction of astrocytes, in contrast, removes all neurite-promoting activity.

These results provide evidence for at least two types of neuronal interactions with cells that promote neurite outgrowth. One involves adhesive proteins present in the ECM and ECM receptors on neurons. The second is mediated through detergent-extractable macromolecules present on non-neuronal cell surfaces and different, uncharacterized receptor(s) on neurons. Schwann cells and skeletal myotubes appear to promote neurite outgrowth by both mechanisms.

During development, selective interactions between nerve growth cones and their environment may help direct growing axons to their appropriate targets. The substrates that growth cones contact in vivo are a complex mixture of neuronal and non-neuronal cell surfaces as well as extracellular matrices (Rogers et al., 1986; Tosney and Landmesser, 1985). Experiments in tissue culture demonstrate that differential adhesion of growth cones to the substrate can lead to patterned axon growth (Letourneau, 1975; Hammarback et al., 1985; Collins and Lee, 1984). The establishment of axon pathways may depend, in part, on the spatiotemporal distribution of adhesion and growth-promoting molecules in the environment of motile growth cones. The degree to which such factors stimulate axon elongation must, in turn, depend on the presence and functional state of neuronal cell surface receptors which interact selectively with them. The identities of substrate factors that influence nerve growth in vivo or the neuronal receptors through which they exert their effects are unknown.

Recent studies in culture have focused on the role of the extracellular matrix (ECM)[1] glycoproteins laminin (LN) and fibronectin (FN) in neurite elongation. In contrast to many conventional tissue culture substrates (i.e., plastic, glass, collagen, or polycations), substrate-bound LN and FN stimulate rapid and profuse neurite elongation from a wide variety of central and peripheral neurons (Baron-Van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983; Faivre-Bauman et al., 1984). This fact, coupled with the presence of LN and FN immunoreactivity in embryonic

1. *Abbreviations used in this paper*: BCE, bovine corneal endothelial; CG, ciliary ganglion; CM, conditioned medium; E, embryonic day; ECM, extracellular matrix; EHS sarcoma, Engelbreth-Holm-Swarm sarcoma tumor; FN, fibronectin; LN, laminin; NOPF, neurite outgrowth-promoting factor.

tissues through which axons grow (Rogers et al., 1986; Adler et al., 1985) suggests that these ECM molecules may be important for nerve growth in vivo.

In addition to the ECM, growth cones migrate in proximity to neuronal and non-neuronal cell surfaces (Al-Ghaith and Lewis, 1982; Tosney and Landmesser, 1985). In vitro, neurites grow preferentially on monolayers of non-neuronal cells that are specialized to interact with neurons (Noble et al., 1984; Fallon, 1985 *a*,*b*). Although some of these cells (i.e., Schwann cells, astrocytes, and muscle cells) are known to synthesize LN and/or FN, (Sanes, 1982; Cornbrooks et al., 1983; Liesi et al., 1983, 1986; Price and Hynes, 1985) it is not known whether these or other surface-associated components stimulate neuronal process outgrowth.

A complementary issue concerns the identity of the neuronal cell surface receptors that mediate the interactions of neurons with their substrate. Progress has been made in identifying these receptors with the generation of two monoclonal antibodies, CSAT (Neff et al., 1982) and JG22 (Greve and Gottlieb, 1982), which interfere with the attachment of several cell types to LN- and FN-coated substrates (Horwitz et al., 1985). The two antibodies appear to recognize overlapping epitopes on molecules that are expressed on the surface of a variety of chick embryonic cells, including muscle cells, fibroblasts, and neurons (Damsky et al., 1985; Bozyczko and Horwitz, 1986). Immunoprecipitation of chick embryo extracts with CSAT or JG22 antibodies identifies a set of three noncovalently associated glycoproteins of ~140 kD (Horwitz et al., 1985; Chapman, 1984). This purified set of glycoproteins has some properties expected of a FN and a LN receptor (Horwitz et al., 1985; Akiyama et al., 1986) and appears to belong to a family of receptors for ECM molecules (Tamkun et al., 1986; Pytela et al., 1986). Recently it has been shown that the CSAT antibody inhibits LN- and FN-stimulated neurite regeneration from chick sensory and ciliary ganglion neurons, suggesting that proteins bound by the CSAT antibody have a role in neuron/matrix interactions (Bozyczko and Horwitz, 1986).

In this study, we have compared neurite outgrowth by ciliary ganglion (CG) neurons on simple and complex LN- and FN-containing substrates with that on non-neuronal cell surfaces. Although neurons extend processes on both classes of substrate, ECM-stimulated neurite outgrowth is distinguished from that on cell surfaces in its susceptibility to blockade by CSAT and JG22 antibodies. Our results suggest that (*a*) factors distinct from LN or FN function to stimulate outgrowth on cell surfaces and (*b*) other neuronal receptor systems, independent of proteins recognized by the CSAT and JG22 antibodies, are involved in cell surface-stimulated process outgrowth. Some of these results have been reported in abstract form (Bixby et al., 1986).

Materials and Methods

Animals

Fertile White Leghorn chicken eggs were purchased from Feather Hill Farm (Petaluma, CA) and kept at 38°C and 95% humidity until use. Newborn Sprague-Dawley rat pups were purchased from Bantin and Kingman (Fremont, CA).

Chemicals and Reagents

Laminin was purified from Engelbreth-Holm-Swarm (EHS) sarcoma tumors using published methods (Kleinman et al., 1982; Timpl et al., 1982). Fibronectin was purified from human plasma and was a generous gift of Dr. Janet Winter. Partially purified glial growth factor and forskolin used in Schwann cell cultures were generously provided by Dr. Greg Lemke. Rat tail collagen was prepared as described (Bornstein, 1958). DEAE-cellulose (DE-52) was from Whatman, Inc. (Clifton, NJ), DEAE Affi-Gel Blue was from Bio-Rad Laboratories (Richmond, CA), and EHS tumor extract (Matri-gel) was purchased from Collaborative Research, Inc. (Lexington, MA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Antibodies

The CSAT hybridoma cell line was kindly provided by Dr. Alan F. Horwitz, and the JG22 and 1A6 hybridomas were kindly provided by Dr. David Gottlieb. IgG was purified from ascites fluid on DEAE Affi-Gel Blue (Bruck et al., 1982) or by ammonium sulfate precipitation and ion exchange chromatography on DEAE-cellulose as described elsewhere (Hudson and Hay, 1980). F_{AB} fragments of JG22 and 1A6 IgG were produced by digestion with papain followed by chromatography on DEAE cellulose (Hudson and Hay, 1980). Rabbit antiserum to N-CAM and IgG from this serum were prepared as described (Bixby and Reichardt, 1987). Anti-LN antiserum was raised in New Zealand White rabbits against LN purified from the EHS sarcoma by Dr. Janet Winter. An antiserum to a partially purified, LN-containing rat myotube conditioned medium (CM) neurite outgrowth-promoting factor (NOPF) was raised in New Zealand White rabbits. The rat myotube CM-NOPF was partially purified using a combination of steps from the purification and characterization of other CM-NOPFs (Lander et al., 1985*b*). Briefly, rat myotube CM was incubated with gelatin-Sepharose to remove fibronectin, followed by centrifugation at 40,000 rpm for 13 h in a 45 Ti rotor (Beckman Instruments, Palo Alto, CA) to pellet the NOPF. The resuspended pellet was chromatographed on Sepharose CL-4B and the NOPF was recovered in fractions just behind the void volume (Winter, J., and L. Reichardt, unpublished observations). In addition to recognizing LN in protein blots of myotube CM, this serum blocks the neurite outgrowth-promoting activities of both LN- and myotube CM-coated substrates (Winter, J., and L. Reichardt, unpublished observations). Both anti-LN and anti-NOPF sera were heated at 56°C for 15 min to inactivate serum complement components before use. Rabbit anti-*alpha*-melanocyte stimulating hormone, which cross-reacts with chick neurofilaments (Drager et al., 1983), was purchased from Immuno Nuclear Corp. (Stillwater, MN). Goat anti-rabbit IgG coupled to fluorescein isothiocyanate (goat anti-rabbit FITC) and goat anti-mouse IgG coupled to rhodamine isothiocyanate (goat anti-mouse RITC) were purchased from Cappel Laboratories, Inc. (Malvern, PA).

Cell Culture

Ciliary ganglion neurons were enzymatically dissociated from embryonic day 8 (E8) chick ciliary ganglia (Nishi and Berg, 1977) and cultured as described (Bixby and Reichardt, 1985). Cell densities were 1,000 neurons/0.28 cm^2 well and 10–15,000 neurons/2 cm^2 well (96- and 24-well plates, respectively; Costar, Cambridge, MA). Chick myoblasts were prepared from E11 chick pectoral muscle and cultured as described (Bixby and Reichardt, 1985).

Embryonic chick fibroblasts were prepared from E11 pectoral muscle by culture of cells that bound to tissue culture plastic during the pre-plating step in the myoblast preparation. After growth for 2–4 d, these cells were removed with trypsin/EDTA and replated on collagen-coated coverslips in the same medium used to culture myoblasts.

Schwann cells were isolated from newborn Sprague Dawley rat sciatic nerve following the procedure of Brockes et al. (1979) and were a generous gift from Dr. Greg Lemke. Schwann cells were grown on poly-D-lysine (1 mg/ml in distilled H_2O) coated tissue culture dishes, in DME with 1.0 g/l glucose (DME H-16; UCSF Cell Culture Facility) supplemented with 10% fetal calf serum, partially purified glial growth factor (12 μg/ml), forskolin (2 μM), glutamine (2 mM), and penicillin/streptomycin (100 U/ml), following a procedure kindly provided by Dr. Greg Lemke. The partially purified glial growth factor used in these experiments was prepared from a bovine pituitary extract by differential ammonium sulfate precipitation and carboxymethyl cellulose chromatography (Brockes et al., 1980).

Astrocytes were derived from 1-d-old rat cortex following the procedure of Fallon (1985*b*) and were grown in DME H-16 plus 10% horse serum, 5% newborn calf serum, glutamine (2 mM), and penicillin/streptomycin (100 U/ml).

Rat fibroblasts were obtained by digesting E20 rat leg muscles with 20% trypsin for 10 min at 37°C. Cells in the supernatant were washed in sterile

PBS, plated in 100-mm tissue culture dishes and grown in DME with 4.5 g/l glucose (UCSF Cell Culture Facility) supplemented with 10% horse serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin.

Conditioned Media Preparation

Rat pheochromocytoma (PC12) cells, rat Schwannoma (RN22) cells, and bovine corneal endothelial (BCE) cells were cultured as described by Lander et al. (1985*a*). Schwann cells, astrocytes, and fibroblasts were cultured as described above. Medium that had been conditioned for 5–7 d over nearly confluent cultures was collected, filtered through 0.45-μm nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA), and stored at −20°C until use. Rat myotube CM was a generous gift from Dr. Janet Winter.

Substrate Preparation

LN and FN were diluted in Ca^{++}/Mg^{++}-free PBS to 10 μg/ml and 100 μg/ml, respectively (unless otherwise stated in figure legends). Approximately 100 μl was used to coat the 0.28-cm^2 surface area of a 96-well tissue culture plate well (Costar) by incubation overnight at 4°C. CM substrates were prepared by coating 0.28-cm^2 wells for 1 h at 25°C with 100 μl of a 1 mg/ml solution of poly-D-lysine in distilled water. After being washed extensively with distilled water and sterilized with UV irradiation, the wells were incubated with 100 μl of CM overnight at 4°C. LN-, FN-, and CM-coated wells were washed three times with sterile PBS before use. Glass coverslips coated with BCE cell ECM were a generous gift from Dr. Dennis Fujii (Fujii et al., 1982). Coverslips coated with a 2 M urea extract of EHS sarcoma ECM (Matri-Gel) were prepared following instructions provided by the supplier (Collaborative Research, Inc.). In brief, 500-μl aliquots of frozen matrix extract were thawed and used to coat 13-mm glass coverslips at 25°C for 15–20 min. The EHS sarcoma extract is a viscous liquid when cold, but upon warming, polymerizes into a semi-rigid gel. For experiments done with the anti-myotube CM-NOPF antiserum on EHS sarcoma extract, Matri-Gel was diluted 1:100 in sterile PBS and used to coat microwells as described for the preparation of CM substrates. For antibody-containing CG cultures, growth medium plus 2× concentration of antibody was added (50 μl for 0.28-cm^2 wells; 300 μl for 2-cm^2 wells) 30 min before addition of neurons in an equal volume of growth medium.

Island Co-cultures

Acid-cleaned 13-mm glass coverslips were placed in Costar 24 well plates and coated with LN (0.5 ml of 10 μg/ml) overnight at 4°C. Alternatively, some coverslips were coated with rat tail collagen as described above and sterilized by UV irradiation. After washing the coverslips three times with PBS, 0.5 ml of the appropriate cell culture medium was added to each well. Cloning rings (5 mm in diameter, 1.5 cm high) were made by cutting the bottoms from 500-μl polypropylene microfuge tubes. These were autoclaved and fixed to the center of LN-coated coverslips using autoclaved vacuum grease. Astrocytes, Schwann cells, and fibroblasts were passaged with trypsin/EDTA and 5,000–10,000 cells were added per cloning ring in 100 μl of growth medium. Cells were allowed to attach overnight and cloning rings were removed. These "island monolayers" were always grown at least 24 h before co-culture with CG neurons. For chick skeletal myotube island cultures, about 10,000 myoblasts were plated per cloning ring and allowed to fuse over several days. At the start of co-culture experiments, the culture medium was removed and replaced with 0.3 ml of CG neuron growth medium with a 2× concentration of the desired antibody. Dissociated neurons were then plated in an equal volume of growth medium and cultured for 16–24 h.

Immunofluorescence and Microscopy

Co-cultures were fixed with PBS plus 10% formalin and 5% sucrose for 15 min at 25°C. After permeabilization and blocking in PBS/0.1% saponin/5% goat serum for 15 min, the coverslips were incubated for 1–2 h at 25°C with rabbit anti-*alpha*-melanocyte stimulating hormone (1:500 dilution in PBS 0.1% saponin/1% goat serum) and/or a 1:500 dilution of 1A6 mouse monoclonal anti-N-CAM ascites. After being washed five times in PBS, coverslips were incubated in goat anti-rabbit FITC and/or goat anti-mouse RITC (1:100 dilution in PBS/1% goat serum) for 1 h. After being washed five times in PBS and once in distilled water, coverslips were mounted in Elvanol on microscope slides and viewed with a 63× (NA 1.4) oil immersion lens on a Zeiss microscope equipped with rhodamine and fluorescein optics. Coverslips were examined systematically for neuronal cell bodies, which were identified by their morphology and immunoreactivity with anti-*alpha*-melanocyte stimulating hormone or anti-N-CAM antibodies. Identified neurons were scored as having neurites if a process greater than two cell body diameters in length could be seen.

Detergent Extraction of Non-neuronal Cell Monolayers

Cultures of embryonic chick skeletal myotubes or chick muscle-derived fibroblasts on rat tail collagen-coated glass coverslips were extracted in sterile growth medium plus 0.1% Triton X-100 for 30 min at 37°C and then washed three times with sterile PBS before use. In one experiment, some myotube cultures were instead extracted with 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in the same medium. Cultures of astrocytes and Schwann cells that were extracted with growth medium plus 0.1% Triton X-100 for 10 min at 37°C were often lost from poly-D-lysine-coated glass coverslips during the subsequent PBS washes and culture conditions. Hence, astrocytes and Schwann cells were lightly fixed in PBS plus 1% formalin and 0.5% sucrose for 1 min at 25°C before detergent extraction.

Results

Characterization of the Interactions Required for Neurite Outgrowth on Simple and Complex ECM-derived Substrates

Dissociated CG neurons develop extensive networks of processes in <16 h when grown on LN-coated tissue culture plastic (Fig. 1 *A*). FN-coated substrates also stimulate process outgrowth by these neurons (Fig. 1 *B*), although a smaller percentage of neurons respond over this time period (Fig. 2). While the neurons adhere readily to surfaces coated with poly-D-lysine or rat tail collagen, little, if any, outgrowth is seen on either substrate in 16 h (Fig. 1, *C* and *D*). Rapid process outgrowth by CG neurons appears, therefore, to require more than simple adhesion to the substrate.

Two monoclonal antibodies, CSAT and JG22, which interfere with the attachment of many cell types to LN and FN (Greve and Gottlieb, 1982; Horwitz et al., 1985), essentially eliminate rapid neurite outgrowth by CG neurons on LN- and FN-coated substrates (Fig. 1, *E* and *F*; Fig. 2, *A* and *B*). Similar observations have been reported by Bozyczko and Horwitz (1986). Neurites that grow in the presence of CSAT and JG22 antibodies are rarely more than several cell diameters in length and are most often associated with the non-neuronal cells in the culture. The inhibition of LN-induced process outgrowth by CSAT or JG22 IgG is dose-dependent and is also seen with monovalent fragments of JG22 IgG (Fig. 2 *A*). In contrast, comparable concentrations of monovalent fragments of a monoclonal anti-N-CAM antibody, 1A6, do not affect neurite regeneration over the time course examined (Fig. 2 *A*). The effects of the antibodies are not due to cytotoxicity, since they are reversible (Fig. 1 *G*). Addition of CSAT IgG after process outgrowth on LN has occurred leads to the retraction of virtually all the neurites in the culture (Fig. 1 *H*).

Examination of CSAT- and JG22-containing cultures before fixation shows that the antibodies prevent the attachment of many of the neurons to the LN substrate, and thus could prevent neurite outgrowth as a consequence of their effects on adhesion. To distinguish the effects of the antibodies on adhesion from those on neurite extension, we studied the effects of CSAT antibody under conditions in which neuronal attachment is independent of LN. CG neurons were grown on a mixed LN/poly-D-lysine substrate, one which promotes vigorous process outgrowth (Fig. 1 *I*). In the presence of

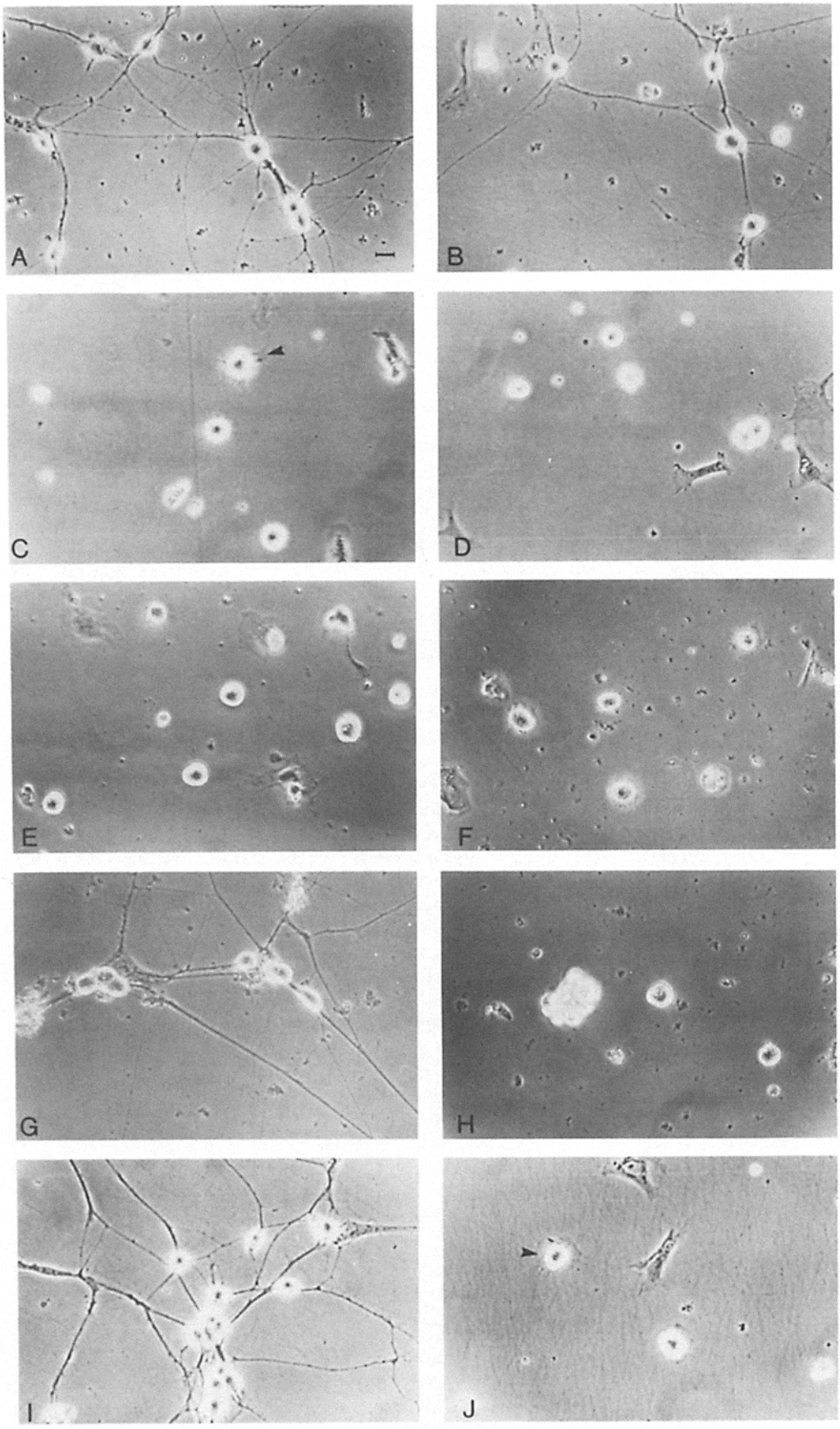

Figure 1. E8 chick CG neurons were grown for 12–16 h on various substrates in the presence or absence of CSAT or JG22 antibodies. After fixation in 2.5% glutaraldehyde/PBS, representative fields were selected and photographed. (*A*) CG neurons grown 16 h on a LN (10 μg/ml) substrate. (*B*) CG neurons grown 16 h on a FN (100 μg/ml) substrate. (*C*) CG neurons grown 16 h on a poly-D-lysine substrate. Arrowhead marks area of adhesion and spreading of neuronal cell soma. (*D*) CG neurons grown 16 h on a rat tail collagen substrate. (*E*) CG neurons grown 16 h on a LN substrate in the presence of 10 μg/ml CSAT IgG. (*F*) CG neurons grown 16 h on a FN substrate in the presence of 50 μg/ml JG22 IgG. (*G*) CG neurons grown 16 h on a LN substrate in the presence of 10 μg/ml CSAT IgG and then grown an additional 24 h after removal of the antibody. (*H*) CG neurons grown 16 h on a LN substrate in the absence of added antibody and then an additional 24 h in the presence of 50 μg/ml CSAT IgG. (*I*) CG neurons grown 16 h on a mixed LN/poly-D-lysine substrate. (*J*) CG neurons grown 16 h on a mixed LN/poly-D-lysine substrate in the presence of 10 μg/ml CSAT IgG. Process outgrowth is blocked despite attachment and spreading of neuronal cell bodies on poly-D-lysine (*arrowhead*). Bar, 10 μm.

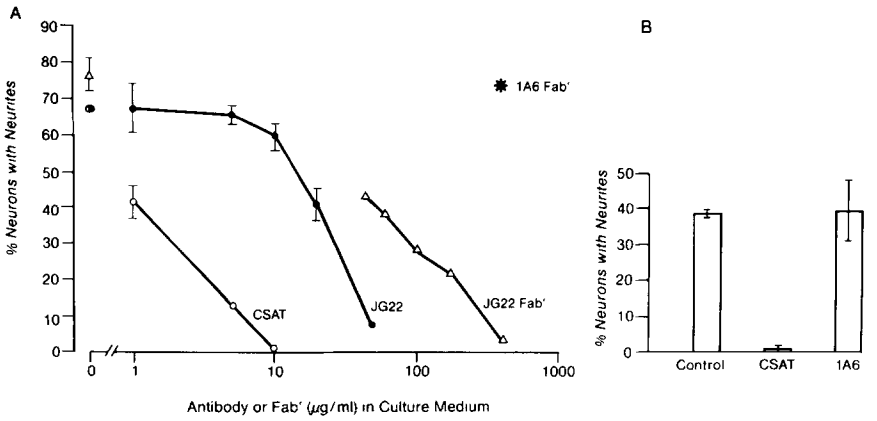

Figure 2. (*A*) Dose-dependent inhibition of LN-induced CG neuron process outgrowth by CSAT and JG22 antibodies. Neurons were cultured for 16 h on LN substrates (10 μg/ml) in varying concentrations of CSAT IgG (*open circles*), JG22 IgG (*closed circles*), JG22 F_{AB} (*open triangles*), or 1A6 monoclonal anti-N-CAM F_{AB} (*asterisk*). After fixation, the percentages of round, phase-bright neurons bearing processes greater than two cell diameters long were determined for duplicate cultures run in parallel and averaged. Error bars indicate the range of determinations made on duplicate cultures. The error bars associated with some data points from cultures containing CSAT and JG22 IgG are smaller than the symbols used to indicate the points. Data points for cultures containing JG22 and 1A6 F_{AB} represent single determinations made on individual cultures. (*B*) CSAT antibody blockade of FN-induced CG neuron process outgrowth. CG neurons were grown for 16 h on a FN (100 μg/ml) substrate in the presence of 10 μg/ml CSAT IgG or a 1:20 dilution of 1A6 ascites (~150 μg/ml IgG). After fixation, the percentages of neurite-bearing cells were determined for duplicate cultures run in parallel and averaged. Error bars indicate the range of determinations made on duplicate cultures.

CSAT IgG, process outgrowth on the mixed substrate is blocked, despite attachment and spreading of the neuronal cell bodies on the substrate (Fig. 1 *J*). Similar observations were made using a mixed LN/anti-N-CAM IgG substrate (not shown).

As LN is associated in vivo with LN-binding proteins and proteoglycans (Timpl et al., 1984; Dziadek and Timpl, 1985) we tested the effects of CSAT and JG22 antibodies on neurite regeneration by neurons grown on more complex LN-containing substrates. In one set of experiments, poly-D-lysine-coated substrates were incubated with medium conditioned by one of several different cell types, each of which has been shown to secrete a substrate-binding NOPF (cf. Adler et al., 1981; Lander et al., 1982). The neurite outgrowth-promoting activities of these factors appear to be associated with LN (Lander et al., 1985*a*,*b*; Davis et al., 1985; Calof and Reichardt, 1985). Neurite regeneration by CG neurons in response to these CM substrates is greatly reduced by CSAT and JG22 IgG at concentrations that block on purified LN substrates (Table I; Fig. 3, *E* and *F*). Adhesion of neurons to these poly-D-lysine/CM substrates is efficient even in the presence of CSAT or JG22 antibodies (compare Figs. 1 *C* and 3 *F*).

Process outgrowth on some of these CM substrates is also inhibited by antibodies to an LN-containing NOPF partially purified from rat myotube CM (Table I). This serum (anti-NOPF) prevents neurite outgrowth on substrates coated with LN, or CM from a variety of cell types, including rat myotubes (Winter, J., and L. Reichardt, unpublished observations), astrocytes, and Schwann cells (Table I). In agreement with previous observations (Manthorpe et al., 1983; Lander et al., 1983, 1985*a*), antibodies to LN are not able to inhibit the outgrowth-promoting activity of these CM substrates, despite the fact that LN is probably the active factor (Table I; for discussion see Lander et al., 1985*a*,*b*).

Two LN-containing substrates that resemble natural extracellular matrices are a 2-M urea extract of the ECM produced by the EHS sarcoma (Kleinman et al., 1986), and the ECM deposited by BCE cells grown in culture (Gospodarowicz et al., 1980). Both of these substrates stimulate vigorous process outgrowth by CG neurons (Table I; Fig. 3, *A* and *B*; see also Lander et al., 1982). In the presence of CSAT IgG, neurite outgrowth is greatly reduced on both ECM substrates (Table I, Fig. 3, *C* and *D*).

The anti-NOPF antibodies are also capable of inhibiting neurite outgrowth on the EHS sarcoma extract (Table I).

Table I. Percent Neurons with Neurites

Substrate	Control	CSAT	JG22	NRS	anti-LN	anti-NOPF
Laminin	67 ± 4	6 ± 0	–	76 ± 4.5	9 ± 2.5	11 ± 2
Rat Schwann cell CM	57 ± 1.5	–	7 ± 0.5	85 ± 0	81 ± 1	2 ± 1
Rat astrocyte CM	55 ± 4	–	13 ± 3	39 ± 3.5	41 ± 2.5	6 ± 0
Rat myotube CM	67 ± 6	17 ± 1.5	–	–	–	–
Rat fibroblast CM	50 ± 3	–	22 ± 0.5	–	–	–
Rat RN22 Schwannoma CM	25 ± 9.5	0	–	–	–	–
Rat PC12 CM	76 ± 3.5	7 ± 3.5	–	–	–	–
BCE CM	68 ± 6	5 ± 1	–	–	–	–
EHS sarcoma extract	74 ± 3	3 ± 0.5	–	61 ± 2.5	–	5 ± 1
BCE ECM	60	5	–	–	–	–

CSAT, JG22, and anti-NOPF antibodies block ciliary ganglion neuron process outgrowth on a variety of CM- and ECM-coated substrates. CG neurons were cultured 12–16 h in the presence or absence of added antibodies on substrates coated with laminin (5 μg/ml), non-neuronal cell-derived CM, the ECM deposited by BCE cells (BCE ECM) or an extract of the EHS sarcoma. After fixation, the percentage of neurite bearing CG neurons was determined. Each value represents the average and range of determinations made on duplicate cultures run in parallel, except for BCE ECM, which represents a single culture. At least 200 neurons were examined for each data point. Not all cultures were run simultaneously. Variability among experiments thus precludes comparison of the neuronal responses on different substrates. Antibodies used and their concentrations were: CSAT IgG (5 μg/ml in CM-substrate cultures; 100 μg/ml in BCE ECM, and EHS sarcoma extract cultures), JG22 IgG (100 μg/ml); normal rabbit serum, anti-LN serum, and an antiserum to an LN-containing CM-NOPF (anti-NOPF) were used at a 1:20 dilution.

Table II. Percent Neurons with Neurites

Non-neuronal cell monolayer/substrate	On cell island monolayer	On cell-free LN surround
Chick skeletal myotubes/LN		
Control	93, 95	94, 97
JG22 (530 μg/ml)	85, 94	2, 7
Rat Schwann cells/LN		
Control	91 ± 2.5	84 ± 2
JG22 (500 μg/ml)	89 ± 6	7 ± 1
CSAT (500 μg/ml)	80	1
Normal rabbit serum (1:20)	86 ± 2	79 ± 3
anti-NOPF (1:20)	82 ± 2.5	11 ± 3.5
anti-LN (1:20)	84 ± 1.5	7 ± 2.5
Rat astrocytes/LN		
Control	62 ± 2	75 ± 2.5
CSAT (100 μg/ml)	62 ± 0.5	1 ± 0.5
JG22 (500 μg/ml)	53 ± 1.5	3 ± 2.5
Normal rabbit serum (1:20)	72 ± 0.5	64 ± 0
anti-NOPF (1:20)	81 ± 2.5	14 ± 5
anti-LN (1:20)	77 ± 3	5 ± 0
Rat fibroblasts/collagen		
Control	32 ± 0.5	–
CSAT (100 μg/ml)	11 ± 2	–
JG22 (500 μg/ml)	11 ± 1	–
Chick fibroblasts/collagen		
Control	87	–
JG22 (530 μg/ml)	48	–

Effects of CSAT, JG22, and anti-NOPF antibodies in CG neuron/non-neuronal island monolayer co-cultures. CG neurons were cultured 16–20 h on substrates consisting of an island of non-neuronal cells 5 mm in diameter centered on a 13 mm LN-coated glass coverslip. Fibroblasts were cultured as confluent monolayers on rat tail collagen-coated coverslips. CSAT IgG, JG22 IgG, normal rabbit serum, anti-LN serum, anti-LN-containing NOPF serum (anti-NOPF), or antibody vehicle solution alone (control) were added to the cultures at the time of plating at the indicated concentrations. After immunofluorescent staining with anti-neurofilament or anti-N-CAM antibodies, the percentages of neurite-bearing CG neurons were counted on the cell island and on the LN-coated surround. Values represent the average and range for determinations made on duplicate cultures run in parallel, except for single values, which represent determinations on individual cultures. At least 200 neurons were examined for each data point.

They were not tested on the BCE ECM, because they do not affect outgrowth on substrates coated with CM from these same cells, probably because of restricted species cross-reactivity (Winter, J., and L. Reichardt, unpublished observations).

In summary, a variety of LN- and FN-containing substrates, both simple and complex, promote rapid and extensive neurite outgrowth by ciliary ganglion neurons. Monoclonal antibodies (CSAT and JG22) to a set of cell surface glycoproteins that mediate the interaction of cells with LN and FN can prevent this outgrowth, even in conditions in which neuronal adhesion does not depend on the function of molecules recognized by these antibodies.

Characterization of the Interactions Required for Neurite Outgrowth on Schwann Cell, Myotube, Astrocyte, and Fibroblast Cell Surfaces

Skeletal myotubes, Schwann cells, astrocytes, and fibroblasts are all potential substrates for axon growth during development and regeneration in vivo. Previous studies have shown that glial cells, compared with fibroblasts, are preferred substrates for process outgrowth in vitro (Noble et al., 1984; Fallon et al., 1985*a*,*b*). Skeletal myotubes, Schwann cells, and astrocytes make LN and/or FN in vitro (Sanes, 1982; Cornbrooks et al., 1983; Liesi et al., 1983, 1986; Price and Hynes, 1985), which could provide a stimulus for neurite outgrowth from neurons cultured on their surfaces. Experiments were designed to determine whether neurite outgrowth on the surfaces of these cells requires the same interactions as those necessary for outgrowth on the ECM and its constituents.

Each of the non-neuronal cell types was cultured as an "island" monolayer 5 mm in diameter on LN-coated 13-mm glass coverslips. CG neurons seeded onto the coverslips were grown for 16–24 h in the presence or absence of CSAT or JG22 IgG. After fixation, neuronal cell bodies and neurites were distinguished from the underlying non-neuronal cell monolayer by immunofluorescence using anti-neurofilament antibodies, and were scored for the presence or absence of neurites. Table II quantifies the results of these island co-culture experiments. In control cultures containing no antibody or antibody vehicle solution, extensive neurite regeneration occurs on myotube, Schwann cell, astrocyte, and fibroblast monolayers as well as on the surrounding LN substrate. In the presence of CSAT or JG22 IgG at concentrations far in excess of those required to block process outgrowth on LN substrates (up to 1 mg/ml of JG22 IgG), neurite outgrowth on the surrounding LN is almost completely blocked while that on myotube, Schwann cell, or astrocyte monolayers remains, and is quantitatively similar to controls (Table II). The possibility of more subtle effects of the antibodies, for example, on neuronal growth rates or branching patterns, was not examined in this study. Representative micrographs of myotube and Schwann cell island co-cultures are shown in Figs. 4 and 5, respectively. That functional CSAT and JG22 antibodies were present throughout the island co-culture period was demonstrated by removing the antibody-containing growth medium before fixation and showing that it could still block CG neurite outgrowth on LN (data not shown). The presence of CSAT or JG22 IgG in the co-cultures appeared to restrict the growth of neurites to cell surfaces. Examples of this were seen at the borders of the island monolayers where neurites growing on cell surfaces in the presence of CSAT IgG were apparently prevented from extending onto the adjacent LN substrate, which they often did in control cultures (data not shown).

In contrast to process outgrowth on Schwann cell, myotube, and astrocyte monolayers, growth on fibroblast monolayers is clearly sensitive to CSAT and JG22 antibodies. The percentage of neurite-bearing cells is reduced by ~50% on both rat and chick muscle-derived fibroblasts in the presence of CSAT or JG22 IgG (Table II). Neurites that grow on fibroblasts in the presence of these antibodies tend to be shorter than those in control cultures (Fig. 6). In co-cultures containing rat non-neuronal cells, any effects of the antibodies could be attributed to their binding to the neurons, since the epitopes recognized by CSAT and JG22 antibodies are not demonstrable on rat cells.

The observations that CSAT and JG22 antibodies block neurite outgrowth on substrates coated with LN-containing CM-NOPFs derived from Schwann cells, myotubes, and astrocytes (Table I), but fail to block growth on these same cell surfaces, suggest that cell-associated factors other than LN

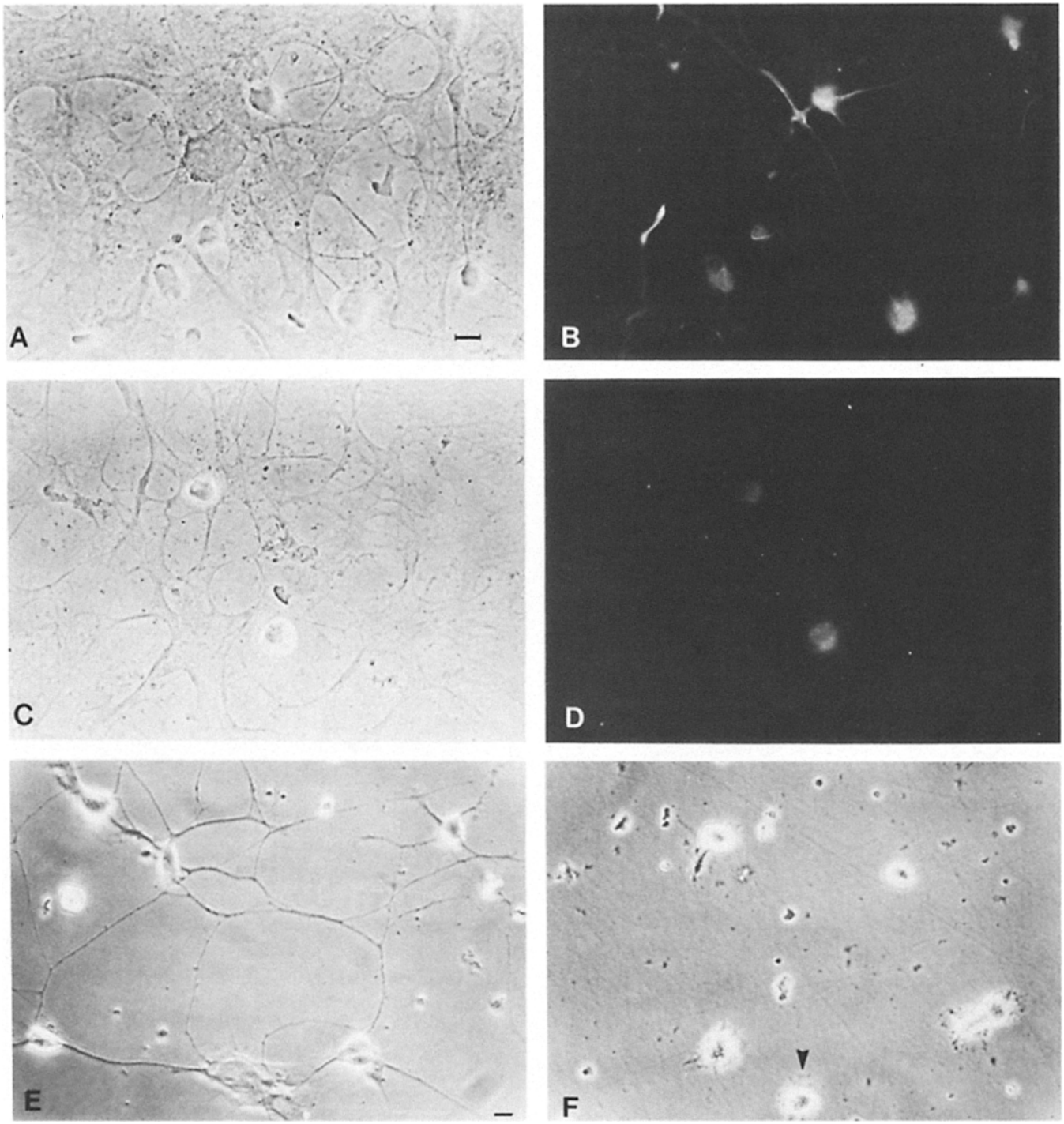

Figure 3. CSAT antibody blocks CG neuron process outgrowth on LN-containing BCE ECM (*A–D*) and BCE CM neurite outgrowth-promoting factor (*E* and *F*). (*A*) Phase contrast micrograph of CG neurons grown 16 h on a BCE ECM-coated glass coverslip in the absence of added antibody. Round, phase bright neuronal cell bodies can be seen against the ECM meshwork. (*B*) Anti-neurofilament immunofluorescent staining of the same field as in *A* to visualize neuronal cell bodies and neurites over the ECM meshwork. (*C*) Phase contrast micrograph of CG neurons grown 16 h on BCE ECM in the presence of 100 μg/ml CSAT IgG. (*D*) Anti-neurofilament immunofluorescent staining of the same field as in *C*. Note the absence of neurites in the CSAT-treated culture. (*E*) CG neurons grown 16 h on a BCE CM-coated substrate in the absence of added antibody. (*F*) CG neurons grown 16 h on BCE CM in the presence of 5 μg/ml CSAT IgG. Note the absence of neurites despite attachment and spreading of neuronal cell bodies (*arrowhead*). Bars, 10 μm.

may function to promote process outgrowth by CG neurons. Alternatively, cell surface–associated LN might stimulate process outgrowth via a CSAT/JG22-independent neuronal receptor system. Antisera capable of blocking the functions of LN in ECM complexes could provide more convincing evidence that something other than LN on glial or muscle cells promotes axon outgrowth. However, antisera raised against purified LN are generally unable to block outgrowth

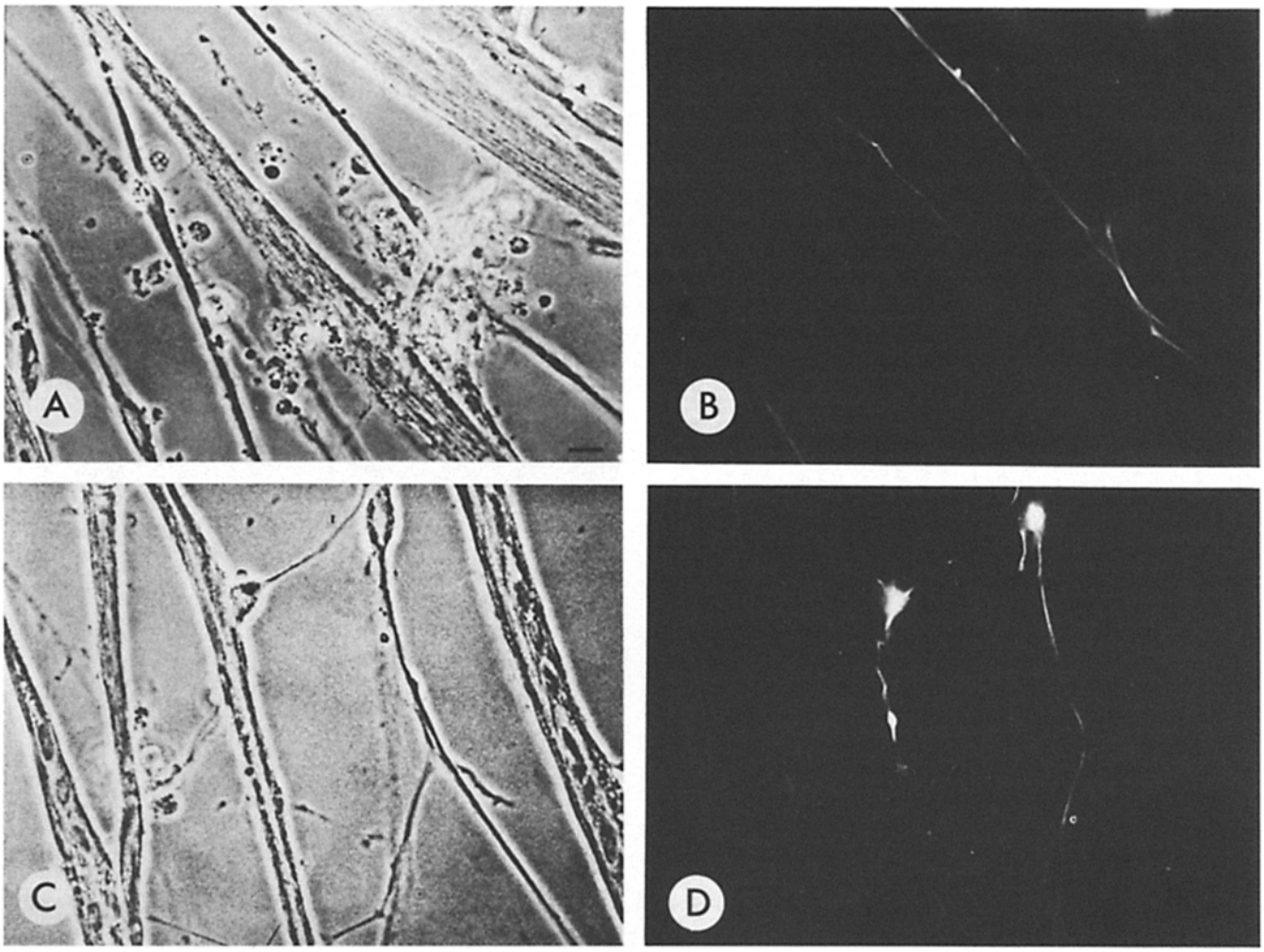

Figure 4. CG neuron process outgrowth on chick myotube cell surfaces is not blocked by high concentrations of JG22 IgG. (*A*) Phase contrast micrograph of CG neurons grown 20 h on embryonic chick myotubes in the absence of added antibody. (*B*) Anti-neurofilament immunofluorescent staining of the same field as in *A* to visualize neurites that have grown out over the myotube surface. (*C*) Phase contrast micrograph of CG neurons grown 20 h on embryonic chick myotubes in the presence of 530 μg/ml JG22 IgG. (*D*) Anti-neurofilament immunofluorescent staining of the same field as in *C*. Neurite outgrowth on myotube cell surfaces persists in the presence of high concentrations of JG22 IgG. Bar, 10 μm.

on more complex, LN-containing NOPFs (Table I; Lander et al., 1983, 1985*a*; Davis et al., 1985; Calof and Reichardt, 1985). We therefore used the anti-NOPF antiserum to examine this issue, since anti-NOPF inhibits neurite outgrowth on CM from non-neuronal cells and on the EHS ECM extract (Table I). The inclusion of anti-NOPF in Schwann cell and astrocyte island co-cultures at sufficiently high concentrations to inhibit outgrowth on either Schwann cell or astrocyte CM fails to inhibit process outgrowth by CG neurons on Schwann cell or astrocyte monolayers (Table II; Fig. 7). Antibodies to LN are also ineffective at blocking CG neurite outgrowth on Schwann cell or astrocyte cell surfaces, despite blocking on the surrounding LN substrate (Table II).

Detergent Extraction of Non-neuronal Cell Monolayers Reveals Two Classes of Neurite Outgrowth-promoting Activity Associated with Myotubes and Schwann Cells

The results of the island co-culture experiments suggest that cell surface–associated NOPFs distinct from LN may be involved in process outgrowth on glial and muscle cells. To determine if additional NOPFs are associated with cell membranes, as opposed to the ECM, we compared the abilities of myotube, Schwann cell, astrocyte and fibroblast monolayers to promote neurite outgrowth before and after extraction with detergent. Cultures of embryonic chick myotubes that have been extracted with the nonionic detergent Triton X-100 are not obviously diminished in their ability to elicit neurite outgrowth from CG neurons (Table III; Fig. 8, *A* and *B*). More than 70% of CG neurons grown on Triton X-100 extracted myotubes regenerate neurites within 20 h. Similar results were seen after extraction with the zwitterionic detergent CHAPS (not shown). However, in contrast to that on unextracted myotube surfaces, process outgrowth on the detergent-insoluble residue is almost completely blocked by JG22 antibodies (Table III; Fig. 8, *C* and *D*). Similarly, chick fibroblasts extracted with nonionic detergent support neurite outgrowth, and this is almost completely inhibited by JG22 antibodies (Table III). Since most of the Schwann cells were lost from the coverslip after even brief detergent extraction,

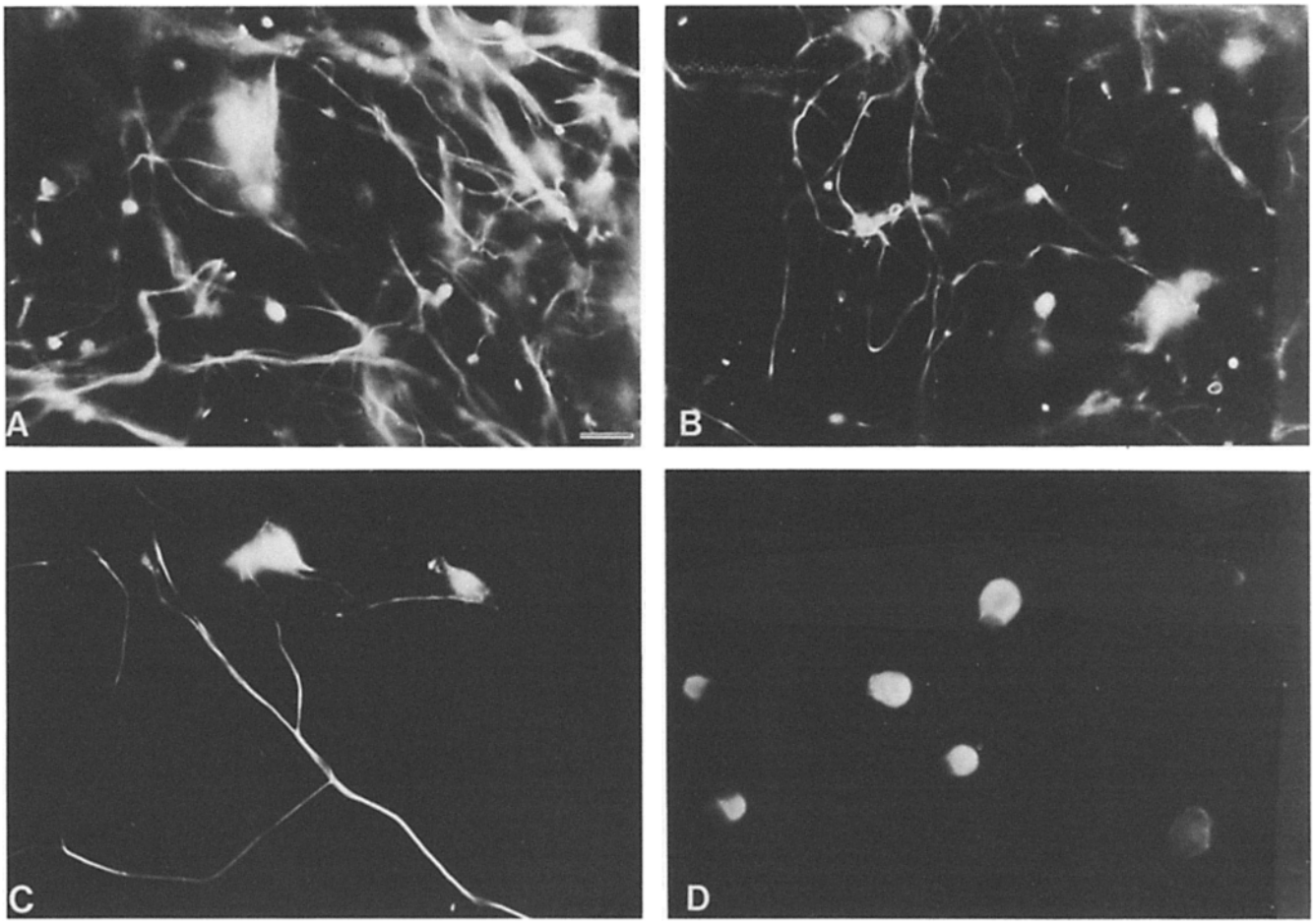

Figure 5. Effects of CSAT IgG on Schwann cell island/CG neuron co-cultures. Schwann cells were cultured as islands 5 mm in diameter on 13-mm LN-coated glass coverslips. 20,000 CG neurons were seeded onto these surfaces and cultured for 20 h in the absence (*A* and *C*) or presence (*B* and *D*) of 50 μg/ml CSAT IgG. Neuronal processes were visualized by immunofluorescent staining with anti-neurofilament antibodies. (*A*) Profuse neurite outgrowth on the Schwann cell island in control cultures. (*B*) Process outgrowth on the Schwann cell island persists in the presence of 50 μg/ml CSAT IgG. (*C*) Neurite growth on the LN substrate surrounding the Schwann cell island in the same culture as in *A* in the absence of added antibody. (*D*) Neurite outgrowth on the surrounding LN substrate in the culture shown in *B* is completely blocked by 50 μg/ml CSAT IgG. Bar, 10 μm.

these cells were lightly fixed (1% formalin for 1 min) before extraction with Triton X-100. Table III shows that light fixation does not noticeably affect the ability of Schwann cells or myotubes to promote neurite outgrowth. A high percentage of neurons regenerate neurites on these cells after fixation and, like that on unfixed cells, this outgrowth is not blocked by CSAT or JG22 antibodies (Table III). Schwann cells that have been fixed and extracted still promote neurite outgrowth, but this activity can now also be inhibited by CSAT IgG (Table III). In contrast, detergent extraction of fixed astrocytes eliminates their ability to support rapid neurite outgrowth (Table III; Fig. 8, *E* and *F*).

Since myotubes and Schwann cells both produce a variety of ECM molecules, including LN, in vitro (Sanes, 1982; Bayne et al., 1984; Cornbrooks et al., 1983; Carey et al., 1983) it is likely that the residual CSAT/JG22-sensitive neurite outgrowth-promoting activity of detergent-extracted myotubes and Schwann cells is associated with the ECM. To examine this issue further, CG neurons were grown on the residue from fixed and extracted Schwann cells in the presence of the anti-NOPF serum. Despite the loss of most of the visible Schwann cell residue from the substrate in these cultures, >60% of CG neurons developed processes on the remaining substrate-associated material (Table III). The outgrowth-promoting activity of this Schwann cell microexudate, like that found in Schwann cell CM, is inhibited by antibodies to the LN-containing NOPF (Table III).

Discussion

Our observations lead to two main conclusions concerning neurite outgrowth in vitro. First, the ability of both simple and complex ECM-derived substrates to stimulate neurite outgrowth depends on the function of the neuronal glycoproteins recognized by CSAT and JG22 antibodies. Second, neuronal process outgrowth on some non-neuronal cell surfaces involves at least two distinct classes of interaction, one of which is independent of receptors recognized by CSAT and JG22 antibodies.

Proteins recognized by CSAT and JG22 antibodies have been identified on embryonic chick peripheral and retinal neurons and appear to be involved in neuronal attachment and growth on LN, FN, and collagen substrates (Bozyczko and Horwitz, 1986; Hall et al., 1987). Our findings are con-

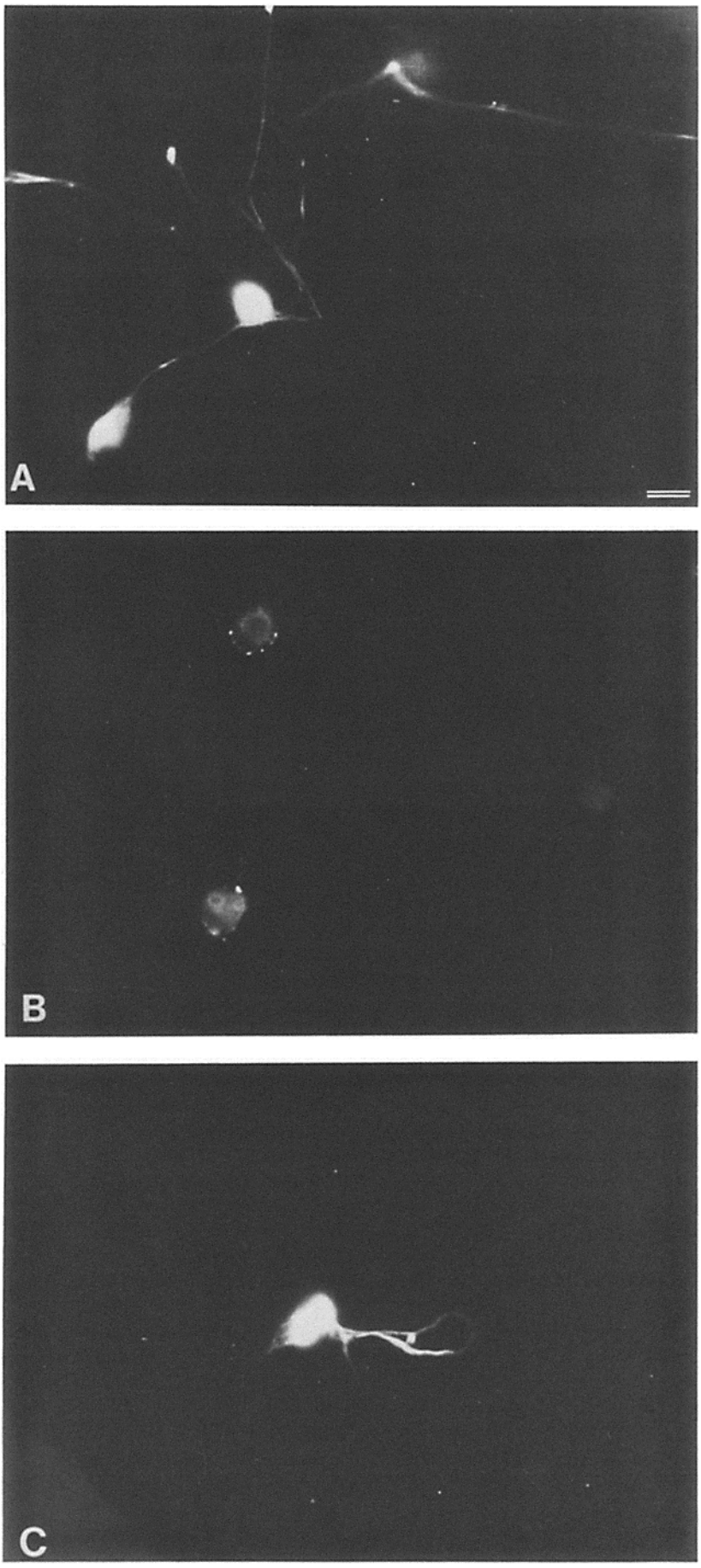

Figure 6. CSAT antibody inhibits neurite outgrowth by CG neurons on fibroblast monolayers. CG neurons were cultured for 20 h on a monolayer of rat muscle-derived fibroblasts in the absence (*A*) or presence (*B* and *C*) of 100 μg/ml CSAT IgG. CG neuronal cell bodies and neurites were visualized by immunofluorescent staining with anti-neurofilament antibodies. (*A*) CG neuron process outgrowth on a fibroblast monolayer in the absence of antibodies (control culture). (*B*) Group of CG neurons cultured on a fibroblast monolayer in the presence of 100 μg/ml CSAT IgG. Process outgrowth from this group of neurons is blocked. (*C*) A neuron from the same culture as those shown in *B*. Neurites that grow on fibroblast monolayers in the presence of CSAT IgG are shorter than in control cultures. CSAT IgG had no noticeable effects on the fibroblast monolayer. Bar, 10 μm.

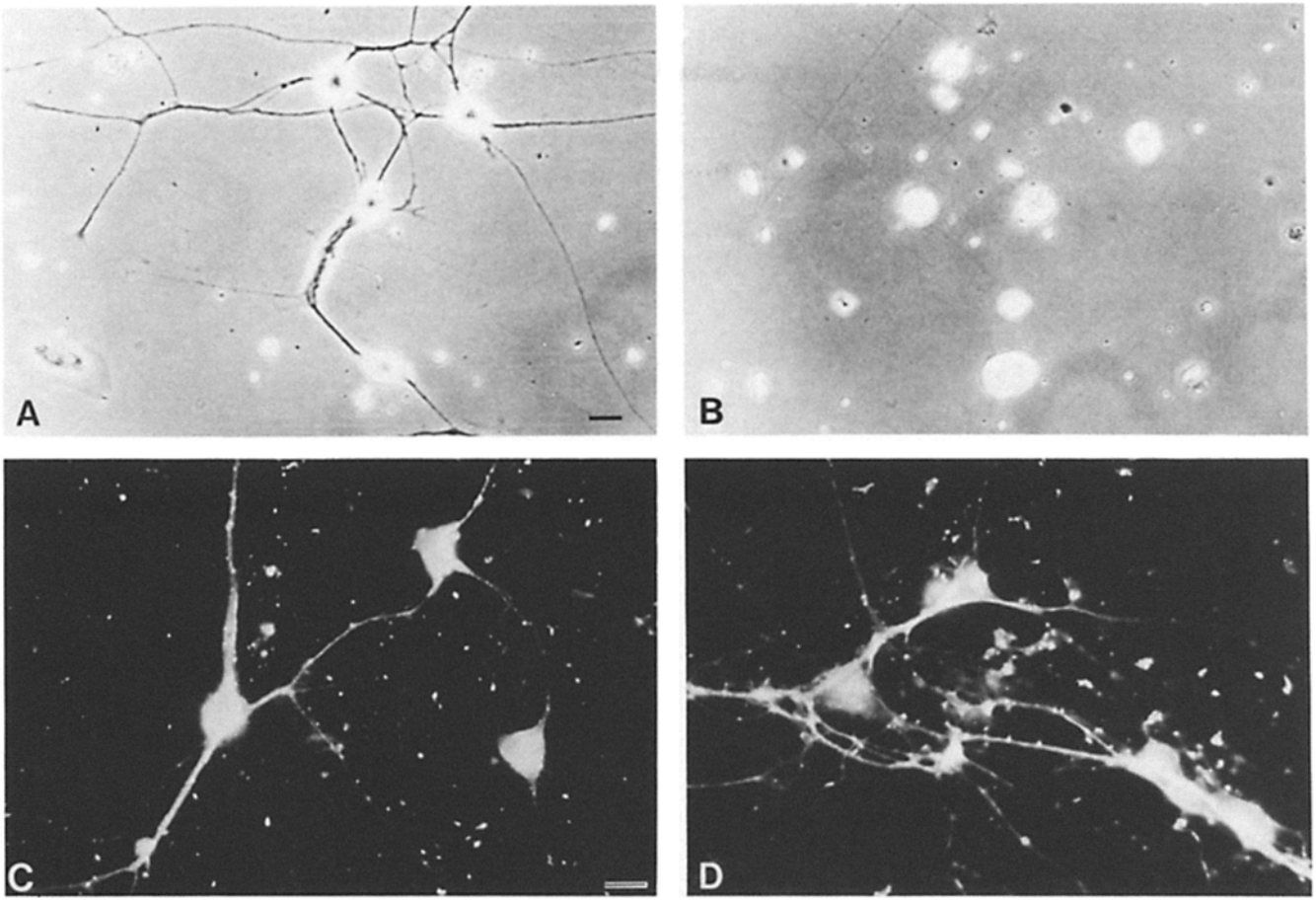

Figure 7. Antibodies to an LN-containing CM-NOPF block process outgrowth on astrocyte CM but not on astrocyte cell surfaces. CG neurons were grown 16–20 h on substrates coated with astrocyte CM (*A* and *B*) or on astrocyte cell surfaces (*C* and *D*). CG neurons grown on astrocyte monolayers were visualized by immunofluorescent staining with the 1A6 monoclonal anti-N-CAM antibody. Neurite outgrowth on astrocyte CM (*A*) is blocked by a 1:20 dilution of anti-NOPF antiserum (*B*). Process outgrowth on astrocyte cell surfaces (*C*) is not noticeably affected by a 1:20 dilution of the anti-NOPF antiserum (*D*). Bars, 10 μm.

sistent with these studies, and extend them in two ways. First, although CSAT and JG22 antibodies largely prevent attachment of neurons and their neurites to LN and FN substrates, our mixed substrate experiments demonstrate that these antibodies also prevent neurite outgrowth induced by LN in conditions where attachment is not noticeably affected. Second, the inhibition by CSAT/JG22 of neurite outgrowth on CM factors from non-neuronal cells as well as on intact ECMs suggests that these proteins are required for the function of receptors capable of recognizing LN and/or FN in a variety of contexts.

Whereas process outgrowth on intact Schwann cells and myotubes is not grossly affected by CSAT/JG22 antibodies, outgrowth on detergent-extracted cells is inhibited. These observations suggest that Schwann cells and myotubes normally have at least two mechanisms for stimulating neurite extension. These mechanisms involve an ECM-associated NOPF whose action depends on the proteins recognized

Table III. Percent Neurons with Neurites

	Fixed only		Fixed and extracted				Extracted only	
Cell monolayer	Control	CSAT/JG22	Control	CSAT/JG22	Normal rabbit serum	anti-NOPF	Control	CSAT/JG22
Chick myotube	96	97	–	–	–	–	82, 60	7, 16
Rat Schwann cell	91	92	62 ± 3.5	13 ± 3.5	66 ± 3	3 ± 0.5	–	–
Rat astrocyte	90	90	9 ± 1.5	9 ± 0.5	–	–	–	–
Chick fibroblast	–	–	–	–	–	–	87	23

CG neuron outgrowth on fixed and/or detergent-extracted cell surfaces was studied in the absence (control) or presence of CSAT IgG (100 μg/ml in astrocyte cultures) or JG22 IgG (500 μg/ml in myotube, Schwann cell, and fibroblast cultures) and in the presence of normal rabbit serum (1:25 dilution), or an antiserum to an LN-containing NOPF (anti-NOPF, 1:25 dilution). Some non-neuronal cell monolayers were fixed in 1% formalin/PBS for 1 min at 25°C before co-culture. Detergent extraction was in growth medium plus 0.1% Triton X-100 for 10 min (Schwann cells and astrocytes) or 30 min (myotubes and fibroblasts) at 37°C. The percentages of neurite-bearing neurons after 16–20 h of growth were counted after immunostaining cell bodies and neurites with anti-neurofilament antibodies. Values represent the average and range of determinations made on duplicate cultures run in parallel, except for single values, which represent separate determinations made on individual cultures. At least 200 neurons were examined for each data point.

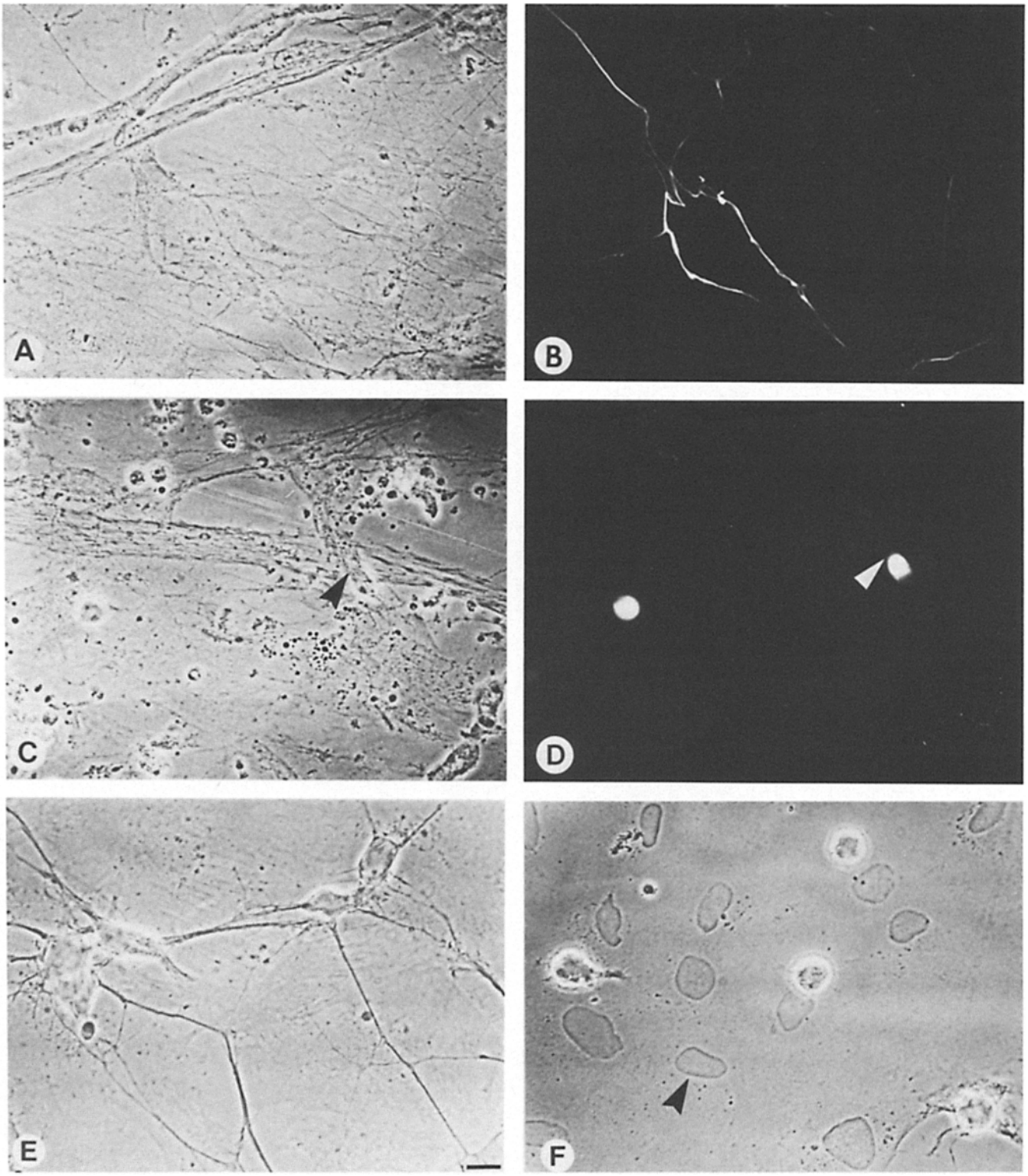

Figure 8. CG neuron process outgrowth on detergent extracted chick skeletal myotubes and on fixed and extracted astrocytes. CG neurons were grown 16–20 h on cultures of chick myotubes that had been extracted with 0.1% Triton X-100 for 30 min at 37°C (*A–D*) or on astrocyte monolayers that were either fixed in 1% formalin/PBS for 1 min at 25°C (*E*) or fixed and then extracted with 0.1% Triton X-100 for 10 min at 37°C (*F*). (*A*) Phase contrast micrograph of CG neurons grown on detergent-extracted myotubes in the absence of added antibodies. (*B*) Anti-neurofilament immunofluorescent staining of the same field as in *A* showing neurites growing on the detergent-insoluble myotube fraction. (*C*) Phase contrast micrograph of CG neurons grown on detergent-extracted myotubes in the presence of 530 μg/ml JG22 IgG. (*D*) Anti-neurofilament immunofluorescent staining of same field as in *C*. JG22 IgG blocks process outgrowth on detergent-extracted myotubes. Arrowheads in *C* and *D* point to a neuronal cell soma in contact with an extracted myotube. (*E*) Phase contrast micrograph of CG neurons grown 16 h on fixed astrocytes. (*F*) Phase contrast micrograph of CG neurons grown 16 h on fixed and extracted astrocytes. No residual neurite outgrowth-promoting activity remains associated with fixed astrocytes after detergent extraction. Arrowhead in *F* marks the nucleus of a fixed and extracted astrocyte. Bars, 10 μm.

by CSAT and JG22 antibodies, and a cell membrane-associated NOPF, whose action is not sensitive to these antibodies. On Schwann cells and myotubes, the membrane-associated NOPF can be extracted with nonionic detergent, leaving behind a matrix-associated NOPF. This is consistent with the observation that skeletal myotubes are capable of synthesizing and secreting an ECM that contains LN and/or FN in vitro (Bayne et al., 1984). In the case of Schwann cells, LN is likely to be the active component of the detergent-resistant NOPF, since neurite outgrowth on the microexudate remaining after fixation and detergent extraction is blocked by an antiserum to an LN-containing NOPF (anti-NOPF). Although Schwann cells apparently do not assemble an intact ECM when cultured in the absence of neurons (Carey et al., 1983), LN is associated with their cell surfaces (Cornbrooks et al., 1983).

Astrocytes appear to differ from myotubes, Schwann cells, and fibroblasts by their lack of a detergent-resistant, CSAT/JG22 antibody-sensitive stimulus for neuronal growth. Though astrocytes can produce LN and FN in vitro, they apparently do not incorporate them under standard culture conditions (Liesi et al., 1983, 1986; Price and Hynes, 1985). The differences observed in vitro between the NOPFs present on astrocytes compared with those on other cell types may reflect the relative contributions of ECM molecules to axon growth in the central nervous system as compared with the periphery. Glial precursors, which are likely to interact with growing axons in the developing central nervous system, may differ from the astrocytes examined in this study.

The demonstration of a potent cell-surface mechanism for the induction of neurite outgrowth raises the question of the identity of the molecules involved. Since neurite outgrowth is clearly dependent on adhesion, cell surface molecules previously identified on the basis of their adhesion-promoting properties are likely candidates for mediating neuronal interactions with non-neuronal cell surfaces. For myotubes these include N-CAM, N-cadherin, and J1, or cytotactin (Rutishauser et al., 1983; Hatta et al., 1985; Kruse et al., 1985; Grumet et al., 1985; Sanes et al., 1986). Similarly, Ng-CAM (or L1) (Grumet et al., 1984; Lindner et al., 1983) and J1, or cytotactin, are present on astrocytes and could be important in neuronal growth on glial cells. The relatively poor growth of neurons on fibroblasts in the presence of CSAT or JG22 antibodies could reflect the paucity of neuronal adhesion molecules associated with the fibroblast surface.

The molecules responsible for neurite outgrowth on defined substrates in vitro are likely to be important for axonal growth and guidance in vivo. For neurons in peripheral sensory and sympathetic ganglia, motoneurons in the spinal cord, and ganglion cells in the neural retina, axon initiation takes place in regions containing the ECM molecules LN and/or FN (Rogers et al., 1986; Adler et al., 1985; see also Easter et al., 1984). Continued axon growth toward the synaptic target occurs along pathways comprising cell surfaces and ECM (Tosney and Landmesser, 1985; Silver and Rutishauser, 1984; Rogers et al., 1986). Recent experiments suggest that both types of neuronal interactions are important in vivo. Antibodies that block cell-matrix interactions and N-CAM function have both been shown to disrupt the development of the chick retino-tectal projection (Halfter and Deiss, 1986; Thanos et al., 1984; Silver and Rutishauser, 1984). Changes in the response of retinal neurons to different substrates may influence axon growth at different stages of development. Between E6 and E11, a time that correlates with the movement of retinal ganglion cell growth cones from eye cup to the optic nerve and tectum, these neurons lose the ability to interact with LN and FN, but remain able to interact with collagen and to extend neurites on astrocyte cell surfaces (Cohen et al., 1986; Hall et al., 1987).

In summary, our studies suggest that molecularly distinct mechanisms for axon growth exist on individual neurons in the form of receptors that are selective for ECM- and cell surface-associated NOPFs. Furthermore, some cells, e.g., Schwann cells and skeletal myotubes, produce at least two classes of NOPFs to which neurons can respond. Developmental regulation of these NOPFs and their corresponding neuronal receptors is likely to play an important part in neuronal growth and pathway selection.

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Note Added in Proof. In subsequent studies we have measured and compared neurite lengths on astrocyte and myotube cell surfaces in the presence or absence of CSAT or JG22 antibodies. The overall rate of process outgrowth on either of these cell types is diminished by these antibodies, implying that molecules recognized by CSAT and JG22 function in neuronal process outgrowth on intact cells as well as on detergent-extracted cells. These results also demonstrate that astrocytes stimulate neuronal process outgrowth by at least two mechanisms, one of which functions through matrix receptors. We are currently studying molecules that may constitute the cell membrane-associated NOPFs present on these cells.

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