Extension of Neurites on Axons Is Impaired by Antibodies against Specific Neural Cell Surface Glycoproteins

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Abstract. We have developed an in vitro assay which measures the ability of growth cones to extend on an axonal substrate. Neurite lengths were compared in the presence or absence of monovalent antibodies against specific neural cell surface glycoproteins. Fab fragments of antibodies against the neural cell adhesion molecule, NCAM, have an insignificant effect on the lengths of neurites elongating on either an axonal substrate or a laminin substrate. Fab fragments of polyclonal antibodies against two new neural cell surface antigens, defined by mAb G4 and mAb F11, decrease the lengths of neurites elongating on an axonal substrate, but have no effect on the lengths of neurites elongating on a laminin substrate. G4 antigen is related to mouse L1, while F11 antigen appears to be distinct from all known neural cell surface glycoproteins.

Our results suggest that the G4 and F11 antigens help to promote the extension of growth cones on axons.

AMONG the many cues which guide the axons of embryonic neurons to their proper destinations, neuronal cell surfaces may be an important source of information. Extending axons are frequently observed to elongate directly upon the surfaces of pre-existing axons. This has been described for developing optic axons of the crustacean *Daphnia magna* (Lopresti et al., 1973), for developing mandibular axons of *Xenopus laevis* (Davies et al., 1982), and for developing peripheral neurons in the grasshopper *Schistocerca nitens* (Ho and Goodman, 1982). In the chick embryo, motoneurons appear to prefer the surfaces of other motoneuron axons as opposed to non-neuronal or extracellular surfaces (Al-Gaith and Lewis, 1982; Tosney and Landmesser, 1985). In the central nervous system of the grasshopper embryo, elongating neurites choose to grow upon the surfaces of specific axons, and the ablation of these axons results in abnormalities in outgrowth (Raper et al., 1983, 1984). Similar results have been obtained in the spinal cord of a fish (Kuwada, 1986).

To identify axonal surface molecules that mediate neurite extension, we have developed an assay which measures the distance growth cones extend on an axonal substrate. Neurite lengths can then be compared in the presence or absence of Fab fragments of antibodies directed against specific neural cell surface molecules. The antibodies used in this study were directed against the neural cell adhesion molecule (NCAM)[1] (for reviews see Edelman, 1984; Rutishauser, 1984); and against two recently purified neural cell surface antigens, defined by mAb's G4 and F11 (for full details, see Rathjen et al., 1987, in this issue). Polyclonal antisera against the G4 and F11 antigens stain components of 135 kD on immunoblots of adult chick brain membrane proteins. G4 antigen is related to mouse L1 (described by Rathjen and Schachner, 1984), but F11 antigen appears to be different from any neural cell surface glycoprotein described to date. We report that antibodies against the F11 and G4 antigens significantly reduce the average length of chick sympathetic neurites growing on chick sympathetic axons. They do not affect the distance sympathetic neurites grow on a uniform laminin substrate. Antibodies against NCAM do not significantly reduce the average lengths of chick sympathetic neurites extending on either sympathetic axons or laminin substrates.

Materials and Methods

Media

F12 complete medium was F12 (Gibco, Grand Island, NY) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, 6 g/liter glucose, 2% nonessential amino acid mix (Gibco), 10^{-5} M cytosine arabinoside, 10^{-5} M fluorodeoxy uridine, 5% FCS (Gibco), 5% chick serum (Gibco), hormones as described by Bottenstein et al. (1980), and 20 ng/ml 7S nerve growth factor (Sigma Chemical Co., St. Louis, MO). A-F12 was F12 supplemented only with glutamine, penicillin, streptomycin, and glucose. 1-d heart-conditioned medium (HCM) was prepared as described

1. *Abbreviations used in this paper*: anti-F11 Fabs, Fab fragments of polyclonal antibodies raised against antigens immunopurified by mAb F11; anti-G4 Fabs, Fab fragments of polyclonal antibodies raised against antigens immunopurified by mAb G4; anti-NCAM Fabs, Fab fragments of polyclonal antibodies raised against NCAM; CMF-Ea medium, Ca^{++}/Mg^{++}-free medium; HCM, heart-conditioned medium; NCAM, neural cell adhesion molecule.

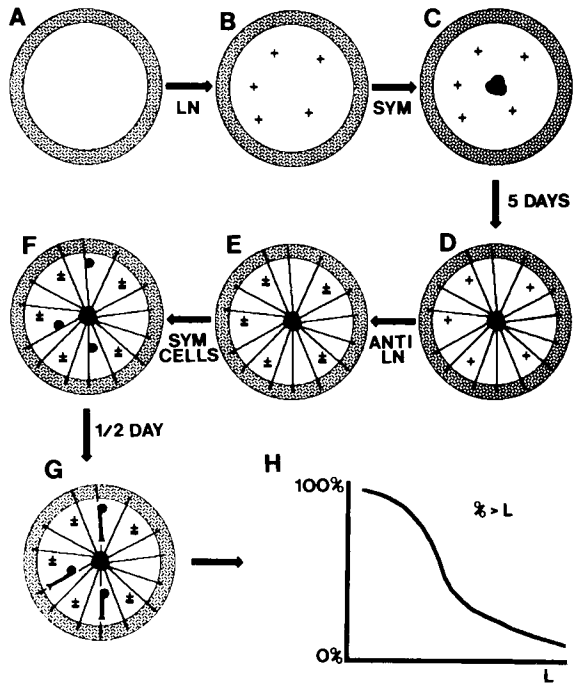

Figure 1. Schematic representation of the axonal substrate bioassay. Shaded area around edge of coverslip indicates ring of fixed cells; laminin is schematically shown as +, anti-laminin as −; an explant is drawn in the middle of the coverslip with fascicles radiating out to the ring of fixed cells. Test neurons are drawn on the fascicles as round dots which have elongated neurites after 0.5 d. Neurite lengths are displayed as the percentage of neurons with neurites (vertical axis) longer than a given length (horizontal axis). For details see text.

by Nishi and Berg (1977) in A-F12 plus fetal calf and chick sera. Ca^{++}/Mg^{++}-free Eagle's (CMF-Ea) medium and Hanks' buffered salt solution were prepared in the Institute and were essentially the same as that available from Gibco, except that Ca^{++} and Mg^{++} were omitted.

Preparation of Axonal Substrates

Round glass coverslips 9 mm in diameter were cleaned in boiling nitric acid, silanized in 10% 3-(triethoxysilyl)-propylamine in DMSO for 3–4 h at 110°C, washed repeatedly with water, and sterilized by UV irradiation. Silicon plugs (Wacker Silicon) 5 mm in diameter and 3 mm thick were placed in the center of each coverslip, and the coverslips dropped into wells of a 48-well plate (Costar, Cambridge, MA).

The brain and retinas of an embryonic day 7 (E7) chick were mechanically dissociated by trituration in 5 ml of CMF-Ea medium, centrifuged at 50 *g* for 10 min, and resuspended in 24 ml of A-F12. 0.5 ml of this cell suspension was added to each well of a 48-well plate containing coverslips prepared as described above. After 30–60 min at 37°C and 5% CO_2, the coverslips were removed from the wells, washed twice in Hank's, and the attached cells were fixed in ice cold methanol/acetone (1:1) for 3–5 min. After fixation, the methanol/acetone was allowed to evaporate, the silicon plugs were removed, and the coverslips were air dried for another 2–4 h. This results in a ring of fixed cells around the periphery of the coverslip.

Laminin (Bethesda Research Laboratories, Bethesda, MD) was applied to the entire surface of coverslips prepared as above by sandwiching 25 μl of laminin solution (20 μg/ml in Hanks') between two coverslips. After 2 h at 37°C, the laminin solution was washed off in Hanks' and the coverslips dropped into wells of a 48-well plate containing 0.5 ml of medium (F12 complete or 50% 1-d HCM and 50% F12 complete). A ganglion excised from E9 sympathetic chains (an age at which neurite extension is vigorous and non-neuronal cells are relatively scarce) was positioned in the center of each coverslip and the explants were cultured at 37°C in 5% CO_2. Explants which had grown symmetrically onto the ring of fixed cells after 5 d in culture were selected for use as axonal substrates. Rabbit anti-laminin antibodies (Bethesda Research Laboratories, or the kind gift of Gerd Klein, Friedrich Miescher Laboratorium, Tuebingen, FRG) were added to these wells at a final dilution of between 1:300 and 1:600. This was sufficient to neutralize the laminin-treated glass without dislodging the tips of the sympathetic axons from the annulus of fixed cells.

Preparation of Test Neurons

Chick E9 sympathetic chains were dissected and stained with a solution of TRITC (Sigma Chemical Co.; isomer "R") prepared as follows: 0.1 mg of TRITC was dissolved in 2 μl DMSO; added, with vigorous vortexing, to 1 ml of Hanks'; and further diluted 1:10 with Hanks' to make the final staining solution. Sympathetic chains were stained for 30 min in the dark at room temperature, and then incubated in F12 complete medium for 1 h at 37°C in 5% CO_2. They were then trypsinized (1 mg/ml trypsin in Hanks') for 15 min at 20°C, washed with Hanks' and soybean trypsin inhibitor (0.1 mg/ml), triturated, centrifuged at 50 *g* for 10 min, and resuspended in F12 complete medium. These cells were plated onto each of the prepared axonal substrates (7.5×10^3/well) and cultured for 15–16 h at 32°C.

Test Antibodies

Antibodies and Fab fragments to G4 and F11 antigens were prepared as in the preceding paper by Rathjen et al. (1987), and stored at −70°C in CMF-Ea medium. They were generally added to the cultures at the same time that the test neurons were added, and to a final concentration of between 0.06 and 2 mg/ml. Control cultures received only the equivalent volume of CMF-Ea medium.

Anti-NCAM Fabs were either the kind gift of Dr. Urs Rutishauser (Case Western Reserve University, Cleveland, OH), Jonathan Covault (Washington University, St. Louis, MO), or were prepared after injection of rabbits with material purified from a monoclonal affinity column also provided by Dr. Rutishauser. Our anti-NCAM Fabs at 1 mg/ml inhibited retinal cell-cell adhesion by 90% over the course of 60 min, and in the assay described in the preceding paper, caused retinal fibers growing on tectal membranes to unbundle. The preparations from Dr. Rutishauser inhibited retinal cell-cell adhesion by 95%. The preparations from Dr. Covault inhibited brain vesicle aggregation, nerve-muscle adhesion, and bundling of chick dorsal root ganglion neurites growing on collagen.

Gel Electrophoresis

SDS PAGE was carried out in 7% acrylamide gels according to Laemmli (1970). The molecular mass markers were from Bio-Rad Laboratories (Richmond, CA). Transfer of proteins from SDS PAGE onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) was performed according to Towbin et al. (1979) and subsequent immunodetection of antigens was according to Wolff et al. (1985).

Data Analysis

Cultures were fixed in 4% paraformaldehyde and 10% sucrose dissolved in PBS. Coverslips were carefully transferred out of the 48-well plates into Petriperm (Heraeus-Amersil, Sayreville, NJ) petri dishes, and viewed on a Zeiss inverted microscope with a 40× neofluor lens. Test neurons were visualized by their rhodamine fluorescence. The distance between the center of each cell body and the tip of its longest neurite was then measured with the aid of a microcomputer (Apricot) equipped with a mouse and a program written by J. A. Raper. Neurites were counted only if they (*a*) were growing exclusively on the axonal substrate, (*b*) were longer than the diameter of the cell body, (*c*) had not collided into other test neurites, and (*d*) did not come from a clump of cells. The straight line measured does not exactly correspond to the actual length of curved neurites, and especially the lengths of longer neurites may therefore be underestimated. Usually 100–200 neurites from a set of four coverslips were measured per experimental condition. The nonparametric Wilcoxon rank test was used to determine whether antibody-treated and control neurite lengths were statistically different. We considered a one-tailed P value of <0.05 to be significant.

Neurite Outgrowth on Laminin

Coverslips were coated with laminin as described above, and placed into the wells of a 48-well plate. Chick E9 sympathetic neurons were stained with TRITC and dissociated as described above. They were then plated at a density of between 7.5×10^3 and 1×10^4 cells per well, and cultured in 0.2 to 0.3 ml of either 50% 1-d HCM/50% F12 complete medium, or 100%

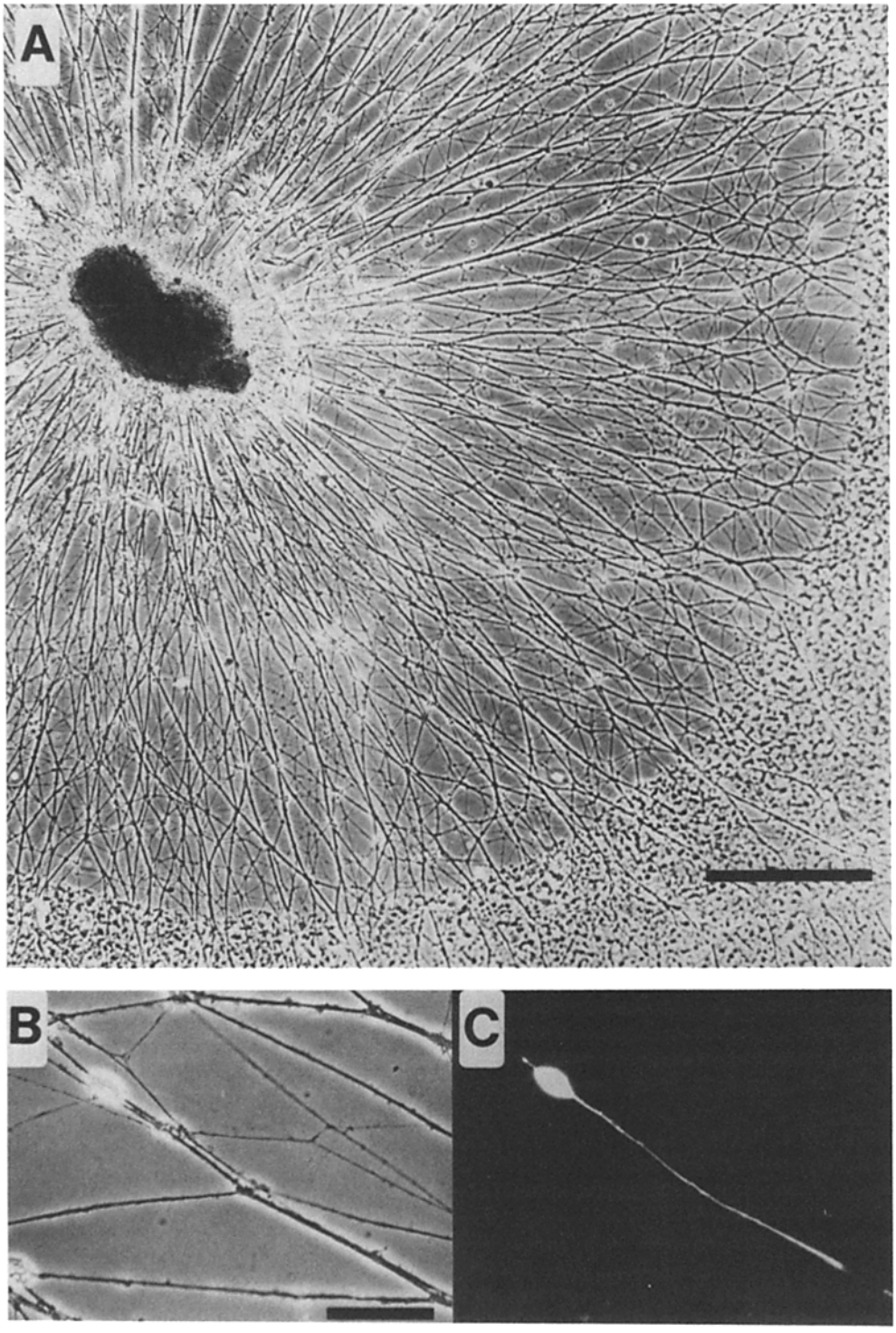

Figure 2. Photomicrograph of a sympathetic explant which has been prepared for use as an axonal substrate (*A*). Higher magnification of the axonal substrate with an attached test neuron elongating upon it, viewed with phase-contrast (*B*) and fluorescence (*C*) optics. Bars: (*A*) 0.5 mm; (*B* and *C*) 50 μm.

1-d HCM. Fab fragments of antibodies were added to a final concentration of 1 mg/ml. An equal amount of carrier was added to control cultures. The cells were fixed after 15–24 h in culture. Neurite lengths were measured in the same manner as experiments on axonal substrates using either rhodamine fluorescence or phase-contrast optics.

Immunofluorescence

Living cultures of sympathetic explants were incubated with Fab fragments of antibodies (50 μg/ml) for 30 min at 37°C and 5% CO_2. They were then gently rinsed with PBS and fixed in 4% paraformaldehyde and 10% sucrose in PBS. Incubation with rhodamine-conjugated second antibody (Cappel Laboratories, Cochranville, PA) was for 30 min at room temperature.

Results

The Growth of Neurites on Axonal Substrates

A schematic representation of the experimental protocol is given in Fig. 1. Briefly, dissociated brain cells are plated

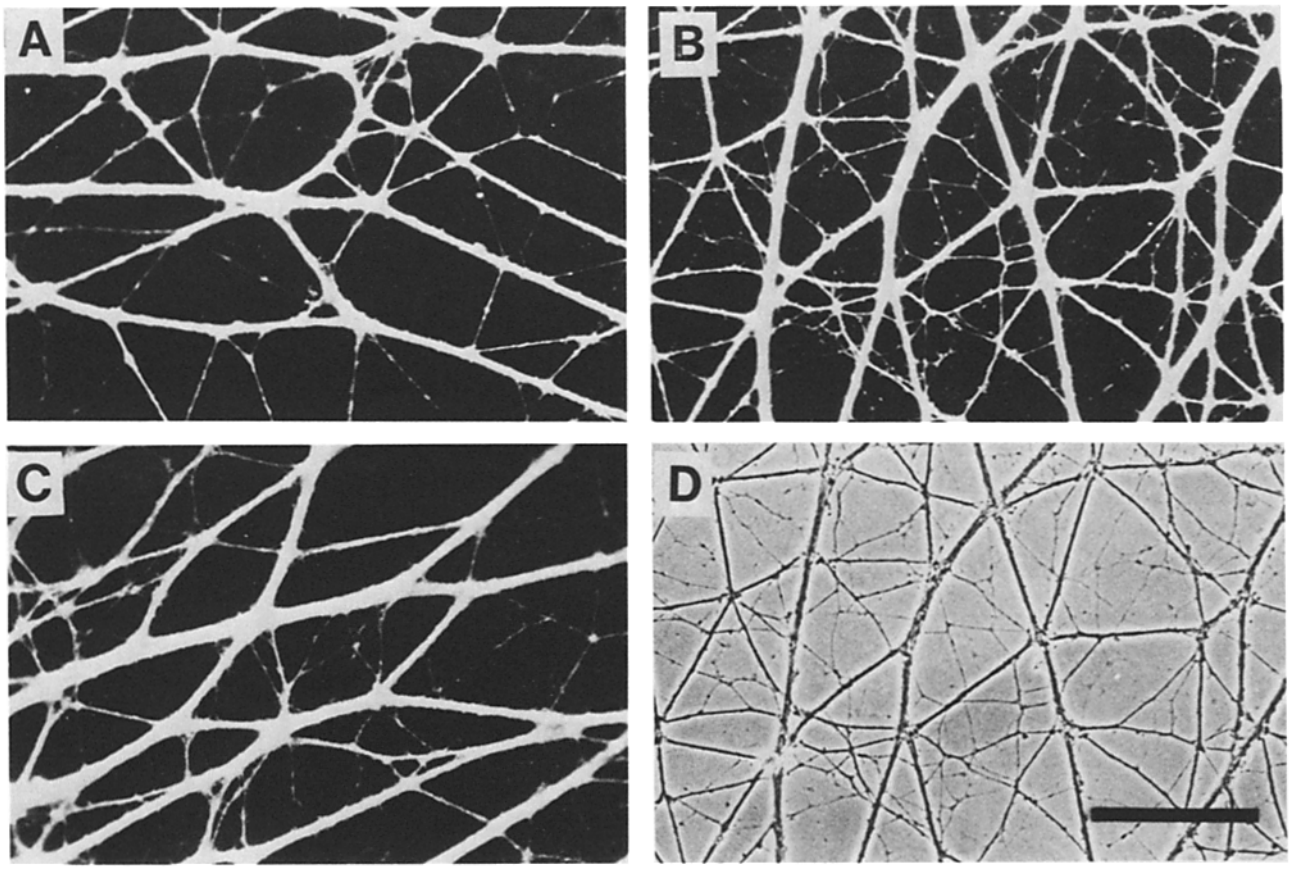

Figure 3. Immunofluorescence of sympathetic axons stained with Fabs against (*A*) F11 antigen, (*B*) G4 antigen, and (*C*) NCAM. *D* is the same field as in *B*, viewed with phase-contrast optics. Bar, 100 μm.

around the perimeter of a coverslip (Fig. 1 *A*) and fixed with methanol/acetone. Laminin is adsorbed to the glass (Fig. 1 *B*) and a chick E9 sympathetic ganglion is explanted onto the center of the coverslip (Fig. 1 *C*). After 5 d in culture, axons radiate from the explant over the laminin substrate and onto the ring of fixed cells (Fig. 1 *D*). Laminin antibodies are then added to the cultures (Fig. 1 *E*). This neutralizes the laminin on the glass, but does not disturb the morphology of the explant, since the annulus of fixed cells anchors the tips of the axonal fascicles. Freshly dissociated, TRITC-labeled E9 test neurons are plated onto the prepared axonal substrate (Fig. 1 *F*). Most or all neurons within the trunk sympathetic chains have initiated processes by this time, and the test neurons therefore contain cells which are regenerating processes. The anti-laminin treatment prevents the test neurons from elongating on the laminin-coated glass, and forces them to use the axonal fascicles as a substrate for outgrowth. Test neurons are grown on the axonal substrate for ~0.5 d (Fig. 1 *G*), and the cultures are then fixed. The lengths of a large number of neurites are measured and the results are displayed as the percentage of the measured population longer than any given length (Fig. 1 *H*). Complete details of the procedure are given in the methods section.

A culture prepared for use as an axonal substrate is shown in Fig. 2 *A*. A dense halo of axons extends from the sympathetic explant, located in the upper left hand corner of the photograph, and into the ring of fixed cells, a portion of which can be seen curving around the lower right hand edge of the photograph. An example of a neurite extending on an axonal fascicle is shown in phase-contrast (Fig. 2 *B*) and in fluorescence (Fig. 2 *C*).

Description of the Antibodies

Sympathetic axons in culture express abundant amounts of the G4, F11, and NCAM antigens on their surfaces. This is demonstrated by applying these antibodies to living preparations, fixing them, and then staining them with a rhodamine-conjugated second antibody (Fig. 3).

Polyclonal antibodies raised against immunoaffinity isolated G4 and F11 antigens react with 135-kD bands when tested on immunoblots of membrane proteins of adult chicken brain (Fig. 4, lanes *3* and *4*). Polyclonal antibodies against NCAM react with a broad smear around 140 kD when tested on immunoblots of membrane proteins of adult chicken brain (Fig. 4, lane *2*).

Effects of Anti-NCAM Fabs

Fab fragments of polyclonal antibodies against NCAM have no significant effect on the lengths of sympathetic neurites growing on sympathetic axons. The lengths of neurites growing on axons in the presence of anti-NCAM Fabs (1 mg/ml), as compared to its absence (*), are shown for one experiment in Fig. 5 *A*. As a group, the neurites growing in the presence of anti-NCAM Fabs are only slightly shorter than those growing in its absence. For any given experiment this dif-

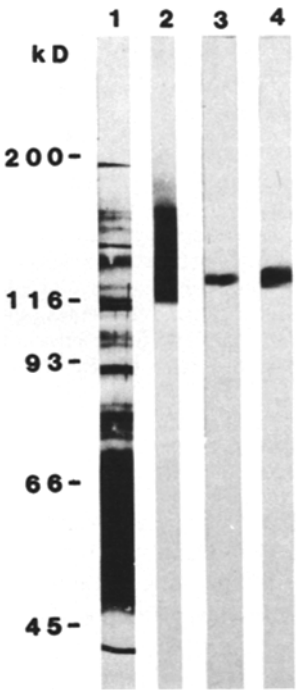

Figure 4. Immunotransfer analysis using polyclonal antibodies against NCAM, F11 antigen, and G4 antigen. Plasma membrane proteins from adult chicken brain were fractionated by SDS PAGE, transferred to nitrocellulose, and stained with india ink (lane *1*), or polyclonal antibodies to NCAM (lane *2*), G4 antigen (lane *3*), and F11 antigen (lane *4*). Binding of antibodies was visualized by peroxidase-conjugated second antibodies. Positions of molecular mass markers are shown to the left of the panel.

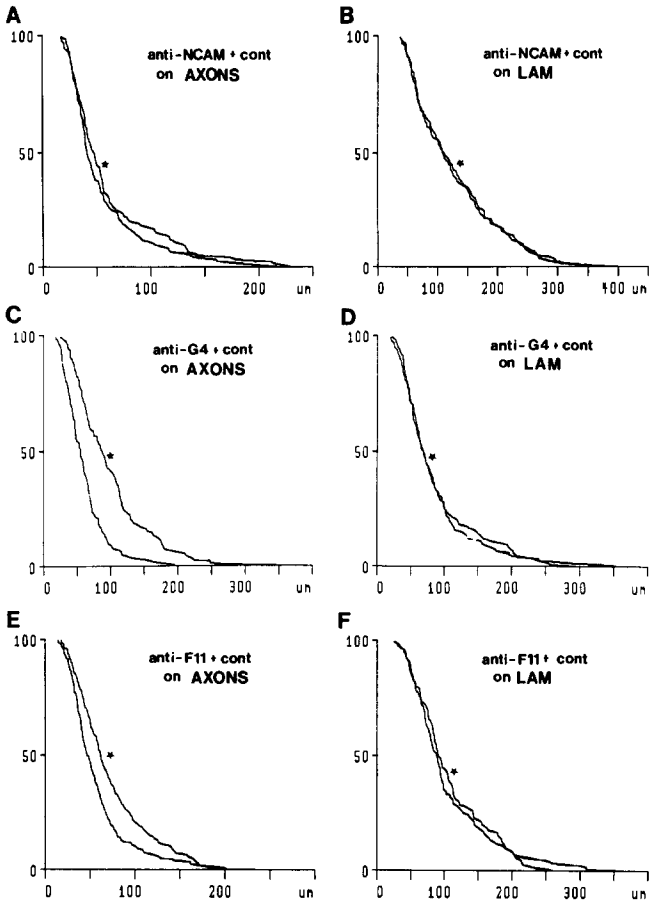

Figure 5. Distribution of neurite lengths in the presence and absence of antibody Fabs. Percentage of neurons with neurites (vertical axes) longer than a given length (horizontal axes). The distribution of lengths in the presence and absence (*) of 1 mg/ml anti-NCAM Fabs on (*A*) axonal substrate, and (*B*) uniform laminin substrate. The distribution of lengths in the presence and absence (*) of 1 mg/ml anti-G4 Fabs on (*C*) axonal substrate, and (*D*) uniform laminin substrate. The distribution of lengths in the presence and absence (*) of 1 mg/ml anti-F11 Fabs on (*E*) axonal substrate, and (*F*) uniform laminin substrate.

Table I. Effect of Anti-NCAM Fabs on Average Neurite Lengths

	N_T/N_C	L_T/L_C	L_T/L_C
		μm	%
Axonal substrate			
SYM/SYM			
1.	131:175	81:83	98
2.	147:195	63:73	87
3.	167:92	82:81	101
4.	225:198	95:97	97
5.	205:325	57:63	90
6.	112:120	52:54	96
7.	120:120	56:62	90
8.	141:131	83:92	90
9.	117:148	95:101	94
DRG/DRG			
1.	84:85	128:121	106
2.	105:85	101:92	110
3.	232:187	91:95	96
4.	118:83	109:102	107
Laminin substrate			
1.	160:120	103:107	96
2.	180:180	127:118	107
3.	101:120	130:128	102

Cultures containing antibody (1 mg/ml anti-NCAM Fabs) or the equivalent amount of carrier solution (CMF-Ea) were grown 15 h at 32°C, fixed, and neurite lengths measured on axonal and laminin substrates. SYM/SYM indicates sympathetic neurites growing on a substrate of sympathetic axons; DRG/DRG indicates dorsal root ganglion neurites growing on a substrate of dorsal root ganglion axons. N_T is the number of neurites measured in antibody-treated cultures; N_C is the number of neurites measured in control cultures. L_T is the average neurite length of antibody-treated cultures; L_C is the average neurite length of control cultures. There were no experiments in which the average neurite lengths of antibody-treated and control cultures were significantly different ($P < 0.05$).

ference is not statistically significant. The lengths of sympathetic neurites growing on laminin in the presence or absence (*) of anti-NCAM Fabs (1 mg/ml) are shown for one experiment in Fig. 5 *B*. The populations of neurite lengths are superimposable, suggesting that anti-NCAM Fabs have no toxic or any other general motility inhibiting effects. The same results were obtained on axonal substrates in seven experiments, and on laminin substrates in three experiments (Table I). We used antisera from four different rabbits and prepared by three different laboratories. In addition, we tested the effects of anti-NCAM Fabs on dorsal root ganglion neurons growing on a dorsal root ganglion substrate. Again the antibodies had no significant effect (Table I).

Effects of Anti-G4 Fabs

Fab fragments of polyclonal antibodies against G4 antigens interfere with the extension of sympathetic neurites growing on sympathetic axons. The lengths of neurites growing on axons in the presence of anti-G4 Fabs (1 mg/ml), as com-

Table II. Effect of Anti-G4 Fabs on Average Neurite Lengths

	N_T/N_C	L_T/L_C	L_T/L_C
		μm	%
Axonal substrate			
1.	123:147	59:83	71*
2.	160:143	67:99	68*
3.	137:143	64:99	65*
4.	138:143	61:99	62*
5.	126:153	59:89	66*
6.	92:120	67:93	72*
7.	130:136	64:109	59*
Laminin substrate			
1.	120:120	94:91	103
2.	120:120	87:86	101

Cultures containing antibody (1 mg/ml anti-G4 Fabs, except in the experiment of line 3 where the concentration was 0.5 mg/ml, and of line 4 where the concentration was 0.25 mg/ml) or control cultures containing the equivalent volume of carrier solution (CMF-Ea) were grown 15 h at 32°C, fixed, and neurite lengths measured on axonal and laminin substrates. N_T is the number of neurites measured in antibody-treated cultures; N_C is the number of neurites measured in control cultures. L_T is the average neurite length of antibody-treated cultures; L_C is the average neurite length of control cultures. Experiments where the average neurite lengths of antibody-treated and control cultures were significantly different ($P < 0.05$) are marked by *.

pared to its absence (*), are shown for one experiment in Fig. 5 *C*. Sympathetic neurites are shorter in the presence of anti-G4 Fabs. The same result was obtained in five experiments in which the Fab concentration was 1 mg/ml, and in two other experiments in which lesser amounts of antibody were used (Table II). Average neurite length decreased to between 60 and 70% of control values; a decrease which is statistically significant to below the 0.05 level in every case.

The lengths of neurites growing on laminin in the presence of anti-G4 Fabs (1 mg/ml), and in its absence (*), are shown

Table III. Effect of Anti-F11 Fabs on Average Neurite Lengths

	N_T/N_C	L_T/L_C	L_T/L_C
		μm	%
Axonal substrate			
1.	78:175	66:83	80*
2.	336:176	70:87	80*
3.	257:204	58:74	80*
4.	152:262	64:80	80*
5.	200:200	69:87	79*
6.	256:304	54:70	77*
7.	200:200	70:87	80*
Laminin substrate			
1.	120:120	105:107	98
2.	80:180	126:118	106
3.	249:197	89:87	102

Cultures containing antibody (1 mg/ml anti-F11 Fabs, except in the experiment of line 7 where the concentration was 2 mg/ml) or control cultures containing the equivalent amount of carrier solution (CMF-Ea) were grown for 15 h at 32°C, fixed, and neurite lengths measured on axonal or laminin substrates. N_T is the number of neurites measured in Fab-treated cultures; N_C is the number of neurites measured in control cultures. L_T is the average neurite length of Fab-treated cultures; L_C is the average neurite length of control cultures. Experiments where the average neurite lengths of Fab-treated and control cultures were significantly different ($P < 0.05$) are indicated by *.

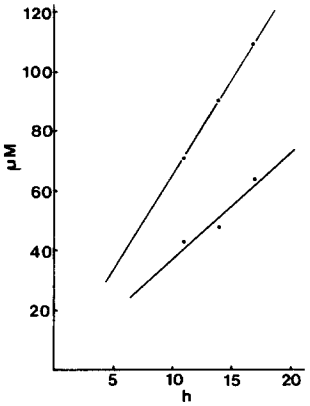

Figure 6. Time course experiment, showing the average lengths of neurites growing on axonal substrates at different times in control cultures (*upper line*) and in the presence of 1 mg/ml anti-G4 Fabs (*lower line*).

for one experiment in Fig. 5 *D*. The two populations of neurite lengths are indistinguishable. The same results were obtained in two experiments (Table II), suggesting that anti-G4 Fabs have no toxic or other general motility inhibiting effect.

Effects of Anti-F11 Fabs

The lengths of sympathetic neurites growing on axons in the presence and absence (*) of 1 mg/ml anti-F11 Fabs are shown in Fig. 5 *E*. Neurites are shorter in the presence of Fab fragments of polyclonal antibodies against F11 antigen. In six experiments, the average length of sympathetic neurites growing on sympathetic axons was consistently found to be 80% of control values in the presence of anti-F11 Fabs (Table III). These results were significant to below the 0.05 level. Increasing the concentration of anti-F11 Fabs to 2 mg/ml does not have a greater effect on average neurite length (line 7 in Table II). When the concentration of anti-F11 Fabs is decreased to 0.5 mg/ml, average neurite lengths are shorter than control values, but they are not statistically different (data not shown).

The lengths of sympathetic neurites extending on a laminin substrate in the presence and absence (*) of 1 mg/ml anti-F11 Fabs are shown in Fig. 5 *F*. The two populations are not significantly different. The same result was obtained in three experiments (Table III), suggesting that anti-F11 Fabs have no toxic or other general motility inhibiting effect.

Mechanisms of Action by Anti-G4 Fabs

Anti-G4 and anti-F11 Fabs may cause shorter average neurite lengths on axons by (*a*) interfering with the ability of test neurons to adhere to axons, (*b*) interfering with their ability to initiate processes once they have adhered, or (*c*) by decreasing the actual rate of outgrowth. Time course studies with anti-G4 Fabs suggest that they reduce the rate of neurite outgrowth. The results from one experiment are shown in Fig. 6. The average neurite lengths measured in the presence of anti-G4 Fabs increase over the course of 5 h at a slower rate than in matched control cultures. Neurites elongate on axons about two-thirds as fast in the presence of anti-G4 Fabs as under control conditions (Table IV).

Discussion

We have introduced a new in vitro bioassay which quantifies neurite extension on axonal substrates. In contrast to culture

Table IV. Rate of Outgrowth in Control and Fab-treated Cultures

Control	Anti-G4	Anti/control
		%
6.0 μm/h	4.0 μm/h	67
6.3 μm/h	3.6 μm/h	57

Control and Fab-treated cultures (1 mg/ml) were fixed at 3-h intervals starting after 10–11 h in culture, and neurite lengths were measured. Three time points were taken per experiment and the rate of outgrowth calculated.

systems in which growth cones extend on nonphysiological materials such as polylysine, or on single proteins such as fibronectin or laminin, this assay offers growth cones a complex, multi-component substrate approximating one which they encounter in vivo: the surfaces of other axons.

Antibodies against two recently purified neural cell surface glycoprotein antigens, G4 and F11 (Rathjen et al., 1987), label the surfaces of sympathetic axons grown in culture. Sympathetic growth cones growing on sympathetic axons are significantly shorter in the presence of Fab fragments of polyclonal antibodies against the G4 or F11 antigens as compared to controls. Anti-G4 Fabs, at saturating levels, result in average neurite lengths which are 60–70% of control values. Anti-F11 Fabs, at saturating levels, result in average neurite lengths which are 80% of control values. Time course studies using anti-G4 Fabs show that they reduce average neurite lengths by decreasing the rate of neurite outgrowth to a value which is about two-thirds of the control rate.

Anti-G4 and anti-F11 Fabs have no significant effect on sympathetic outgrowth on laminin. Consequently, the shortening they cause on axonal substrates probably can not be ascribed to general effects on cell health or growth cone motility. Due to the fact that our assay ignores neurons without neurites, it is possible that these antibodies could have small effects on sympathetic neuron viability or on their propensity to produce neurites. We also can not rule out the possibility that anti-G4 and anti-F11 Fabs may have additional effects on neuron–neurite adhesion. However, the data do suggest that anti-G4 and anti-F11 Fabs significantly impair the ability of sympathetic neurites to extend on sympathetic axons, and that this effect is substrate dependent.

The preparations of anti-NCAM antibodies we used strongly inhibit retinal cell aggregation, the original functional definition of NCAM activity (Brackenbury et al., 1977). However, since they had no effect in our assay, NCAM is not significantly involved in the extension of sympathetic growth cones on sympathetic axons, or in the extension of dorsal root ganglion growth cones on dorsal root ganglion axons. This is true even though NCAM is plentiful on both sympathetic and dorsal root ganglion axons in culture. Therefore, simply covering axonal surfaces with Fab fragments is not deleterious to neurite extension.

The sera from five naive, uninjected rabbits had no effect in our assay (data not shown). Antibodies against C5, a molecule which is not present in high amounts on sympathetic axons, have no effect (data not shown). All rabbits injected with G4 antigens (2 out of 2) produced antisera that were functionally identical. The same was true for all rabbits injected with F11 antigens (4 out of 4).

The above considerations suggest that the effects of anti-G4 and anti-F11 Fabs are most likely due to the recognition of specific components involved in neurite extension on axons.

The antisera used in this study were directed against antigens prepared from monoclonal antibody affinity columns, each of which purify a major component of 135 kD and other minor components. The 135-kD components of the G4 and F11 antigens do not appear to be biochemically or immunologically related to each other. The 135-kD component of G4 antigen is, however, related to mouse L1 (see the preceding paper by Rathjen et al. for details). Since the polyclonal antisera seem to react only with the 135-kD bands, it is probably these components which are involved in the extension of sympathetic neurites on sympathetic axons.

The relationship of G4 to L1 is interesting in light of the biological functions reported for L1. Antibodies to mouse L1 have been shown to interfere with adhesion between neural cells and between neuroblastoma N2A cells (Rathjen and Schachner, 1984). This suggests that L1 is a cell adhesion molecule. Antibodies to L1 also inhibit the migration of granule cells in cultured cerebellar slices (Lindner et al., 1983), and de-bundle neurites growing from cerebellar explants on polylysine (Fischer et al., 1986). Similarly, anti-G4 and anti-F11 Fabs cause chick retinal axons growing on a substrate of tectal membranes to de-bundle (Rathjen et al., 1987). Antibodies against the L1-related molecule NILE debundle rat neurites grown on polylysine (Stallcup and Beasley, 1985). Antibodies against the L1 related molecule Ng-CAM also de-bundle chick dorsal root ganglion neurites growing on collagen (Hoffman et al., 1986).

The apparent fasciculation of neurites in tissue culture could be the result of at least two different mechanisms. True fasciculation involves the extension of growth cones on preexisting axons. This is the process our assay measures and the process which anti-G4 and anti-F11 perturb. In addition, the side-to-side adhesion of neuritic shafts behind growth cones can lead to the bundling of neurites. This is the mechanism Rutishauser et al. (1978) originally proposed for the role NCAM may play in bundle formation.

Another antibody which has been shown to inhibit neurite outgrowth in vitro is the T61 monoclonal antibody (Henke-Fahle and Bonhoeffer, 1983). It inhibits retinal neurite outgrowth on collagen, laminin, and a retinal basement membrane preparation (Henke-Fahle et al., 1984). The antigen is a glycoprotein of 170–180 kD, and appears not to be expressed by peripheral axons. Thus T61 inhibits growth cone motility of a particular class of neurons, and is not substrate specific. Its effects are in contrast to the G4 and F11 antigens, whose effects are substrate specific.

We have shown that Fab fragments of antibodies to G4 and F11 antigens compromise neurite elongation on axonal surfaces in vitro. The antisera recognize major proteins of 135 kD, which are probably the active molecular species. Since these antibody preparations only partially block neurite extension, it might be expected that other molecules will be found which participate in the same phenomenon. Both G4 and F11 antigens appear on axons very early in vivo, and in some brain regions there are differences in their distribution (Raper, J. A., E. B. Grunewald, F. G. Rathjen, and S. Chang, unpublished observations). It is therefore possible that they also play a role in growth cone extension on axonal substrates in vivo.

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