

Neuronal Inhibition of Astroglial Cell Proliferation Is Membrane Mediated

Mary E. Hatten

Department of Pharmacology, New York University School of Medicine, New York 10016

Abstract. Previously we have used a microwell tissue culture assay to show that early postnatal mouse cerebellar astroglia have a flattened morphology and proliferate rapidly when they are cultured in the absence of neurons, but develop specific cell-cell contacts and undergo morphological differentiation when they are co-cultured with purified granule neurons (Hatten, M. E., 1985, *J. Cell Biol.*, 100:384-396.)

In these studies of cell binding between neurons and astroglia, measurement with light and fluorescence microscopy or with [³⁵S]methionine-labeled cells indicated that the kinetics of the binding of the neurons to astroglial cells are rapid, occurring within 10 min of the addition of the neurons to the growing glia. 6 h after neuronal attachment, astroglial DNA synthesis decreases, as shown by a two- to fivefold decrease in [³H]thymidine incorporation, and glial growth ceases.

No effects on astroglial cell growth were seen after adding medium conditioned (a) by purified cerebellar neurons cultured in the absence of astroglia, (b) by astroglia cultured in the absence of neurons, or (c) by a mixed population of cerebellar cells. This result was unchanged when any of these media were concentrated up to 50-fold, or when neurons and astroglia were cultured in separate chambers with confluent medium.

Two groups of experiments suggest that membrane-

membrane interactions between granule neurons and astroglia control astroglial cell growth. First, neurons fixed with dilute amounts of paraformaldehyde (0.5%) bound to the astroglia with the same kinetics as did living cells, inhibited DNA synthesis, and arrested glial growth within hours. Second, a cell membrane preparation of highly purified granule neurons also bound rapidly to the glia, decreased [³H]thymidine incorporation two- to fivefold and inhibited astroglial cell growth. The rate of the decrease in glial growth depended on the concentration of the granule neural membrane preparation added. A similar membrane preparation from purified cerebellar astroglial cells, PC12 cells, 3T3 mouse fibroblasts, or PTK rat epithelial cells did not decrease astroglial cell growth rates.

Living neurons were the only preparation that both inhibited glial DNA synthesis and induced the astroglial cells to transform from the flat, epithelial shapes they have when they are cultured without neurons to highly differentiated forms that resemble Bergmann glia or astrocytes seen in vivo.

These results suggest that membrane-membrane interactions between neurons and astroglia inhibit astroglial proliferation in vitro, and raise the possibility that membrane elements involved in glial growth regulation include neuron-glial interaction molecules.

THREE areas of brain development that have gone largely unexplored are the regulation of astroglial proliferation, its role in the architectonics of the young brain, and its relevance to injury or tumorigenesis. Astroglial cells appear very early in central nervous system development (7, 16, 20) and a variety of glial forms emerge through proliferation of precursor and progenitor cells (24, 27). Whereas neurons are generated in proliferative zones, generally at the ventricular surface (20), astroglia are generated in all layers of the embryonic brain.

In the immature brain, the proliferation of astroglial cells appears to be coordinated with the number and maturation of the neurons. As the proliferation of the neurons concludes, glial growth also slows (7) and most radial astroglia differentiate into astrocytes (1, 27). In some regions of the early post-

natal brain, bursts of glial proliferation continue, especially in axon tracts (2). By adulthood, astroglial growth is reduced to a very slow rate, stimulated primarily by injury (15, 28) or tumorigenesis (25). It is not clear whether these cases of increased astroglial growth arise via glial mitogens (19, 21, 23), disruptions in neuron-glial contact relationships (9), or changes in the levels of expression of oncogenes.

Our laboratory has previously developed an in vitro model system with which to study neuron-glial relationships (11). When cells are harvested from early postnatal mouse cerebellar tissue during the period when neuronal migration occurs in vivo, two forms of astroglial cells are made evident by staining with antisera raised against the glial filament protein (11). The predominant form has a stellate shape with numerous arms which anchor several dozen neurons. The

second glial form has a highly elongated shape and generally binds several neurons. Neuronal migration along these elongated glial processes can be seen in real time in vitro with time-lapse video microscopy (5, 12).

Recently we have separated neurons and astroglia from mouse cerebellum into highly purified cell fractions and have studied the regulation of astroglial form and proliferation (9). Such experiments demonstrate that astroglial cells lose their highly differentiated forms when neurons are absent and collapse into highly flattened, epithelial-like cells which proliferate rapidly. When neurons are added back to cultures of purified astroglia, the neurons bind rapidly to glia, glia differentiate into the complex forms normally seen in cultures of postnatal cerebellum (9), and extensive neuronal migration along astroglial processes commences (5, 13).

In the present report we have measured the kinetics of the neuronal regulation of glial proliferation and morphology and analyzed whether the mechanism of this control is via trophic factors or cell-cell contacts.

Materials and Methods

Purification of Cerebellar Neurons and Astroglia

Cells were harvested from C57BL/6_J mouse cerebellar tissue at postnatal days 0–4 (P0–P4) and dissociated into a single cell suspension as described (10). Immediately thereafter the suspension was applied to a step gradient of Percoll (35/60%; Pharmacia, Uppsala, Sweden), centrifuged for 10 min, and two fractions collected as described (9). After a brief (30 min) preplating on a polylysine-coated culture dish, two fractions, one a highly purified culture of astroglial cells and the other a suspension of highly purified granule neurons, were used for culture studies. The details of this method are as described (9).

Cultures were maintained in one of three types of dishes. First, cells were plated into Labtek 4048 8 chamber/slides (Miles Scientific, Div. Miles Lab., Inc., Naperville, IL), 4- or 24-well multidishes (Nunc, InterMed/InterLab, Thousand Oaks, CA), or into microcultures prepared as described (10) by drilling a hole in a 50-mm dish and placing a No. 1 coverslip over the opening with a mixture of vaseline and paraffin. In all cases the cells were maintained in Eagle's basal medium (Gibco, Long Island City, NY) supplemented with horse serum (10%, Gibco), glutamine (30 mM), glucose (8 mM), and penicillin-streptomycin.

In all experiments, an aliquot of the granule neuron fraction was cultured separately and immunostained with anti-glial filament protein antibodies (AbGFP) (11, 12) 24–48 h after plating to measure the number of contaminating astroglial cells. In experiments where this number exceeded 5% of the total astroglial cells present in the culture to which the neuronal fraction was added, the experiment was discarded.

Cells from the clonal PC12 line, a line originally isolated from a pheochromocytoma and shown to have neuronal properties when cultured in the presence of the nerve growth factor (8), and primary cultures of mouse sympathetic neurons were generously provided to us by our colleague, Dr. Lloyd Greene. BALB/c 3T3 cells and kangaroo rat PTK cells were provided to us by our colleague Dr. R. K. H. Liem.

Preparation of Conditioned Medium

Spent medium was taken from mixed cultures of cerebellar cells or from cultures of purified neurons or astroglia after 24–48 h in vitro. In some experiments, the medium was centrifuged to remove debris; in others the medium was filtered through a filter (0.4 µm) (Millipore Corp., Bedford, MA). In other experiments, spent medium was concentrated up to 50-fold by vacuum dialysis.

1. *Abbreviations used in this paper:* AbGFP, anti-glial filament protein antibodies; CFDA, carboxymethyl fluorescein diacetate; P0–P4, postnatal days 0–4.

Co-Culture of Purified Neurons and Astroglia

Two different types of co-cultures were made, one where the cells were in direct contact and others where the cells were physically separated but where the medium was intermixed. In the first case, purified granule neurons were added to cultures of isolated astroglial cells at a cell ratio between 1:1 and 4:1 and cultures were maintained as described (9).

In the second type of culture, the two cell types were kept separated by plating the cells in four types of culture vessels. First, we drilled two holes in a 50-mm tissue culture plate and plated neurons in one microculture and astroglia in the other one. 2 h after plating, the dish was flooded so that the medium intermixed, but the cells remained in their original compartment. Second, we plated cells in adjacent wells of a 24-well cluster plate (Costar, Cambridge, MA), drilled a small hole through the wall of the culture well, and inserted a 3–5-mm piece of Tygon tubing through the hole so that the medium would exchange. Third, we made a culture dish out of a piece of Plexiglas (2 cm × 5 cm × 7 mm-thick) by boring two holes 5–7 mm in diameter and 7 mm deep, affixing a glass coverslip to the two chambers with vaseline/paraffin as described (10), and then cutting a groove 2–3 mm above the bottom of the culture wells so that the medium would flow between the two chambers but the cells would be kept separated. Finally, we plated astroglia in a 24-well cluster dish (Costar) and then placed a filter (Millicell, 12 mm; 0.45 µm; Millipore Corp., Bedford, MA) on which neurons were plated above the glia. In this case the medium intermixed, but was filtered. In all cases, we plated cells for 1–3 h before the medium was flooded to levels where it would intermix between the chambers.

Kinetics of Binding of Neurons to Astroglial Cells

Three different assay methods were used to measure the time course of binding of granule neurons to astroglial cells. In the first, we plated astroglial cells and added neurons to a series of duplicate wells for time periods of 15 min–1 h, after which the dish was shaken vigorously on a rotary platform shaker (Thomas Scientific, Philadelphia, PA), and unbound cells were removed. The number of granule neurons, recognized by their relatively small size (6–8 µm) and phase brightness, were counted by phase-contrast microscopy. 10 fields were counted for each time point.

Second, we filled granule neurons with the fluorescent dye carboxymethyl fluorescein diacetate (CFDA) (29) immediately after their purification and before their addition to astroglial cultures. The neurons were incubated with CFDA (10 mM in PBS, pH 6.0; Molecular Probes, Inc., Junction City, OR) for 10 min after which the free CFDA was removed by washing. The uptake and leakage of the dye was monitored by fluorescence microscopy with excitation at 490 nm. Significant leakage of the dye did not occur for more than 12 h after it was added to the cells. Labeled neurons were then added to unlabeled astroglia as described above for periods of 15 min–1 h, after which unbound cells were removed by vigorously shaking the dish and by pipetting off free cells. Bound cells were counted by light microscopy with epifluorescent illumination. In some experiments, the astroglial cells were filled with CFDA as described and unlabeled neurons were added.

In both methods monitored by light microscopy, we counted neurons bound by or within 20 µm of an astroglial cell, recognized either by its larger, flat morphology as compared with neurons or by the presence or absence of CFDA, depending on whether we had filled granule neurons or the astroglia.

Third, we metabolically labeled granule neurons immediately after their purification for 6 h with 20 µCi/ml of L-³⁵S]methionine (970 Ci/mmol; New England Nuclear, Boston, MA) in methionine-free medium. We then washed the cells free of unincorporated methionine, and measured their adhesion to a confluent monolayer of astroglial cells. The number of bound cells was assayed after 15 min–1 h by solubilizing the cells in Hydrofluor (National Diagnostics, Inc., Sommerville, NJ) and by counting the amount of bound radioactivity using the method of Cole and Glaser (4).

Measurements of Glial Growth Rate

Two different types of experiments were carried out to measure the influence of neurons on glial growth. First, we added neurons to astroglia at cell ratios of 4:1 to 1:1, and after 0–96 h in vitro, the cultures were immunostained with AbGFP to identify and count the number of astroglial cells (9, 11). Second, we plated duplicate cultures with a neuron/astroglial ratio of 1:1 to 4:1, and after 0–96 h in vitro we removed the cells by a gentle trypsinization (0.25%, 5 min, 37°C) and counted the cells with cell diameters of 6–8 µm

versus those of larger diameter with either a Coulter counter equipped with sizing channels or with Cytofluorograf System 50H (Ortho Diagnostic Systems, Inc., Raritan, NJ)

Measurement of Glial DNA Synthesis

To analyze DNA synthesis, we cultured the cells for 2–5 d in the presence or absence of neurons and then measured the amount of [³H]thymidine incorporation by radiolabeling the culture for 3–5 h with 100 μCi/ml of [6-³H]thymidine (5 ci/mmol, Amersham International, United Kingdom), washing out unbound thymidine, removing the cells from the dish, counting the number of glial cells, precipitating for protein with cold trichloroacetic acid, solubilizing in Hydrofluor, and counting in a scintillation counter. Control experiments, where we labeled neurons in the absence of astroglia or added labeled neurons to astroglia, indicated that neurons incorporated [³H]thymidine for no more than 12–24 h after they were placed in culture.

Finally, we used autoradiography of [³H]thymidine-labeled cultures to count the percentage of cells with heavy or light labeling, and correlated the level of thymidine incorporation with the number of neurons bound per glial cell. All measurements were made with light microscopic images projected onto a Hipad digitizing tablet with a drawing tube and recorded by an image analysis system (Bioquant, Nashville, TN).

Addition of Fixed Neurons to Isolated Astroglial Cultures

To assess the influence of fixed neurons on glial differentiation, we prepared astroglial cultures as described above and added the neuronal fraction after it had been lightly fixed with paraformaldehyde. The concentration of paraformaldehyde was varied between 0.01 and 4.0%. Cells were fixed for 30 min at room temperature and then washed three times with calcium- and magnesium-free PBS before they were counted with a hemocytometer and added to the astroglial cultures at a cell/cell ratio of 4:1 (neurons/glia).

We measured the kinetics of binding of fixed neurons to astroglial cells as described above for living cells.

Preparation of Granule Cell Membranes

A membrane fraction highly enriched in plasma membranes was prepared by the method of Brunette and Till (3). The length of time that the cells had to be kept in the ZnCl₂ buffer to induce swelling was longer than that seen for fibroblasts, 12 h at 4°. Under light microscopy, the preparation consisted of large sheets of membrane. In general, we purified 5 × 10⁶ granule neurons, prepared the membranes, washed the membranes free of polyethylene glycol and dextran by three low speed spins, and then resuspended the preparation in 2.0 ml of culture medium supplemented as described for cell culture. A typical membrane preparation contained 65–75 μg of total protein. Similar membrane preparations were made with BALB/c 3T3 cells, PTK

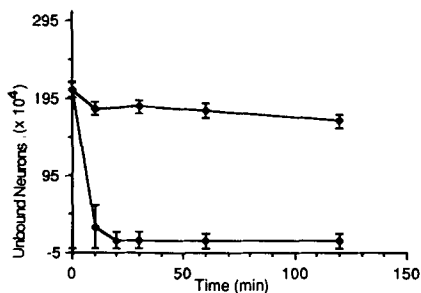


Figure 1. Kinetics of neuronal binding to monolayers of astroglia. Astroglia purified from P2 cerebellum (9) were cultured in chamber slides for 4 d, at which time the glia were >80% confluent. Granule neurons (2 × 10⁶ cells/ml) purified at P2 (9) were added to the astroglial monolayer (solid diamond) or to an empty culture chamber (open diamond) and incubated at 37° for 0–150 min, after which the dish was shaken vigorously on a rotary shaker for 5 min, the supernatant was removed, and the number of unbound neurons was counted with a Coulter counter.

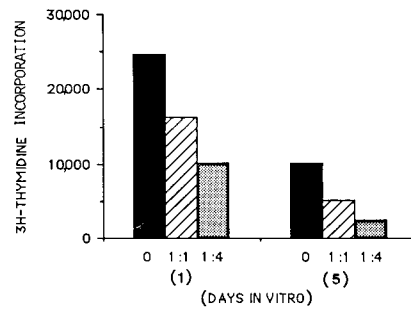


Figure 2. Granule neurons inhibit astroglial DNA synthesis. Purified astroglial cells were labeled with 20 μCi/ml of [³H]thymidine for 3–5 h, after which the free [³H]thymidine was removed by washing and purified granule neurons were added at a ratio of one neuron per glial cell or four neurons per glial cell. After 1–5 d in culture, the cells were precipitated with TCA, solubilized in Hydrofluor, and counted with a scintillation counter. Counts of incorporated thymidine were averaged in each of four duplicate cultures per neuron/glial ratio, each containing identical numbers of astroglial cells (within 5%). The standard error (not shown) was 5–7%.

cells, PC12 neurons, and purified cerebellar astroglial cells. The total membrane preparation of these cells was diluted in culture medium to an equivalence with the granule cell membrane preparation before being added to cell cultures.

In some experiments, astroglia were plated in the presence of medium taken from tubes to which amounts of gradient material were added and then washed as described for cells as a control.

Results

The binding of cerebellar neurons to astroglia was rapid, occurring within 10–15 min of the addition of the neurons (Fig. 1). Under microscopy and by the addition of [³⁵S]methionine-labeled cells, the neurons remained bound to the astroglia and could not be removed by vigorous shaking, washing, or treatment of the culture with trypsin or EDTA. In the two latter cases, all of the cells lifted off of the dish before cell–cell contacts between neurons and glia were disrupted.

Two other events occurred along with the binding of neurons to glia: a decrease in cellular incorporation of [³H]thymidine and the emergence of astroglial processes. Thymidine incorporation dropped two- to sixfold within 6 h of the addition of the neurons to astroglial cultures. In the continued presence of neurons, thymidine incorporation remained low for 5–7 d (Fig. 2). After that time, many of the neurons detached from the astroglial cells and flattened glial forms were seen again, cells which proliferated rapidly. When purified astroglial cells were cultured in isolation from neurons, a rise in thymidine incorporation continued for up to a week, after which thymidine incorporation dropped. The drop in thymidine incorporation appeared to coincide with the point at which the glial cells reached confluency.

The effect of neurons on astroglial proliferation and morphological differentiation depended on the number of neurons added and on the time elapsed since the glial cells were plated. A neuron/glial ratio of 4:1 was optimal, since at lower concentrations of neurons, some of the astroglial cells remained free of bound neurons and continued to proliferate

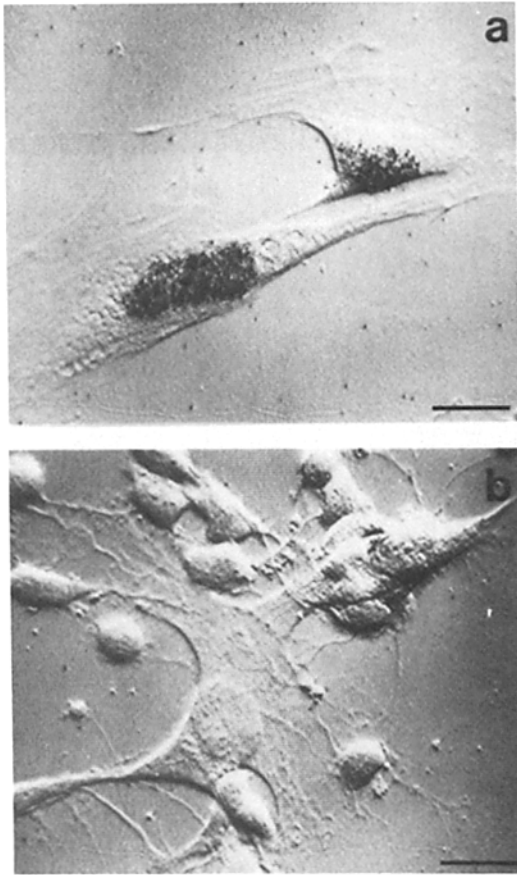


Figure 3. Neurons inhibit [³H]thymidine incorporation of astroglial cells. Growing astroglial cells purified on P2 were cultured in the absence (a) or presence (b) of granule neurons (4:1 ratio of neurons to glia) for 48 h, after which the cultures were radiolabeled with 20 μ Ci of [³H]thymidine for 3–5 h, washed, fixed with paraformaldehyde, and processed for autoradiography. (a) A heavily labeled astroglial cell; (b) unlabeled astroglia co-cultured with neurons. A number of granule neurons (5–8 μ m) cluster around the astroglial cell in the center of the field. Nomarski optics enhanced with a Hamamatsu C1965 Chalnicon camera. Photographs were taken from the video monitor as described (5) Bar, 10 μ m.

(Fig. 2). To assess whether all of the astroglial cells in the recombination culture that bound neurons had lower levels of thymidine incorporation, we combined autoradiographic localization of thymidine with immunostaining with AbGFP. These experiments indicated that >90% of the astroglia in the culture incorporated less thymidine when co-cultured with neurons (Fig. 3).

Within an hour of the time when the neurons were added to the glial culture, the astroglial cells began to change their form into complex, elongated figures (Fig. 4). The responsiveness of the astroglia to the neurons depended on the time the cells had been cultured in isolation from neurons and on the developmental stage of the glial cells at the time they were dissociated and placed into culture. Astroglia purified from cerebellar tissue taken on P0–P3 were very responsive to neurons, even after several days of culture in the absence of neurons; those harvested on P4 often took 12–18 h to begin to extend processes in the presence of neurons. The age of the neurons was also important, older neurons being more effective at inhibiting glial growth. Neurons purified on P4

were the most effective inhibitors of astroglial proliferation when the glia had been allowed to proliferate for several days before the neurons were added. The details of the age dependence of neuron–glial interactions and glial growth control will be considered elsewhere.

Two features of glial process outgrowth after recombination of purified neurons and astroglia were notable. First, within 6–12 h of the addition of the neurons, the astroglial cells extended extremely long processes, often as long as several hundred micrometers. Thereafter, progressively shorter processes were seen, until by 48 h after the addition of the neurons, two glial forms predominated: a stellate form and a cell with processes 100–150 μ m in length (Fig. 4). The latter cells were of interest because there was an unusually high percentage of these elongated glial forms, a rise from \sim 10% of the glial in a mixed culture (10) to 70–80% in recombination cultures. With time-lapse video microscopy, numerous migrating neurons were seen along these elongated glial cells (5).

To assess the specificity of the effects of granule neurons on cerebellar astroglial process extension, we tested the influence of PC12 cells, a clonal cell line of neuron-like cells derived originally from a pheochromocytoma (7), on glial growth and form. No effects were seen, even when the PC12 cells were added at a ratio of 20:1 to the glia. These results are consistent with our earlier finding that PC12 cells do not bind to cerebellar astroglia in microcultures (11). Similarly, primary sympathetic neurons isolated from mouse superior cervical ganglia did not inhibit cerebellar astroglial growth.

To test whether granule neurons would arrest the growth of fibroblasts or epithelial cells, in addition to astroglial cells, we added purified granule neurons to cultures of 3T3 fibroblasts and PTK epithelial cells at various stages of confluency. Although many of the granule neurons bound to these cells rather than to the culture dish surface, they did not inhibit the growth of 3T3 or PTK cells.

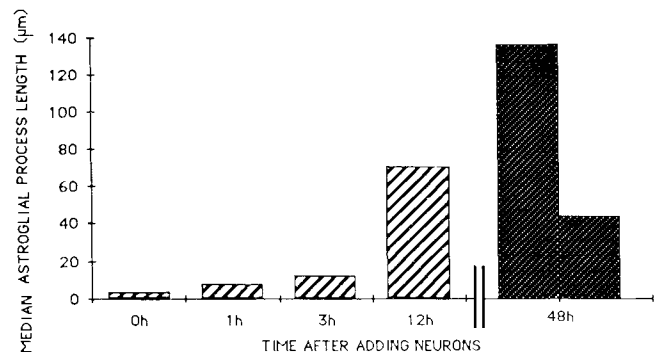


Figure 4. Astroglial process extension follows addition of neurons. Neurons were recombined with astroglial cells immediately after their purification (9). After 0, 1, 3, 12, or 48 h, the cultures were fixed with paraformaldehyde and immunostained with antibodies against the glial filament protein by the peroxidase–anti-peroxidase method (11). The length of the processes of \sim 500 glial cells was measured by projecting the microscope image onto a tablet and an image analysis system. Mean values for glial process length are given for each time point. Process extension is rapid after the addition of the neurons (Mason, C. A., J. C. Edmondson, and M. E. Hatten, manuscript submitted for publication). After 48 h, the cells fall into two distinct populations, one with long processes, resembling Bergmann glia, the other with shorter processes such as those seen for astrocytes.

To assess whether the effects of neurons on astroglial shape and proliferation were due to cell-cell contact with neurons or to trophic factors released by the neurons, we carried out co-culture experiments and tested the effects of medium conditioned by neurons on astroglial cells grown in isolation. No effects on glial shape or proliferation were seen when medium was removed from cultures of purified cerebellar neurons, of mixed cultures of cerebellar cells, or of cultures of purified astroglia. Similarly, medium taken from 3T3 fibroblasts and PC12 cells did not inhibit astroglial growth, as assessed by measuring the cell density of the glia in the culture after immunostaining with AbGFP.

To further test the effects of conditioned medium, we concentrated conditioned medium 50-fold and added it to cultures of isolated astroglia. Concentrated medium taken from neuronal, mixed, or astroglial cultures had no effect on glial morphology or proliferation *in vitro*. In all cases, the morphology and number of astroglial cells were measured by immunostaining with antibodies against the glial filament protein.

Four configurations of co-cultures between isolated cerebellar astroglia and either cerebellar neurons or a mixed culture of neurons and astroglia were tested. Co-culture of neurons and astroglia in separate compartments with confluent medium did not affect glial growth rate.

Although these experiments do not rule out the possibility that cerebellar neurons emit factors that modify glial shape, it seemed highly unlikely that the rapid, profound changes

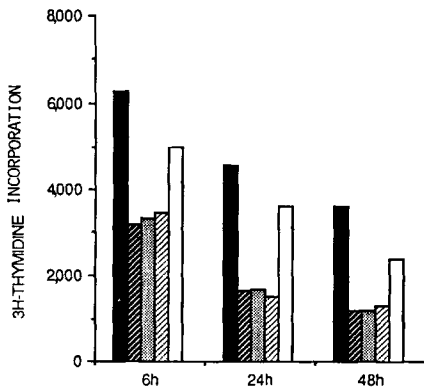


Figure 5. Fixed granule neurons inhibit astroglial DNA synthesis. Purified granule neurons were fixed with 0, 0.1, 0.5, and 1.0% paraformaldehyde as described in the text, and added to astroglial cells that had been purified from P2 cerebellar tissue and grown in microcultures for 2 d. After 6, 24, or 48 h, the cells were removed, washed, precipitated with TCA, solubilized in Hydrofluor, and counted in a scintillation counter. Astroglia cultured without neurons (*solid bar*); with living, unfixed neurons (*heavy, diagonally striped bar*); with neurons fixed with 0.1% paraformaldehyde (*stippled bar*); with 0.5% paraformaldehyde (*thin, diagonally striped bar*); or with 1.0% paraformaldehyde (*open bar*). In each case, duplicate samples of neurons without astroglia were run and the number of counts incorporated in the absence of astroglial cells was subtracted from the number measured when the cells were co-cultured. In general, this number was <10% of the total number of counts precipitated from the mixed culture. Astroglial [³H]thymidine incorporation is markedly inhibited in the presence of neurons or of neurons fixed with 0.1 or 0.5% neurons, but not in the absence of neurons or in the presence of neurons fixed with 1.0% paraformaldehyde.

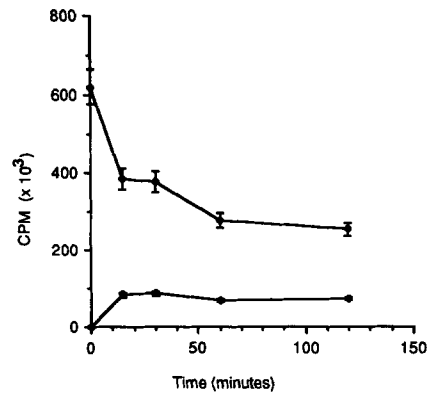


Figure 6. Binding of a [³⁵S]methionine-labeled neuronal membrane preparation to astroglial cells. Purified granule neurons were radiolabeled with [³⁵S]methionine for 5 h, after which a membrane fraction was prepared by the method of Brunette and Till (3). The radiolabeled granule cell membrane material was then added to chamber slide microcultures (100 μ l) of purified astroglia that had been grown in culture for 4 d, reaching 80–100% confluency. 0–150 min after the membrane preparation was added to the cells, the culture dish was shaken vigorously on a rotary platform shaker for 5 min and the culture medium (*open symbols*) or the cells (*solid symbols*) were removed, TCA precipitated, and counted. The binding of [³⁵S]methionine-labeled membrane material to the cells and its coincident disappearance from the culture medium were rapid. For each point, mean counts from six duplicate microcultures are given.

in the number and form of the astroglia seen in this culture system could be explained by trophic factors. We therefore tested the effects of fixed neurons and/or membranes purified from cerebellar neurons on astroglial growth and morphological differentiation.

When granule neurons were lightly fixed with paraformaldehyde, washed, and added to isolated astroglia, the kinetics of their binding to astroglial cells closely paralleled that of living neurons. Within 12–24 h, thymidine incorporation dropped (Fig. 5) and glial proliferation ceased. The effects of fixed neurons on astroglia depended on the amount of paraformaldehyde used to fix the cells. When concentrations >0.5% were used, the neurons failed to bind to the astroglia and effects on glial growth were not seen.

When a membrane fraction prepared from purified cerebellar granule neurons was added to cultures of isolated astroglial cells, [³⁵S]methionine-labeled membrane material rapidly bound to the astroglial cells (Fig. 6) and glial proliferation was arrested within 12–24 h (Fig. 7). The binding of [³⁵S]methionine-labeled granule neuron membrane material to astroglia is shown in Fig. 6. Approximately 20% of the labeled membrane material bound to the glia and was recovered when the glial cells were removed from the culture dish, TCA precipitated, and counted. Approximately half of the counts remained in suspension in the culture medium, as shown when the medium containing membranes was removed from the glial cells, TCA precipitated, and counted (Fig. 6). The balance of the membrane material adsorbed to the culture dish.

The effects of neuronal membranes on glial proliferation depended on the amount of the membrane preparation added to the glial cultures (Fig. 7). In contrast to living cells, astroglial form did not change after the addition of neuronal

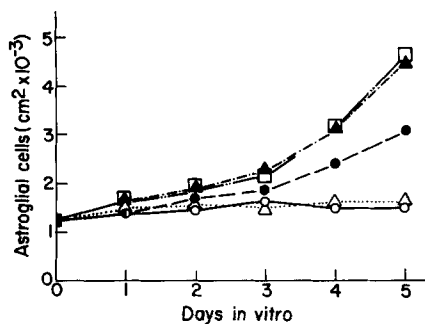


Figure 7. A granule neuron membrane preparation inhibits astroglial cell growth. The growth rate of astroglial cells cultured without any addition (*open square*), with granule neurons at a ratio of four neurons per glial cell (*open triangle*), or with 5 μ l (*solid triangle*), 10 μ l (*solid circle*), or 20 μ l (*open circle*) of a granule neuronal membrane preparation (70 μ g protein/ml) resuspended in culture medium. The number of cells was measured by counting 20 fields on each of five consecutive days, averaging the counts, and expressing the cell density per square centimeter of culture surface.

membranes. This result was unchanged when the granule neuron membranes were added to astroglial cells in the presence of medium conditioned for 24–48 h by P7 cerebellar cells. Thus granule neuron membranes bound to astroglial cells and inhibited glial growth, but did not induce morphological differentiation of the glia.

To assess the specificity of the effects of cerebellar neurons and to control for toxic effects of the procedure used to prepare the membrane fraction, we tested membrane preparations from PC12 neurons, BALB/c 3T3 fibroblasts, and PTK2 epithelial cells. None of these membrane preparations inhibited astroglial growth (Table I).

To measure the effect of the granule neuron membrane preparation on astroglial DNA synthesis, we purified astroglial cells, labeled them for 5 h with [3 H]thymidine, and then added granule cells, granule cell membranes, or PC12 cell membranes for 5 d before quantitating the amount of thymidine incorporation by autoradiography (Fig. 8). These experiments showed that astroglial cells proliferated rapidly, diluting the thymidine incorporated at the time they were plated, in the absence of neurons, but remained heavily labeled when granule cells or their membranes were added.

Table I. Specificity of Astroglial Growth Inhibition by Plasma Membrane Fractions

Cellular source of membranes	Inhibition of astroglial cell growth
Cerebellar granule neurons	+
Cerebellar astroglia	—
PC12 neurons	—
BALB/c 3T3 fibroblasts	—
PTK2 epithelial cells	—

Membranes were purified by the method of Brunette and Till (3) and added to growing astroglial cells. The number of astroglial cells was measured by removing the cells with trypsin and counting them with a Coulter counter or a hemocytometer. The plus sign indicates a difference of >30% between cells grown in the presence and absence of (40 μ l) of a membrane preparation containing 60–70 μ g protein/ml.

Discussion

The results presented in this study suggest that neuron–glia contacts inhibit the DNA synthesis of purified astroglial cells harvested from early postnatal mouse cerebellum, and control their cell growth rate. Along with the establishment of neuronal contacts and inhibition of glial cell growth, astroglial cells extend processes that organize neuronal positioning in the cultures (11).

The present report did not explain the relationship between the developmental stage of the neurons, the number of neurons bound, and astroglial morphological differentiation. Both stellate and elongated forms were seen in the presence of neurons, with a disproportionately large number of elongated forms appearing in the first 24 h after the neurons were added. The large number of elongated glia with migrating neurons seen when P4 neurons were added to P0 astroglia cultured for 2 d before the addition of the neurons turned out to be fortuitous, as it provided a method for preparing cultures enriched in migrating neurons (5).

The experiments with conditioned medium and with culturing neurons and astroglia in separate cultures having confluent medium did not support a role for trophic factors in glial growth control. Instead, glial proliferation appeared to depend on cell–cell contacts with neurons. This interpretation was supported by the finding that fixed cells and neuronal membranes arrested the proliferation of astroglial cells.



Figure 8. Astroglial DNA synthesis is inhibited by a granule neuron membrane preparation. Astroglial cells were purified from P2 cerebellar tissue (9) and labeled with [3 H]thymidine (30 μ Ci/ml) for 5 h after the cells were plated. Unbound [3 H]thymidine was removed by washing and either purified neurons (4:1 per glial cell), a membrane preparation from granule neurons, or a membrane preparation from PC12 cells was added. After 5 d in vitro, the cultures were immunostained with AbGFP by the peroxidase–anti-peroxidase method and processed for autoradiography. The number of heavily labeled (see Fig. 3), AbGFP-positive cells was counted and expressed as a percentage of the total number of immunostained astroglial cells present. Cells were counted in each of 50 fields at a magnification of 400 \times and the mean values were used to generate the percentages. In the absence of neurons (*open bar*), or in the presence of PC12 cell membranes (*vertically striped bar*), very few astroglial cells are labeled, each successive round of cell division having diluted the [3 H]thymidine radiolabel incorporated just after plating (20). In the presence of granule neurons (*solid bar*) or their membranes (*horizontally striped bar*), the vast majority of the astroglia remain heavily labeled after 5 d, indicating that they have not undergone subsequent cell division.

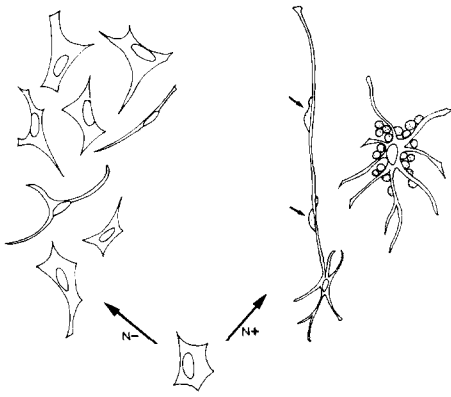


Figure 9. Model for influence of neurons on growing astroglial cells. In the absence of granule neurons, cerebellar astroglial cells divide rapidly and have flattened cell shapes. When neurons are added to growing astroglia, DNA synthesis is inhibited, growth is arrested, and the astroglia transform into complex shapes that support neuronal migration (*elongated forms*) or anchor granule neurons (*astrocytes*) (12). The inhibition of astroglial growth occurs via membrane-membrane interactions between the neuron and glial cells.

The changes in astroglial morphology seen in the presence of neurons occurred by the extension of glial processes. Glial cells extend their processes and attain specific shapes in the same general fashion as do neurons, by projecting a process with a highly motile growth cone at their leading edge. The details of the glial growth cone and of glial process extension have been described recently (Mason, C. A., J. C. Edmondson, and M. E. Hatten, manuscript submitted for publication). One point of interest in that study was that the outgrowth of glial arms coincided with the onset of specific cell-cell interactions with neurons (C. A. Mason, J. C. Edmondson, and M. E. Hatten, manuscript submitted for publication).

The morphological differentiation of cerebellar astroglial cells was complex. Three findings are notable. First, trophic factors did not inhibit glial growth and alone were insufficient to induce glial maturation to shapes resembling those seen for astroglial cells *in vivo*. Second, fixed cells and membrane preparations controlled astroglial growth, but failed to induce the glia to express complex morphologies, even when they were added to glial cells in the presence of conditioned medium. Finally, intact neurons arrested glial growth and induced the outgrowth of processes and the expression of highly differentiated glial forms that resemble cells seen *in vivo*.

In complementary studies, Bunge and co-workers have shown that peripheral nerve axons stimulate the proliferation of Schwann cells via a mitogen that can be purified from neuronal membranes (26, 33). Thus at early stages of development, before myelination, axon-Schwann cell contacts promote Schwann cell growth. Later, after an appropriate number of Schwann cells is attained, contacts between axons and Schwann cells coincide with a cessation of Schwann cell growth.

The results presented in this study suggest a model for astroglial growth regulation, shown in Fig. 9. In the absence of an appropriate number of cell-cell contacts with neurons, early postnatal cerebellar astroglial cells have a flat morphol-

ogy and proliferate rapidly. When neurons are added, they bind to the astroglial cells and arrest astroglial growth. Living neurons induce astroglial process extension, an event which provides the neuron with a support for complex behaviors, including neuronal migration. Two general mechanisms seem likely for neuronal control of glial growth. In one, the neuron binds to a receptor on the glial surface that signals a positional or density-dependent cessation of growth. In the other, the neuron binds to a receptor for a mitogen, thereby blocking the mitogenic signal.

The finding that cell-cell contacts regulate astroglial growth is reminiscent of the density-dependent growth of many cells in culture, most notably 3T3 fibroblasts (17, 22, 30) and a variety of epithelial cells. Primary astroglial cells, harvested from early postnatal mouse cerebellum, when cultured alone, will also undergo density-dependent growth regulation (our unpublished observation), having progressively slower growth rates as they reach confluency. What is striking about the present study is that cell-cell contact between two types of cells, a neuron and a glial cell, is involved, and that growth regulation occurs rapidly after cell contact is established, even when the cells are at very low density.

The finding that granule cell membranes inhibited astroglial cell DNA synthesis is consistent with a number of studies on 3T3 fibroblasts, showing that membranes from 3T3 cells, or proteins extracted from the membrane preparation (31), inhibit 3T3 cell DNA synthesis by a reversible mechanism (31, 32). Another notable example of growth regulation by cell-cell contact is found in endothelial and epithelial cells, where a complex pattern of positional signals apparently controls cell growth rates (6). Recently Heimark and Schwartz (14) have shown that a cell surface preparation of epithelial cells can inhibit DNA synthesis of actively growing epithelial cells. The receptors and ligands that control epithelial cell growth are, as yet, uncharacterized.

One of the possible *in vivo* correlates of this study is the response of astroglial cells to injury. In the adult brain, injury, especially wounds that sever axons or disrupt neuron-glial contacts, is accompanied by a local gliosis, a proliferation of astroglia (15, 28). This gliosis can be visualized by staining with antisera against glial filament protein, which is intensely localized in areas around wounds. In addition, many degenerative central nervous system diseases have a secondary gliotic response. In most cases, these episodes of glial proliferation end with the formation of a glial scar, a tight web of astroglial cells often rich in extracellular molecules such as laminin (18). The present results suggest a new hypothesis for some of these events, namely that glial growth can be initiated by an interruption in neuron-glial contacts or axon-glial contacts.

In some instances, glial proliferation does not end with a glial scar, and astrocytomas arise (25). Recently we have added purified cerebellar granule neurons to a number of human and rodent glioma cell lines. The results of these experiments with transformed astroglial cells closely parallel the studies reported here. Granule neurons and a preparation of granule neuron membranes arrest glioma cell proliferation of three human glioma cell lines and three rodent astrocytomas (Hatten, M. E., J. Sanchez, and M. L. Shelanski, manuscript submitted for publication).

These results underscore the importance of cellular relationships with neurons to the control of astroglial growth, and suggest that neurons induce cerebellar astroglial cells to express the cellular forms needed for their architectonic roles in brain development.

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