

Neuronal Regulation of Astroglial Morphology and Proliferation in Vitro

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ABSTRACT To analyze the interdependence of neurons and astroglia during central nervous system development, a rapid method for purifying early postnatal cerebellar neurons and astroglia, and recombining them in vitro, has been developed. The influence of neurons on astroglial shape and proliferation has been evaluated with an in vitro model system previously used to describe the role of cerebellar astroglia in neuronal migration and positioning (Hatten, M. E., and R. K. H. Liem, 1981, *J. Cell Biol.*, 90:622–630; and Hatten, M. E., R. K. H. Liem, and C. A. Mason, 1984, *J. Cell Biol.*, 98:193–204.

Cerebellar tissue harvested from C57Bl/6J mouse cerebellum on the third or fourth day postnatal was dissociated into a single cell suspension with trypsin, and enriched glial and neuronal fractions were separated with a step gradient of Percoll. Highly purified astroglial and neuronal fractions resulted from subsequently preplating the cells on a polylysine-coated culture surface. In the absence of neurons, astroglia, identified by staining with antisera raised against purified glial filament protein, assumed a flattened shape and proliferated rapidly. In the absence of astroglia, cerebellar neurons, identified by staining with antisera raised against the nerve growth factor-inducible large external (NILE) glycoprotein and by electron microscopy, formed cellular reagggregates, had markedly impaired neurite outgrowth, and survived poorly.

When purified neurons and isolated astroglia were recombined, astroglial proliferation slowed markedly and the flattened shape expressed in the absence of neurons transformed into highly elongated profiles that resembled embryonic forms of cerebellar astroglia. After longer periods (48–72 h) in the presence of neurons, astroglia had “Bergmann-like” or “astrocyte-like” shapes and neurons commonly associated with them. These results suggest that neurons influence the differentiation of astroglia.

Astroglia are thought to guide the positioning of young neurons in the developing brain (32–34, 40) and to organize mature neurons into compartments (28). A central question, which has not yet been addressed, is whether the cellular properties underlying the architectonic role(s) of astroglia are specified by intrinsic genetic information or whether they are induced by the interaction of astroglia with immature neurons.

In the cerebellum, a region that has frequently been used as a model because of its limited number of cell types, repeating structure, and well-understood circuitry (28), studies of the differentiation and transformation of astroglia have been complicated by the appearance at late embryonic stages of a unique astroglial cell, the Bergmann glial cell, in the outer layers of the cerebellar cortex (8, 32, 43). The cell bodies of

the Bergmann glia, located in the Purkinje cell layer, project four or five processes to the pial surface, apparently to provide a guide for the anomalous “outside-in” migration of the granule neurons (32, 34).

The migration of granule neurons along Bergmann glia is actually the second step of granule cell migration, the first step being a morphogenetic movement of proliferating granule neuron precursors from the lateral, caudal edge of the thickening cerebellar plate where they are generated up across the rhombic lip to the pial surface (1, 23, 32). In the mouse, the first immature granule neurons arrive on the surface on embryonic day 13. Between that time and birth, they spread across the pial surface as a layer of proliferating cells, the external granular layer (1, 11, 23). During the last few days of gestation, the Bergmann glia differentiate just beneath the

external granule layer (8), which raises the possibility that the overlying young granule neurons induce the maturation of this unique class of astroglia before granule neuronal migration begins.

The cerebellum, because it presents a unique astroglial cell, Bergmann glia, in concert with an anomalous pattern of neuronal migration, that from the pia inward by the granule neuron, provides a unique opportunity to analyze the influence of neurons on astroglial shape. By separating granule neurons from astroglia at different stages of their development and recombining them, it should be possible to test whether granule neurons influence the differentiation of astroglia.

Others have reported methods to separate cerebellar cells by velocity sedimentation (2) or to provide relatively pure astroglial cell cultures (4, 7, 22, 26). Although these methods have yielded much information on the growth and maturation of astroglia, they are not well suited to studies of the effect of neurons on astroglial differentiation because the glia have to be kept in culture a week or more to completely remove the neurons. Here we present a new method to separate rapidly cerebellar neurons and astroglia before plating, and report the characteristics of astroglia cultured in the absence of neurons and our initial observations on the effect of purified neurons on astroglial shape and proliferation.

MATERIALS AND METHODS

Purification of Cerebellar Neurons and Astroglia: Whole cerebellum was removed from C57Bl/6J mice at postnatal day 2–4 (P2–P4).¹ The meninges were carefully stripped off and whole tissue was washed in calcium- and magnesium-free Tyrode's solution (CMF-PBS) three times and dissociated into single cells as described (14, 45). The starting cell suspension was passed through a monofilament polyester screen (33- μ m mesh size, Tetko Inc., Elmsford, NY) to remove cellular reagggregates (Fig. 1) and applied immediately to a two-step gradient of Percoll (35/60% in CMF-PBS that contained 2 mM EDTA; Pharmacia Fine Chemicals, Uppsala, Sweden). The gradients were centrifuged at 3,300 rpm for 10 min in an IEC clinical centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, MA) and the two resulting bands, one at the interface of the CMF-PBS/35% Percoll (fraction 1) and the other at the interface between the layer of 35% Percoll and that of 60% Percoll (fraction 2), were removed with a fire-polished pasteur pipet, diluted to 10 ml with CMF-PBS, pelleted at 600 rpm for 5 min, washed in medium without serum (Eagle's basal medium with Earle's salts; Gibco Laboratories, Grand Island, NY), glucose (6 mM; Sigma Chemical Co., St. Louis, MO), glutamine (4 mM, Gibco Laboratories), and penicillin-streptomycin (20 U/ml, Gibco), and counted. Subsequently the cell suspension was pelleted again, resuspended at the desired cell density in complete medium (supplemented with 10% horse serum, Gibco), and plated either in a Costar cluster 24-well tissue culture dish (16-mm; Costar Data Packaging, Cambridge, MA) pretreated with 25 μ g/ml polylysine (19) or a Lab-Tek tissue culture chamber/slide (cat. no. 4808, 0.89 \times 0.89 cm, Lab-Tek Div., Miles Laboratories Inc., Naperville, IL) pretreated with 50 μ g/ml polylysine. The plates were incubated at 35.5°C with 5% CO₂ and 100% humidity for 15 min, after which the dish was agitated vigorously for 2 min, the supernatant was removed, and the remaining unbound cells were removed by being washed with Eagle's basal medium lacking serum. In some experiments, the two fractions harvested from the Percoll gradient were preplated on an untreated culture dish for 15 min to remove any remaining fibroblast-like cells before being preplated on a polylysine-coated culture surface.

In most experiments, the unbound cells were removed after preplating fraction 1 (the large neuronal fraction, fraction 1b) counted, and discarded. The cells that remained bound after preplating fraction 1 were covered with

fresh culture medium and used as the purified astroglial fraction (fraction 1a). We calculated the number of bound astroglia by subtracting the number of unbound cells (fraction 1b) from the total number of cells in fraction 1.

The unbound cells removed after preplating fraction 2 were counted and used as the purified small neuron fraction (fraction 2b). The cells that remained bound to the polylysine-coated substratum after preplating fraction 2 were discarded (fraction 2a).

Best results were achieved when 1 ml of CMF-PBS containing 3–5 \times 10⁶ cells was applied to the gradient. At higher cell densities, the effectiveness of the Percoll gradient diminished. In some experiments, the culture surface was pretreated with lectins (14) or fibronectin (15) instead of with polylysine.

Cultures of Purified Neurons, Astroglia, and Meninges

Four types of cultures of purified cells were prepared. First, fraction 1a, the "bound astroglia," was used as a pure astroglial cell culture. For long-term experiments, the cultures were fed with fresh medium (50% of the plating volume) every 3 d. We analyzed the size distribution of the cells in the astroglial cultures by photographing four fields of 300 to 500 cells and measuring the diameter of each cell by placing a piece of graph paper with squares cut out to scales of 4, 6, 8, 10, or 12 μ m to determine the best fit for each cell's size.

Second, fraction 2b, the "unbound, small neuronal fraction" was resuspended at 0.5–2 \times 10⁶ cells/ml in complete Eagle's medium and used as a pure neuronal culture. We determined the number and size distribution of the cells by counting an aliquot of the cell suspension with a hemocytometer prior to plating and photographing at least four planes of the neuronal cell suspension with phase-contrast microscopy; cell size was measured by placing a piece of graph paper with squares cut out to a scale of 2, 4, 6, 8, 10, or 12 μ m to determine the best fit of each cell diameter.

These first two types of cultures were carried as a control for every experiment. The third type of culture was a recombination of neurons and astroglia. After preplating, the number of bound astroglia was counted directly by phase-contrast microscopy or indirectly by counting the number of cells removed after preplating and adjusting the number of astroglia plated. The number of cells in fraction 2b was counted with a hemocytometer, adjusted by dilution to a ratio of 10:1 to 1:1 to the number of astroglia, and added to the well that contained isolated astroglia.

In some experiments, single cell suspensions of unseparated cerebellar cells were plated as described (14, 19) at a cell density of 2 \times 10⁶ cells/ml; after 1–3 d in vitro, the culture medium was removed, centrifuged to remove debris, and used as conditioned medium. In other experiments, purified cerebellar neurons (fraction 2b) were plated at 2 \times 10⁶ cells/ml on a culture substratum preplated with 1 mg/ml polylysine; after 2–3 d in vitro, the medium was removed, centrifuged, and used as conditioned medium.

The fourth type of culture was prepared by removing the meninges from the cerebellar tissue used for the purification of neurons and astroglia, dissociating it into single cells with trypsin (46), and plating the cells at 0.1–1.0 \times 10⁶ cells/ml on untreated Costar cluster plates or Lab-Tek tissue culture chamber/slides. All cultures were maintained at 35.5°C with 100% humidity in 5% CO₂.

Most cultures used for immunocytochemistry were prepared in a Lab-Tek tissue culture chamber/slide, a dish with eight square plastic wells (0.89 \times 0.89 cm) affixed to a standard, glass microscope slide. At the conclusion of the culture experiment, the cells were stained, the plastic reservoirs were peeled off, a No. 1 coverslip was mounted over the eight microcultures, and the set of stained microcultures was stored permanently. Cell fractions were purified from more than 75 litters of P2–P4 animals. Approximately 700 cultures were analyzed.

Electron Microscopy of Purified Neuronal Fraction: Fraction 2b was pelleted and processed for electron microscopy by a modification of the method described previously (19). In brief, the pellet was fixed with glutaraldehyde (3% in 0.1 M Sorenson's phosphate buffer, pH 7.2, 1 h, 20°C), washed with phosphate buffer, postfixed with 1% osmium tetroxide (30 min, 20°C), and washed briefly with water. After dehydration in a series of ethanols, the pellet was transferred to a Beem capsule, embedded in Epok (E. F. Fullam, Inc., Schenectady, NY), thin-sectioned and stained with uranyl acetate and lead citrate, and examined with a JEOL 100S electron microscope. Cell types were identified by their size and nuclear morphology and by the presence of 10-nm filaments as described (17).

Immunocytochemical Characterization of Purified Neurons and Astroglia: Antisera against purified glial filament protein (AbGF) were raised in a rabbit and a guinea pig (20). Antisera raised against purified galactocerebroside, a marker for oligodendroglia (35), were kindly provided by Dr. Barbara Ranscht of the Massachusetts Institute of Technology. Antisera against purified nerve growth factor-inducible large external (NILE) glycoprotein (AbNILE), a marker for neurons (36), were provided by Drs. L. A. Greene and M. L. Shelanski of this department.

Four antisera against purified fibronectin, two polyclonal and two mono-

¹Abbreviations used in this paper. AbFN, AbGF, and AbNILE, antisera raised against purified fibronectin, the major glial filament protein, and the nerve growth factor-inducible large external (NILE) glycoprotein, respectively; P2–P4, postnatal days 2–4; CMF-PBS, calcium- and magnesium-free Tyrode's solution; PAP method, peroxidase-antiperoxidase method.

clonal, were used. The first polyclonal antiserum, kindly provided by Dr. Daniel B. Rifkin of the Department of Cell Biology, was raised in a rabbit using gel-purified human plasma fibronectin as an antigen. The specificity of this antiserum has been described previously (12). Another rabbit polyclonal anti-human plasma fibronectin antiserum was purchased from Bethesda Research Laboratories (catalog no. 6071SA, lot no. 20208, Bethesda, MD). Two mouse monoclonal anti-human fibronectin antisera, one provided by Dr. Rifkin and the other purchased from Bethesda Research Laboratories (catalog no. 9523SA, lot no. 20617) were also used.

Cerebellar cultures were stained with antisera with either fluorescence (16) or peroxidase antiperoxidase (PAP) (18, 42) localization. In some experiments, cultures were double-labeled with antisera against purified fibronectin (AbFN) and AbGF by one of two methods. In the first method, the cultures were first fixed with paraformaldehyde (4% in CMF-PBS, 30 min, 20°C), washed three times with CMF-PBS, stained with mouse or rabbit AbFN (1:20–1:500 in CMF-PBS, 1 h, 20°C), fixed a second time with paraformaldehyde (4% in CMF-PBS, 30 min, 20°C), washed three times with CMF-PBS, and then stained with guinea pig AbGF (1:50–1:500 in CMF-PBS containing 0.01% Triton X-100) as described (16). Fluorescein isothiocyanate-conjugated anti-guinea pig or rhodamine isothiocyanate-conjugated anti-rabbit or anti-mouse second antibodies (Cappel Laboratories, Inc., Cochranville, PA) were used at 1:200.

In the second method, the AbFN and AbGF staining steps were combined: cultures were fixed with paraformaldehyde, stained with a mixture of AbGF and AbFN (1:20–1:500 in CMF-PBS containing 0.01% Triton X-100), washed three times, and incubated with a mixture of the two second antibodies. Fluorescence was observed with a Leitz Diavert microscope with epifluorescent illumination (E. Leitz, Inc., Rockleigh, NJ) and a Zeiss 25× Neofluar 0.8-NA phase-contrast/fluorescence or Zeiss plan apochromat 63× 1.4-NA phase contrast/fluorescence objective (Carl Zeiss, Inc., Thornwood, NY). Excitation was at 490 nm (fluorescein) or 560 nm (rhodamine). A band-pass filter (546 nm, Leitz) was used to prevent bleeding of the rhodamine fluorescence into the fluorescein image.

Staining of fraction 2b with AbNILE was carried out in suspension. The cells were washed once with CMF-PBS and resuspended in paraformaldehyde (4%) for 30 min. After being washed three times in CMF-PBS (5 min, 20°C) and incubated in CMF-PBS containing BSA (Sigma, 1 mg/ml) for 30 min, the cells were incubated with AbNILE (1:50 in CMF-PBS containing 1 mg/ml BSA) for 1 h, washed three times, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (1:200 in CMF-PBS containing 1 mg/ml BSA) for 10 min. After three additional washes with CMF-PBS, the cells were resuspended in CMF-PBS and plated in a glass coverslip microculture (14). Fluorescence was observed with a Leitz Diavert microscope as described. In all washing steps, the cells were centrifuged for three minutes at 1,000 rpm in an IEC clinical centrifuge. In control experiments, staining was performed with preimmune antisera or with antisera preabsorbed with purified antigen as described (15, 16, 18).

Measurements of Astroglial Proliferation: To quantitate the number of astroglia, we prepared a series of paired duplicate cultures of fraction 1a, one of which did not have neurons added back whereas the other had neurons added at a final ratio of four neurons to one astroglia. After 24, 48, 72, or 96 h in vitro, the number of cells in the astroglial culture without neurons was counted with phase-contrast microscopy using a calibrated eyepiece. After the last cell count, the astroglial culture was fixed and stained with AbGF (PAP localization) and the number of AbGF-positive cells was compared with the number counted by phase-contrast microscopy at the final time point. In the experiments reported, these two values were within experimental error.

Recombination cultures with both neurons and astroglia were fixed after the indicated time in vitro and stained with AbGF using the PAP method to visualize astroglia. In control experiments, duplicate cultures of fraction 2b, the neuronal fraction, were plated at the same cell density that was added back to purified astroglia and after 24, 48, 72, or 96 h the number of contaminating astroglia was quantitated by staining with AbGF. Experiments where the number of AbGF-positive cells in fraction 2b was >5% of the number of cells plated as fraction 1a were discarded.

RESULTS

In the absence of cerebellar neurons, purified astroglia lost their characteristic appearance, flattened, and proliferated. The shape and proliferation rate appeared to be "titrated" by the addition of increasing numbers of neurons. In the presence of neurons, the flat astroglia assumed a shape characteristic of astrocytes and interacted with granule neurons. These results suggest that neurons affect astroglial morphology and proliferation.

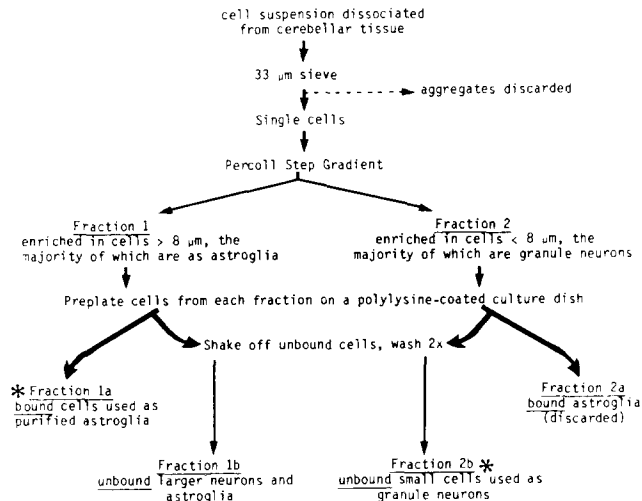


FIGURE 1 Scheme for purification of cerebellar astroglia and neurons. For details, see text.

TABLE I
Distribution of Cells in Purified Fractions

Group	Starting cell sus-	Fraction			
		1	2	1a	2b
I	100	22 ± 4.0	59 ± 19	12 ± 2.5	48 ± 15
II	100	17 ± 7.2	48 ± 16	12 ± 6.0	40 ± 10

The number of cells in each fraction was counted with a hemocytometer and expressed as the percentage of cells in the starting cell suspension. In group I, fractions 1 and 2 from the Percoll gradient were preplated on a Costar 24-well culture dish that had been pretreated with 10 μg/ml of polylysine. In group II, the two fractions were preplated on a Lab-Tek chamber/slide pretreated with 50 μg/ml of polylysine. Each group contained at least 13 separate experiments.

Separation of Astroglia and Neurons

The most successful purification method combined a gradient separation based on cell size and buoyancy with preplating the cells on a culture surface treated with polylysine (50 μg/ml) (Fig. 1). The preplating was the more critical step, exploiting the finding that astroglia attach to a polylysine-coated surface more rapidly than neurons (our unpublished observation). After screening various culture substrates (polylysine, lectins, fibronectin) and time intervals (10 min to 24 h), we found that the optimal condition for separating astroglia from neurons was to preplate the cells for 15–20 min on a polylysine-coated culture surface, after which unbound neurons could be removed by vigorous shaking. As reported earlier (19), the optimal amount of polylysine depended on the batch and type of culture dish used. (If the method is to be adapted for other types of culture plates, a range of polylysine concentrations should be screened). The number of cells in each fraction is given in Table I.

Four other parameters were critical to the purification. First, it was important to use mice on either the second, third, or fourth postnatal days. Markedly poorer separation was achieved with older animals. Second, it was critical to remove completely remove the meninges before the dissociation of the tissue. This was also easier with younger animals. When meningeal cells were present, they could be removed by an additional preplating step (15 min) on an untreated "tissue culture" plate (astroglia required polylysine or some other adhesive ligand to attach rapidly). This additional preplating

step was not necessary when the meninges were removed properly. Third, it was important to wash out the Percoll as it was toxic when the cells were exposed to it for long periods (12–24 h). Finally, the procedure had to be carried out as rapidly as possible, especially the initial steps: sieving of the cells through a 33- μm mesh, application to the gradient, and centrifugation. Best results were obtained when the entire procedure required <45 min.

The most efficient separation of astroglia from neurons was accomplished with culture surfaces >1 mm²; incomplete separation was seen with microtest plates or with microcultures. We obtained an increased local density of astroglia by plating a drop (100 μl) of the cell suspension (fraction 1) onto the center of a Costar well or a Lab-Tek chamber/slide, preplating, and then washing off any unbound cells. In general, the procedure did not generate large quantities of astroglial cells; rather it yielded a small, but highly purified astroglial population more suited for studies on astroglial differentiation and cell-cell interactions than for large-scale biochemical studies.

Purity of Astroglial and Neuronal Cell Fractions

The purity of the cell fractions was analyzed by phase-contrast microscopy, electron microscopy, and immunocytochemical localization of cell markers. Analyses of the distribution of cell sizes in the starting cell suspension and in purified astroglial (fraction 1a) and small neuronal (fraction 2b) fractions were made by phase-contrast microscopy immediately after the preplating step. The vast majority of the cells in the astroglial fraction were 8–12 μm diam (Fig. 2), a size that corresponds to that of cerebellar astroglia (17, 28). Fewer than 5% of the cells in the astroglial fraction were <8 μm diam, a size characteristic of granule neurons, and <2% were >12 μm diam, a size characteristic of most fibroblast-like cells. The distribution of cell sizes in the small neuronal (fraction 2b) was markedly different, most cells being 6–8 μm diam with <5% measuring 10–12 μm diam.

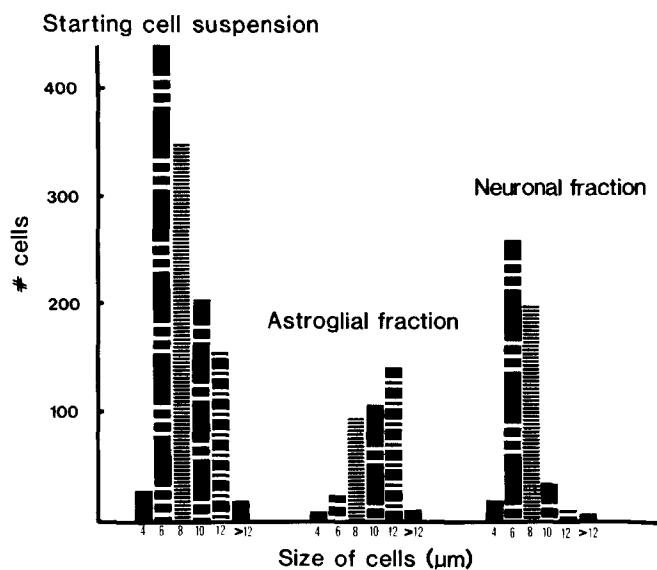


FIGURE 2 Size distribution of cells in purified astroglial and neuronal fractions. The size of each cell in light micrographs of the starting cell suspension, fraction 1a (astroglial fraction), and fraction 2b (neuronal fraction) was measured with a calibrated rule as described in the text, and the number of cells with a diameter of 4, 6, 8, 10, 12, or >12 μm was tabulated.

The relative number of cells in each fraction is given in Table I. Fraction 2 and then fraction 2b contained the largest number of cells, generally three to four times as many as were in fraction 1. In turn, fraction 2b usually had five times as many cells as did fraction 1a. Approximately 15–20% of the cells in the starting cell suspension were lost during the Percoll gradient step, presumably because they were distributed away from the major bands of cells that we harvested for the preplating step. Fewer cells were lost in the preplating step, usually 5–10%; these cells were probably discarded in the washing steps.

The majority of astroglial cells in fraction 1 appeared to bind to the polylysine-coated substratum rapidly, since serial preplating of fraction 1 did not significantly increase their numbers. After preplating for >15–20 min, many more neurons were seen in fraction 1a; after 1 h, most of the neurons were bound to astroglia and were not removed by shaking. Short preplating periods thus optimized the purity of the astroglial fraction.

The removal of the astroglia from fraction 2 by the preplating step (Fig. 3a), and the homogeneity of cell size in the neuronal fraction (Fig. 3b) could also be visualized with phase-contrast microscopy. More than 98% of the cells were AbNILE positive (data not shown). With electron microscopy, most of the cells in fraction 3b had sparse cytoplasm filled with ribosomes and dark, indented nuclei and resembled granule neurons (Fig. 3c). Very few, if any, cells had 10-nm filaments characteristic of astroglial cells. The distribution of the size and types of neurons in fraction 2b remains to be quantitated, but the predominant type of neuron resembled granule neurons.

When purified astroglia (fraction 1a) were stained with AbGF or AbFN after 1–7 d in vitro, all of the cells were AbGF positive and AbFN negative (Fig. 4), which suggests they were astroglial in origin (24). The same staining pattern was seen with four different antifibronectin antibodies (two rabbit anti-human plasma fibronectin and two mouse monoclonal anti-human plasma fibronectin antibodies) even at high antibody dilutions (1:20); no AbFN-positive cells were present. In contrast, when cells dissociated from the meninges were stained, they were AbGF negative with both the rabbit and guinea pig AbGF, at antiserum dilutions up to 1:20 and AbFN positive at antiserum dilutions down to 1:500. These results were seen with both staining protocols, one where the cells were fixed, stained for AbFN in the absence of Triton X-100, fixed again, and then stained for AbGF in the presence of dilute amounts of detergent; or when the cells were fixed and then stained with a mixture of the two antibodies in a buffer containing 0.01% Triton X-100.

Staining of cultures of fractions 1a with antisera against purified galactocerebroside (data not shown) suggested that oligodendroglia were not present. In addition, no cells with the morphological characteristics of oligodendroglia (17, 31, 35, 41) were seen in purified astroglial or neuronal cultures or in recombination cultures. These cells were either discarded with fraction 1b or survived poorly after the cell separation procedure.

Characteristics of Astroglia and Neurons Cultured in Isolation

When plated in microcultures, isolated astroglia had a flattened shape (Figs. 5 and 6a) and all were AbGF positive

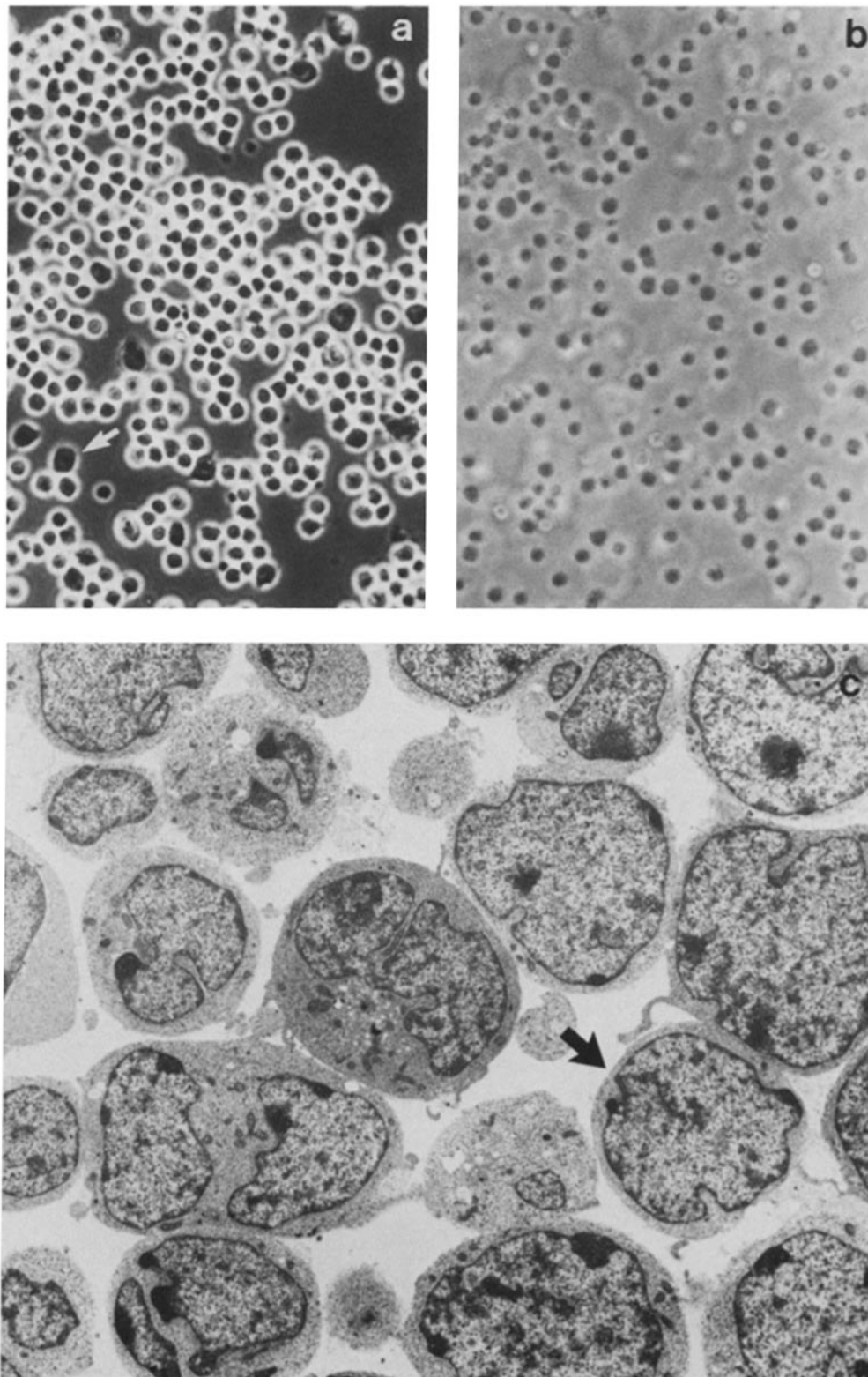


FIGURE 3 Characterization of purified small neurons (fraction 2b) with light and electron microscopy. (a) At the end of the preplating step, fraction 2b was photographed by phase-contrast light microscopy. A number of large cells (white arrow), some of which have already begun to sprout processes, are evident among the more numerous, small, phase-bright neurons. $\times 270$. (b) After the dish is shaken, the large cells are left on the culture surface and a relatively homogeneous population of small, phase-bright cells is seen in suspension. Phase-contrast microscopy, $\times 270$. (c) The purified neuronal fraction was pelleted and processed for electron microscopy. The vast majority of cells resemble granule neurons (arrow), with a diameter of 5–8 μm , sparse cytoplasm, dense heterochromatin, and lacking intermediate filaments. $\times 4,000$.

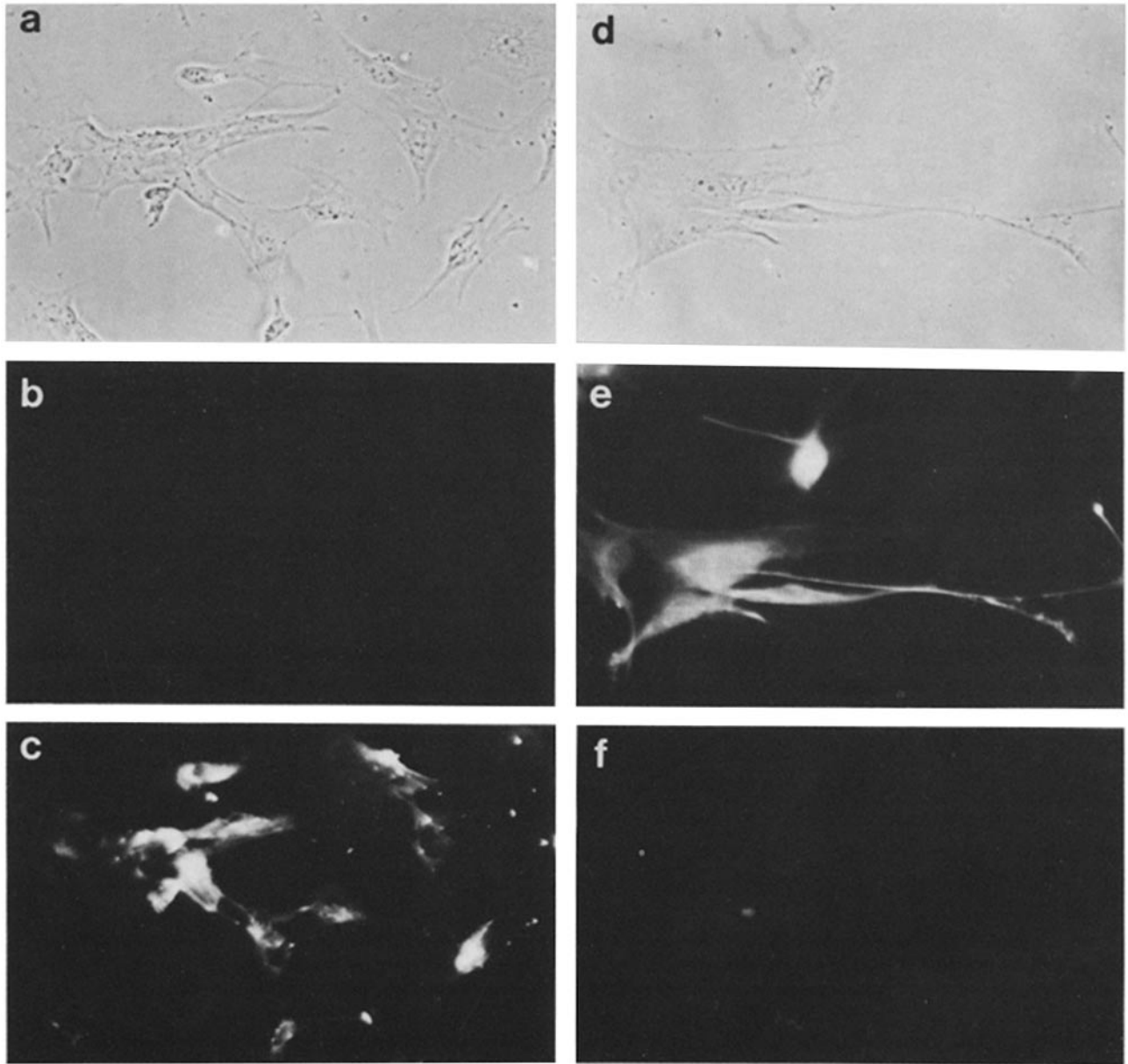


FIGURE 4 Immunological characterization of purified meningeal cells and astroglia. After 5 d *in vitro*, cells dissociated from the meninges of the cerebella used to prepare the cultures of isolated astroglia have a flattened shape (a) and, when double-labeled with guinea pig AbGF (1:200) and rabbit AbFN (Rifkin antibody, 1:50), are AbGF negative (b) and AbFN positive (c). Cells in cultures of isolated astroglia have a more flattened form than that of astroglia typically seen in cerebellar cultures (d); when double-labeled with AbGF (e) and AbFN (f) under the same conditions used for cells from the meninges, astroglial cells show the opposite staining characteristics. (a and d) Phase-contrast microscopy; (b and e) epifluorescent excitation at 490 nm (AbGF); (c and f) epifluorescent excitation at 540 nm (AbFN). Paired fields (a and d; b and e; c and f) were photographed and printed under identical conditions. $\times 190$.

and AbFN negative (not shown). The predominant form of AbGF-positive cell had a shape more characteristic of epithelioid cells than of astroglia seen previously in cultures of dissociated mouse cerebellum (17). After 24–48 h *in vitro*, many of these astroglial forms had a dense, perinuclear bundle of stained filaments; these included mitotic cells as well as cells that were well spread on the polylysine substratum (Fig. 5, a and b). About a third of the flat astroglia had diffuse cytoplasmic AbGF staining. “Ruffled” edges were seen on some of the cells and these often contained stained glial filaments (not shown).

Some isolated astroglial cells had a bipolar shape (Fig. 5c)

with stained filaments coursing through their thick processes and flattened terminations. A few stained cells were multipolar (Fig. 7d) with densely stained filamentous material in their processes. Multipolar astroglia also had endings that were enlarged and thinly spread on the polylysine-coated substratum (Fig. 5d). Although these cells more closely resembled astroglia seen in mixed cultures of neurons and astroglia, their processes were fewer, markedly thicker, and shorter than astroglial cells previously seen in cerebellar cultures (16, 17).

In contrast to isolated astroglia, cells from fraction 2b formed cellular reagggregates that remained in suspension (Fig. 6b) on a culture substratum treated with 10–50 $\mu\text{g}/\text{ml}$ of

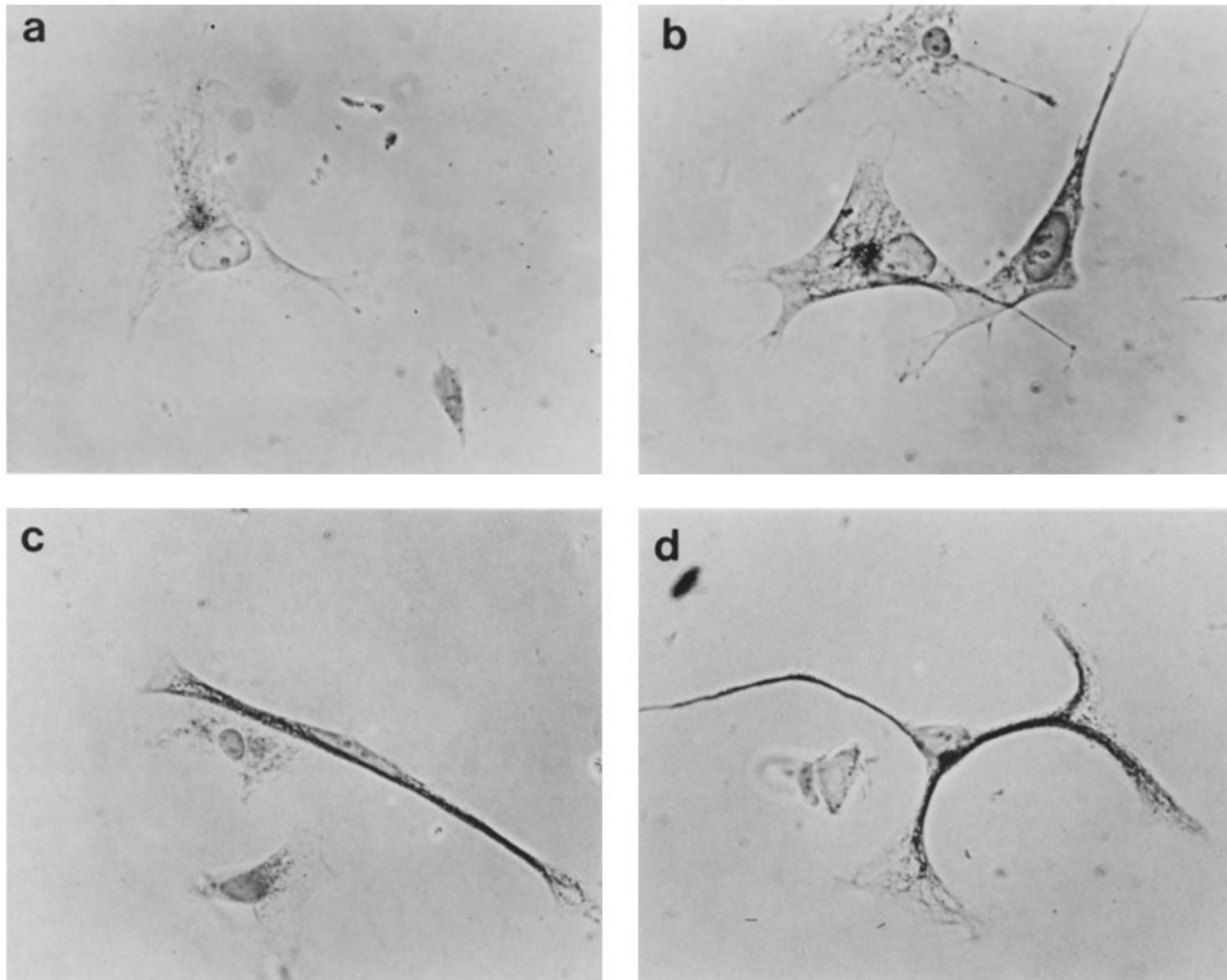


FIGURE 5 Morphology of astroglia cultured in the absence of neurons. After 48 h *in vitro*, cells from fraction 1a were stained with rabbit AbGF (1:2,000), visualized by PAP localization. The predominant form of AbGF-positive cell has a flattened morphology and a dense, perinuclear bundle of stained filaments (a and b). Some cells have a more elongated form (c) with stained filaments and enlarged "end feet." A few stained cells are multipolar (d) with flattened endings. Phase-contrast microscopy. $\times 475$.

polylysine. Very little process outgrowth was seen and cell survival, as measured by exclusion of the dye Trypan blue, was poor after a few days in culture. Contaminating astroglia, when present, adhered to the substratum. Markedly improved plating efficiency (measured as the percentage of trypan blue-negative cells after 24 h *in vitro*), neuronal adhesion, and neurite outgrowth were seen when the culture dish was pre-treated with much higher amounts of polylysine (500–1,000 $\mu\text{g}/\text{ml}$, data not shown). Very few, if any, non-neuronal cells were seen when the cells were plated on surfaces coated with more polylysine.

Recombination of Astroglia and Neurons

When purified neurons were recombined with purified astroglia 1–6 h after the separation procedure, a monolayer formed (Fig. 6) and instead of the flattened forms characteristic of isolated astroglia, elongate or stellate forms were seen (Fig. 7), having a cell soma 9–12 μm diam and long, slender arms that appeared to organize the arrangement of the neurons in the culture (Fig. 7a). Stained filamentous material was seen both in the processes and in the perinuclear regions of these astroglia (Fig. 7b). A relatively high ratio, more than

3:1, of neurons to glia was needed to induce the shape changes from flat to complex forms. At lower ratios of neurons to astroglia, many "flat" astroglial forms remained.

The morphology of the astroglia depended on the amount of time they were co-cultured with neurons. 12–24 h after the neurons were added to purified astroglia, few if any "flat" astroglia were seen. Instead, many AbGF-positive cells had short arms, and others had a stellate profile or an elongated shape (Fig. 8). Some AbGF-positive cells had extremely long arms (200–600 μm), a shape that resembled radial glia (Fig. 8b). Most of these stained astroglia had fewer, longer arms than astroglia typically seen in cultures of postnatal cells and thereby resembled embryonic forms of cerebellar astroglia (13).

After longer periods (2–4 d) in recombination with neurons, cerebellar astroglia had shapes that more closely resembled postnatal astroglia. Two forms predominated: one with long, slender arms and the other with short, stellate arms (Fig. 9). These two *in vitro* forms of postnatal cerebellar astroglia and their support of different neuronal behaviors have recently been described in detail (17).

The changes in astroglial morphology seen after neurons were added were independent of the concentration (10–1,000

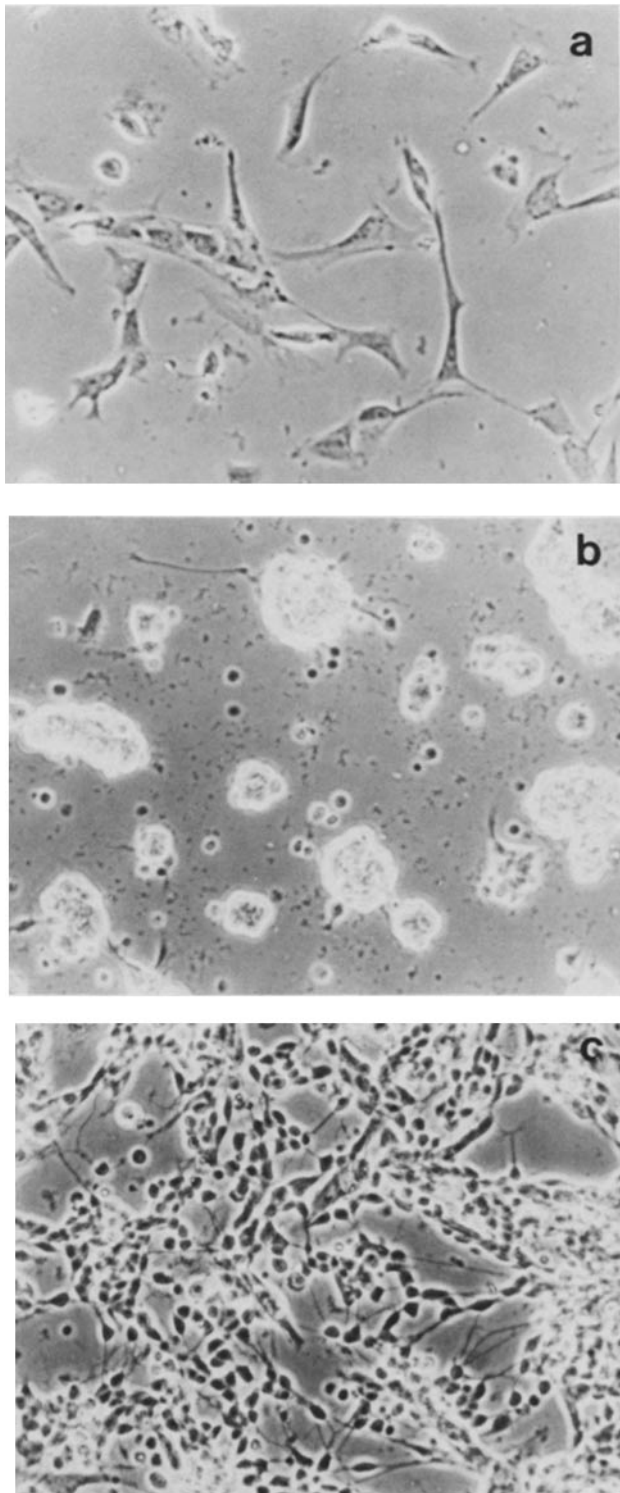


FIGURE 6 Phase-contrast microscopy of purified astroglia (a), purified neurons (b), and purified astroglia recombined with purified neurons (c) after 1 d *in vitro*. In a, presumed astroglia have flat shape and form a monolayer. In b, presumed, phase-bright neurons form cellular aggregates from which little, if any, process outgrowth is visible. (c) When the two cell types are recombined, a monolayer forms. $\times 200$.

$\mu\text{g/ml}$) of polylysine used to treat the culture surface. Thus it is unlikely that the morphological differentiation of the astroglia related exclusively to the strength of their adhesion to the culture dish.

In a preliminary attempt to explore whether the effect of neurons was induced by contact interactions or trophic factors, we tested the effects of adding medium conditioned by cerebellar cells or by purified cerebellar neurons on cultures of isolated astroglial cells, either just after plating or 12–24 h after plating. No changes in astroglial shape or number were seen when the medium was replaced with one of four different “concentrations” of conditioned medium—0, 25, 50, and 100% (prepared in fresh medium supplemented as described).

The most important control for the recombination experiments was to demonstrate that the astroglia whose shape we measured derived from fraction 1a and were not added as contaminant of fraction 2b. Although the analysis of the size distribution of cells in fraction 2b made this possibility unlikely, we evaluated it for each experiment with two controls. First, one duplicate aliquot of fraction 2b was added to a culture of isolated astroglia and another was added to an empty well. After an identical interval *in vitro*, the cultures were stained with AbGF and the number of positive cells was counted. In cases where the number of AbGF-positive cells in the well without astroglia amounted to $>5\%$ of the total number of AbGF-positive cells in the recombination culture, the experiment was discarded. In the second control experiment, a range of cell densities of fraction 2b was added to isolated astroglia and the cultures were later stained with AbGF to determine whether the proportion of AbGF-positive cells increased with the number of neurons. In all of the experiments reported, the variation in the number of AbGF-positive cells over a series of neuron glial ratios between 0 and 10:1 was $<5\%$. The results of these controls were consistent with the interpretation that the astroglia studied were not added to the purified astroglia as a contaminant of fraction 2b.

Influence of Neurons on Astroglial Proliferation

Mitotic figures were evident in cultures of isolated astroglia and cell counts suggested that purified astroglia proliferated rapidly (Fig. 10). When neurons were added back to purified astroglia, the rate of astroglial proliferation was dramatically reduced (Fig. 10), suggesting that neurons inhibit the proliferation of cerebellar astroglia. This finding depended on the plating density of the astroglia. At very low densities (less than 10 cells/mm^2), the onset of glial proliferation was delayed for several days. At higher cell densities, rapid proliferation commenced within 24 h of plating.

DISCUSSION

These studies suggest that neuron–glia interactions influence astroglial differentiation. In particular, neurons appear to induce the expression of astroglial shapes associated with neuronal migration and compartmentalization; and neurons appear to markedly slow the rate of astroglial proliferation.

Among the purification methods that we evaluated, a combination of a Percoll gradient and preplating on a polylysine-coated substratum to remove astroglia provided the most highly purified neuronal and astroglial cell populations. The enrichment by the Percoll gradient is probably best explained by the large difference in size between granule neurons and astroglia. Granule neurons are among the smallest cells in the brain, the size of granule neurons in fixed tissue generally given as 5–6 μm , sometimes 5–8 μm , in diameter (28); *in vitro*, unfixed granule cells are 6–8 μm diam (17). Several

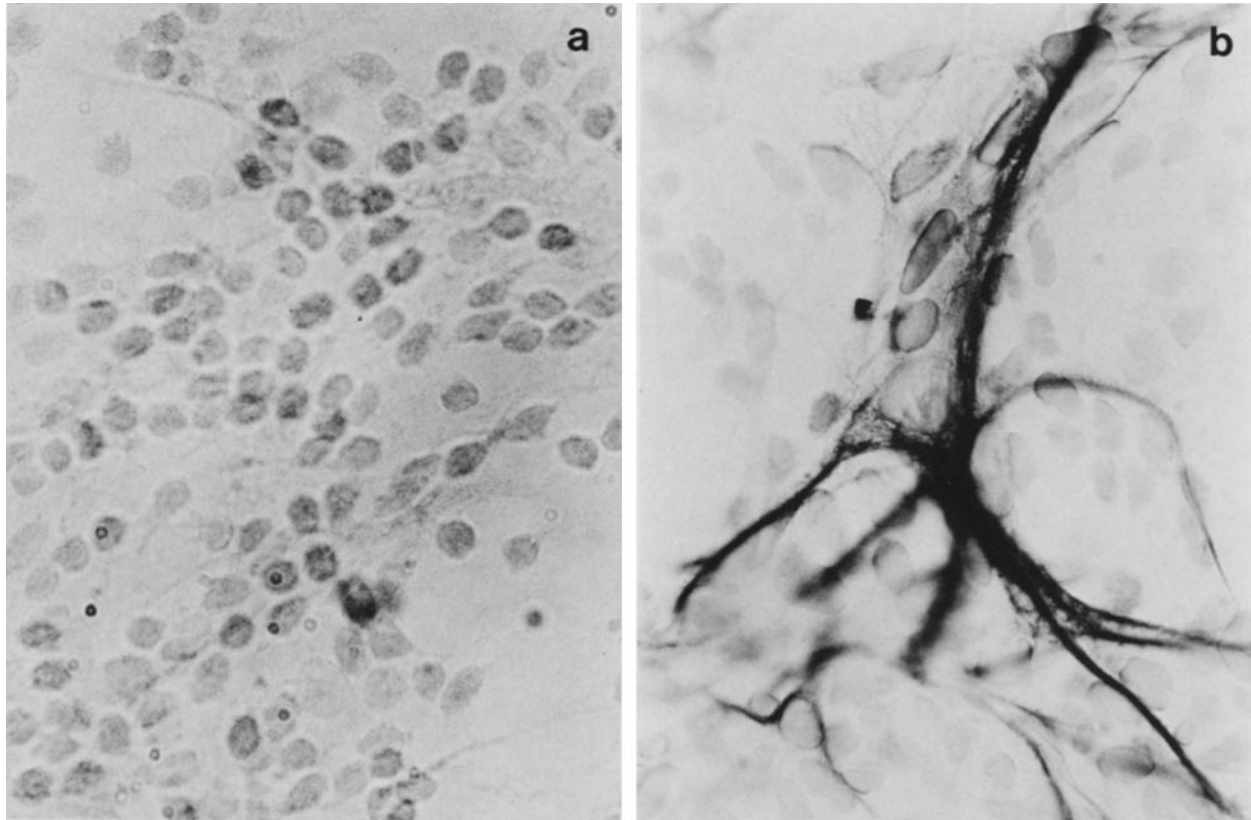


FIGURE 7 Effect of neurons on astroglial shape. Purified neurons were added to purified astroglia immediately after plating at a ratio of 4:1; paired cultures were kept in vitro for 48 h and stained with AbNILE (1:200) to visualize neurons (a) or with rabbit AbGF (1:1,000) to visualize astroglia (b). (b) An AbGF-positive cell clusters unstained, presumed neurons among its processes. Bright-field microscopy. $\times 600$.

other cerebellar neurons are within the upper size range of granule neurons, the basket and stellate neurons (28) generally being $8 \mu\text{m}$ diam.

Young Purkinje neurons and Golgi neurons are $>8 \mu\text{m}$, generally ~ 8 – $10 \mu\text{m}$ diam. By size characteristics alone, it is likely that fraction 2b was highly enriched in granule neurons, but also contained a few basket and stellate neurons, and that fraction 1b was enriched in larger neurons.

By ultrastructural analysis, most neurons present in fraction 2b resembled granule neurons. A few larger neurons were present, but their identity was difficult to assign by morphological criteria alone. More extensive electron microscopy and ^3H -gamma-amino butyric acid uptake studies will be required to quantitate the distribution and types of cerebellar neurons in the fractions.

In contrast to granule neurons, astroglia are one of the largest cerebellar cells, their cell bodies usually being 9 – $12 \mu\text{m}$ diam in vitro (17). Oligodendroglia are also much larger than granule neurons, generally 10 – $15 \mu\text{m}$ diam after 12 – 24 h in culture (17, 35, 41). It is likely, based on the size data alone, that most of the glial cells were present in fraction 1.

The results of the preplating step suggest that the astroglia that contaminated fraction 2 could be removed by a short preplating step on a polylysine-coated surface. The relatively complete purification of astroglia from the fractions by preplating on a polylysine-coated culture surface is consistent with our earlier observation that astroglia rapidly adhere to a polylysine-coated surface (10 – 20 min) and that the neurons, in turn, bind to the astroglial cells and their processes after

much longer periods (30 min to 3 h) (our unpublished observation).

The immunostaining experiments were consistent with the interpretation that whereas the astroglia bound to the polylysine surface with fraction 1a during the preplating step, very few oligodendroglia co-purified with the astroglia. Other experiments not presented here suggest that oligodendroglia can be removed from fraction 1 by preplating on a culture surface treated with the lectin RCA_1 rather than polylysine. Astroglia do not bind to this lectin substratum rapidly (our unpublished observation). Thus in the present purification scheme, oligodendroglia were probably discarded in fraction 1b.

The number of cells in each of the four fractions was consistent with established values on the relative numbers of different cerebellar cell types (6, 28). Granule neurons are by far the most numerous of cerebellar neurons, outnumbering Purkinje neurons by $28:1$ in the mouse at P4 (6). Astroglia are also far less numerous than granule neurons, constituting roughly 10 – 12% of the total cell population dissociated from early postnatal mouse cerebellar tissue (16). At P3, only $\sim 1\%$ of the cells seen in cultures of dissociated cerebellum are oligodendroglia (17, 35, 41). These generalizations are consistent with our result that fraction 2b contained 40 – 50% of the dissociated cerebellar cell population, fraction 1a had 10 – 12% , and the remaining cells were a mixture of larger neurons, immature oligodendroglia, and astroglia that did not bind rapidly to the polylysine surface.

The present study did not characterize the cells present in fraction 1b, a fraction we assume was enriched in Purkinje

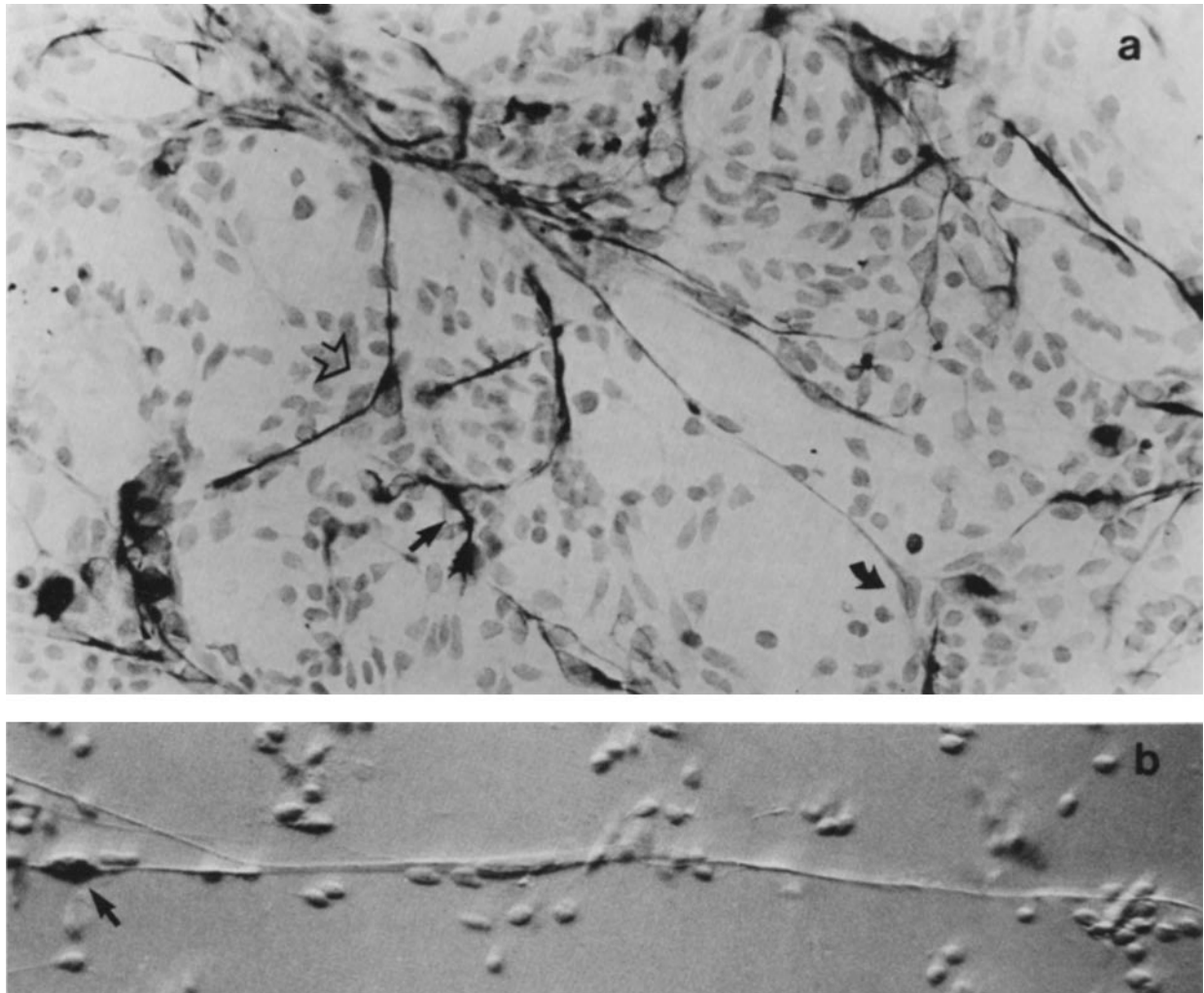


FIGURE 8 Morphology of cerebellar astroglia co-cultured 1 d with neurons. Purified cerebellar neurons were recombined with purified astroglia at a ratio of 5:1 4 h after the astroglia were plated. After 24 h in co-culture, the cells were stained with AbGF (1:1,000) to visualize the shape of the astroglia. In a, many AbGF-positive cells have short processes (arrow); others have fewer, more elongated arms that are characteristic of embryonic forms of cerebellar astroglia (open arrow, curved arrow). Some unstained, presumed neurons are distant from stained astroglial arms. Phase-contrast microscopy. $\times 330$. (b) A highly elongated form of AbGF-positive cell whose processes stretch $>250 \mu\text{m}$ across the culture surface is seen. Nomarski optics. $\times 468$.

neurons, Golgi neurons, and oligodendroglia. Preliminary electron microscopic evidence suggests that this fraction may indeed contain the bulk of the larger cerebellar neurons (C. A. Mason, unpublished observation).

The finding that the cells in the purified astroglial fraction were AbGF positive and AbFN negative also suggests that they were glial in origin. Since cells taken from the meninges had the opposite immunostaining characteristics, it is unlikely that the cells in the astroglial fraction were pial cells.

The flattened shape seen in the absence of neurons is consistent with other *in vitro* studies on purified astroglial cells (4, 7, 22, 26). The flat forms of astroglia that we observed in the absence of neurons appeared to reflect different patterns of organization of the astroglial cytoskeleton. Mitotic cells and cells with epithelial shapes often had perinuclear caps of stained glial filaments; cells with bipolar or stellate profiles had stained glial filaments in their processes. All of these forms of purified astroglia had broader processes bearing more enlarged endings than their counterparts in co-culture with neurons. It remains to be shown whether these different

astroglial shapes represent different types of astroglia, different stages of astroglial differentiation, or the progressing of one or more types of immature glia through the cell cycle.

Removing neurons from the astroglia thus appeared to direct the immature, yet partially differentiated, early postnatal cerebellar astroglia to a more primitive flat form (30). In turn, adding neurons to the isolated astroglia rapidly induced the morphological differentiation of astroglia; all of the astroglia in the culture had complex shapes in the presence of sufficient numbers of neurons. Concomitant with the transformation of astroglial shape, specific associations of neurons with astroglia, previously described for embryonic (13) and postnatal cerebellar cells (16, 17), were seen.

It was of special interest that a minimum number of neurons appeared to be required to induce astroglial shape changes and that postnatal astroglia seemed to revert to embryonic forms before expressing shapes characteristic of postnatal glia. The observation that a certain number of neurons is needed to promote or perhaps sustain astroglial shape could relate to cerebellar pathologies such as the weaver

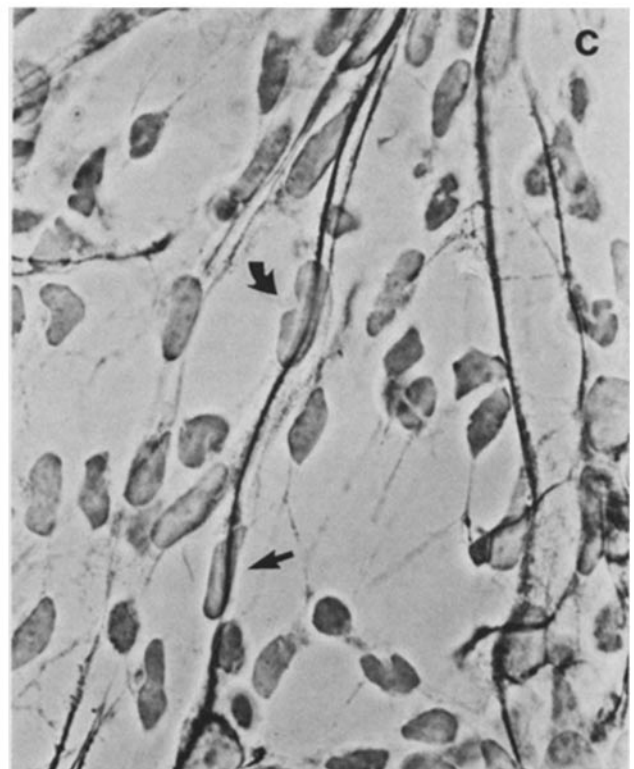
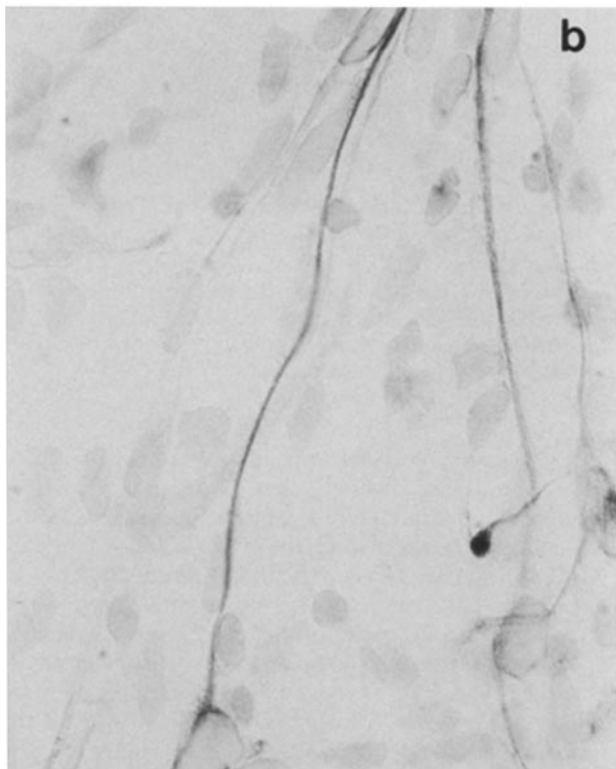
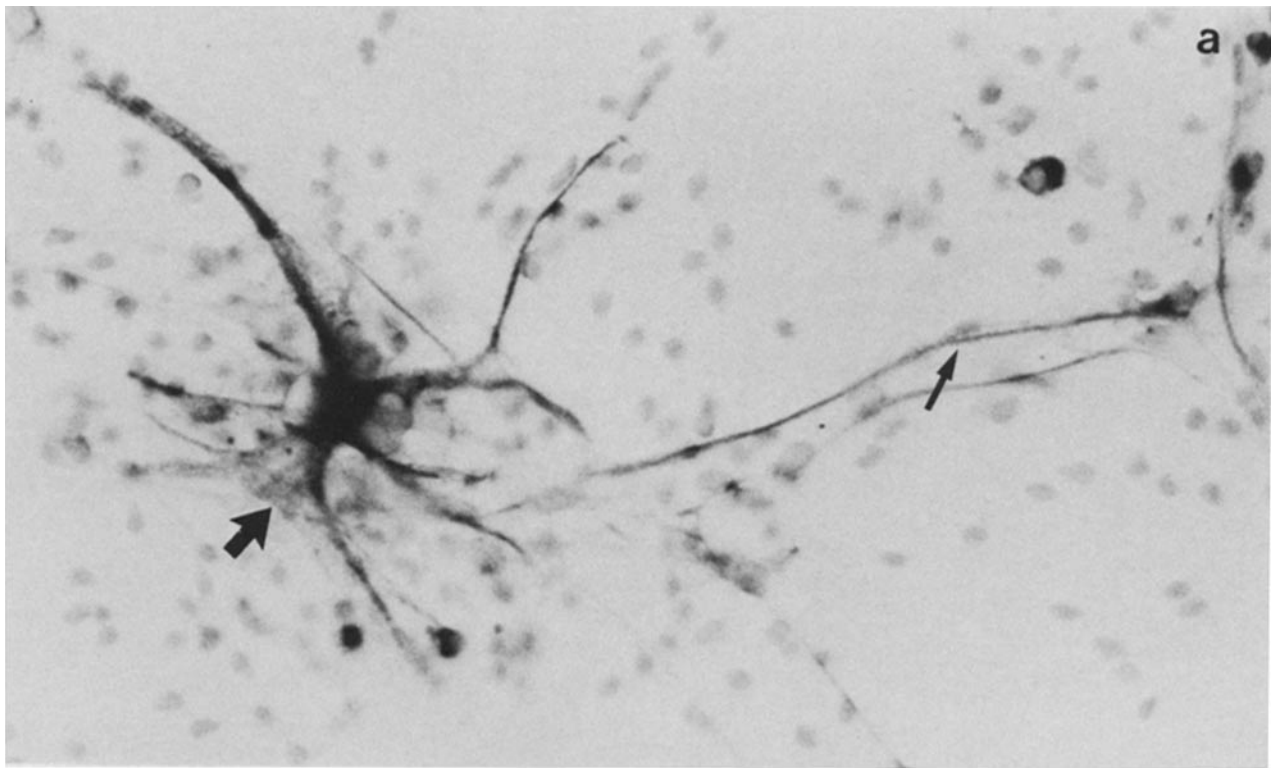


FIGURE 9 Morphology of cerebellar astroglia after longer periods of co-culture with neurons. Purified neurons were recombined with purified astroglia at a ratio of 5:1 4 h after the astroglia were plated. After 48–72 h *in vitro*, the cultures were stained with rabbit AbGF (1:1,000), visualized by PAP localization. Stained cells had more complex shapes than AbGF-positive cells seen 0–24 h after the neurons were recombined with the astroglia. Two forms predominated, both similar to postnatal forms of AbGF-positive cells seen *in vitro* and *in vivo*, one with a stellate shape (thick arrow) that clusters a large number of unstained, presumed neurons among its processes (a) and another with fewer, longer processes (a, thin arrow; b). With phase-contrast microscopy (c), the relationship of unstained neurons to astroglia is more evident; unstained, presumed neurons spiral around (arrow) or appose (curved arrow) a long AbGF-positive process. (a) Bright-field microscopy. $\times 430$. (b) Bright-field microscopy. $\times 600$. (c) Phase-contrast microscopy. $\times 600$.

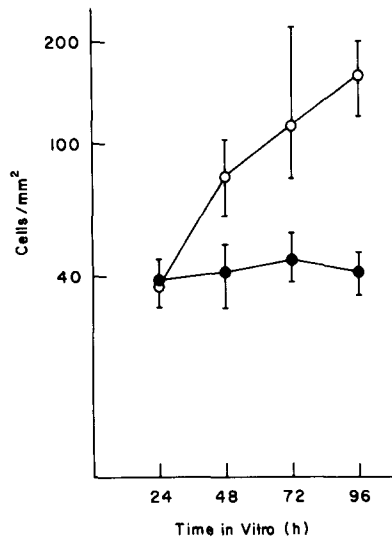


FIGURE 10 Influence of neurons on the proliferation of astroglia. After 1 d to 1 wk in vitro, the number of cells in a culture of purified astroglia (○) was counted by phase-contrast microscopy. After identical times in vitro, duplicate cultures of purified astroglia to which purified neurons had been added after plating at a ratio of four neurons/one glia 2 h were stained with AbGF and the number of AbGF-positive cells was counted with bright-field light microscopy (●).

mutant mouse where a loss of granule neurons has been correlated with an abnormal astroglial shape (3, 18, 33). The finding that isolated astroglia appear to transit through a series of immature forms (5, 13, 16) suggests that a discrete series of steps might be required for the expression of astroglial shape and that an interruption in the sequence, or simply a loss of cell associations with neurons, leads to a reversion to immature forms.

The present experiments did not distinguish whether the morphology (bipolar or multipolar, highly elongated, etc.) of the astroglial cells seen after increasing periods of co-culture with cerebellar neurons related to timing per se, to a particular ratio of neurons to individual astroglial cells, to a particular initial plating density of astroglia, or to other parameters of the culture system. Similarly, we could not distinguish whether all of the astroglial cells seen, especially stellate versus elongated shapes, were interconvertible or whether they represented distinct types of cells. Time-lapse studies on single astroglial cells will be required to settle this issue.

In turn, these experiments raise the possibility, which we did not explore, that astroglia promote neuron survival and neurite outgrowth. Since a more adhesive substratum (higher concentrations of polylysine) improved the poor neuron survival and neurite outgrowth seen in the absence of astroglia, astroglia may provide a naturally occurring adhesive substratum that promotes neurite outgrowth. This finding is consistent with two recent studies, one showing that glia from different brain regions induce different patterns of neurite branching (9) and another suggesting that astroglia promote neurite outgrowth in vitro more than nonglial cells (10, 27); and with studies on neurite outgrowth by cerebellar neurons from the mutant mouse weaver, the latter having impaired neurite outgrowth in the presence of abnormal astroglia (3, 18, 33, 44, 47).

Two mechanisms, one via neuron–glia contact and the other via a soluble differentiation factor, seem likely for

neurons in modulating astroglial differentiation. At present, the former interpretation is supported by two results. First, astroglial cells with elongated processes most often bind only one or two neurons and stellate cells harbor many neurons (18), which suggests a correlation between the number of bound neurons and astroglial shape. Second, medium conditioned by isolated neurons or by a mixed population of neurons and astroglia did not induce astroglial shape changes, which suggests that neuron–glia interactions are needed for the rapid induction of astroglial differentiation. Either of these mechanisms probably involves the binding of a ligand (a neuron–glia adhesion molecule or a soluble factor) to an astroglial cell surface receptor that modulates growth.

Others have reported the rapid proliferation of isolated astroglia in vitro and the morphological differentiation of astrocytes in the presence of brain extracts (39), the glial maturation factor (21, 29), or elevated levels of dibutyryl cyclic AMP (25, 46). It remains to be analyzed whether the neuron-induced differentiation of cerebellar astroglia that we observed relates to similar factors or to neuron–glia contacts.

The finding that neurons inhibited the proliferation rate of astroglia was of special interest. Others have reported that neurons have a mitogenic effect on glia from the peripheral nervous system in culture (37, 38, 48). These two findings suggest that neuron–glia interactions are linked to the control of glial growth, inhibiting the proliferation of central nervous system astroglia and stimulating that of Schwann cells.

The present studies underscore the interdependence of neurons and astroglia during mammalian brain development. The mechanism(s) of the influence of neurons on astroglial shape and proliferation is unknown. However, the described purification method should facilitate these and other studies on the differentiation and interrelationship of neurons and astroglia during development.

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