

Astroglial Cells Provide a Template for the Positioning of Developing Cerebellar Neurons In Vitro

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ABSTRACT Indirect immunocytochemical staining with antisera raised against purified glial filament protein and a neurofilament polypeptide was used to study cell interactions between astrocytes and neurons dissociated from embryonic and early postnatal cerebellum. Staining with antibodies raised against purified glial filament protein revealed that >99% of all processes present in cerebellar cultures during the 1st wk in vitro were glial in origin. After 1 wk in culture, unstained processes that were presumably neuronal were observed.

Stained astroglial processes formed a dense network that served as a template for cerebellar neurons, identified by indirect immunocytochemical localization of tetanus toxin. More than 90% of the neurons from postnatal days 1 or 7 were positioned within one cell diameter of a glial process. In contrast, <40% of the neurons dissociated from early embryonic cerebellum were located adjacent to a glial process.

Staining with antibodies raised against purified glial filament protein also revealed differences in astroglial morphology that were under developmental regulation. Astroglial cells from embryonic cerebellum were fewer in number and had thick, unbranched processes. Those from postnatal day 1 were more slender, branched, and stellate. Those from postnatal day 7 were highly branched and stellate. Some veil-like astroglial processes were also observed in cells from postnatal animals. These morphological changes were also observed when cells from embryonic day 13 were maintained for a week in vitro.

No specific staining of embryonic or postnatal cerebellum cells was observed with antibodies raised against purified neurofilament polypeptides.

In the developing mammalian brain, the fibers radiating from astroglial cells have been suggested to guide the proper positioning of immature migrating neurons and establish the architectonics of most brain regions (12). In the immature cerebellum, Bergmann glia, a specialized type of astrocyte, extend thick, umbellate, radial processes that have been proposed to guide the migration of postmitotic granule cells from the pial surface down into the internal granule cell layer (5, 16, 17). Within the internal granule cell layer, velate protoplasmic astrocytes extend numerous, slender, branched radial fibers that organize the mature granule cells and their dendrites into compartments (15, 16). In the mature cerebellum, neuroglial fibers persist and may sustain the proper cellular geometry of the region (14).

None of the tissue culture systems developed to maintain cerebellar cells in vitro has addressed the specific interactions between astrocytes and neurons evident in the developing cerebellum. Cerebellar cells can be maintained in culture either

as monolayers or as aggregates. Both culture systems support the outgrowth of abundant processes that have been assumed to be neuronal in origin.

Cerebellar astrocytes can be identified ultrastructurally by the presence of densely packed bundles of glial filaments (14, 15), and immunocytochemically by antibodies against a glial fibrillary acidic protein (GFA) (1–4, 6, 12, 19, 20). In the cerebellum, some neurons, stellate and basket cells, can similarly be identified by the presence of neurofilament protein (14, 15), which is antigenically distinct from the glial filament protein (GF). Both GF and neurofilament protein can be isolated by biochemical means.¹ Glial filaments consist of a single major subunit (51,000 daltons) whereas neurofilaments consist of three polypeptide chains (200,000, 150,000, and 70,000 daltons).

¹ Liem, R. K. H. Simultaneous separation and purification of neurofilament and glial filament proteins. Submitted for publication.

In the present study, indirect immunofluorescence staining with antibodies raised against the major subunit of glial filaments (51,000 daltons) and against a neurofilament polypeptide (150,000 daltons) has been used to define the interactions between astrocytes and neurons isolated from embryonic and early postnatal mouse cerebellum, and maintained in microcultures.

MATERIALS AND METHODS

Antibodies against Purified Glial and Neurofilaments

Antisera (AbGF) against the major glial filament protein (GF, 51,000 daltons) and antisera (AbNF) against the neurofilament polypeptide (NF, 150,000 daltons) were raised in a guinea pig and a rabbit, respectively, using gel-purified material as antigen. The gamma globulin fractions were prepared by ammonium sulfate precipitation and redissolved in phosphate-buffered saline (PBS) to the original volume of the serum. The characterization of the antibodies is described elsewhere.¹ They did not cross-react by indirect immunofluorescence studies or by radioimmunoassays.¹ In some experiments, a 1:100 dilution of AbNF and a 1:200 dilution of AbGF were absorbed overnight with 0.2 mg/ml of reassembled and purified filaments (final concentration). The filaments were removed by centrifugation in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, N. Y.), and the absorbed antibodies were used for immunofluorescence experiments. In all cases, the experimental samples of AbNF and AbGF were absorbed with the reassembled GF and NF, respectively, to remove any possible cross-contamination in the two antibodies. The control AbNF and AbGF were absorbed with reassembled NF and GF, which removed all specific staining. These absorptions were effective when tested on brain sections by indirect immunofluorescence.¹

Cerebellar Cultures

All studies were carried out with C57Bl/6J mice derived from a breeding colony in this department. Females were checked daily for the presence of vaginal plugs, the day of impregnation being designated E0. Whole cerebellums were removed from embryos at E14 (9) or from postnatal animals at P0, P1, or P7, the day of birth being P0. In all >10 litters of embryos and 50 postnatal animals were studied.

To facilitate fluorescence microscopy, cerebellar cells were grown on glass cover slips in microcultures (8, 10). The culture dishes were prepared by drilling out a 4-mm hole in a 50-mm Petri dish (Falcon 1006, Falcon Labware Div., Becton, Dickinson & Co., Oxnard, Calif.) and mounting a glass cover slip (20 mm Diam, round, Thomas Scientific, Co., Philadelphia, Pa.) as a false bottom with a mixture of paraffin and petroleum jelly (three parts Tissue Prep: one part Vaseline, Fisher Scientific Co., Pittsburgh, Pa.). The plates were sterilized by short wave ultraviolet irradiation (1 h, 20°C), washed with H₂O, and treated with poly-D-lysine (25 µg/ml in H₂O) before use as described (9).

Single cell suspensions of embryonic and postnatal cerebellar tissue were prepared as described (8, 9). The cells were resuspended at a final density of $1-2 \times 10^6$ cells/ml in Eagle's Basal medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with horse serum (Kansas City Biologicals, Lenexa, Kansas lot 269107, 10%), penicillin-streptomycin (20 U/ml, Gibco Invenex Div., Lawrence, Maine), glucose (6 mM, Fisher Scientific Co.) and glutamine (4 mM, Gibco). Cells were plated in microcultures to a final volume per culture of 30 µl. Before plating, cell viability, as measured by exclusion of the dye, trypan blue (0.4%, Gibco), was >85%. Plating efficiency was assayed 24, 48, and 168 h after plating by counting the total number of cells per microculture, either by phase contrast microscopy or by removing the cells and counting them with a hemocytometer, and expressing the total as a percentage of the total number of cells plated. Throughout the time period studied, plating efficiency was >95%. Cells were maintained at 35.5°C with 5% CO₂ and 100% humidity. For cultures maintained longer than 48 h in vitro, 10 µl of fresh medium was added 3, 4, and 7 d after plating. In all, >650 microcultures were studied.

Indirect Immunofluorescence Staining with Antibodies against Glial and Neuronal Filament Proteins

The procedure followed was a modification of that reported by Osborn and Weber (13). In brief, cultures were washed three times with calcium- and magnesium-free PBS (CMF-PBS, 10 min, 20°C), fixed in paraformaldehyde (3.7% in 0.18 M CMF-PBS, 5 min, 20°C) and washed three times in CMF-PBS

(5 min, 20°C). The cultures were extracted with Triton (0.1%, 20–30 min, 20°C), washed three times with CMF-PBS (5 min, 20°C) and the primary antibody was applied (30 µl, 30 min, 37°C) at a dilution of 1:100 (AbNF) or 1:200 (AbGF) in CMF-PBS. The cultures were then washed with three changes of CMF-PBS (10 min, 20°C) before the addition of the second antibody (30 µl, 30 min, 37°C). For AbNF, the second antibody was rhodamine-isothiocyanate-conjugated (RITC) goat anti-rabbit IgG (1:100, N.L. Cappel Laboratories, Inc., Cochranville, Pa.). For AbGF, the second antibody was fluorescein-isothiocyanate-conjugated (FITC) goat anti-guinea pig IgG (1:100, Antibodies Inc., Davis, Calif.).

Immunofluorescence staining was assessed with a Leitz Diavert inverted microscope fitted with phase contrast and epifluorescent optics, a Zeiss $\times 25$ oil/water immersion phase/fluorescence objective and a Zeiss $\times 63$ oil/water immersion phase/fluorescence objective. Results were recorded with an RCA TC 1030/H image intensifier video camera (RCA Closed Circuit Video Equipment, Lancaster, Pa.), an Ikegami 9-inch monitor (model PM-950, Ikegami Electronics [USA], Inc., Maywood, N. J.), and a Panasonic video tape recorder (model 8030 Panasonic Co., Secaucus, N. J.). Photographs were made from the television monitor.

Measurement of Distances between Glial Processes and Neurons

Video tape recordings of phase and fluorescence images of the cultures were used to quantitate the distance between glia and neurons. The sequence of immunofluorescence staining with AbGF was replayed and all glial processes and glial cell bodies were traced on the television monitor screen. The sequence of phase contrast for the identical field was replayed and measurements of the distance between the center of the glial fiber and the center of the nucleus of neuronal cells were made with a ruler on the television screen. Distances on the screen were calibrated with a Leitz stage micrometer.

Specific Staining of Neuronal Cells with Tetanus Toxin

Purified tetanus toxin and antisera against tetanus toxin were the generous gifts of Dr. William H. Habig, National Institutes of Health, Bethesda, Md. Cultures were fixed with paraformaldehyde as described, and treated with tetanus toxin (0.5 µg/ml, 30 min, 20°C) (7). After washing (three times, CMF-PBS, 10 min, 20°C) the cultures were treated with goat antitetanus toxin (1:50 dilution, 30 min, 20°C). The cultures were washed three times (CMF-PBS, 10 min, 20°C) and treated with RITC-labeled rabbit antigoat antibody (N. L. Cappel, Laboratories, Inc., 1:50 dilution, 30 min, 20°C). After washing in CMF-PBS (three times, 10 min, 20°C), toxin binding was visualized by fluorescence microscopy. Results were recorded by video tape as described.

RESULTS

Cells from Postnatal Day 7 Cerebellum

After 24–48 h in vitro, ~10–15% of the cells in the monolayer cultures of cells dissociated from postnatal day 7 cerebellar tissue were intensely stained with antisera raised against purified glial filament protein (AbGF) (Fig. 1 a–c). No cells were stained with antisera directed against the purified neurofilament protein polypeptide (AbNF). Stained cells were slightly larger than the more numerous phase-bright cells present in the cultures and had the morphological characteristics of astrocytes; a bean-shaped nucleus and a large number of slender, smooth, branched processes. Both the perikaryon and the processes of these cells were brightly stained with AbGF. Some cells had fewer, thicker processes, one to four in number. Other cells had more numerous, slender, highly branched, stellate processes. A third cell type had lamellar processes that were veil-like.

The processes projected by the astrocytes produced a thick network that appeared to provide a matrix for the organization of phase-bright neuronal cells in the culture (Fig. 1 c). More than 99% of the processes present in the cultures were stained with AbGF. After 24–48 h in vitro, >95% of the phase-bright cells in the culture were positioned within a distance of two cell diameters of a stained astrocytic process (Fig. 2). Measure-

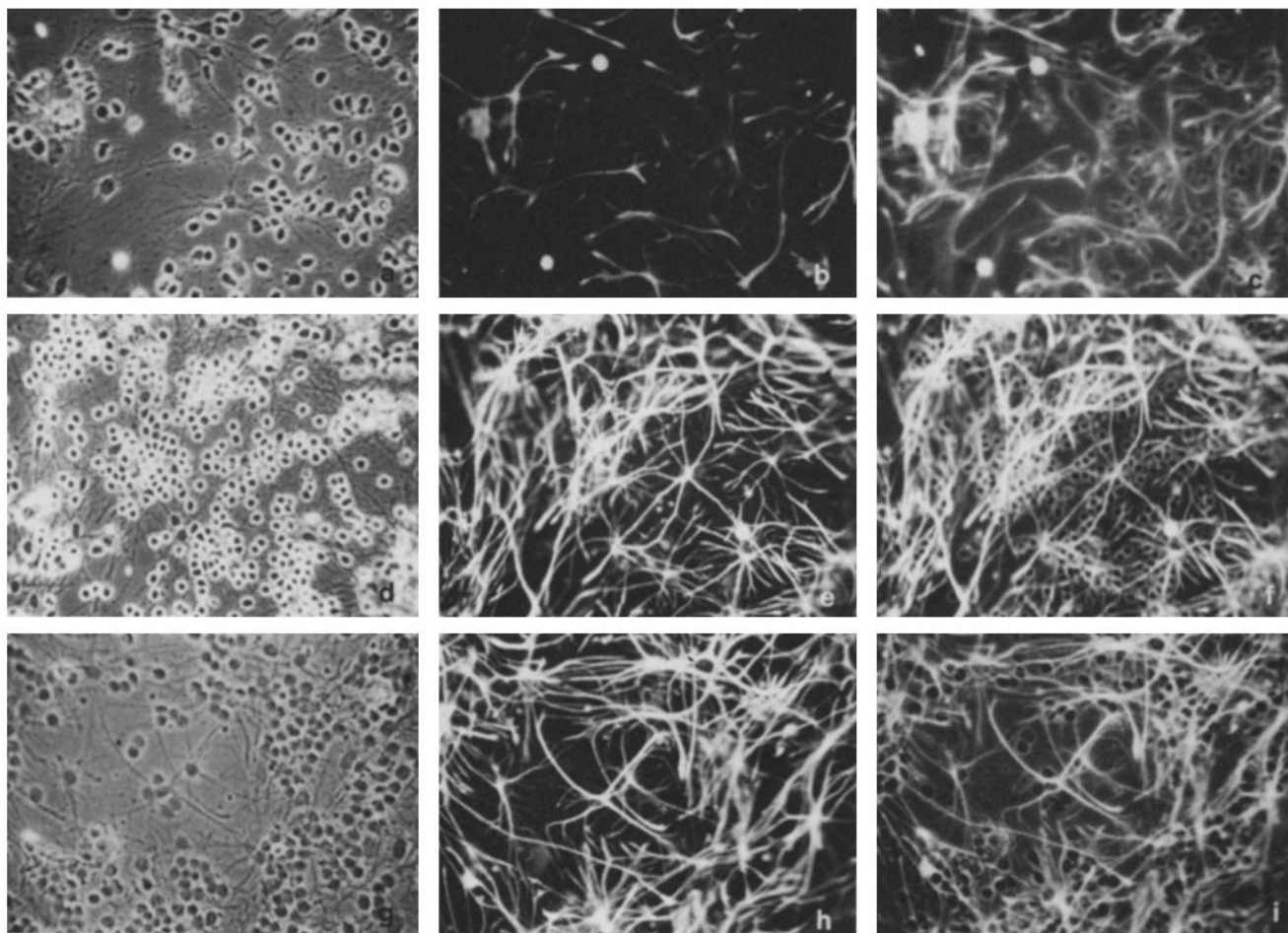


FIGURE 1 Immunofluorescence staining of P7 cerebellar cells in microculture with antibodies against glial filament protein (AbGF). The identical field was photographed with phase contrast (a, d, g), epifluorescence (b, e, h), or a double image (c, f, i) illumination. After 48 h (a, b, c), 5 d (d, e, f), or 8 d (g, h, i) in vitro. $\times 210$.

ments of the distance separating the phase-bright cells from the center of glial processes stained with AbGF (Fig. 3) revealed that no cells were positioned at distances $>60 \mu\text{m}$ from a glial process. All of the phase-bright cells were specifically stained with tetanus toxin and were therefore assumed to be neuronal (Fig. 4).

To demonstrate that the association between astroglial processes and neurons was nonrandom, the number of neurons per square millimeter of the area within $20 \mu\text{m}$ of a glial process was compared with areas $>20 \mu\text{m}$ from a glial process. This was measured by replaying the video tape recordings of phase and fluorescence images for identical fields of cultures stained with AbGF as described. The area within $20 \mu\text{m}$ of a stained astroglial process was traced onto paper from the television screen, and the number of phase-bright, unstained, neuronlike cells within that area was counted. The area was measured by cutting out the tracing of the area within $20 \mu\text{m}$ of a stained glial cell or process and weighing it. The total number of phase-bright, unstained, neuronlike cells outside the area within $20 \mu\text{m}$ of the stained glial process was counted and the area was calculated by weighing the paper remaining after the area within $20 \mu\text{m}$ of the stained glial processes was removed. Distances on the screen were calibrated with a Leitz stage micrometer and used to calculate the area per unit weight of tracing paper. For P7 cells after 48 h in vitro, there were 48×10^3 neurons/ mm^2 in the area within $20 \mu\text{m}$ of a stained glial

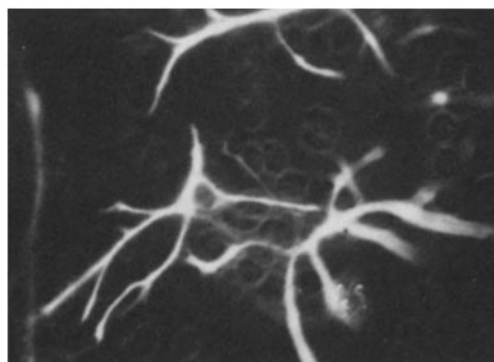


FIGURE 2 Immunofluorescence staining with AbGF of the interaction of P7 astroglia with neurons. A double image of phase and epifluorescence illumination is shown, 48 h in vitro. $\times 526$.

process and 1.2×10^3 neurons/ mm^2 in the area beyond that distance.

Specific staining of phase-bright neuronal cells occurred only at low concentrations of tetanus toxin ($0.5 \mu\text{g}/\text{ml}$). Small neuronlike cells were intensely stained with the toxin. Some of the processes were weakly stained with tetanus toxin. These processes were also stained with AbGF. Specific staining of neuronlike cells was observed for cells dissociated from E14 (Fig. 4), P1, or P7, and carried in microcultures for 48 h, 5 d,

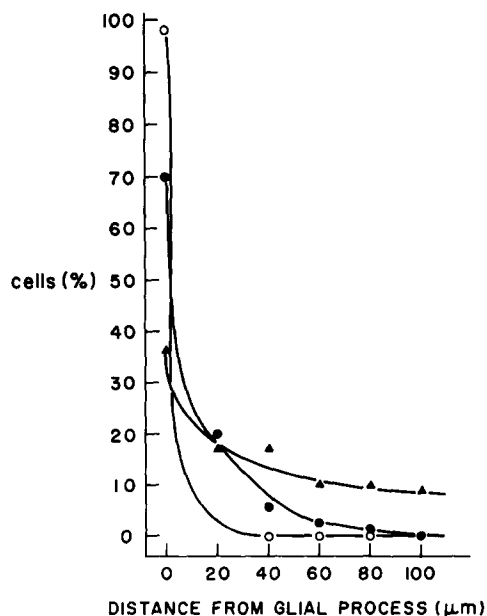


FIGURE 3 Distribution of neurons dissociated from E14 (\blacktriangle), P1 (\bullet), or P7 (\circ) cerebellum along astroglial processes. Cells were plated at low cell density (1×10^6 cells/ml) in microcultures and stained with AbGF after 48 h in vitro as described in the text. At this density, the area within $20 \mu\text{m}$ of stained astroglial cells and their processes was $<20\%$ of the total area of the culture dish. The videotape recording of phase and fluorescence images of the cultures were replayed for identical fields and the distance between the center of the stained astroglial fiber and the center of the nucleus of phase-bright unstained neuronal cells was measured with a ruler. Distances on the screen were calibrated with a Leitz stage micrometer and expressed in microns.

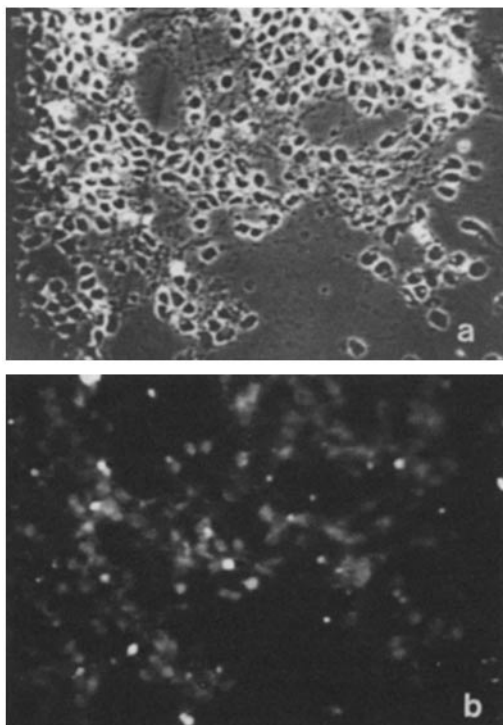


FIGURE 4 Immunofluorescence staining with tetanus toxin of E14 cerebellar cells after 48 h in vitro. The same field was recorded and photographed with (a) phase and (b) epifluorescent illumination. $\times 210$.

or 1 wk. No staining of astroglia was observed at that dilution. At higher concentrations of the toxin, intense staining of phase-bright cells and weak staining of astroglial cell bodies and processes were recorded (data not shown).

In cultures of low cell density ($<1 \times 10^6$ cells/ml), large vacant areas were present on the culture substratum. These areas were devoid of both astrocytes and neuronal cells. Neuronal cells were present only in areas where astrocytes were located.

In cultures with high cell density ($2-5 \times 10^6$ cells/ml), cells formed aggregates as well as monolayer regions on the substratum. Thick networks of glial fibers were evident in the aggregates (Fig. 5). Neuronal cells were positioned within the aggregates and along glial fibers at the edge of aggregates.

After 5 d in vitro, $>99\%$ of all of the processes were stained with AbGF (Fig. 1 d, e). No specific staining was observed with AbNF. The network of glial processes was thicker after 5 d than after 48 h, and had formed a three-dimensional lattice in which neuronal cells were located (Fig. 1 f).

After 8 d in vitro, a dense network of glial processes persisted (Fig. 1 g, h). A few (1–2%) of the processes were unstained and were presumably neuronal in origin. Weak specific staining of some processes was observed with AbNF and with tetanus toxin (data not shown). In regions of high cell density, the vast majority of neuronal cells were still associated with a dense network of stained glial filaments, but in regions of low cell density, neuronal cells were often present at large distances ($>40 \mu\text{m}$) from stained, glial fibers than was seen earlier (Fig. 6).

Cells from Postnatal Day-1 Cerebellum

The results obtained depended on the developmental stage

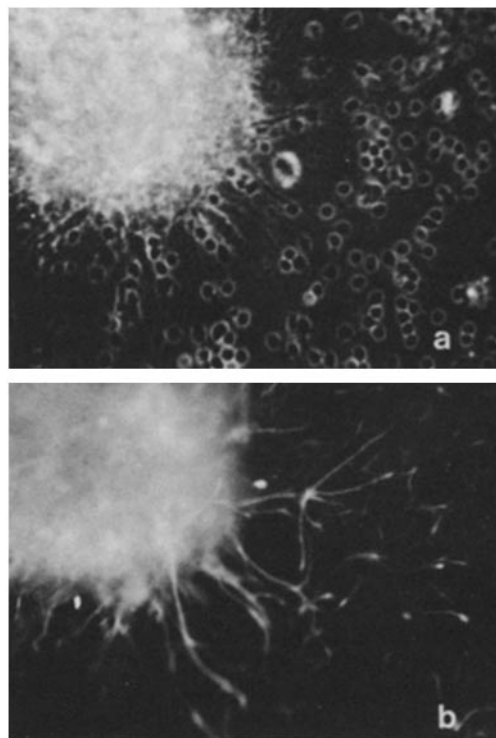


FIGURE 5 Immunofluorescence staining with AbGF of P7 cerebellar cells plated at high density. The same field was recorded and photographed with (a) phase and (b) epifluorescence illumination. $\times 210$.

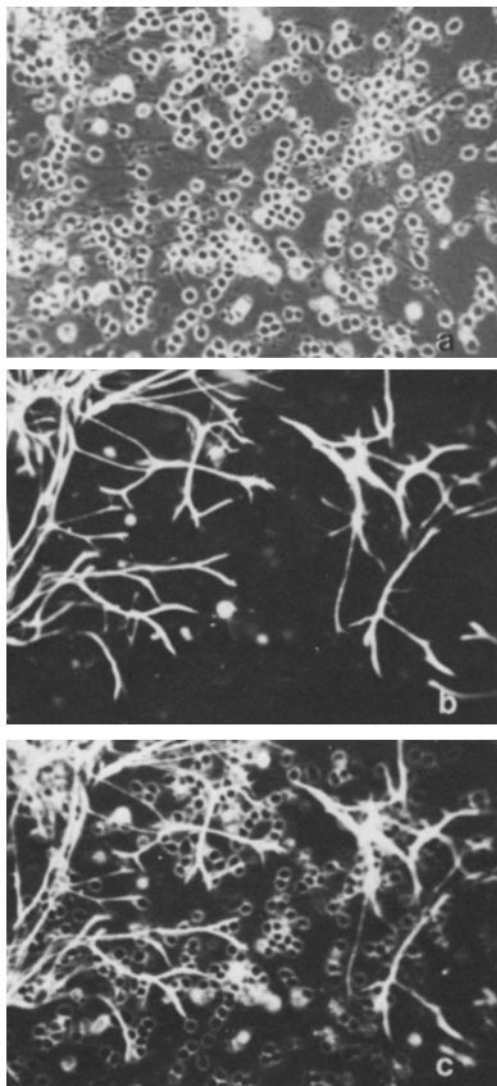


FIGURE 6 Dissociation of P7 cerebellar neurons after 8 d in vitro from processes stained with AbGF. The same field recorded and photographed with (a) phase, (b) epifluorescence, and (c) phase and epifluorescence illumination. $\times 210$.

of the cerebellar tissue from which the cells were dissociated. When cells were dissociated on the day of birth (P0) or on the first postnatal day (P1), staining after 24–48 h in vitro again revealed numerous astrocytes and thin processes in the cultures (Fig. 7 *a, b*). The astrocytes constituted 12–15% of the total cell population. As was the case for cells dissociated from cerebellar tissue at postnatal day 7, the vast majority (99%) of the processes in the culture were glial in origin and no staining was seen with AbNF. Both the perikaryon and processes of astrocytes were stained. The processes of astrocytes dissociated at postnatal days 0 or 1 were generally more slender than those observed for cells dissociated 6 d later. These processes established a dense network in the culture and tetanus-toxin positive neuronal cells were invariably closely associated with them (Fig. 3 and Fig. 7 *c*).

After 5 d in culture, results from P1 animals were identical to those reported for 24–48 h in vitro (Fig. 7 *d, e*). All processes were stained with AbGF, none with AbNF, and the neuronal cells were aligned along the processes or very close to them (Fig. 7 *f*).

After 8 d in culture, nearly all of the processes were stained with AbGF (Fig. 7 *g, h*). None was stained with AbNF. A few were stained with tetanus toxin. Extremely few processes were unstained. All of the cells were still closely associated with glial processes (Fig. 7 *i*).

Cells from Embryonic Day 14 Cerebellum

In contrast, after 24–48 h fewer cells (5–7%) dissociated at E14 were stained with AbGF (Fig. 8 *a, b*). Most of the processes in the culture were stained; however, there were more unstained processes than were observed for postnatal cells. No staining was observed with AbNF. Specific staining of glial cells with AbGF was observed for both the perikaryon and processes of astrocytes. The morphology of these cells was distinct from that of cells stained in cultures of postnatal cells (Fig. 9). Whereas postnatal astrocytes had numerous, slender, branched, radiating processes, embryonic astrocytes extended only several thick, rarely branched, smooth processes. No cells were observed with stellate processes.

E14 neuronal cells were not strictly associated with glial processes (Fig. 8 *c*). Numerous cells were positioned at distances $>20 \mu\text{m}$ from glial fibers (Fig. 3). Phase-bright, tetanus toxin-positive cells constituted the major (95%) cell population (Fig. 4).

After 5 d in vitro, the morphology of the cells stained with AbGF was similar to that for cells dissociated from P0 or P1 tissue. Astrocytes had branched processes (Fig. 8 *e, f*). Most of the neuronal cells ($>85\%$) were located within one cell diameter of a glial process (Fig. 8 *g*). Nearly all of the processes in the culture were stained with AbGF. None was stained with AbNF (data not shown).

After 8 d in vitro E14 astrocytes had numerous, slender, radiating processes and appeared more like astrocytes from postnatal tissue (Fig. 8 *g, h*). More than 85% of the neuronal cell population was within one cell diameter of a glial process (Fig. 8 *i*). Specific staining with AbGF was observed for all the processes in the culture. No processes were stained with AbNF (data not shown). A few processes were stained with tetanus toxin.

For all ages and periods in vitro the specificity of cellular staining with AbGF and AbNF was assayed by preabsorbing the antisera used with purified glial filament protein or purified neurofilament polypeptides.¹ No staining was observed when AbGF was preabsorbed with purified glial filament protein or when AbNF was preabsorbed with purified neurofilament polypeptide (Fig. 10). Preabsorption of AbGF with purified neurofilament polypeptide or of AbNF with purified glial filament protein had no effect on the cellular staining patterns of the antisera. No fluorescent staining was observed with preimmune sera. No background staining occurred.

Identical cellular staining patterns were obtained when the cultures were double labeled with AbGF and AbNF or labeled with either AbGF or AbNF. Identical results were also obtained when AbNF staining was visualized with either fluorescein or rhodamine-conjugated goat anti-rabbit IgG.

DISCUSSION

These results suggest that after several days in culture, almost all, if not all, of the processes present in monolayer cultures of dissociated cerebellar cells are astroglial in origin. These processes have always been assumed to be neuronal. The cells stained with AbGF are probably astrocytes, either Bergmann

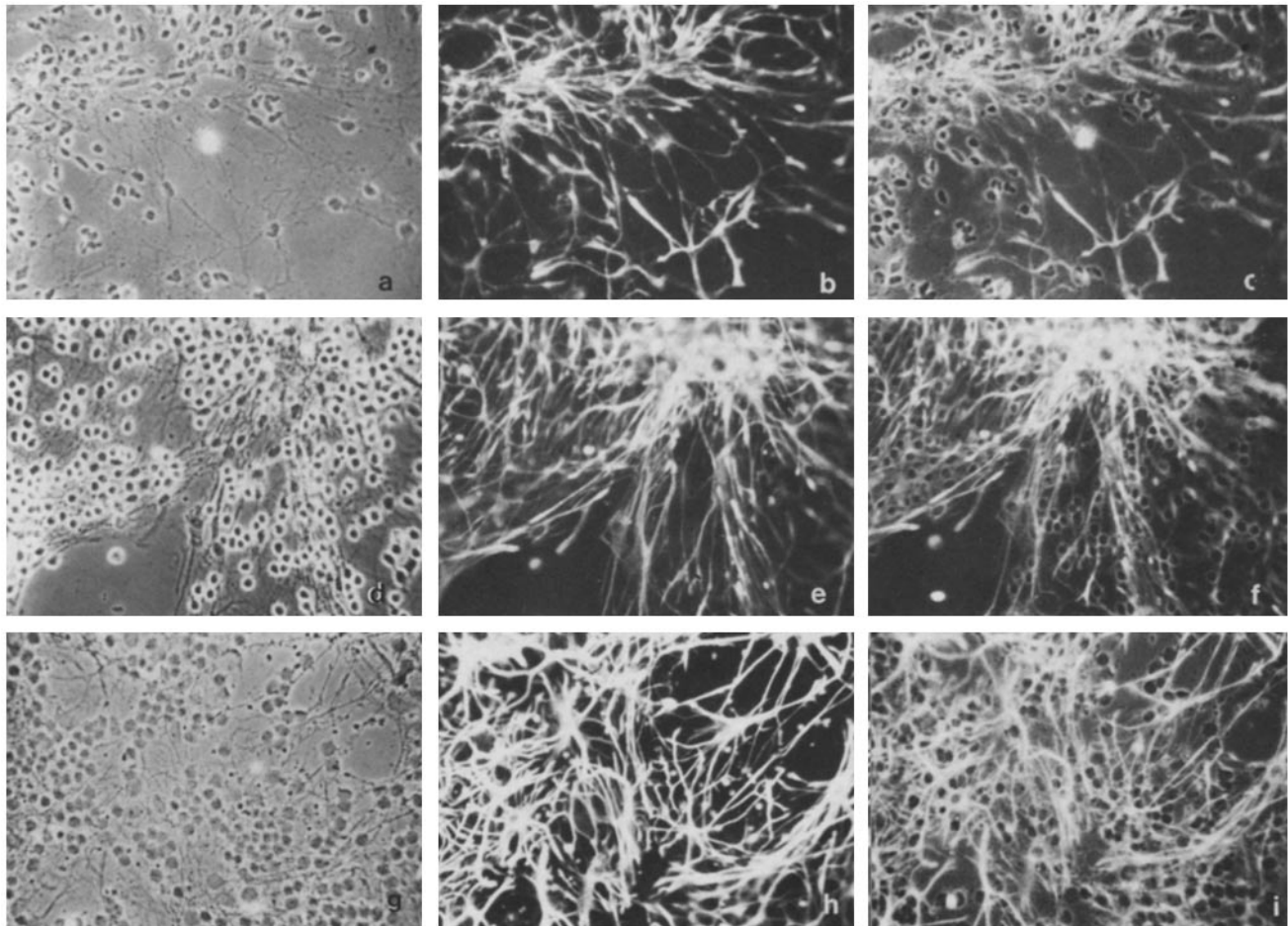


FIGURE 7 Immunofluorescence staining with AbGF of P1 cerebellar cells. The identical field was recorded and photographed with phase contrast (a, d, g), epifluorescence (b, e, h) or double image (c, f, i). After 48 h (a, b, c), 5 d (d, e, f), or 8 d (g, h, i) in vitro. $\times 210$.

glia or velate protoplasmic astrocytes, because these are the only cells in the cerebellum that contain densely packed bundles of glial filaments (1, 2, 4, 12, 14, 15, 19). The astrocyte processes set forth a template that determines the positioning of neuronal cells in the cultures. More than 95% of the cells dissociated from early postnatal cerebellum were aligned along astrocyte processes. Glial processes were absent in areas of the culture that were devoid of neuronal cells and present in great abundance in aggregates of cells. The dense network of processes projected by the relatively few astrocytes present in the cultures organized the arrangement of dissociated postnatal cerebellar cells.

After a week in culture, finer processes that were not stained with AbGF were observed. These were probably neuronal processes. This finding suggests a two-step mechanism for cerebellar neuronal differentiation in vitro. The first step is cell patterning of immature neurons along glial processes. The second, neuronal process extension, follows later. With light microscopy, it is difficult to assess whether neuronal processes begin to extend along the thicker astroglial processes soon after plating or after a number of days in vitro. This issue as well as the distribution of neuronal cell types along glial processes will have to be addressed with electron microscopy. The dynamic interactions between glial processes and neuronal cells, especially right after plating, and the onset of neurite extension will be addressed by light microscopy with time-lapse video recordings.

The absence of specific staining with antisera raised against purified neurofilament polypeptides probably reflects the very small number of basket and stellate cells present in the immature cerebellum. Apparently very few, if any, of these cells either survive or produce their typical axonal baskets in microcultures.

Because very few processes in the cerebellum contain NF, even in adult animals, tetanus toxin was used to resolve neuronal elements. The weak staining of some processes with tetanus toxin in cultures from E14, P1, or P7 cerebellum raises the possibility that some neurons do, in fact, project neurites along the astroglial arms. This was the case even after 24–48 h in culture, a period when all of the processes are intensely stained with AbGF. Either the tetanus toxin staining was nonspecific, or small neurites track along the glia. This issue could not be resolved with light microscopy.

Two distinct properties are under developmental regulation. First, the morphology of the astrocytes depended on the age of the tissue from which they were isolated. Astrocytes from embryonic cerebellum were fewer in number, although present, and had thick, smooth processes with very few branchlets. Astrocytes from early postnatal tissue were characterized by longer, more slender processes that were highly branched. The morphology of astrocytes, dissociated at E14 and maintained in culture for 8 d, changed with a time-course that approximated the described changes of astrocyte morphology in intact cerebellar tissue. The microculture system, therefore, provides

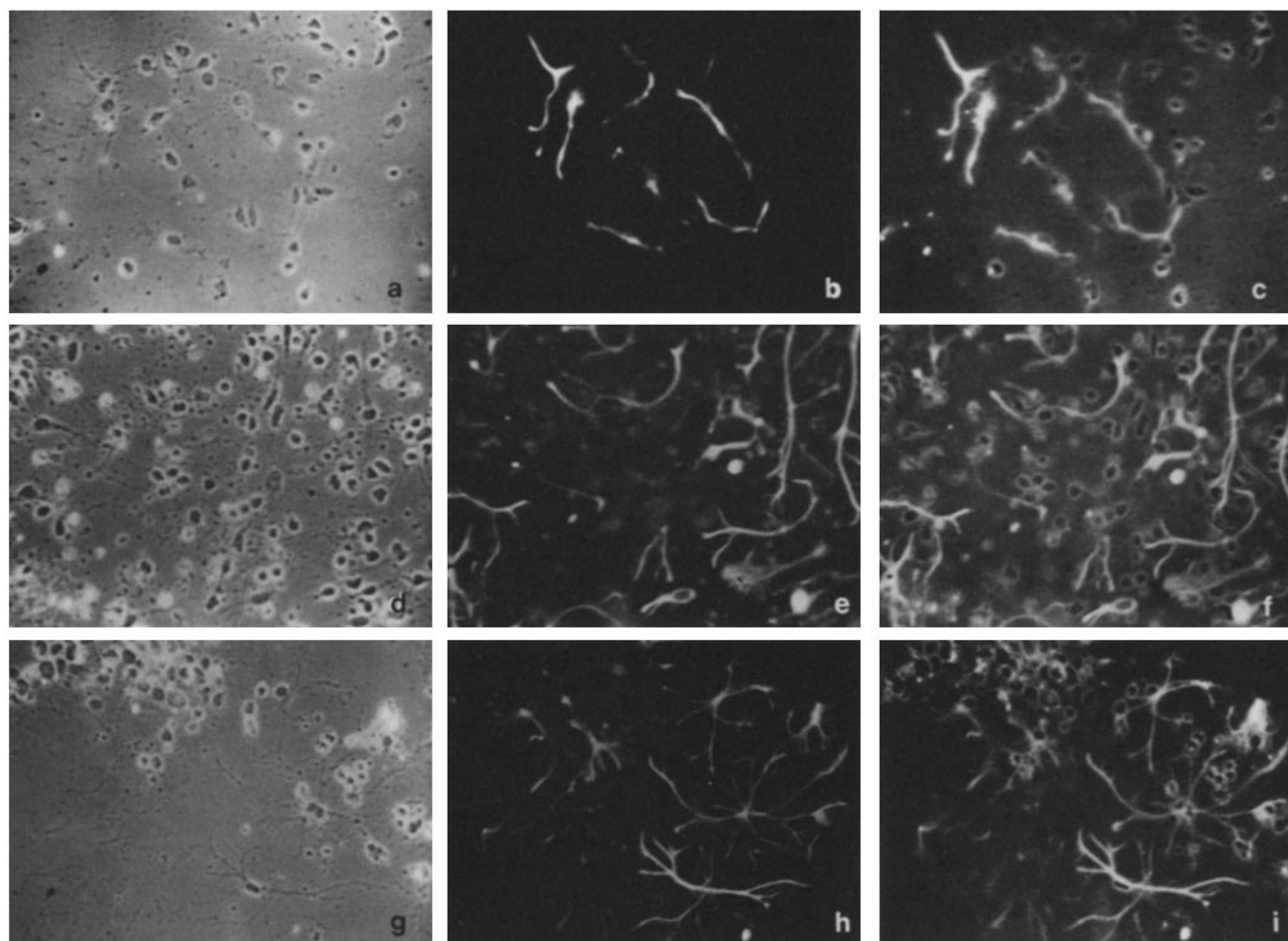


FIGURE 8 Immunofluorescence staining with AbGF of E14 cerebellar cells. The identical field was recorded and photographed with phase contrast (a, d, g), epifluorescence (b, e, h), or a double image (c, f, i). After 48 h (a, b, c), 5 d (d, e, f), or 8 d (g, h, i) in vitro. $\times 210$.

an excellent model system to explore the differentiation of astrocytes.

The second event that appeared to be under developmental regulation was the association of neuronal cells with astrocyte processes extended in vitro. Cells dissociated from E14 cerebellum did not strictly align with the glial processes in the culture, whereas those from early postnatal tissue seemed to be organized by the network of astrocyte processes projected on the culture substratum. Thus, the association of neuronal cells with astrocyte processes in microcultures followed a time schedule that was very similar to that observed in the intact cerebellum. Namely, in E14 cerebellum, few glial cells are present and immature granule neurons have not yet begun their migrations along Bergmann glial processes to the position that they will occupy in the mature cerebellar cortex (5, 12). By early postnatal stages, however, Bergmann glia are in place and have projected processes that seem to guide the specific migration of the most numerous cell type present in the cerebellum, the granule cells, down into the deeper layers of the cortex (16–18). At the completion of the migration, granule cells detach from the Bergmann glial processes and later extend neurites. The microculture system, therefore, provides an excellent model system for the study of the specific cellular interactions between astrocytes and developing neurons.

These findings suggest that astrocytes are required for the

attachment and survival of postnatal cerebellar cells in culture. Embryonic cerebellar cells are less dependent on the presence of astroglia, but a subpopulation of embryonic neuronal cells may require astroglial processes. A limiting number of astroglial cells may explain the previous finding that postnatal cerebellar cell populations do not survive at extremely low ($<5 \times 10^5$ cells/ml) cell densities.²

A number of studies of cell patterning and cell surface properties of developing cerebellar cells in vitro, as well as other studies, will have to be reconsidered in light of the finding that almost all of the processes in cerebellar cultures are glial in origin for at least a week in vitro. It is the attachment of the glia to the substratum that apparently sets forth the patterning of cerebellar cells in culture. This attachment is strongly dependent on the substratum provided. Specific changes in glial cell attachment, process extension, and neuronal patterning are observed when the cells are plated on polylysine or on carbohydrate-derivatized (8) and lectin-derivatized (11) substrata. Future studies of cell surface moieties involved in glial-neuronal cell interactions should resolve the mechanism of recognition between these two cell types.

These studies underscore the importance of neuroglia to the early development and organization of the cellular geometry

² M. E. Hatten, and R. K. H. Liem. Unpublished observations.

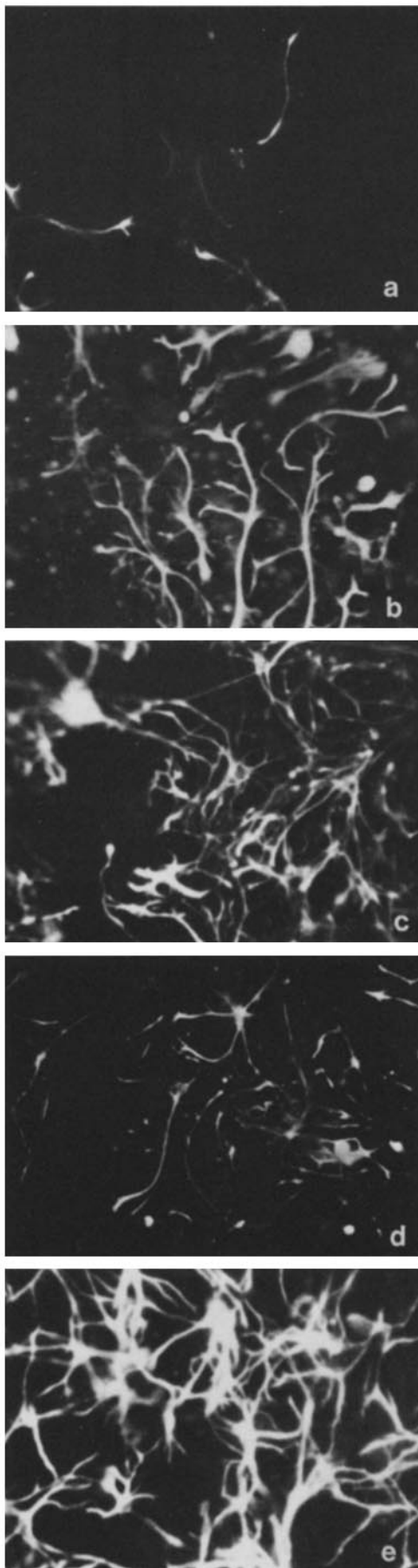
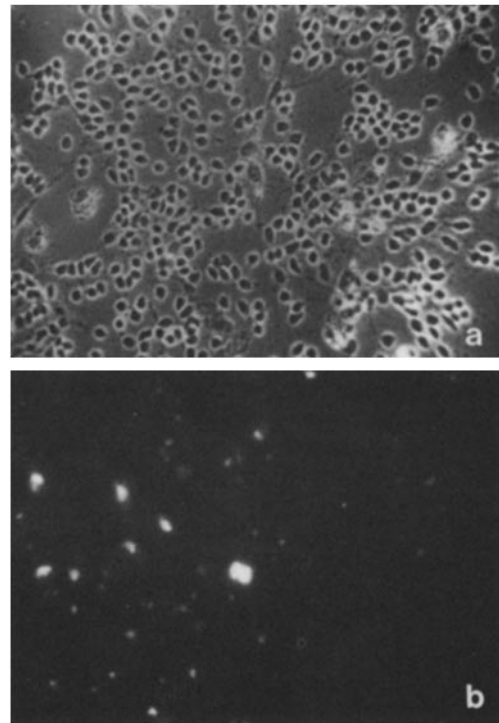


FIGURE 10 Immunofluorescence staining of P7 cerebellar cells after 48 h in vitro with absorbed serum. The same field was recorded and photographed with (a) phase and (b) epifluorescence illumination. $\times 210$.



in the cerebellar cortex. The microculture system offers an ideal culture system for the study of glial-neuronal interactions and differentiation, and for the isolation of surface components responsible for these specific associations.

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REFERENCES

1. Antanitus, D. S., B. H. Choi, and L. W. Lapham. 1975. Immunofluorescence staining of astrocytes *in vitro* using antiserum to glial fibrillary acidic protein. *Brain Res.* 89:363-367.
2. Bignami, A., and D. Dahl. 1973. Differentiation of astrocytes in the cerebellar cortex and the pyramidal tracts of the newborn rat. An immunofluorescence study with antibodies to a protein specific to astrocytes. *Brain Res.* 49:393-402.
3. Bignami, A., and D. Dahl. 1974. Astrocyte-specific protein and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. *J. Comp. Neurol.* 153:27-38.
4. Bignami, A., L. F. Eng, D. Dahl, and C. T. Uyeda. 1972. Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res.* 43:429-435.
5. del Cerro, M., and J. R. Swartz. 1976. Prenatal development of Bergmann glial fibers in rodent cerebellum. *J. Comp. Neurocytol.* 5:669-676.
6. Eng, L. F., J. J. Vanderhaeghen, A. Bignami, and B. Gerstl. 1971. An acidic protein isolated from fibrous astrocytes. *Brain Res.* 28:351-354.

FIGURE 9 Development of astroglia in vitro. Immunofluorescence staining with AbGF of (a) E14 cells after 48 h in vitro, (b) P1 cells after 48 h in vitro, (c) P7 cells after 48 h in vitro, (d) E14 cells after 5 d in vitro, and (e) E14 cells after 8 d in vitro. $\times 210$.

7. Fields, K. L., J. P. Brockes, R. Mirsky, and L. M. B. Wendon. 1978. Cell surface markers for distinguishing different types of dorsal root ganglion cells in culture. *Cell*. 14:43-51.
8. Hatten, M. E. 1981. Cell assembly patterns of embryonic mouse cerebellar cells on carbohydrate-derivatized polylysine culture substrata. *J. Cell Biol.* 89:54-61.
9. Hatten, M. E., and R. L. Sidman. 1978. Cell reassociation behavior and lectin-induced agglutination of embryonic mouse cells from different brain regions. *Exp. Cell Res.* 113: 111-125.
10. Hüek, S., and M. E. Hatten. 1981. Histochemical localization of lectin binding to embryonic and early postnatal mouse cerebellar cells in microcultures. *Neuroscience*. In press.
11. Hatten, M. E., and A. M. Francois. 1981. Cell assembly patterns of developing cerebellar cells on lectin-derivatized culture substrata. *Dev. Biol.* In press.
12. Leviitt, P., and P. Rakic. 1980. Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing Rhesus monkey brain. *J. Comp. Neurol.* 193:815-840.
13. Osborn, M., and K. Weber. 1976. The display of microtubules in transformed cells. *Cell*. 12:561-571.
14. Palay, S. L., and V. Chan-Palay. 1974. Cerebellar Cortex, Cytology and Organization. Springer-Verlag, Berlin. 289-316.
15. Peters, A., S. L. Palay, and H. d. F. Webster. 1976. The Fine Structure of The Nervous System. W. B. Saunders Co., Philadelphia. 231-248.
16. Rakic, P. 1971. Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electron microscopic study in macacus rhesus. *J. Comp. Neurol.* 141:283-312.
17. Rakic, P., and R. L. Sidman. 1973. Sequence of developmental abnormalities leading to granule cell deficit in cerebellar cortex of weaver mutant mice. *J. Comp. Neurol.* 152:103-132.
18. Rakic, P., L. J. Stensas, E. P. Sayre, and R. L. Sidman. 1974. Computer-aided three dimensional reconstruction and quantitative analysis of cells from serial electron microscopic montages of foetal monkey brain. *Nature (Lond.)*. 250:31-34.
19. Schachner, M., E. T. Hedley-White, D. W. Hsu, G. Shoonmaker, and A. Bignami. 1977. Ultrastructural localization of glial fibrillary acidic protein in mouse cerebellum by immunoperoxidase labeling. *J. Cell Biol.* 75:67-73.
20. Yen, S.-H., and K. L. Fields. 1981. Antibodies to neurofilament, glial filament, and fibroblast intermediate filament proteins bind to different cell types of the nervous system. *J. Cell Biol.* 88:115-126.