

Astrotactin: A Novel Neuronal Cell Surface Antigen that Mediates Neuron–Astroglial Interactions in Cerebellar Microcultures

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Abstract. A microculture system for mouse cerebellar cells has been used to identify an immune activity, raised in rabbits against postnatal cerebellar cells, that blocks neuron–glial interactions in vitro. In the presence of blocking antibodies, stable neuron–glial contacts did not form and neuronal induction of glial process outgrowth did not occur. Subsequently, neurons were randomly arranged in the cultures rather than organized along the arms of astroglia. We have named the immune activity that blocks neuron–astroglial interactions anti–astrotactin.

Partial purification of the anti–astrotactin blocking antibodies was obtained by cellular absorption with PC12 cells, a clonal cell line which expresses both the N-CAM and NILE (Ng-CAM, LI) glycoproteins. Subsequent absorption with purified cerebellar granule cells, but not with astroglial cells, removed the blocking activity, suggesting that the antigen(s) bound by blocking antibodies are neuronal.

Immunoprecipitation of [³⁵S]methionine- or [³H]fucose-radiolabeled Triton extracts of early postnatal cerebellar cells showed that the unabsorbed antiserum recognized a large number of proteins. Among these were bands with apparent molecular masses of N-CAM

(180 and 140 kD) and NILE (230 kD). After absorption of the immune serum with PC12 cells, the number of bands recognized by the antiserum was reduced to a prominent band at 100 kD and a diffuse smear of material between 80 and 90 kD. The prominent band at 100 kD was removed by subsequent absorption of the immune serum with granule cells, a step which removed the blocking activity in the cerebellar microculture assay.

Further evidence suggests that the astrotactin activity is missing or defective on granule cells from the neurological mutant mouse weaver, an animal that suffers a failure of glial-guided neuronal migration. When anti–astrotactin Fab fragments were pre-absorbed with weaver cerebellar neurons and then tested in the functional assay of neuron–glial interactions, the immune blocking activity was not removed. In contrast, wild-type cerebellar neurons removed the anti–astrotactin blocking activity under the same conditions. Subsequently, when [³H]fucose-radiolabeled Triton extracts of weaver and normal cells were immunoprecipitated with whole or PC12-absorbed anti–astrotactin antiserum, the intensity of the band at 100 kD was reduced by 95% in weaver cells.

To study the molecular basis of neuron–glial interactions, our laboratory has developed a cerebellar microculture system, in which specific cell–cell interactions between one neuronal type, the granule neuron, and several forms of astroglial cells, elongated forms and stellate forms, can be studied (14, 15). One striking neuron–glial interaction seen in the microcultures is neuronal migration along highly elongated glial processes, an event which can be visualized in real time with high resolution video microscopy (5).

The other prominent neuron–glial interaction seen in microcultures is astroglial organization of neuronal positioning. The location of the neurons relative to glial processes (14) can be measured after immunostaining cultures with an-

tibodies against the glial filament protein. After 24 h in vitro, under culture conditions where only 20% of the surface area of the culture dish is occupied by cells, over 90% of granule neurons are located within 20 μ m of a glial process. Neurons are not randomly distributed in microcultures, but are arranged along glial processes. Quantitation of the distribution of neurons relative to glial processes in microcultures provides a functional assay for neuron–glial interactions.

The neurological mutant weaver mouse has been studied extensively in vivo and in vitro as a test of the hypothesis that Bergmann fibers guide the migration of young granule neurons from the external to the internal granular layers of the developing cerebellum (18, 24–27, 35). In weaver, young granule neurons fail to appose their cell somata against or to migrate along Bergmann fibers, which are abnormally thickened, vacuolated and improperly aligned in the cerebellar cortex (2, 9, 16, 17, 25).

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When weaver cerebellar cells are placed into microwell cultures, the neurons fail to associate with glial cells, and the glia are flattened and vacuolated (16). In contrast, in cultures from normal cerebellum, astroglia attain differentiated shapes resembling those *in vivo*, and granule neurons bind to and migrate along the glial processes (5, 14, 15, 38). Development of a technique for rapid separation of granule neurons and astroglia into pure populations has allowed comparison of the behavior of mutant neurons on mutant or normal glia, and vice versa (12, 17). Weaver granule neurons fail to bind to or to migrate on either weaver or normal glia, and the glia in either case are flattened and stunted. In contrast, normal neurons bind to and migrate on both weaver and normal glia, and the glial forms in each case resemble those seen in the intact normal cerebellum. Thus, the studies with separated cells support the hypothesis that the weaver mutation primarily affects granule neurons. An explanation for defects in astroglial morphology in weaver is also suggested by these studies, as astroglial differentiation into forms that support migration appears to rely on proper interactions with normal granule neurons (12).

We report here on an antiserum we have raised against whole cerebellar cells that blocks specific neuron–glial associations, an activity which we have named anti–astrotactin. This activity is directed against antigen(s) distinct from the common adhesion molecules N-CAM; (BSP-2), NILE (L1, Ng-CAM) and Thy-1 (7, 22, 30, 33, 34). Immunoprecipitation of metabolically-labeled cerebellar glycoproteins with the absorbed antisera suggests that the blocking antibodies recognize a novel cell surface glycoprotein with an apparent molecular mass of 100 kD present on granule neurons, harvested from normal but not weaver mice.

Materials and Methods

Preparation of Anti–Astrotactin Serum, IgG Fraction and Fab Fragments

Cerebellar cells from 7-d-old mice were dissociated as described (14) and plated on untreated tissue culture plastic for 6 h in medium containing 1% horse serum. This was to allow recovery of surface components after trypsinization. Cells were collected, washed with PBS, mixed with complete Freund's adjuvant (Gibco, Grand Island, NY) and injected subcutaneously into six rabbits. Incomplete Freund's was used for 10 subsequent biweekly injections, followed by 10 biweekly bleeds of 20 ml/rabbit; rabbits were then exsanguinated. An IgG fraction from each rabbit antiserum was made by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography (Sigma Chemical Co., St. Louis, MO). Fab fragments were produced by controlled digestion of the purified IgG fraction using papain (Sigma Chemical Co.), followed by removal of Fc fragments using CM-cellulose chromatography (8). Purity of IgG and Fab preparations was tested using 10% SDS–PAGE run under reducing conditions (not shown).

Other Antisera

Antisera against NILE glycoprotein were kindly provided by Drs. Lloyd Greene and Michael Shelanski of this department. Antisera against the L1 antigen (28), the large molecular mass component of which is apparently identical to NILE (32) and Ng-CAM (10, 11), were generously provided by Dr. Melitta Schachner of the University of Heidelberg (Federal Republic of Germany). Antisera and Fab' against BSP-2, which is apparently identical to N-CAM (3, 19, 23, 37), were kindly provided by Dr. Christo Goridis of the Centre d'Immunologie, Institut Nationale de la Santé et de la Recherche Médicale–Centre National de la Recherche Scientifique (INSERM–CNRS) de Marseille-Luminy (France).

Cerebellar Cultures

Microcultures of C57BL/6J mouse or rat cerebellar cells dissociated from cerebellar tissue on P6 or P7 were prepared as described (14, 15). The culture surface was treated with poly-D-lysine (50–100 µg/ml). Cells were plated at $0.5\text{--}2.0 \times 10^6$ cells/ml in a final volume of 40 µl.

Primary monolayer cultures of cells from 5-d-old B6CBA-A⁺-J-vol/vol (+/+) or (wt/vol/wt/vol) mice were prepared as described (16). Homozygous weaver mice and their homozygous normal littermates were identified as described (16). The midline portions of each were used to prepare cultures as described for videomicroscopic observation and cellular absorption. In all, cultures were prepared from 400 litters of normal and 20 litters of weaver mice.

Immunocytochemistry

Glial cells were identified by immunostaining with anti–glial filament protein (anti–GFP)¹ antibodies; neurons by immunostaining with anti–NILE antibodies (14, 15). Cultures were fixed after 24 h *in vitro* for 30 min in 4% paraformaldehyde in 0.1 M Sorenson's buffer, pH 7.4; washed three times with PBS; incubated 30 min with 10% normal goat serum in PBS with 0.05% Triton X-100; incubated 1 h in guinea pig antiserum against purified bovine glial filaments (1:100 with 10% NGS and 0.01% Triton X-100); washed three times with PBS; incubated 30 min with FITC-conjugated goat anti–Guinea pig Ig, and washed extensively with PBS. The procedure was varied for diaminobenzidine staining as follows: instead of fluorescein-conjugated goat anti–Guinea pig IgG, peroxidase-conjugated goat anti–Guinea pig IgG was used, followed by washes in 50 mM Tris, pH 7.6, incubation in diaminobenzidine (0.1 mg/ml) in Tris with 0.003% hydrogen peroxide, and extensive washes in PBS. Stained cultures were mounted in glycerol for microscopic observation.

Assays for Neuron–Glial Interactions

Two assays were used to test the biological effects of the anti–astrotactin Fab fragments. In the first, the cells were plated in the presence or absence of antibodies and after 24 h *in vitro*, the cultures were fixed, immunostained with anti–glial filament protein antiserum, and the distribution of neurons relative to stained glial processes was measured. In the second, the cells were plated in the presence or absence of antibodies and direct observations on the influence of the immune reagents on cell–cell interactions in living cultures were made by time-lapse video microscopy. In all, more than 700 microcultures were analyzed.

Quantitation of Neuron–glial Interactions by Immunocytochemistry.

Neuron–glial interactions were measured by a modification of the method described previously (14). After immunostaining with antibodies against the glial filament protein, an image of concentric rings, calibrated with a microscope stage micron marker, was projected onto the microscopic image of the stained cultures by means of a camera lucida. Using the movable microscope stage, each neuron in the selected field was maneuvered in turn to the center of the concentric rings and the distance between neuronal cell bodies and the nearest glial process was measured.

A modeled random distribution of neurons relative to glia was generated by spacing neurons every 0.6 cm and glia every 2.0 cm in a square matrix 20 × 20 cm. The density of the neurons on this modeled matrix was 10 times that of astroglial cells, the ratio of neurons to glia that was found in the cultures.

The methods described above were then used to measure the lengths of glial processes.

Visualization of the Formation of Neuron–glial Contacts by High Resolution Video Microscopy of Living Cultures.

Cultures were plated on glass coverslip dishes (14) in medium with or without 0.5 mg/ml Fab fragments and were placed in a 5% CO₂ incubator (Napco) for 30 min. Before observation, cultures were sealed in a closed chamber formed by placing a second coverslip over the culture well and sealing it with silicon grease (Dow Corning Corp., Midland, MI). Cultures were placed on the heated stage (maintained at 35.5°C) of a Zeiss IM microscope equipped with differential interference contrast optics, attached to a Hamamatsu C1965-01 chalnicon video camera according to the method of Allen (1, 5). The video-enhanced image was recorded on a Panasonic optical memory disc recorder and was viewed on a Sony 12" PVI22 monitor (Tokyo, Japan). Granule neurons were identified on the basis of their small cell soma (6–8 µm) and scant cytoplasm, while astroglia were identified on the basis of their large cell

1. Abbreviation used in this paper: Anti-GFP, anti–glial filament protein.

soma (10–15 μm) and extension of large, paddle-like processes (21). At the conclusion of some experiments, video-observed cultures were fixed and stained with the anti-gial filament protein serum to confirm the identification of glial cell type made in the videomicroscope.

Absorption of Anti-Astrotactin Antiserum

Two different techniques were used to absorb the starting antiserum:

Absorption on Monolayers of Cultured Cells. Purified cerebellar granule neurons and astroglia, prepared as described (12), cultures of weaver cerebellar cells, prepared as described (16) and the cell lines C6, A-172, PC12, mouse 3T3, G26-24, BRK2-F, PTK2 and L cells, kindly provided by Drs. D. Bigner (University of Connecticut, Hartford, CT), S. Pfeiffer (Duke University, Durham, NC), R. Dalla-Favera, L. Greene, F. Maxfield, M. Rosenfeld, and M. Shelanski, respectively, were grown in five 40- μl wells for 24 h. Anti-astrotactin Fab fragments were mixed with culture medium at twice the final concentration and 20 μl was incubated serially on the five monolayers before being placed in an empty well to which 20 μl of freshly dissociated cells were added. To confirm that absorption was exhaustive, we immunostained with FITC goat anti-rabbit antiserum (1:100).

Absorption with Pellets of Purified Cells. The starting antiserum was absorbed overnight at 4°C with PC12 cells or with purified cerebellar granule neurons harvested on the third or fourth postnatal day, followed by centrifugation in an Eppendorf microfuge for 5 min to pellet particulate material. In some experiments, the absorptions were sequential.

Radiolabeling with [³H]Fucose, [³H]Glucosamine and [³⁵S]Methionine and Solubilization of Glycoproteins From Cerebellar Cells

To label the cells with [³H]fucose or [³H]glucosamine, two 24-well plates of cerebellar cells dissociated from P7 mouse or rat cerebellum were maintained in vitro for 4 d in medium containing either 20 μCi [³H]fucose or 20 μCi [³H]glucosamine (New England Nuclear, Boston, MA) per ml of medium. The cultures were then washed three times with calcium- and magnesium-free phosphate buffered saline (CMF-PBS) and lysed with 200 μl per well of a disruption buffer (50 mM Tris-HCl containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.025% Na₂S₂O₈, 2.0 mM PMSF) for 30 min at 4°C. The cells were removed by scraping with a rubber policeman and the suspension was centrifuged at 100,000 g for 45 min, after which the pellet was discarded, and the supernatant was used for immunoprecipitation experiments.

To label the cells with [³⁵S]methionine, two 24-well plates of cerebellar cells dissociated from P4-P7 cerebellum were cultured for 2–6 h in methionine-free medium (Gibco; selectamine culture medium) containing 30–100 μCi per ml medium of [³⁵S]methionine (New England Nuclear). Membrane-enriched material was solubilized by adding disruption buffer for 1–2 min. Cytoskeletal-enriched ghosts of the cells remained on the culture dish. After centrifugation at 100,000 g for 45 min, the supernatant of solubilized material was used for immunoprecipitation experiments.

Immunoprecipitations

Triton extracts of cerebellar cells (500 μl) were preincubated with preimmune rabbit serum (4 μl) for 3–4 h at room temperature, after which 1,600 μg of Protein A-Sepharose (Sigma Chemical Co.) was added and the sample was incubated with shaking for 2–3 h. To remove Protein A-Sepharose, the samples were centrifuged 5 min in an Eppendorf Microcentrifuge and the pellets were discarded.

Either the starting polyclonal antiserum, PC12 cell-absorbed antiserum, PC12 cell plus granule neuron-absorbed antiserum, anti-BSP-2 antibody or anti-NILE antiserum were diluted 1:80 with the cerebellar extract, rotated overnight at 4°C, after which 2 mg Protein A-Sepharose was added to each 400- μl sample and the mixture was incubated for 1–2 h at room temperature. The samples were centrifuged as for preimmune serum and washed twice with CMF-PBS containing 0.05% NP40 and once with CMF-PBS.

Disruption buffer (25 μl , 0.5 M Tris-HCl, pH 8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.2% bromphenol blue) was added and the samples were placed in a boiling water bath for 3 min after which the entire sample was loaded onto a 7.5% SDS-polyacrylamide gel prepared by the method of Laemmli (20). After electrophoresis, the gels were fixed in 50% Methanol with 10% acetic acid, stained with Coomassie Blue in the fixing solution, destained in 10% Methanol/10% acetic acid, soaked in distilled water, followed by 1 M sodium salicylate (Sigma Chemical Co.) for 1 h.

The gels were dried under vacuum and exposed to preflashed X-AR X-ray film (Kodak) for 3–7 d before developing.

Affinity Purification of Blocking Fab Fragments on Sepharose-Linked Granule Neuron Membrane Proteins

An affinity column of immobilized granule neuron membrane proteins was prepared by coupling Triton extracts of purified granule neurons harvested from 40 P7 cerebella to cyanogen bromide-activated Sepharose CL-4B. PC12-absorbed antiserum was applied to the column, after which flow was stopped for 30 min to allow maximal binding. After washing with 100 mM NaCl in 50 mM Tris buffer, pH 8.0, the column was eluted with 100 mM glycine, pH 3, after which the pH of the eluate was immediately adjusted to 7.0 with concentrated Tris. Antibodies were concentrated to 20 $\mu\text{g}/\text{ml}$ by vacuum dialysis.

Gel Scanning

Autoradiograms of immunoprecipitations were scanned on a Hoefer GS300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA) in the transmittance mode and were recorded on a Linear chart recorder.

Photomicrography

Video-enhanced differential interference contrast images were photographed directly from the monitor onto Kodak Technical Pan film using a 35 mm Nikon camera with a 55-mm macro lens set at ASA 80 and a 1/4 s exposure time.

Results

Of the six rabbit antisera raised against whole postnatal mouse cerebellar cells, one altered the arrangement of the cells in the microculture. By phase-contrast microscopy, the treated cells appeared to form small cellular reagggregates rather than a monolayer. No effects were seen with the preimmune serum. We then prepared Fab fragments of this rabbit serum and assayed the effects of the fragments on neuron-gial interactions in the microcultures.

Quantitation of Neuron-Gial Associations in Microcultures: A Functional Assay for Immune Blocking Activity

The Fab fragments were added to growing cultures at 0.5 mg/ml. In untreated cultures, as judged by exclusion of Trypan Blue (Gibco), plating efficiency was greater than 90%. Addition of the Fab fragments into the medium at the time of plating had no effect on cell viability.

To assay for changes in the astroglial organization of neuronal positioning, the Fab fragments prepared from the immune sera (0.5 mg/ml) were added at the time the cells were plated and the cultures were immunostained with anti-GFP 24 h later. We chose the 24-h time point because previous studies (14) have shown that after 24 h in vitro, the vast majority of neurons harvested from early postnatal mouse cerebellum are positioned within 20 μm of a stained glial process.

Neuron-gial interactions were quantitated by measuring the distance of each neuron from the nearest stained glial cell process and then plotting the distribution of the neurons relative to glial processes in the culture. In cases where a non-random distribution (where more than 90% of the neurons were within 20 μm of a glial process) failed to develop, and a random distribution (where fewer than 50% of the cells were close to a glial process) was therefore seen, the immune

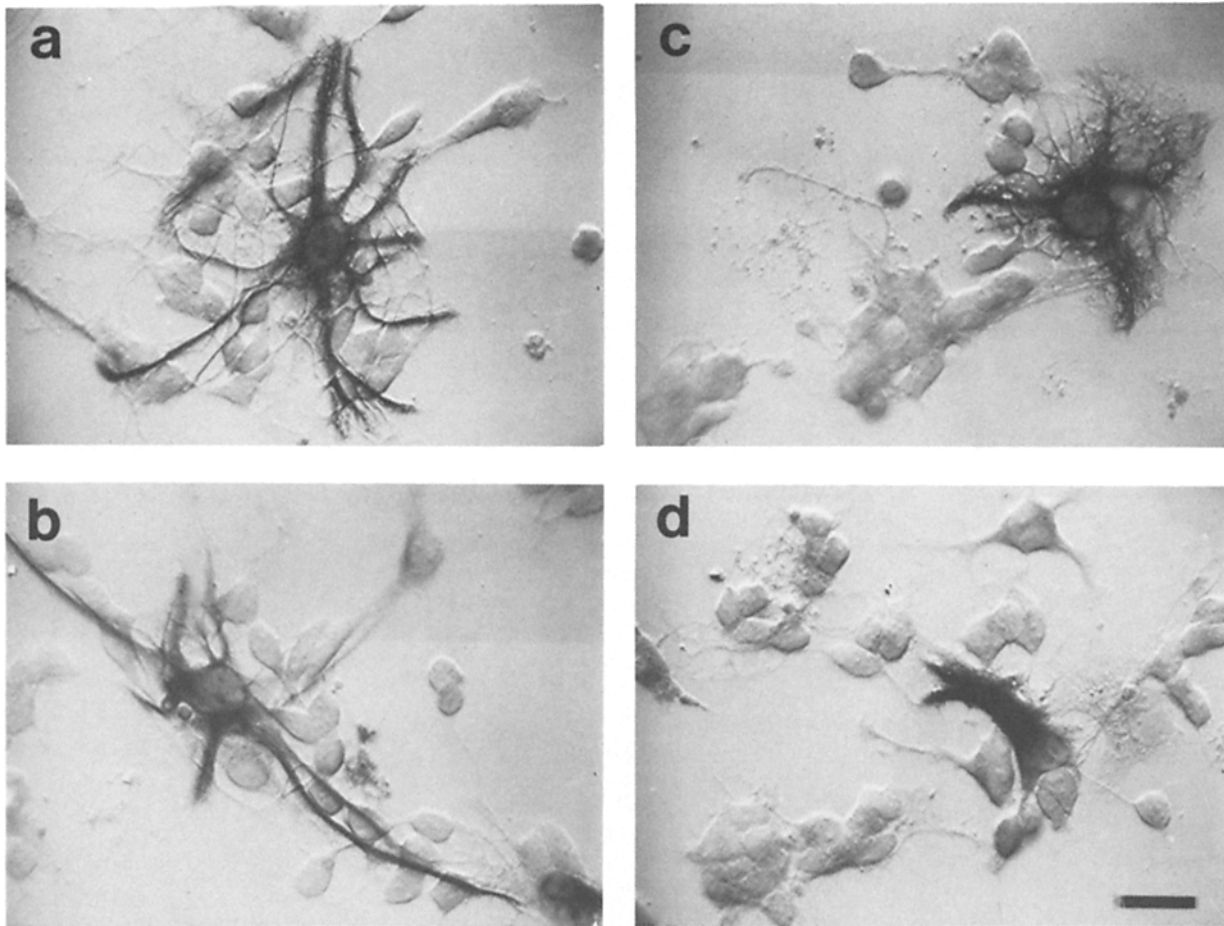


Figure 1. Immunostaining of astroglial cells with anti-glial filament protein in cultures grown for 24 h in the absence, (*a* and *b*), or presence, (*c* and *d*), of anti-astrotactin Fab fragments (0.5 mg/ml). In *a* and *b* astroglial cells are highly differentiated, bearing long, thin processes that organize the neurons. Unstained neurons are positioned close to stained glial arms. In *c* and *d*, the processes of the astroglial cells are severely stunted and many neurons are positioned away from the glial arms. Bar, 20 μ m.

activity was called "blocking activity." In the Fab-treated cultures, such a change in distribution occurred, whereas it did not in untreated cultures or in cultures treated with Fab fragments prepared from the preimmune serum (Figs. 1 and 2).

Immunostaining with anti-GFP revealed that in the presence of Fab fragments of the immune serum, glial processes were shorter and thicker than those normally seen in control cultures (Fig. 1). In this regard, they resembled astroglial cells cultured in the absence of neurons (12). Quantitative measurements of glial process extension in the presence of anti-astrotactin antibodies confirmed the general impression from the micrographs (Fig. 3).

To analyze whether anti-astrotactin antibodies affected the cell-substrate adhesion of neurons or astroglia, three experiments were carried out. First, we treated the culture surface with anti-astrotactin antisera, with Fab fragments of the antiserum or with a mixture of either the antisera or Fab fragments and polylysine, using the same concentration of reagents as in the blocking assays. We then plated cerebellar cells (14) onto these antibody-treated substrates and measured neuron-glial interactions as described above. No changes in the distribution of the neurons vis a vis the glia were seen on antibody-treated culture surfaces. The astroglial cells attached to the culture surface and the neurons,

in turn, attached to the glia (14, 15). Second, when we plated purified granule cells (12) onto antibody-treated culture dishes, the cells did not adhere to the antibody-coated substratum. Third, we plated purified neurons or astroglia on a polylysine-coated culture surface and added Fab fragments of anti-astrotactin antiserum to the culture medium. No effects on neuronal attachment or astroglial cell form were seen when the antibody was added to purified cells. These experiments suggested that the astrotactin antibodies primarily affected neuron-glial contacts rather than neuron-substratum or glial-substratum adhesion.

In other experiments (not shown), we tested the influence of anti-astrotactin antibodies on cerebellar cells harvested from postnatal rat cerebellum. Identical results were seen with rat cells. Anti-astrotactin antibodies disrupted neuron-glial interactions and impaired glial process extension.

Purification of the Anti-Astrotactin Blocking Antibodies

To purify the blocking activity from the polyspecific starting antiserum, we absorbed the serum with monolayers of cells, or pellets of intact cells, and tested the effect of the absorbed serum on neuron-glial interactions in microcultures.

With monolayers of cells, the antiserum was absorbed by

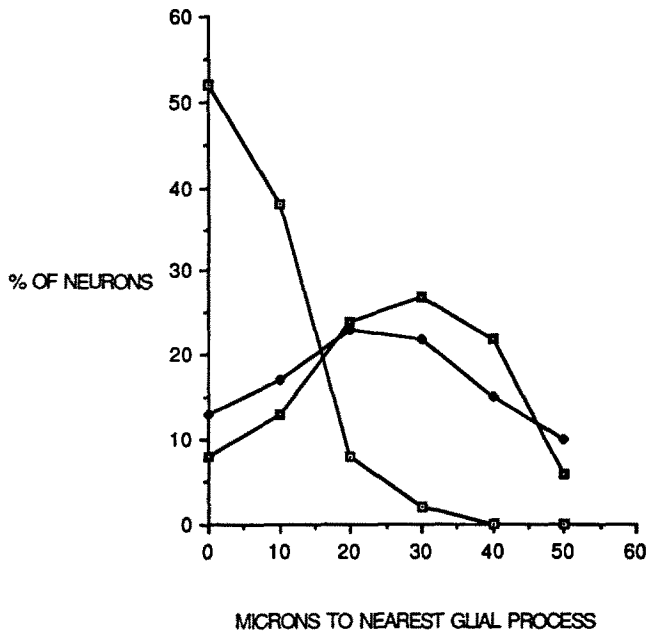


Figure 2. Graph of the distribution of distances from each neuron to the nearest glial process (□) at the time of plating, and at 24 h in the (○) absence or (◆) presence of anti-astrotactin Fab fragments. At the time of plating, the distribution of distances is a bell-shaped random distribution. After 24 h in untreated cultures, over 90% of neurons are within 20 μm of an astroglial process, as reported previously (14). In contrast, after 24 h in Fab-treated cultures, the distribution remains bell-shaped, indicating that the Fab fragments blocked the glial organization of neuronal position. The SD was <10% (not shown).

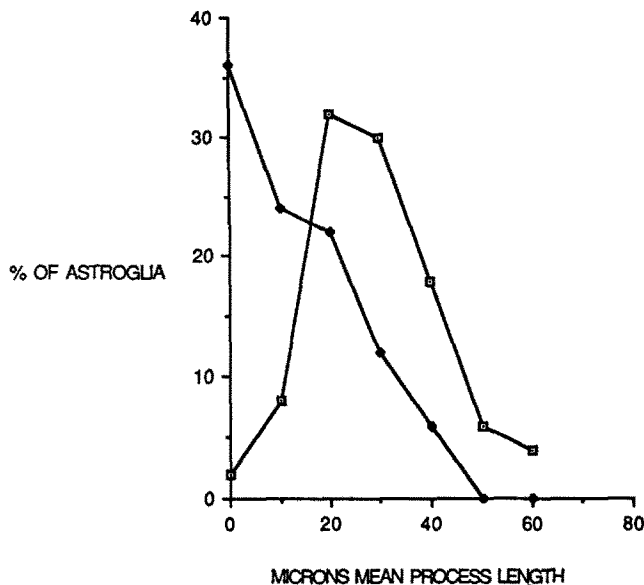


Figure 3. Graph of distribution of lengths of glial processes after 24 h in the absence (□) or presence (◆) of anti-astrotactin Fab fragments. In the untreated cultures, astroglial processes attain a range of lengths with a mean of 25 μm. In Fab-treated cultures, in contrast, 40% of astroglia lack processes and very few have processes in excess of 30 μm. The SD (not shown) was <10%.

Table I. Summary of Cellular Staining and Absorption Experiments

Cell type	Binds antiserum	Neutralizes activity
Cerebellar neurons	+	+
Cerebellar glia	+	-
PC12 cells	+	-
G26-24	+	-
A-172	+	-
C6	-	-
PTK-2	-	-
BRK2-F	-	-
Mouse 3T3-F	+	-
Mouse L	+	-

passage over a series of replicate monolayer cultures of clonal cell lines or of cerebellar neurons or glia. Immunostaining of the last replicate culture used to absorb the antibody was used to confirm that the absorption was exhaustive.

When the immune Fab fragments were preabsorbed with cultures of cerebellar cells, the biological blocking activity was removed, suggesting that the blocking antibodies were bound and removed by the cells (Table I). Absorption with purified granule neurons also removed the blocking activity, suggesting that the immune activity was present on granule neurons.

To determine whether the blocking antibodies were bound by granule neuron membrane material, we passed the antiserum through an affinity column of solubilized granule neuron glycoproteins coupled to CNBr-activated Sepharose 4B and tested the effects of the Fab fragments eluted from the column on neuron-glial interactions in the cultures. The affinity-purified Fab fragments blocked neuron-glial interactions at a concentration of 1 μg/ml, representing a 500-fold increase in the activity of the serum (Fig. 4, a and b).

We then immunostained clonal cell lines derived from different tissues with anti-astrotactin antiserum. Cells of five of the lines tested, including the PC12 neuronal cell line derived originally from pheochromocytoma tissue, the mouse glioma line G26-24 and human glioma line A-172, mouse 3T3 fibroblasts, and mouse L cells were stained with the antiserum. Cells of three other lines, the rat glioma cell line C6, the kangaroo rat epithelial cell line PTK-2, and the rat BRK2-F cell line were unstained (Table I). When we absorbed the immune serum with these clonal cell lines, none removed the blocking activity (Table I), indicating that the staining was not related to the anti-astrotactin activity.

The finding that PC12 cells did not remove the blocking activity (Fig. 5) was of special interest because PC12 neurons do not bind to cerebellar astroglial cells and because they carry several molecules proposed to be involved in neuron-neuron and neuron-glial cell adhesion, including BSP-2 (N-CAM) and NILE (L1, Ng-CAM) (7, 22, 30-34). To test directly whether anti-BSP-2, anti-NILE glycoprotein or anti-L1 antigen Fab fragments blocked neuron-glial interactions in microcultures, we added these antibodies in microcultures at the time the cells were plated and measured neuronal associations with astroglial cells 24 h later. None of these reagents blocked astroglial organization of neuronal

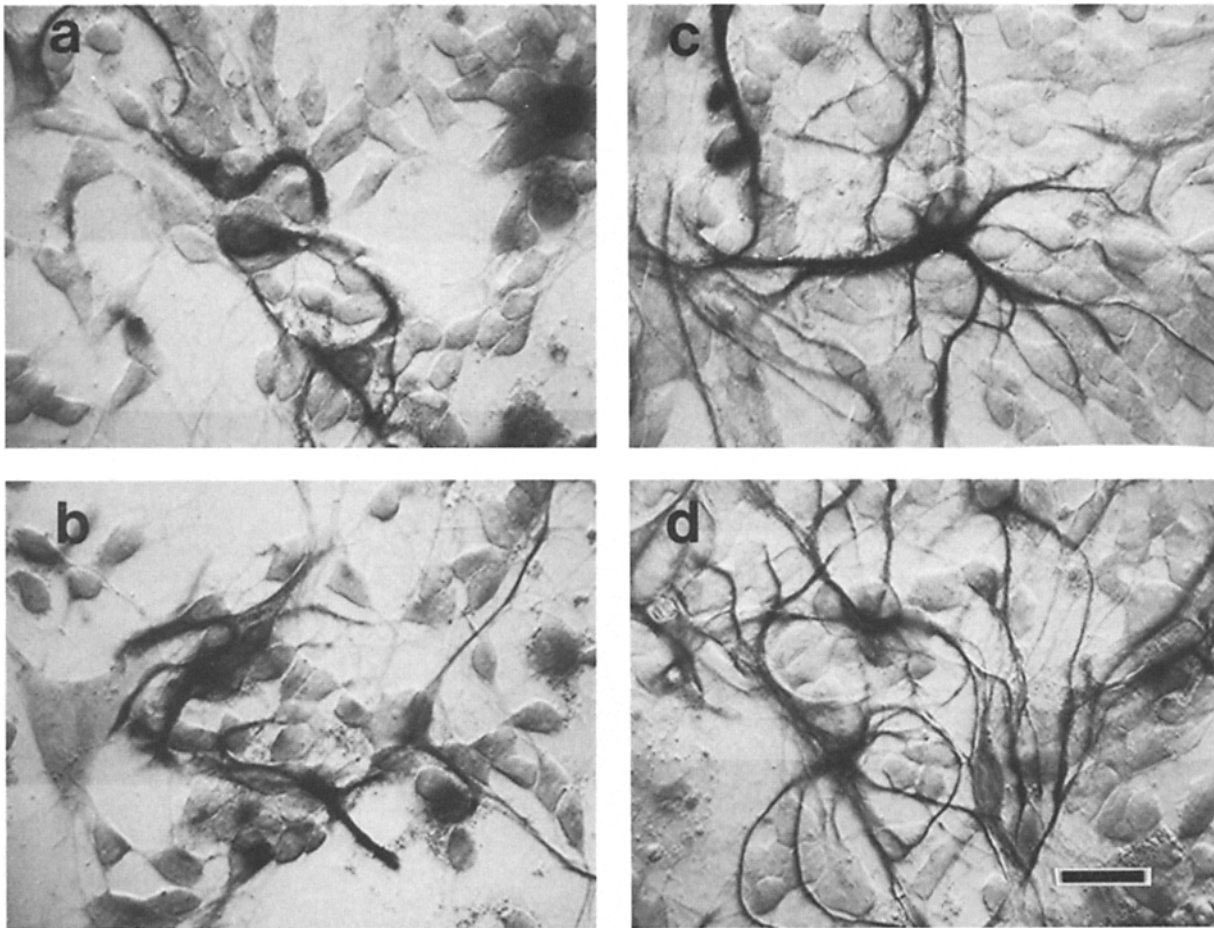


Figure 4. Anti-glial filament staining after 24 h in the presence of affinity-purified anti-astrotactin Fab fragments (*a* and *b*), or anti-NILE glycoprotein Fab fragments (*c* and *d*). The concentration of affinity purified Fab fragments needed to achieve blocking was 500-fold less than that of the unpurified Fab fragments. In the anti-NILE-treated cultures, astroglia form stable contacts with granule neurons, extend processes and organize the position of granule neurons. Bar, 20 μ m.

position over the first 24 h (shown for anti-NILE Fab fragments in Fig. 4, *c* and *d*).

Biochemical Characterization of Anti-Astroctactin Activity

Immunoprecipitation of [³⁵S]methionine-radiolabeled mate-

rial from cerebellar cells cultured for 48 h in vitro, showed that the starting antiserum recognized a large number of bands (Fig. 6 *a*). Among these were antigens with apparent molecular masses of 180, 140 (N-CAM, BSP-2), and 230 kD (NILE, L1, NgCAM). Absorption of the antiserum with PC12 cells removed activity against most of the bands recog-

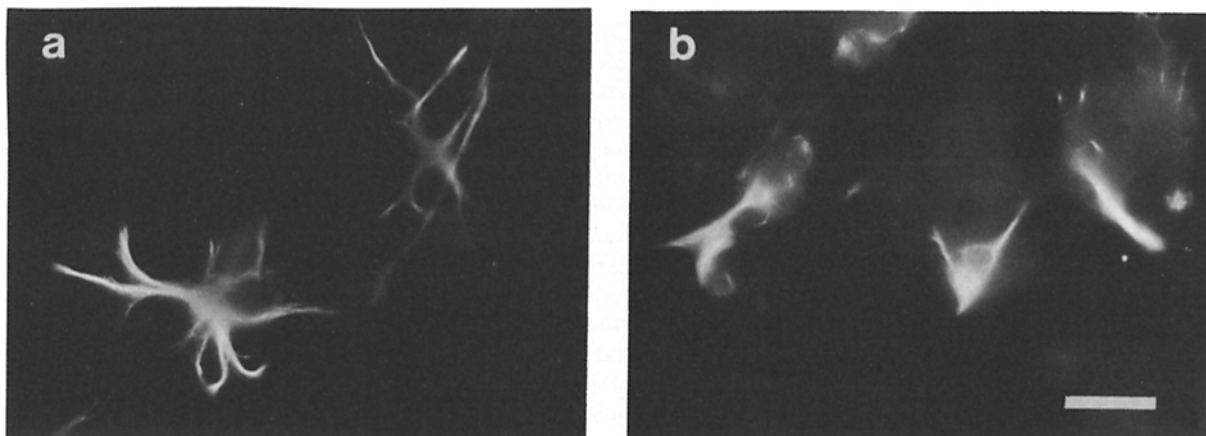


Figure 5. Glial filament staining of cultures treated with (*a*) cerebellar-neuron or (*b*) PC12-absorbed anti-astrotactin Fab fragments. In (*a*), astroglia extend processes as in untreated cultures. In (*b*), astroglia lack processes and are flattened. Bar, 20 μ m.

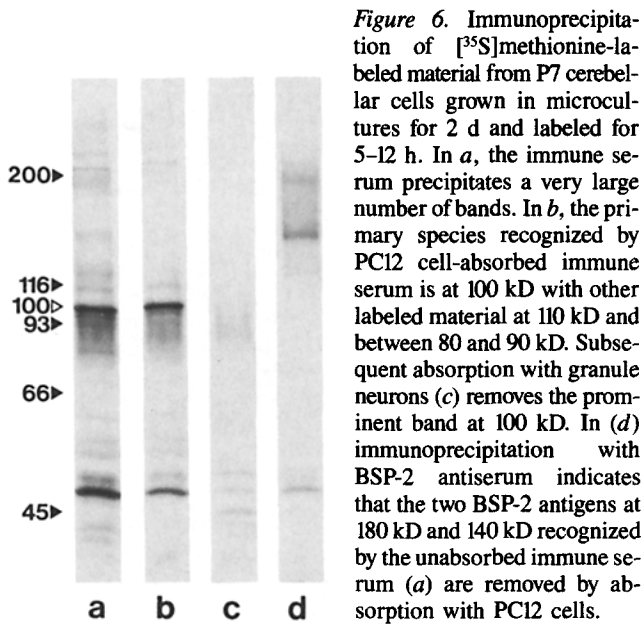


Figure 6. Immunoprecipitation of [^{35}S]methionine-labeled material from P7 cerebellar cells grown in microcultures for 2 d and labeled for 5–12 h. In *a*, the immune serum precipitates a very large number of bands. In *b*, the primary species recognized by PC12 cell-absorbed immune serum is at 100 kD with other labeled material at 110 kD and between 80 and 90 kD. Subsequent absorption with granule neurons (*c*) removes the prominent band at 100 kD. In (*d*) immunoprecipitation with BSP-2 antiserum indicates that the two BSP-2 antigens at 180 kD and 140 kD recognized by the unabsorbed immune serum (*a*) are removed by absorption with PC12 cells.

nized by the immune serum, leaving a prominent band at 100 kD and a smear of material at 80–100 kD (Fig. 6 *b*). Subsequent absorption of the antibody with purified cerebellar granule neurons, a step that removed the blocking antibodies in the microculture assay, removed activity against the band at 100 kD, suggesting that this antigen was bound by blocking antibodies (Fig. 6 *c*).

Similar results were seen when Triton extracts of cerebellar cells labeled with [^3H]fucose or with [^3H]glucosamine were immunoprecipitated with the same set of antibodies. The starting antiserum recognized a large number of bands (Fig. 7 *a*), most of which were removed by absorption with PC12 cells (Fig. 7 *b*). Subsequent absorption with granule neurons removed the prominent band at 100 kD, the same apparent molecular mass as the moiety identified in the [^{35}S]methionine immunoprecipitation, suggesting that this antigen is a glycoprotein (Fig. 7 *c*).

In addition to the removal of the band at 100 kD, two less reproducible changes were noted after absorption with granule cells. First, in fluorograms of [^3H]fucose-radiolabeled cells, some reduction was seen in the amount of a heavily glycosylated band with an apparent molecular mass of 93 kD, after absorption with granule neurons. Second, immunoprecipitation of [^{35}S]methionine-labeled Triton extracts of cells showed a reduction in a minor band at \sim 110 kD after granule cell absorption. The band at 110 kD was not seen when the cells were labeled with [^3H]fucose or [^3H]glucosamine, suggesting that it is not a glycoprotein.

Immunoprecipitation of [^3H]fucose-radiolabeled Triton extracts of cerebellar cells with antiserum that was first absorbed with PC12 cells and then affinity purified with cerebellar granule neuron membranes coupled to CNBr-activated Sepharose, showed a pattern of bands that was identical to that in Fig. 7 *b* (not shown). Affinity purified antibodies did not bind to the BSP-2 (N-CAM) or NILE (L1, Ng-CAM) antigens.

To compare directly the immune activities removed by absorption with PC12 cells with anti-N-CAM or anti-NILE

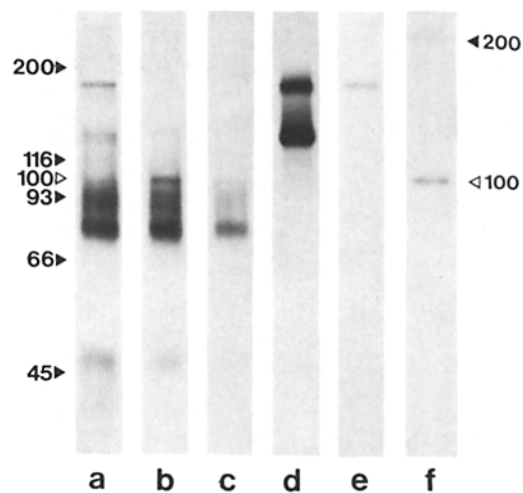


Figure 7. Immunoprecipitation of [^3H]fucose-labeled material solubilized from mouse cerebellar cells grown in microcultures for 5 d. In lane *a*, the immune serum precipitates a very large number of bands. In *b*, PC12 cell-absorbed antibodies recognize a major band at 100 kD and a diffuse smear of material of lower molecular mass. Subsequent absorption of the antiserum with purified granule cells (*c*) removes the prominent band at 100 kD, but leaves the material at 80–90 kD. Immunoprecipitation of duplicate samples with antibodies against BSP-2 (*d*) and NILE (*e*), respectively, show that these antigens are recognized by the unabsorbed immune serum, and are among the bands removed by absorption with PC12 cells. In lane *f*, Triton extracts of rat cerebellar harvested at P7 and radiolabeled with [^3H]fucose as described for weaver cells were immunoprecipitated with PC12 cell-absorbed anti-astrotactin antiserum. A single prominent band is seen at 100 kD. The molecular mass markers for lanes *a*–*e* are shown at left, those for lane *f*, at right.

activity, we immunoprecipitated Triton extracts of [^{35}S]methionine-radiolabeled cerebellar cells with anti-BSP-2 antiserum and with anti-NILE antiserum. As shown in Fig. 6 *d*, anti-BSP-2 antiserum precipitated two bands, one at 180 and another at 140 kD. The anti-NILE antiserum precipitated a single band at 230 kD (not shown). All three of these bands were also labeled with both [^3H]fucose and [^3H]glucosamine (Fig. 7, *d* and *e*).

To test whether antibodies against BSP-2 or NILE would remove the bands recognized by the immune serum, we sequentially immunoprecipitated a single sample with antibodies against BSP-2, antibodies against NILE and then by PC12-absorbed antiserum. The results of this experiment were identical to those seen in Figs. 6 and 7. Anti-BSP-2 antibodies precipitated two bands, one at 180 kD and the second at 140 kD; anti-NILE antibodies precipitated a band at 230 kD; PC12 cell absorbed immune serum then bound two bands, the band at 100 kD and a smear of material between 80–90 kD.

For comparison, since identical biological effects of the antiserum were seen for mouse and rat cells, we immunoprecipitated Triton extracts of [^3H]fucose-labeled rat cerebellar cells (Fig. 7 *f*). In contrast to the results with mouse cells, this showed a single prominent band at 100 kD after absorption of the serum with PC12 cells. Subsequent absorption with granule cells removed the band at 100 kD and removed blocking activity in the microculture assay.

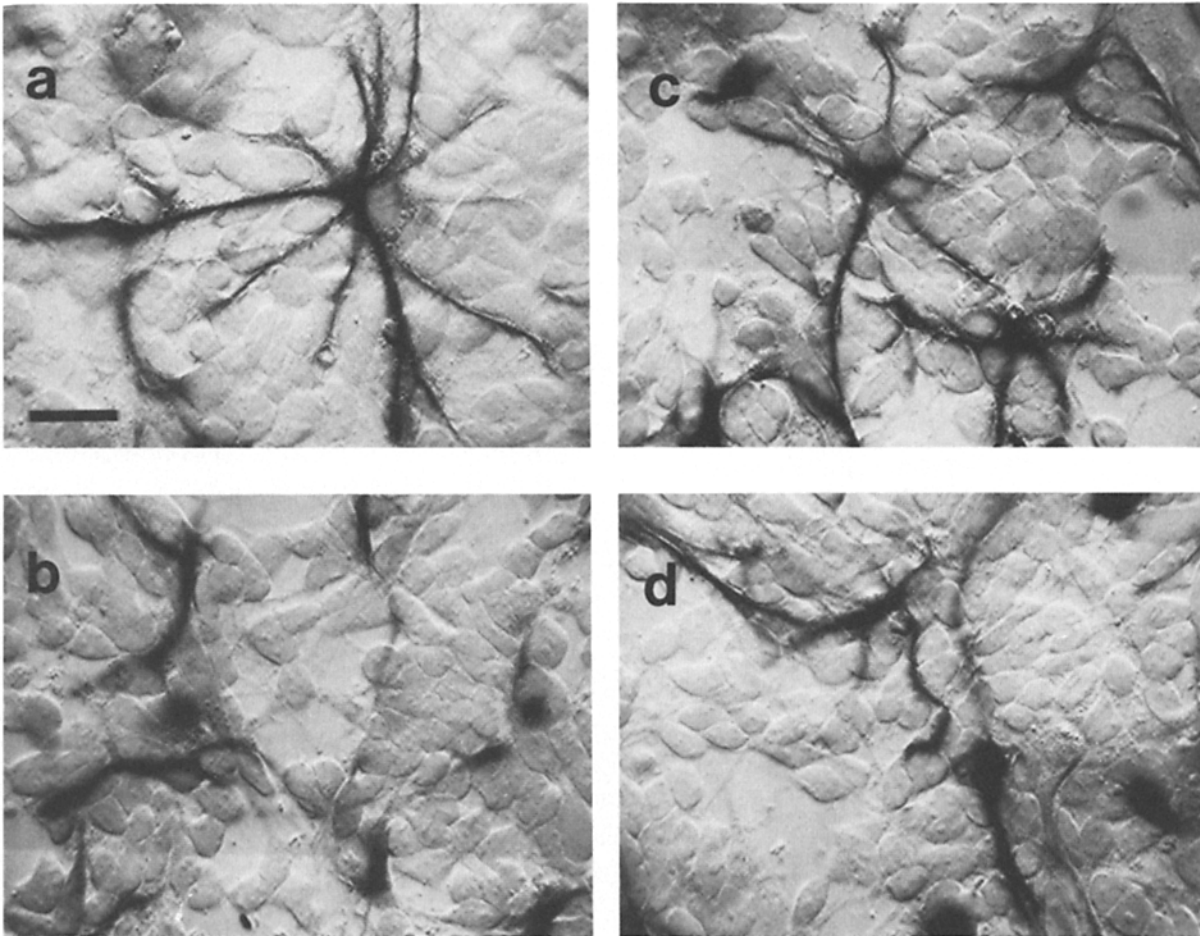


Figure 8. Immunocytochemical localization of glial filament protein at the 24-h time point in cultures from untreated normal cerebellum (*a*) or normal cerebellum treated at the time of plating with unabsorbed anti-astrotactin Fab fragments (*b*) or anti-astrotactin Fab fragments absorbed with normal (*c*) or weaver (*d*) cells. Note reduced number of neuron-glia contacts and stunted astroglial process outgrowth in *b* as compared with *a*. In *c*, pre-absorption with normal cerebellar cells restores formation of neuron-glia contacts and astroglial process outgrowth, whereas in *d*, in contrast, pre-absorption with weaver cells fails to neutralize the anti-astrotactin blocking activity. Bar, 20 μ m.

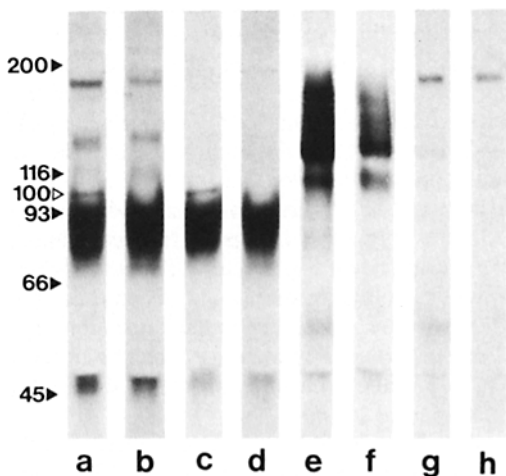


Figure 9. Immunoprecipitation of [3 H]fucose labeled, detergent solubilized glycoprotein material with starting anti-astrotactin, PC12-absorbed anti-astrotactin, anti-BSP-2 and anti-NILE antisera. (Lanes *a*, *c*, *e*, and *g*) normal; (lanes *b*, *d*, *f*, and *h*) weaver. (Lane *a*) Wild-type material immunoprecipitated with the starting serum. (Lane *b*) Weaver cellular material immunoprecipitated with

Weaver Cerebellar Cells Fail to Remove the Anti-Astrotactin Blocking Activity

In contrast to the results with normal cerebellar cells, sequential absorption with weaver cerebellar cells did not remove the anti-astrotactin blocking activity (Fig. 8). This result was unchanged at comparatively high concentrations of

starting antiserum. The major difference between lanes *a* and *b* is the greatly reduced level of the 100-kD band in *b*. (Lane *c*) Wild-type material immunoprecipitated with PC12-cell absorbed antiserum. (Lane *d*) Weaver material immunoprecipitated with PC12-absorbed anti-astrotactin antiserum. The reduced levels of the 100-kD band in weaver (*d*) are also seen with the PC12-absorbed serum. (Lane *e*) Wild-type material immunoprecipitated with anti-BSP-2 antiserum. (Lane *f*) Weaver material immunoprecipitated with anti-BSP-2 antiserum. Slightly reduced amounts of BSP-2 were immunoprecipitated from weaver cells. (Lane *g*) Wild-type material immunoprecipitated with anti-NILE antiserum. (Lane *h*) Weaver material immunoprecipitated with anti-NILE antiserum. No difference in the amounts of NILE was seen between wild-type and weaver, in agreement with the studies of Faissner et al. on the L1 antigen (6).

weaver granule cells, concentrations that would offset cell loss from the death or degeneration of weaver granule cells. The finding that weaver cells failed to remove blocking antibodies from the immune preparation suggested that the astrotactin activity was missing or defective on weaver granule neurons.

Biochemical Studies of Weaver Cerebellar Cells

When cerebellar cultures from weaver cerebellum were solubilized and immunoprecipitated with the unabsorbed serum, the most striking difference in the pattern of bands was that the band at 100 kD was greatly reduced in intensity (Fig. 9). By scanning densitometry, levels of the 100-kD band were shown to be reduced by more than 95% in weaver.

When the radiolabeled Triton extracts of weaver and normal cells were immunoprecipitated with anti-BSP-2 antisera a decrease in the amount of BSP-2 (N-CAM) was seen in weaver cells. Immunoprecipitation with anti-NILE antisera, in contrast, revealed comparable amounts of labeled glycoprotein material in weaver vs normal cerebellar cells. In all experiments, the weaver cells were taken from the midline portion of the cerebellum.

Time-Lapse Video Microscopy of the Formation of Neuron-Glial Contacts in Anti-Astrotactin-Treated Cells and in Untreated Weaver Cells

To determine whether the anti-astrotactin Fab fragments interrupted the initial steps in the establishment of normal neuron-glia interactions in the cerebellar microculture, we added the Fab fragments (0.5 mg/ml) at the time of plating and observed cell-cell interactions between neurons and glia with high resolution time-lapse video microscopy (21).

In untreated normal cultures ($n = 8$) when growing glial processes contacted granule neurons, stable neuron-glia contacts formed rapidly (21), generally within the first minute after the initial encounter between the glial process and a filopodial extension of the granule cell (Fig. 10, *a* and *b*; Fig. 11, *a* and *b*). These contacts resulted in a stable association of the neuron with the glial process and the extension of a short neuronal process onto the glial arm. When we followed the cells for two 5-h intervals thereafter, the neuron-glia contact remained stable. In contrast, when the cells were plated in the presence of Fab fragments of anti-astrotactin antibodies ($n = 8$), the glial process contacted the neuron, via respective filopodial or lamellopodial extensions (see arrow, Fig. 10, *c* and *d*; Fig. 11, *c* and *d*), but rapidly withdrew, followed by repeated advances and withdrawals that never resulted in contacts that persisted for more than a few seconds. After we observed the cells with video microscopy, we scribed the field recorded with the video camera and immunostained the cells with antisera against the glial filament protein to confirm the identity of glial processes.

To compare initial neuron-glia contacts of anti-astrotactin Fab-treated normal cells with untreated weaver cells, cultures from the midline weaver cerebellum were observed with high-resolution time-lapse videomicroscopy at 1 h after plating (Fig. 10, *e* and *f*; Fig. 11, *e* and *f*). In weaver cultures ($n = 6$) filopodia on neuronal and glial processes touched and withdrew repeatedly, without forming a stable contact.

This sequence of forming and releasing cell-cell contacts between neurons and astroglia, seen for weaver neurons and glia, resembled the behavior of normal cells in cultures that had been treated with anti-astrotactin Fab fragments.

In addition to defects in the establishment of cell-cell contacts between neurons and glia, the cytology of growing weaver astroglial processes differed from that of normal astroglial cells (21). The most prominent differences were a less organized central core of mitochondria and organelles and a less regular pattern of extension of lamellopodia.

Discussion

In these experiments, the cerebellar microculture system has been used to identify and partially purify an immune activity that blocks neuron-glia interactions. The assay that formed the basis of the purification was a measurement of the position of neurons relative to astroglial processes after 24 h in vitro (14). The blocking activity prevented neuronal positioning along glial processes during the first 24 h, resulting in a random distribution of neurons relative to glia. The cellular absorption experiments indicated that the blocking antibodies recognized a novel granule neuron cell surface antigen, which we have named astrotactin because of its apparent role in mediating neuron-astroglial contacts.

The cellular absorption experiments suggested that cerebellar granule neurons, but not cerebellar astroglia, removed the blocking activity. The finding that PC12 cells, 3T3 fibroblasts and PTK-2 cells did not remove the blocking activity was not surprising given the results of prior studies showing that none of these cell types bind to cerebellar astroglia in vitro (14).

The direct visualization of neuron-glia interactions in living microcultures by high resolution video microscopy suggested that the anti-astrotactin Fab material blocked the formation of stable neuron-glia contacts. Previous studies (12, 13) have shown that astroglial have flattened, epithelioid shapes in the absence of neurons, but rapidly extend processes and attain complex, process-bearing forms after forming contacts with neurons. In the present study, glial process extension was inhibited in the presence of anti-astrotactin antibodies. One explanation for this result is that after the antibody blocks the formation of stable contacts between neurons and glia the structure of the emerging glial process becomes disorganized and glial process extension is inhibited by, as yet, unknown mechanisms.

The immunoprecipitations are consistent with the interpretation that a major species recognized by the immune blocking antibodies is the band at 100 kD. The finding that two other antigens, including a heavily glycosylated protein with an apparent molecular mass 93 kD and a protein of apparent molecular mass 110 kD, were reduced after absorption with granule cells cautions against equating the astrotactin activity with the band at 100 kD.

The results of our experiments with rat cerebellar cells support the interpretation that the band at 100 kD is the prominent activity in the anti-astrotactin serum. Whereas anti-astrotactin antibodies had identical functional effects on rat and mouse cells, the immunoprecipitation of rat cells with PC12-cell absorbed serum showed a single prominent band at 100 kD. The intense smear seen at 93 kD in Triton extracts of mouse cells was not seen for rat cells.

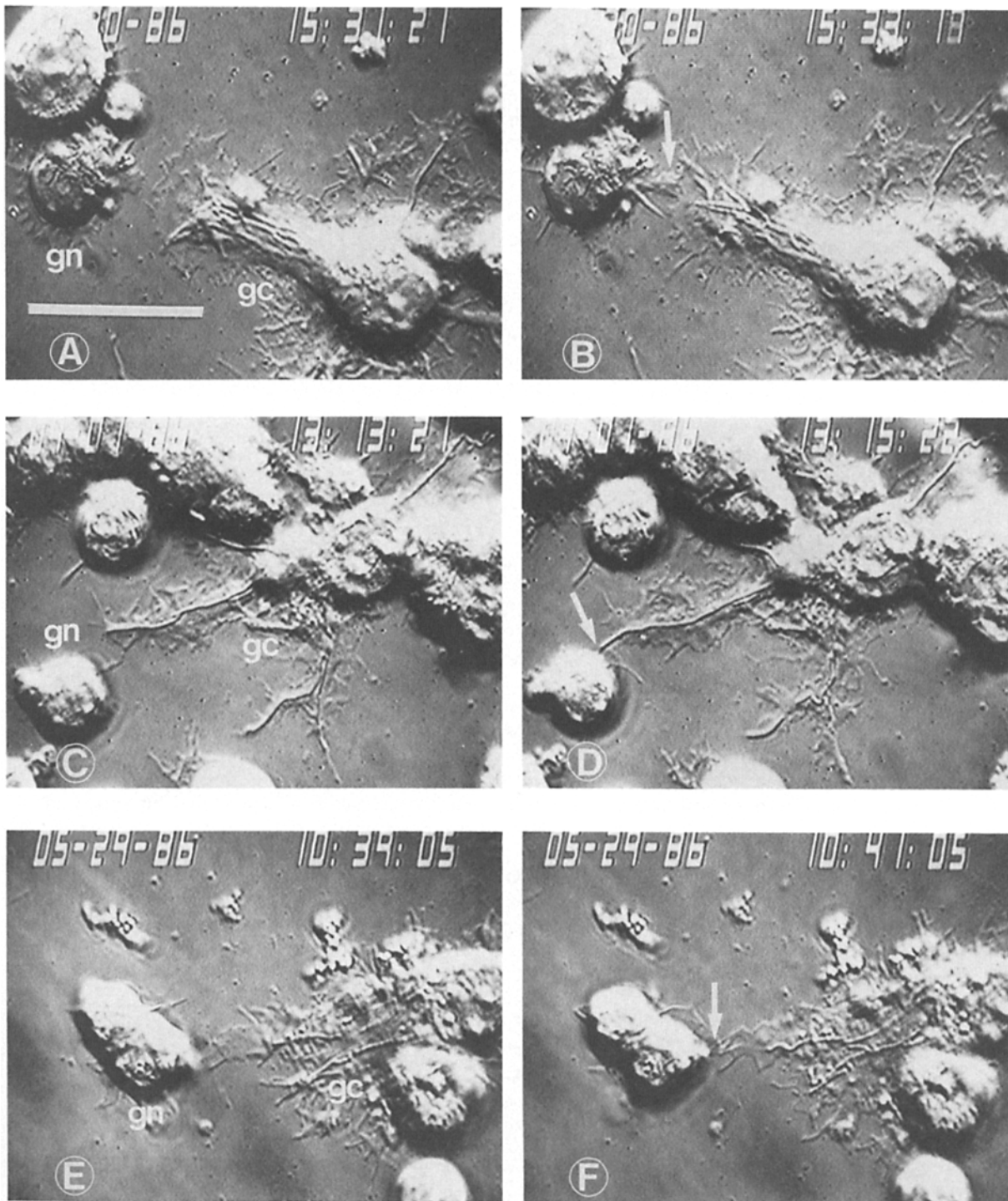


Figure 10. High resolution time-lapse videomicroscopy of astroglial process outgrowth and initial neuron-glia interactions in untreated normal, anti-astrotractin Fab-treated normal and weaver cultures, part I (continued in Fig. 11). (*gn*) Granule neuron; (*gc*) glial cell. *A* and *B*: Untreated normal cultures. *C* and *D*: Anti-astrotractin Fab-treated normal cultures. *E* and *F*: Untreated weaver cultures. In the untreated normal cultures, a stable contact forms between the astroglial process and the granule neuron (*arrow*). In contrast, in both the anti-astrotractin Fab-treated normal culture and the weaver culture, the astroglial process and the granule neuron contact and detach repeatedly, but do not form a stable binding. Time in hours/minutes/seconds at top of field. Bar, 20 μ m.

Several different experiments in the present study suggest that the anti-astrotractin activity is not against the BSP-2 (N-CAM) or NILE (L1, NgCAM) glycoproteins. First, the immunoprecipitation experiments suggest that the BSP-2 (N-CAM) and NILE (L1, NgCAM) antibodies were removed

from the starting immune serum by absorption with PC12 neurons, cells that Friedlander et al. (7), McGuire et al. (22), and Sajovic et al. (32) have shown express N-CAM and NILE on their cell surfaces. Second, the sequential immunoprecipitation of the immune serum with antibodies against

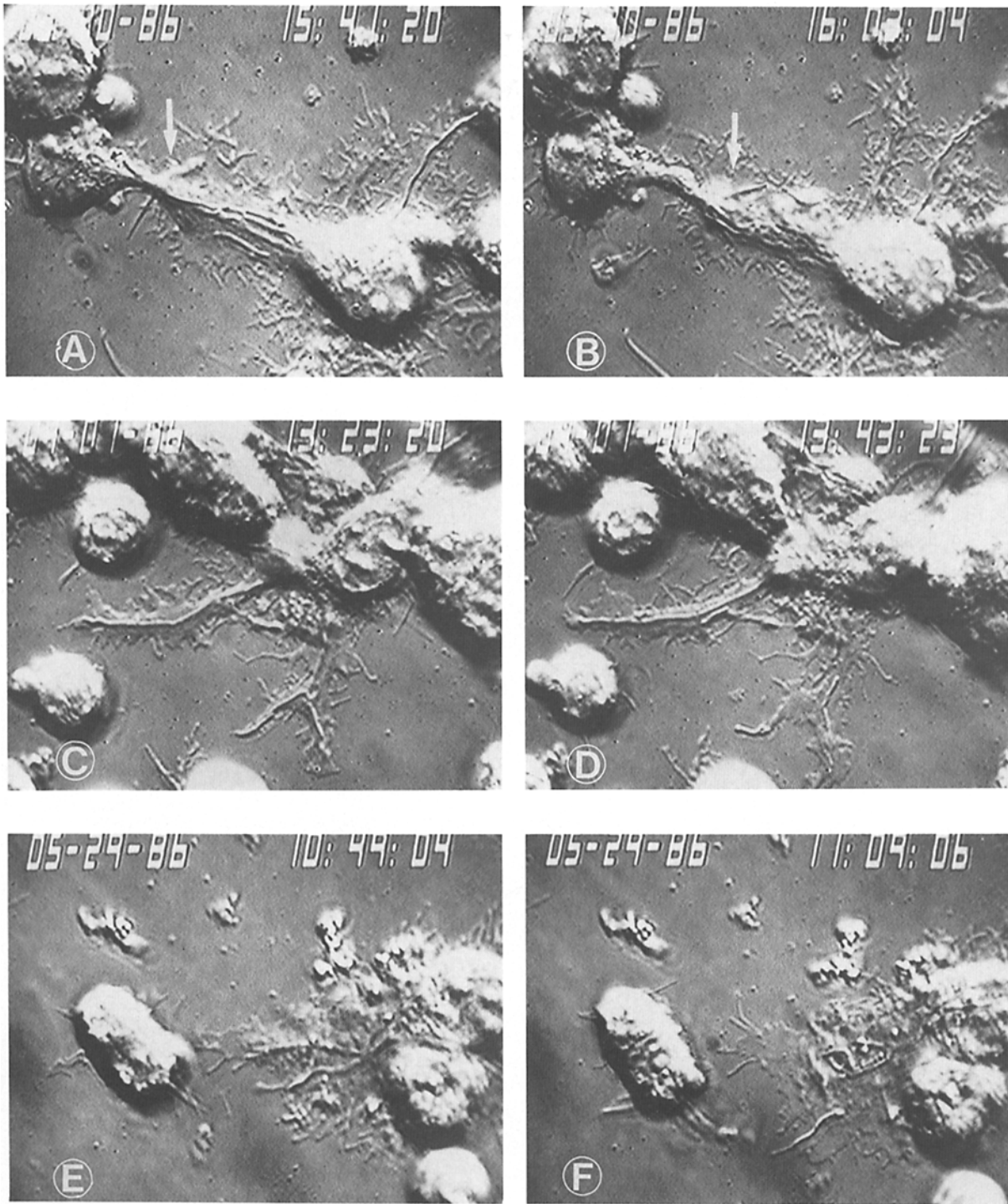


Figure 11. High resolution time-lapse videomicroscopy of astroglial process outgrowth and initial neuron-glia interactions in untreated normal, anti-astrotactin Fab-treated normal and weaver cultures, part II. (A and B): Untreated normal cultures. (C and D) Anti-astrotactin Fab-treated normal cultures. (E and F) Untreated weaver cultures. Continuation of Fig. 10; see Fig. 10 for explanation.

BSP-2, with anti-NILE antibodies and then with PC12 cell-absorbed anti-astrotactin antibodies suggested that the band at 100 kD is not BSP-2 or NILE. Finally the direct studies of the effects of these antibodies on neuron-glia interactions in microcultures showed that neither anti-BSP-2, anti-NILE, nor anti-L1 antibodies affected the neuron-glia associations we measured.

The fact that anti-BSP-2 antibodies did not disrupt neu-

ron-glia interactions in the microculture assay system is consistent with studies by others showing that BSP-2, a mouse antigen that is apparently identical to N-CAM (3, 19, 23, 37), does not mediate neuron-glia contacts (31). This is not surprising since available evidence shows that N-CAM is more prominent in neuron-neuron interactions and has been suggested to play a role in axon fasciculation (36).

The presence of the astroctactin antigen on granule neurons

from weaver cerebella was tested using two complementary techniques: cellular absorption and immunoprecipitation of metabolically labeled cerebellar cells. The cellular absorption experiments suggested that weaver cells either failed to bind or bound markedly lower amounts of the anti-astroctactin antibodies than did cells from normal cerebellum. As a result, the weaver-absorbed anti-astroctactin material still possessed immune activity that blocked neuron-glial interactions in the microculture assay. The cellular absorptions were performed on cultured cells from either normal or weaver cerebella harvested at postnatal day five, an age after the weaver phenotype becomes apparent but before massive granule neuron cell death occurs (29). Thus, it is unlikely that the failure of weaver cultures to remove the anti-astroctactin blocking antibodies was due to the absence of granule neurons in weaver cultures. Rather, the absorption results appeared to reflect differences in the expression of cell surface antigens by the granule neurons which were present in the weaver cultures. The immunoprecipitation findings in the present study suggested that a major deficit was a reduced amount of the 100-kD band among glycoprotein material solubilized from weaver cerebellar cells.

The present findings did not implicate the NILE glycoprotein (L1 antigen, Ng-CAM) in the mechanism of the weaver pathology, as similar amounts of NILE were immunoprecipitated from weaver and normal cells. This finding is consistent with the report of Faissner et al. (6), who did not detect differences in the levels of L1 antigen in weaver. Slightly lower amounts of BSP-2 (N-CAM; see reference 19) were immunoprecipitated from some of the [³H]fucose-labeled weaver cultures in the present experiments. Edelman and Chuong (4) reported that the conversion from the embryonic to the adult form of N-CAM is undisturbed in weaver. However, they did not comment on the levels of N-CAM, specifically in the midline region of the cerebellum. The finding that weaver cells did not neutralize the anti-astroctactin blocking activity, although they expressed normal levels of BSP-2 and NILE, is consistent with our prior finding that preabsorption of the anti-astroctactin antiserum with PC12 cells, which express both N-CAM and NILE glycoprotein, did not remove the anti-astroctactin blocking activity.

The videomicroscopic observations in the present study suggested that anti-astroctactin Fab-treated cerebellar neurons resembled untreated weaver neurons, because they did not form stable neuron-glial contacts at the 1-h point and had impaired astroglial process outgrowth in vitro. Two likely functions for astroctactin suggested by the in vitro experiments are therefore the binding of granule neurons to astroglia and the maintenance of cerebellar astroglial form.

The present studies suggest the usefulness of in vitro model systems, in which complex neuronal behaviors occur, as functional assays to identify molecules involved in cell-cell interactions important to brain development. Here the microculture system has been used to identify and partially purify a novel neuronal antigen, astroctactin, which appears to play an important role in the formation of neuron-glial cell contacts. Preliminary evidence indicates that the astroctactin activity is needed for both neuronal migration along astroglia and for neuronal regulation of astroglial cell growth (our unpublished observations). Thus it is likely that astroctactin is important for a variety of contact-mediated relationships between neurons and astroglia.

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