The Journal of Neuroscience, May 1994, 14(5): 3195–3207

Astroglial Differentiation Is Required for Support of Neurite Outgrowth

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Models of astrocyte differentiation stress a lineage program that involves a progressive loss of astroglial support of neuronal differentiation. These models predict that astroglial promotion of neurite extension declines with the "age" of the astrocyte. An alternative view is that astroglial support of neurite growth is regulated by epigenetic factors that induce the cells either to differentiate and support neuronal functions or to undergo cell proliferation and fail to support neurons. To compare the contribution of astroglial cell "age" to astroglial support of neurite extension, mouse cerebellar astroglia were maintained *in vitro* for 3–90 d, and assayed for their ability to support neurite formation. When cultured in isolation, astroglial support of neurite extension declined with time *in vitro*, as assayed by quantifying outgrowth from explants of pontine nuclei, falling from a robust level just after the astroglia were harvested to negligible levels 21–90 d later. Since previous studies have shown that neurons can change the state of astroglial cells (Hatten, 1985), we tested the neurite promoting activity of astroglia that were cultured for 21–90 d *in vitro* and subsequently induced to differentiate by the addition of neurons. When granule neurons were added to aged astroglia and pontine explants plated 2 d later, neurite growth from the explants was exuberant, regardless of the time astroglia spent *in vitro* prior to the addition of neurons.

The state of astroglia that were growth promoting or growth inhibiting was examined by bromodeoxyuridine staining and with antisera to glial filament protein. Aged astroglia cultured alone and thus inhibitory to axon growth, proliferated at high rates and had polygonal shapes. In contrast, aged astroglia to which neurons had been added, proliferated at low rates and developed process-bearing stellate shapes. To test further whether proliferation levels related to the growth-supporting properties of astroglia, astroglia were plated alone in medium without serum, or with the addition of transforming growth factor-β1, each treatment known to arrest proliferation. In both cases, promotion of neurite growth was restored in aged astroglia, but the morphology of astroglia did not correlate with the ability to support neurite growth. Finally, the growth-inhibiting properties of aged astroglia do not appear to be mediated by diffusible factors, and require close apposition with living astroglial cells.

We conclude that astroglial support of neurite extension depends on the state of differentiation of astroglial cells, and that these properties can be modified by coculture with neurons or conditions that arrest of astroglial proliferation, irrespective of astroglial "age."

[Key words: astroglia, axon outgrowth, glial differentiation, glial proliferation, transforming growth factor-β1]

During brain development, astroglia are thought to aid neurite extension, either by providing a scaffold, or by trophic support (Hatten et al., 1990). During periods of axon growth in developing brain, the astroglia present in axon pathways are immature, and differ from mature astroglia in their cytoskeletal composition and morphology (Smith et al., 1986; Bovolenta et al., 1987; Hankin et al., 1988; Hatten et al., 1990; Silverman et al., 1991). In contrast to their assumed function in developing brain, in normal mature brain and after injury, astroglia are thought to be particularly unsupportive of axon growth, especially in the vicinity of a glial scar (Hatten et al., 1991). From these studies, based primarily on analysis of the brain *in vivo*, the question arises as to whether astroglia and their neurite growth-promoting properties are, as in neurons, progressively restricted as development proceeds, irrevocably becoming less supportive of axon growth. An alternative view is that the state of astroglial cell differentiation, including expression of components that promote neuronal growth, is regulated by extrinsic signals (see Levison and Goldman, 1993).

In vitro, astroglia also possess neuronal growth-promoting properties including cell adhesion receptor systems that support neurite extension (Hatten et al., 1984; Noble et al., 1984; Fallon, 1985; Neugebauer et al., 1988). In these experiments, astroglia were taken from young brain. In other experimental studies on injury, astroglia from young brain cultured for short periods and transplanted to mature injured brain will suppress scar formation and promote axon growth, but lose this capacity if maintained in culture for long periods (Smith et al., 1986).

The hypothesis that support of axon growth declines with age was tested by Smith et al. (1990), who attempted to reproduce aging of astroglia *in vivo*, by maintaining young astroglia in culture for increasing periods of time. These studies demonstrated that astroglia that were "old," that is, kept *in vitro* for several weeks, supported less outgrowth than freshly plated as-

Received May 13, 1993; revised Oct. 28, 1993; accepted Nov. 17, 1993.

We are grateful to Drs. Wei-Qiang Gao, Carlos Baptista, Susana Vidan, and Steve Levison for advice and assistance in some of the experiments and to Drs. Kim Hunter, Steve Levison, Ron Liem, and Riva Marcus for critically reading the manuscript. This work was supported by NIH Grants NS 16951 and 27615 to C.A.M. and Program Project 21457.

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troglia, and that molecules expressed on astroglia kept *in vitro* varied over time. They thus concluded that astroglial cells must have a scheduled, age-dependent expression of properties that promote axon growth. Such a model would equate astroglial development with the regulatory model proposed for neural identity, involving a "progressive restriction" of gene expression that would lead to a particular cell phenotype (Anderson, 1989; Hatten, 1993).

An alternative model, based upon our previous *in vitro* analyses (Hatten, 1985; Hatten et al., 1991), is that astroglia are dependent not on age-related schedules of development but on regulatory elements provided by cell–cell interactions. This view of astroglia as highly plastic cells is supported by our *in vitro* studies demonstrating that astroglia maintained in culture in the absence of neurons exhibit high rates of proliferation and fail to develop process-bearing morphology common in more differentiated states. Addition of purified neurons to such astroglia will arrest proliferation and lead to the development of stellate shapes. We thus asked whether the state of differentiation of astroglia, influenced by the presence or absence of neuron–glial contacts and irrespective of "age" or time in culture, relates to their growth-supporting properties.

To test this, we repeated the approach of Smith et al. (1990) and examined the extent of neurite outgrowth on astroglia kept in culture for different periods. We then determined whether the growth-supporting state could be altered by the addition of neurons to the astroglia (Hatten, 1985). In addition, since growth factors such as transforming growth factor-$\beta 1$ (TGF-$\beta 1$) are thought to mediate the effects of neurons on astroglial differentiation (Vidan et al., 1990), we further examined the growth support of astroglia cultured in the presence of TGF-$\beta 1$. Third, we correlated the state of morphological differentiation of astroglia under these various conditions with their ability to support neurite outgrowth. Finally, we determined whether the growth-inhibiting properties of aged astroglia were mediated by cell contacts with neurons and/or by diffusible factors, and to what extent living astroglial cells are required. These experiments confirm previous findings that astroglial support of neurite growth declines with "age," but stress that aged glia can be converted to a state in which they can support neurite growth, irrespective of the time spent in culture.

Some of the results presented here have been reported in abstract form (Wang et al., 1990).

Materials and Methods

Preparation of astroglial monolayers. All studies were performed with cerebellar tissue harvested from C57BL/6J mice or Sprague–Dawley rats at postnatal days 3–6 (P3–P6). Purified astroglia, prepared by a two-step Percoll step gradient separation and subsequent preplating, described previously (Hatten, 1985), were maintained in 60 mm poly-D-lysine–coated bacteriological plastic petri dishes (Becton Dickinson, Lincoln Park, NJ) for 3 d to 3 months in serum-containing medium. After 1 d *in vitro* (DIV), the dishes were washed twice with cold (4°C) calcium/magnesium-free Tyrode's buffered saline (CMF-PBS) to remove remaining neurons, and then kept in the same medium to allow astroglial proliferation and induce neuronal detachment (Gilad et al., 1990).

At different "ages," that is, after different times in culture ranging from 3 to 7 d for "young" glia, and from 21 to 90 d for "aged" glia, astroglia were harvested with trypsin/EDTA (Sigma) and replated at a final cell density of 500 cells/mm^2 in a final volume of 30 μl in glass coverslip microcultures (Hatten and Francois, 1981). The culture surface was treated with poly-D-lysine (50 or 500 μg/ml; Sigma), or polylysine (50 μg/ml) followed by laminin (E-Y Laboratories; 20 μg/ml, 45 min at 35°C). Initial plating was carried out in Eagle's basal medium (Grand Island Biologicals, Grand Island, NY) supplemented with horse serum (10%; GIBCO), L-glutamine (2 mM; GIBCO), glucose (32 mM), and penicillin/streptomycin (20 U/ml each; GIBCO). After several hours or 1 d, the medium was exchanged for a chemically defined medium, consisting of Eagle's basal medium supplemented with 1% BSA, 2 mM L-glutamine, 32 mM glucose, 5 mg/liter insulin, 5 mg/liter transferrin, 5 μg/liter sodium selenite (Sigma medium supplement, I-1884), and 20 U/ml penicillin/streptomycin (each). We refer to this medium as "serum-free medium." All cultures were incubated at 35.5°C with 100% humidity and 5% CO_2.

Addition of granule neurons back to astroglia. Granule neurons, purified from P3–P6 mice or rats by the Percoll step gradient method (Hatten, 1985), were used to induce astroglial differentiation by adding them to the astroglia. The neurons had a purity of 95–98% 1 d after the subculture step as judged by the small size, characteristic morphology, and expression of L1 (NILE, Hatten, 1985), and will be referred to hereafter as pure granule neurons. Pure granule neurons were added to astroglia and this mixture was plated at a ratio of 1:1 to 1:10 astroglia: neurons, the total number of neurons in each dish being 500–5000 cells/mm^2. As a control for each experiment, cultures of the same density of granule neurons without astroglia were made.

In some cases, especially in higher ratios of neurons to astroglia, rat granule neurons were used, and mouse pontine explants were added, the latter visualized with the mouse-specific antibody M6 (see below) to distinguish granule neuron axons from pontine neurites, the major afferent to granule neurons (Lund et al., 1985; Baird et al., 1992).

Other means of inducing astroglial differentiation. (1) Astroglia grown in serum-containing medium for more than 21 d were harvested and transferred to microculture dishes with serum-free medium for 1–3 d. Removing serum arrests cell division and induces morphological differentiation, as revealed by immunostaining with antisera against glial filament protein (AbGFP; Ard and Bunge, 1988). (2) TGF-$\beta 1$ is thought to decrease the responsiveness of astroglia to mitogens and direct the astroglial cell toward a differentiation pathway (Vidan et al., 1990; Lindholm et al., 1992). Aged astroglia were replated in microculture dishes in serum-containing medium with 2–10 ng/ml TGF-$\beta 1$ (R&D Systems) for 2–7 d. After washing several times with CMF-PBS, pontine explants were then immediately added. For control experiments, pontine explants were grown on a polylysine-coated substrate with addition of the same concentration of TGF-$\beta 1$.

Basilar pontine nuclei explants. Neurite extension from explants of pontine nuclei was used to assay the astroglial promoting ability in this study. Pontine explants taken from P0 mice were prepared and cultured as described (Baird et al., 1992). Briefly, basilar pontine nuclei were dissected from the ventral surface of the brainstem in CMF-PBS and cut into 100–300-μm-diameter pieces. Before transferring to microculture dishes, explants were washed in serum-free medium. Approximately 10 explants per dish were added to either cellular monolayers (astroglia or granule neurons, plated individually or together), or to noncellular substrata (polylysine, laminin), both in serum-free medium. After plating pontine explants, a second coverslip was placed over the culture well to increase explant attachment.

Visualizing pontine neurites and astroglial cells. For simultaneous localization of pontine neurites and astroglial cells, the cultures were fixed 2 d after the coculture began with 4% paraformaldehyde at room temperature for 30 min. After a brief wash, the cultures were immunostained with NF2 monoclonal antibodies against the 200,000 Da neurofilament protein (AbNF2; Liem et al., 1985) and/or antisera against glial filament protein (AbGFP), both generously provided by our colleague Dr. Ronald K. H. Liem (Columbia University). Secondary antibodies conjugated with peroxidase, fluorescein, or rhodamine were applied. In rat granule neuron/mouse pontine explant experiments, the pontine neurites were labeled with the mouse-specific antibody M6, generously provided by Dr. Carl Lagenaur (Lund et al., 1985; Baird et al., 1992). To test if other cell types contaminated astroglial cultures, several cell type–specific antibodies were used. Fibronectin (Sigma) was used to reveal fibroblasts (Fallon, 1985), O4 (gift of Drs. Steven Pfeiffer and Rashmi Bansal, University of Connecticut) and galactocerebroside (gift of Dr. Cedric Raine, Albert Einstein Medical College) to reveal oligodendrocytes (Raff et al., 1978), RC2 to reveal mouse radial astroglia (Misson et al., 1988), and F4/80 or Pan Mac (Serotec) to reveal mouse microglia (Valentino and Jones, 1981; Perry et al., 1985). The preparations were viewed and photographed on a Leitz Orthomat microscope equipped with phase optics or Nikon Optiphot microscope equipped with epifluorescence illumination.

Measurement of astroglial proliferation. To measure astroglial pro-

liferation, the astroglia were incubated in 1:100 bromodeoxyuridine (BrdU; Amersham) for 5–8 hr, and then fixed in ethanol with 5% acetic acid. The astroglial cultures were treated with 2 *N* HCl for 40 min in order to denature the DNA. After a brief wash in Sorenson's buffer to neutralize the HCl and a brief PBS wash to rinse off the Sorenson's buffer, the cultures were incubated at 4°C overnight or at 35°C for 1 hr with 1:20 mouse monoclonal antibody against BrdU sera (Becton-Dickinson, Mountain View, CA), followed by 1:100 peroxidase-conjugated secondary goat anti-mouse antibody. After the peroxidase reaction, the preparations were dehydrated and mounted in Permount.

For triple labeling for BrdU and Abs M6 and GFP, control and experimental cultures were fixed with 4% paraformaldehyde for 30 min at room temperature and labeled with AbM6 using the indirect peroxidase method. Since the paraformaldehdye fixation prevents the detection of anti-BrdU binding sites, the cultures were then dehydrated and rehydrated in a graded ethanol series to facilitate exposure of BrdU binding sites. Then, another two antibodies (Abs GFP and BrdU) were added together overnight at 4°C. After three washes with PBS, rhodamine-conjugated goat anti-mouse IgG (for BrdU) and fluorescein-conjugated goat-rabbit IgG (for GFP) were applied for 30 min at room temperature.

Fixation and heat treatment of astroglial monolayers. To assess the influence of live astroglia and membranes on pontine neurite outgrowth, astroglia cells were heat killed by incubation in culture medium at 60°C for 45 min, and then washed once with fresh serum-free medium prior adding explants. Astroglial cultures were also fixed with 0.5–1% paraformaldehyde for 30 min at room temperature, and then extensively washed in CMF-PBS and left in serum-containing medium overnight before adding pontine explants.

Preparation of substrates of astroglial extracellular matrix. To prepare a substrate of astroglial extracellular matrix, aged astroglia were replated into microculture dishes with or without neurons for more than 1 week in order to increase the amount of extracellular matrix assembled by astroglia, and then lysed by hypoosmotic shock. The cultures were incubated in sterile, distilled water for 48 hr at room temperature, and then the lysed substrate rinsed once with sterile CMF-PBS and once with culture medium. All visible cell debris were washed from the cultures by this treatment, leaving only the extracellular matrix of the astroglia and the original substrate of poly-D-lysine (Sakaguchi et al., 1989).

Preparation of conditioned medium. To test whether the diffusible factors secreted by astroglia in different states of differentiation affect neurite outgrowth from pontine explants, conditioned medium was collected from either pure astroglia cultures, or mixed cultures of astroglia plus granule neurons (Hatten, 1987). For studies on soluble factors, this medium, either diluted 1:1 (volume) with fresh serum-free medium or undiluted, replaced normal medium at the time of plating explants.

"Wall" and "ceiling" cultures. In another two sets of experiments, the pontine explants were physically separated from the dissociated cerebellar cells. A plastic (Aclar) "wall" was put in the middle of the microculture dish, pontine explants plated on one side, and other cells (astroglia, granule cells, or both) on the other side. One hour after plating, the "wall" was removed, the dish was flooded so that the medium intermixed, but the cells remained attached to the substrate in their original compartment (Baird et al., 1992). "Ceiling" cultures were made by growing pontine explants on the bottom coverslip coated with polylysine and inverting another coverslip on which other cells were plated across the top of microwells, forming unsealed chambers (Baird et al., 1992). To control for the few cells that fell off the upper coverslip, landing among the explants, some cultures were incubated in an inverted fashion (cells beneath explants). Similar results were obtained in either situation.

Assays of pontine neurite outgrowth. Quantitation of pontine neurite outgrowth was performed as previously described (Baird et al., 1992). Cultures were fixed 2 d after adding pontine explants and stained with AbNF2 or AbM6 to identify pontine neurites. The length of stained processes was measured using a Leitz Dialux 20 EB microscope, a Hipad Digitizer, a Zeos 286 computer, and the Bioquant System IV (R & M Biometrics Inc., Nashville, TN). A set of concentric rings of radii 200, 300, and 400 μm was centered on the image of the explants as projected by a drawing tube. The number of neurites of each corresponding length was then measured and the primary data were statistically analyzed with the Bioquant System IV (R & M Biometrics Inc., Nashville, TN). The graphs were created on a Macintosh IIsi with CRICKET GRAPH (Cricket Software) and the CANVAS program (Deneba System, Inc.). At least three experiments (six coverslips per experiment and 5–10 explants per coverslip) were used to obtain the data presented for each condition. Significant differences between the means were tested by using Student's *t* test.

Results

Young astroglia support neurite outgrowth better than aged astroglia

In a previous study (Baird et al., 1992), we compared cerebellar afferent growth on cerebellar astroglia and target neurons, using mossy fibers from the pontine nuclei and target granule neurons. When pontine explants were placed on a monolayer of cells enriched for astroglia from young brain, neurite outgrowth was extensive. The length of pontine neurites and rate of neurite outgrowth on enriched astroglial monolayers were greater than that observed on a polylysine-coated surface (Baird et al., 1992).

To address whether the chronological age of the astroglial cells determines its capacity to support neurite extension, astroglia were purified and maintained *in vitro* for 3–90 d. To confirm purity of the cultures of both young and aged astroglia, cells were immunostained with a variety of cell type–specific markers. More than 97% of the cells forming the monolayers were labeled with AbGFP, and therefore considered to be astrocytes. No cells were stained with AbRC2, a marker for radial glia, and no microglia were seen. Only 2.4% and 0.1% of the cells expressed fibroblast and oligodendrocyte markers, respectively.

On monolayers of purified astroglia maintained *in vitro* from 3 to 5 d, robust pontine neurite outgrowth was seen, with little tendency to fasciculate (Fig. 1*A*). In addition, plating efficiency and long-term survival were good. However, as astroglia became "old" from 21 d after plating onward, outgrowth on the surface of aged astroglia was dramatically reduced compared to growth on young astroglia, with the majority of explants extending short neuritic processes (Fig. 1*C,E*). These results confirm that aged astroglia do not support neurite outgrowth as well as astroglia taken from young brain (Smith et al., 1990).

The morphology of astroglia at different ages also varied. Young astroglia, in culture for no more than 5 d, displayed both polygonal and stellate morphologies and expressed GFP (Fig. 1*B*). After 21 DIV, cultures contained primarily astroglia with polygonal morphology (Fig. 1*D,F*).

The addition of granule neurons can reverse the neurite-inhibiting properties of aged astroglia

When astroglia harvested from early postnatal brain are maintained in serum-containing medium, they proliferate and express a polygonal morphology with low levels of GFP. The addition of purified granule neurons to such cultures rapidly inhibits DNA synthesis, increases GFP synthesis, and induces a process-bearing morphology (Hatten, 1985). We reasoned that the addition of neurons to aged astroglia might also bring about a reversion of the growth-inhibiting properties of astroglia maintained long-term in culture. To address this, granule neurons were added to 21–28-d-old astroglia, and pontine explants were plated on the astroglia/neuron monolayers 1 d later.

On aged astroglia to which neurons had been added, outgrowth from pontine explants was vigorous, in the form of a network of fine fibers (Fig. 2; see also Fig. 4). Although neurites grew well on both astroglia and the polylysine substrate, they showed a preference for growth along the astroglia. In addition to the change from growth inhibition to growth support after addition of neurons to aged astroglial cultures, astroglia devel-

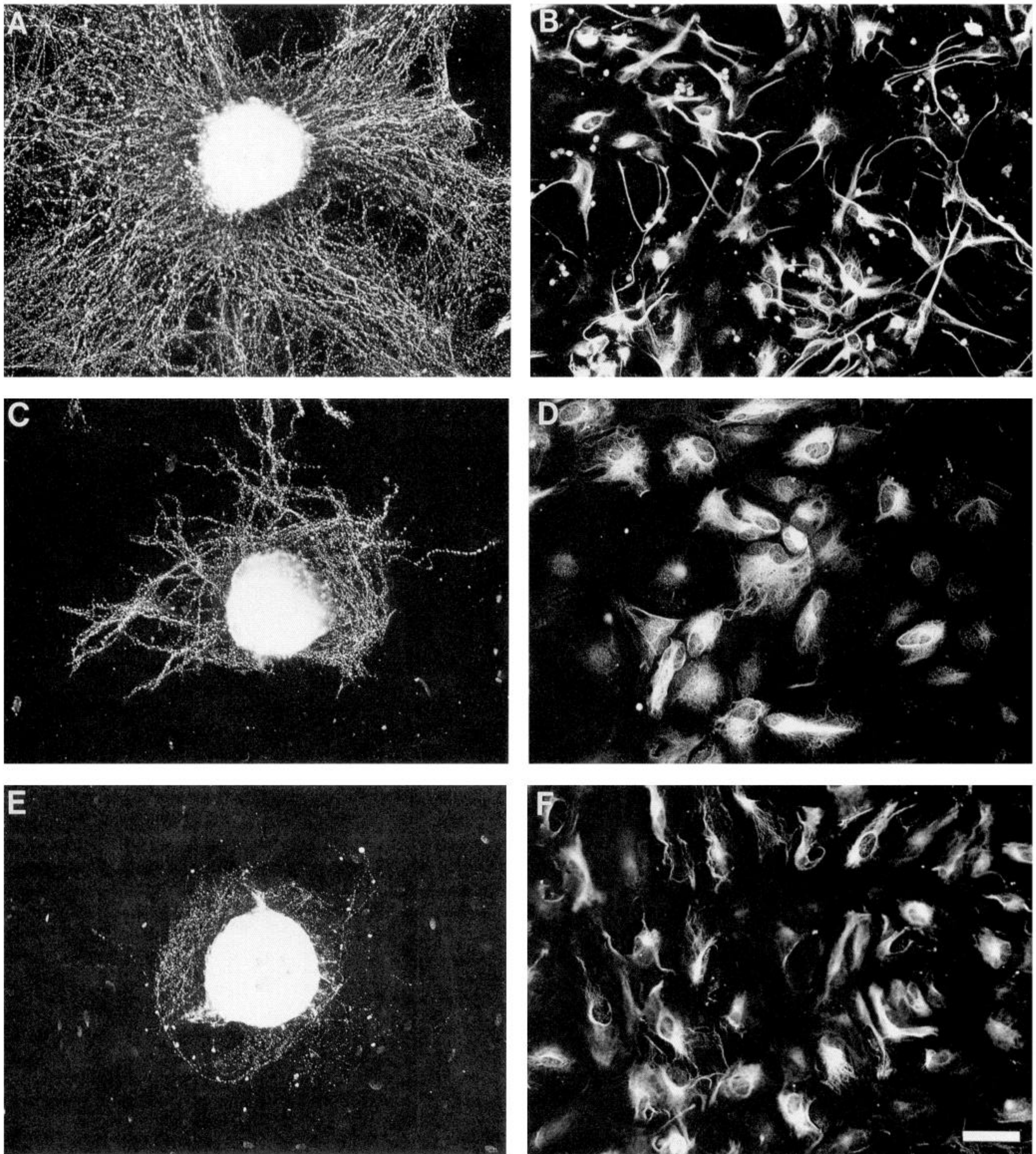


Figure 1. Extent of neurite outgrowth from pontine explants placed on monolayers of astroglia maintained for different times *in vitro*. Neurite growth is most extensive on young astroglia. *A* and *B*, 3 DIV; *C* and *D*, 34 DIV; and *E* and *F*, 91 DIV. *A*, *C*, and *E*, Pontine explants from mouse cultured on monolayers of astroglia, fixed and labeled with anti-neurofilament (NF2) antibodies. *B*, *D*, and *F*, Sister cultures labeled with anti-glial filament (GFP) antibodies, showing that astroglial morphology changes over time from process-bearing to polygonal and flat shapes. Scale bar: 100 μm for *A*, *C*, and *E*; 50 μm for *B*, *D*, and *F*.

oped stellate processes intensely stained by antibodies to GFP (compare Figs. 1*B*, 4*F*).

One question arising from the previous experiments is whether granule neurons themselves might support neurite outgrowth from pontine explants. To test this, pontine explants were plated on monolayers of purified granule neurons (Hatten, 1985) at a density of 500–5000 cells/mm². On monolayers of purified granule neurons at a density of 500–2000 cells/mm², pontine neurites

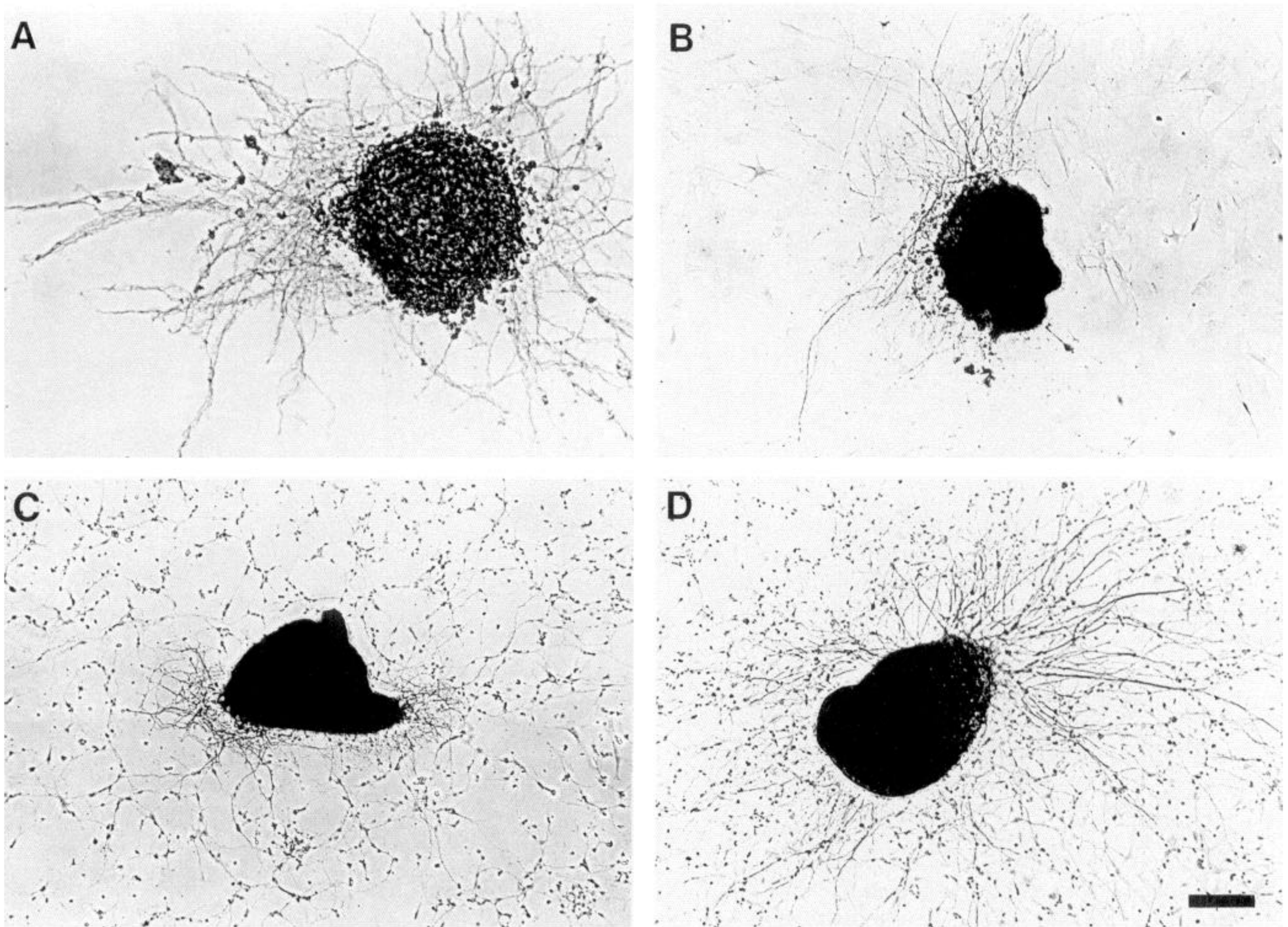


Figure 2. Comparison of pontine neurite outgrowth on different substrates. *A*, polylysine; *B*, aged astroglia maintained for 31 DIV; *C*, 1 DIV granule neurons alone plated at 3000 cells/mm²; *D*, the same density of granule neurons plus 31 DIV astroglia. Astroglia and granule neurons were taken from rat cerebellum, and pontine explants were taken from mouse and labeled with mouse-specific antibody M6. Scale bar, 100 μm.

extended distances comparable to controls on polylysine alone (Fig. 2*A*). At 3000 cells/mm² or higher, growth on purified granule cells was reduced from control levels (Fig. 2*C*; compare to Fig. 2*A*). The growth arrest of pontine neurites by granule neurons was demonstrated in our previous work on axon–target interactions *in vitro*. Granule neurons present a stop-growing signal to extending pontine neurites, their normal presynaptic mossy fiber afferents (Baird et al., 1992).

The effect of adding neurons to astroglia to induce growth-supporting properties was dependent on the ratio of neurons to glia. When neurons were added at low density (500 cells/mm²), in a 1:1 ratio, many astroglial cells remained free of neurons and continued to proliferate (Hatten, 1985; Hatten and Shelanski, 1988). In these cultures, neurite growth was reduced, similar to pontine explants in culture with aged astroglia alone. If granule neurons were added to aged astroglia at a density of 1000–3000 cells/mm², a dramatic increase in pontine neurite outgrowth was seen (Fig. 2*D*). However, neurite growth arrest is seen again if neurons were added at higher density (5000 cells/mm²), or 10:1 granule neurons:astroglia, similar to the ratio used in our studies to demonstrate that the stop-growing signal can override growth-supporting signals from astroglia (Baird et al., 1992). Thus, a ratio of 2:1 to 4:1 granule neurons:aged astroglia resulted in the most extensive pontine neurite outgrowth (Fig. 3). Below this ratio, the state of aged astroglia is not altered and they remain growth inhibiting; above this ratio, granule neurons arrest pontine outgrowth by a stop-growing signal.

Astroglial state, not age, is critical for pontine neurite outgrowth

We next determined the parameters of the state of differentiation of aged astroglia when they inhibit neurite growth. AbGFP was used to label astroglia to determine their morphology, and AbBrdU revealed dividing cells. There was a negative correlation between astroglial division and growth-promoting activity. In cultures with more BrdU-labeled astroglia, less pontine neurite outgrowth occurred, whereas astroglia that displayed a low level of proliferation were more effective in supporting pontine neurite outgrowth (Fig. 4*B,E,H,K*, Table 1). Moreover, there was also a correlation between astroglial morphology and growth support. When granule neurons were added to aged astroglia, astroglia became process-bearing with high levels of GFP, and supported extensive neurite growth (Fig. 4*F*). In contrast, aged astroglia alone were flat and polygonal, with weak expression of GFP, and they inhibited pontine neurite outgrowth (Fig. 4*C*).

Based on other reports that withdrawal of serum results in a

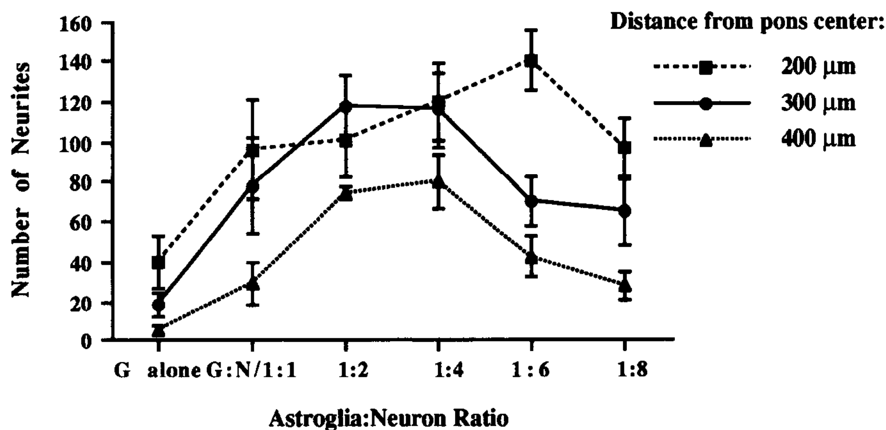


Figure 3. Pontine neurite outgrowth on aged astroglia plus neurons, with astroglial density constant but neuron density varied. Astroglia maintained for 27 DIV were harvested and mixed with granule neurons at a ratio of 1:1 to 1:10 astroglia:neurons, and then replated in microculture dishes in serum-free medium. Pontine explants were added 1 d later. Outgrowth is most robust at a ratio of between 1:2 and 1:4 astroglia: neurons. At very high density, granule cells interrupt the growth of their mossy fiber afferents from pontine nuclei (Baird et al., 1992).

decrease of astroglial proliferation (Ard and Bunge, 1988), we attempted to change the proliferation levels, and putatively, the growth-inhibiting properties of aged astroglia, by simply plating astroglia in serum-free medium. Aged astroglia were grown in serum-containing medium for 21–28 d, and then transferred to microcultures in serum-free medium for a further 2 d. Withdrawal of serum resulted in the morphological change from a confluent monolayer of polygonal astroglia to a population of stellate cells, and a reduction in proliferation (Fig. 4*H,I*; Table 1). After 2 d in serum-free medium, pontine explants were added and neurite growth was extensive, similar to the levels seen when neurons were added to aged astroglia (Figs. 4*G*, 5).

We then further tested whether the rate of proliferation related to neurite growth support. As TGF-β1 is also known to reduce levels of proliferation (Moses et al., 1990; Lindholm et al., 1992), we asked whether this growth factor would also lead to a reversion of growth inhibition in aged astroglia. TGF-β1 (2–10 ng/ml) was added to astroglia in serum-containing medium for 2–7 d. Astroglia were then washed with CMF-PBS; pontine explants were plated in serum-free medium. TGF-β1 led to a decrease in astroglial proliferation (Table 1) and induced aged astroglia to become supportive of neurite outgrowth (Figs. 4*J–L*, 5). Addition of TGF-β1 to pontine explants in control cultures on polylysine had no effect on neurite outgrowth (Fig. 5). However, in contrast to the effect of addition of granule neurons to aged astroglia or withdrawing serum, with TGF-β1, most astroglial shapes did not change from flat and polygonal to process bearing.

These experiments confirm that the state of astroglia, in particular, proliferation, or lack thereof, is an important correlate of growth support of astroglia, and that astroglial morphological alterations are not by themselves required.

The mechanism of inhibition of neurite growth by aged astroglia requires living cells and is contact mediated

Effects of inhibition of aged astroglia require living astroglia. To test whether the growth reduction on aged astroglia was dependent on active expression of molecules, astroglia were fixed and their growth-support activity tested by adding pontine explants after fixation. Surprisingly, paraformaldehyde-fixed aged astroglia supported greater neurite outgrowth better than living aged astroglia. On fixed cocultures of aged astroglia and neurons, neurite outgrowth was extensive, similar to growth in cultures of living astroglia plus neurons (Fig. 6).

In a second experiment, aged astroglia were heat killed by incubating both proliferating and differentiated (plus neurons) astroglial cultures in a 60°C oven for 45 min, and then rinsed with CMF-PBS and culture medium, and pontine explants subsequently added. The results were similar to those with fixed astroglia; that is, heat-killed aged astroglia supported axon growth better than living aged astroglia (Fig. 6).

To determine whether factors are simply on the membrane of the astroglial cells, cell-free substrates were created to test for growth inhibition. Confluent monolayers of aged astroglia with or without neurons were lysed by hypoosmotic shock. Cultures were incubated in sterile, distilled water for 48 hr at room temperature, and then rinsed with sterile CMF-PBS and culture medium. The results showed that pontine neurites grow better on the cell-free extracellular matrix from aged astroglia than on live aged astroglia, similar to growth on fixed or heat-killed aged astroglia (Fig. 6).

Taken together, these results demonstrate that the growth-inhibitory activity from aged astroglia is presented to growing neurites by contact with living intact astroglia.

Table 1. BrdU labeling index of aged astroglia in different culture conditions

Culture condition	Days *in vitro*	BrdU labeling index (%)
Serum-containing medium	22	39.3 ± 2.9
Granule neurons added	22	3.3 ± 1.3*
Serum-free medium	22	8.1 ± 1.9*
Treated with TGF-β1	25	10.8 ± 3.1*

Astroglia, purified from mouse cerebellum on P5 and maintained in serum-containing medium for 20 DIV, were considered aged astroglia. Astroglia were replated and treated as follows. (1) Serum-containing medium: astroglia were cultured in the same serum-containing medium for an additional 2 DIV. (2) Granule neurons added: granule neurons at a density of 2000 cell/mm^2 were added to aged astroglia, resulting in a ratio of 1:4 astroglia : neurons, and cultured for another 2 DIV. (3) Serum-free medium: aged astroglia were replated and maintained in serum-free medium for an additional 2 DIV. (4) Treated with TGF-β1: aged astroglia were maintained in serum-containing medium to which 5 ng/ml TGF-β1 (R & D Systems) was added, and maintained for an additional 5 DIV. For each group, fresh medium containing BrdU (diluted 1:100; Amersham) was added for 8–12 hr. Cultures were fixed in ethanol with 5% acetic acid, and immunostained with AbBrdU and AbGFP. A random sample of eight fields and approximately 1000–1500 GFP-positive cells was counted for each value. The BrdU-labeled astroglia were calculated as a percentage of the total number of GFP-positive cells. Primary data were statistically analyzed with the Bioquant System IV (R & M Biometrics Inc., Nashville, TN) to obtain the mean and SEM.

* $P < 0.005$ versus aged astroglia maintained in serum-containing medium.

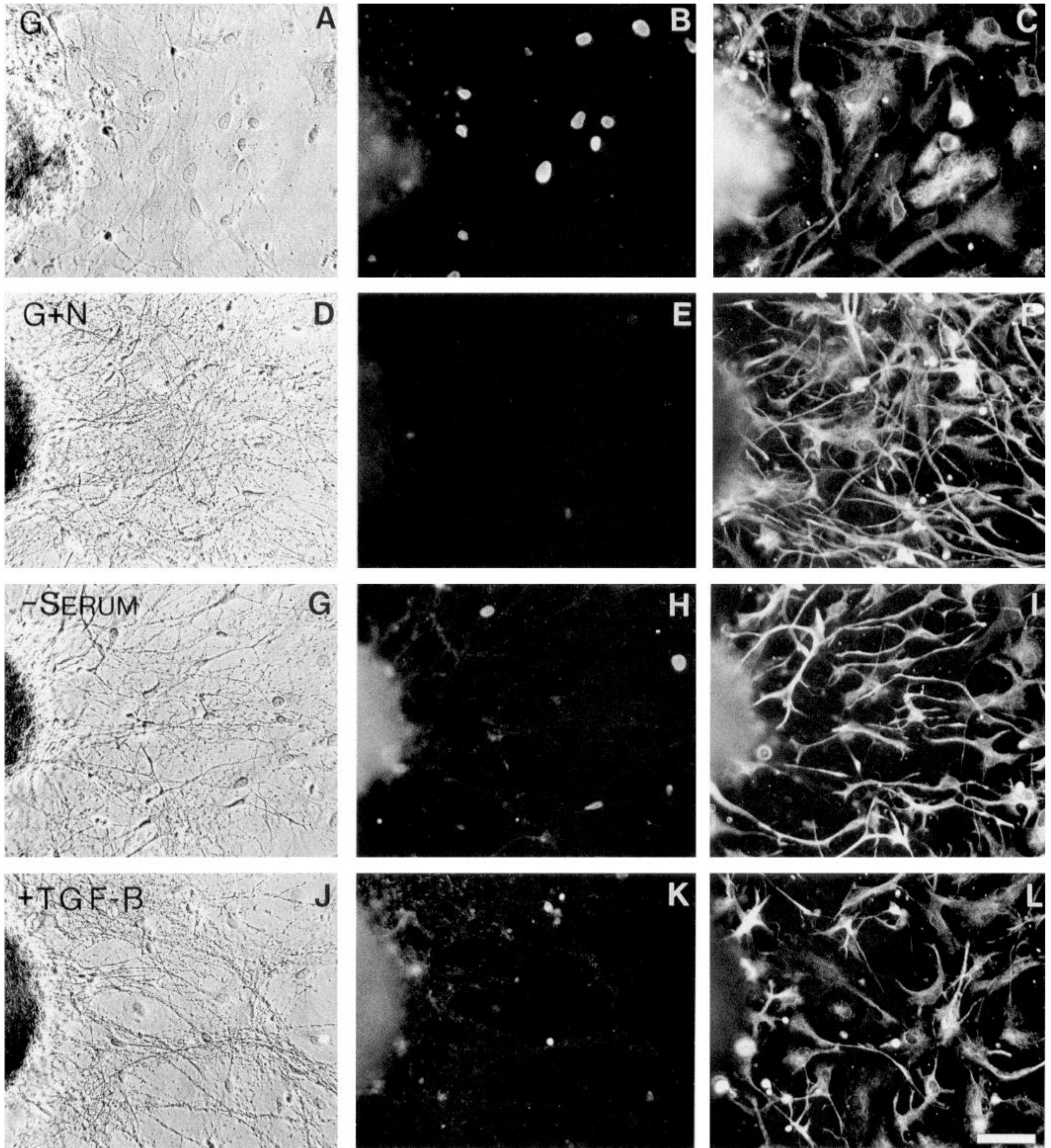


Figure 4. Neurite outgrowth is directly correlated with decreased astroglial proliferation and enhanced astroglial differentiation. Cultures were triple stained with antibodies to M6 (*A, D, G, J*), BrdU (*B, E, H, K*), and GFP (*C, F, I, L*). Pontine neurite outgrown on 25 d aged astroglia alone (*A–C*), on cultures to which neurons were added to aged astroglia maintained previously for 24 DIV (*D–F*), on the same age old astroglia but kept in serum-free medium for another 2 d (*G–I*), and on the same age old astroglia treated with TGF-β1 for 5 d (*J–L*). Note that astroglial morphology transits from flat and polygonal in *C* to process bearing after addition of neurons (*F*) and withdrawal of serum (*I*). A minority of cells have processes after addition of TGF-β1 (*L*). Scale bar, 50 μm.

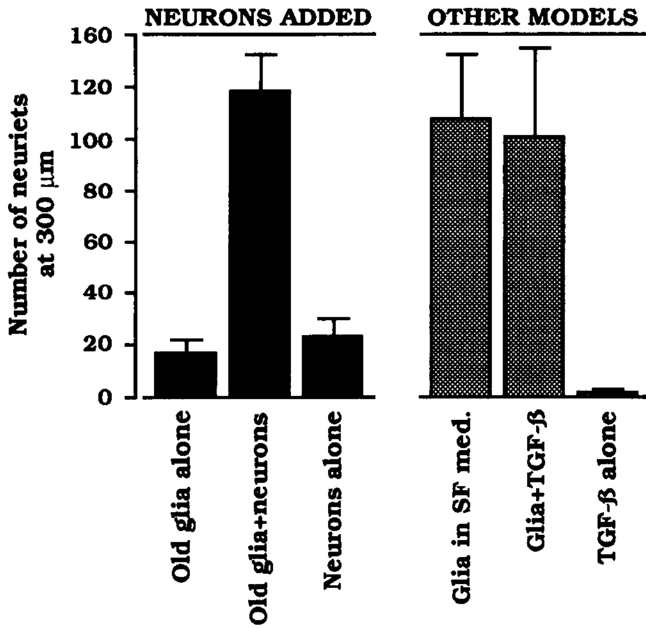


Figure 5. Neurite growth increases when astroglial state is changed. *NEURONS ADDED,* Histograms of pontine neurite growth on aged astroglia maintained for 28 DIV (*Old glia alone,* 11 explants), on astroglia of the same age to which neurons were added (*Old glia+ neurons,* 17 explants), and on neurons alone (*Neurons alone,* 10 explants), at the same plating density (3800 cells/mm^2) added to the astroglial cultures depicted by the *middle bar. OTHER MODELS,* Histograms of pontine neurite growth on astroglia kept in cultures for 23 DIV, and then changed to serum-free medium for 2 DIV before explants were added (*Glia in SF med.,* 15 explants), or treated with TGF-β1 in serum-containing medium for 7 DIV before explants were added (*Glia+TGF-β,* 10 explants), and on polylysine with TGF-β1 added (*TGF-β alone,* 10 explants). Error bars are SEM.

Inhibition of neurite growth by aged astroglia is not mediated by soluble factors. To test whether the growth inhibition of pontine explants by aged astroglia was mediated by molecules that were either secreted into the medium or secreted and deposited in the extracellular matrix, two experiments were performed. First, conditioned medium from aged astroglia with or without neurons was added to explants grown on polylysine substrates (500 μg/ml). There was no significant difference between growth on polylysine alone and on polylysine in the presence of conditioned medium from aged astroglia with or without neurons (Fig. 7*A*).

Second, pontine explants were cultured on polylysine with a coverslip of aged astroglia with or without neurons, suspended as a "ceiling" above the explants. As in the previous experiment, pontine explants with a ceiling of aged astroglia or with a ceiling of astroglia plus neurons showed outgrowth equal to control explants grown on polylysine alone without ceilings (Fig. 7*B*). These experiments suggest that if factors are released from astroglia, they are not inhibitory.

Contact with aged astroglia is important for mediation of growth inhibition. The previous experiments argue that aged astroglia do not release soluble factors that inhibit neurite outgrowth. To investigate whether contact with aged astroglia is necessary to mediate growth inhibition, we prepared wall cultures, that is, microculture coverslips with a temporary wall; on one side astroglia are plated on a polylysine surface, and the other side remains coated with polylysine only (Baird et al., 1992). Some hours after plating, the wall was removed, and the well-defined border between the astroglial cells and polylysine was stable for at least 3 d. When pontine explants were plated onto the polylysine side, the neurites grew freely in all directions until they reached the border of aged astroglia, where they grew very little or not at all (Fig. 8*A,B*). Pontine explants that were positioned entirely on the aged astroglial side exhibited little neurite outgrowth (not shown). In contrast, neurites grew profusely across the border of a half-field of aged astroglia to which neurons were added (Fig. 8*C,D*).

The experiments involving a substrate–astroglial border, together with experiments in which explants grew in the presence of conditioned medium, either soluble or deriving from "ceilings" of cells, suggest that the reduction of growth of neurites on aged astroglial monolayers is contact mediated.

Discussion

The present experiments provide support for previous findings that the chronological "age" *in vivo* or *in vitro* relates to the level of astroglial support of CNS neurite extension (Baorto and Shelanski, 1990; Smith et al., 1990). Moreover, our analysis goes further to demonstrate that aged astroglia can revert to a growth-supporting state by the addition of neurons, by reducing serum levels, or by the addition of TGF-β1, shown by other experiments in our lab to arrest astroglial growth (Vidan et al.,

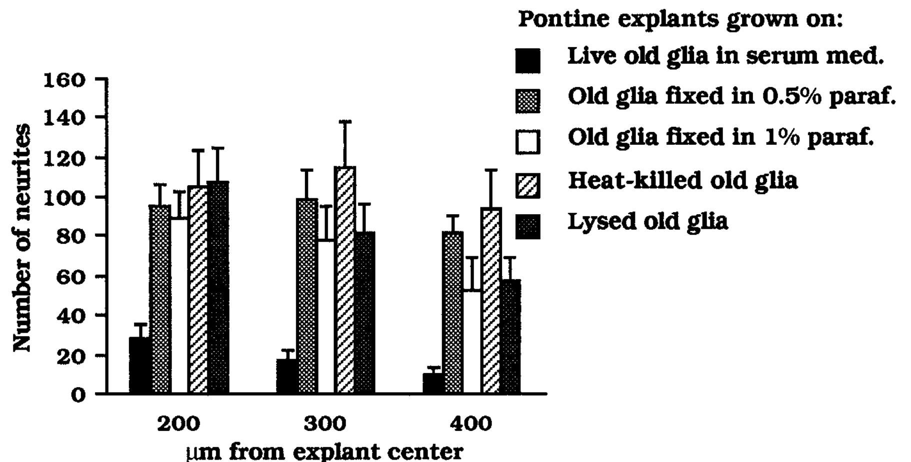


Figure 6. Inhibition of neurite growth by aged astroglia requires living cells: comparison of neurite growth on aged astroglia alone, after fixation, heat treatment, or lysis. Astroglia were kept in serum-containing medium for 22 DIV and treated as indicated. At least 12 explants were quantitated for each condition. Error bars are SEM.

1990). In addition, our results show that the growth-inhibitory factors are produced only by live astroglia. Finally, the growth-inhibiting effects of aged astroglia require close apposition of neurites and astroglia.

The present data argue that aged astroglia display a considerable degree of plasticity and can be altered to a growth-supporting state with the appropriate cell–cell interactions. Thus, unlike neurons, which show a progressively restricted program of gene expression during development (Anderson, 1989; Hatten, 1993), astroglial cells remain responsive to epigenetic regulation of differentiation even in the adult animal. Whereas dedifferentiation of astroglia and reentry into the cell cycle decreases support of neuronal function, as reported after injury (Hatten, 1991), differentiation of astroglial cells induces a maintained support of neuronal functioning. In sum, the ability of astrocytes to support neurite outgrowth is not totally regulated by cell intrinsic mechanisms, but is regulated by epigenetic factors.

Plasticity of astroglia: interconversion of growth-supporting and growth-inhibiting states

The view that the state of differentiation is important in determining the growth-supporting properties of astroglia is supported in studies by Barres et al. (1990), who demonstrated that the expression of astroglial ion channels in "type 1" optic nerve astrocytes is not strictly age dependent but is regulated by extrinsic factors, which they suggest are neuronal in origin. Earlier experiments showing that astroglia support neurite outgrowth (Noble et al., 1984; Fallon, 1985) in fact used astroglia that had been in serum for long periods, by our standards "aged" astroglia. However, antimitotic agents or other methods to prevent cell division were employed, preventing these astroglia from dividing. Thus, these were similar to the astroglia in our study that were rendered growth promoting by a variety of means that also lead to an arrest of proliferation.

A principal feature of astroglia when they are growth supporting is a low level of proliferation. Proliferation can be inhibited either by the addition of neurons, TGF-β1, or withdrawing serum. Growth-supporting and -inhibiting molecules may be differentially regulated in proliferating and resting astroglia. Other studies in our laboratory indicate that neurons influence astroglial differentiation by releasing TGF-β1, a growth factor that inhibits astroglial cell DNA synthesis (Vidan et al., 1990; Hunter et al., 1993). TGF-β1 is likely to function by arresting astroglial proliferation, repressing expression of growth-inhibiting molecules, and inducing expression of growth-supporting molecules or unmasking the already present growth-supporting molecules.

As with the effects of TGF-β1 on astroglia in our experiments, other studies on growth factor mediation of neurite growth have demonstrated that the growth factors act on astroglia, which in turn promote neurite growth. For example, mesencephalic astroglia are thought to provide a neurotrophic factor(s) for dopaminergic neurons that is distinct from acidic fibroblast growth factor (FGF), basic FGF, interleukin-1, or platelet-derived growth factor (Engele and Bohn, 1991). The apparent effects of acidic and basic FGF on dopaminergic neurons probably occur through increasing the amount of this astroglial factor. In other work, Petroski et al. (1991) have shown that basic FGF acts on astrocytes, not on the hypothalamic neurons growing on them, arresting the proliferation of embryonic astroblasts and inducing morphological differentiation of astrocytes. Neurons subsequently differentiate well on astrocytes treated with this growth factor.

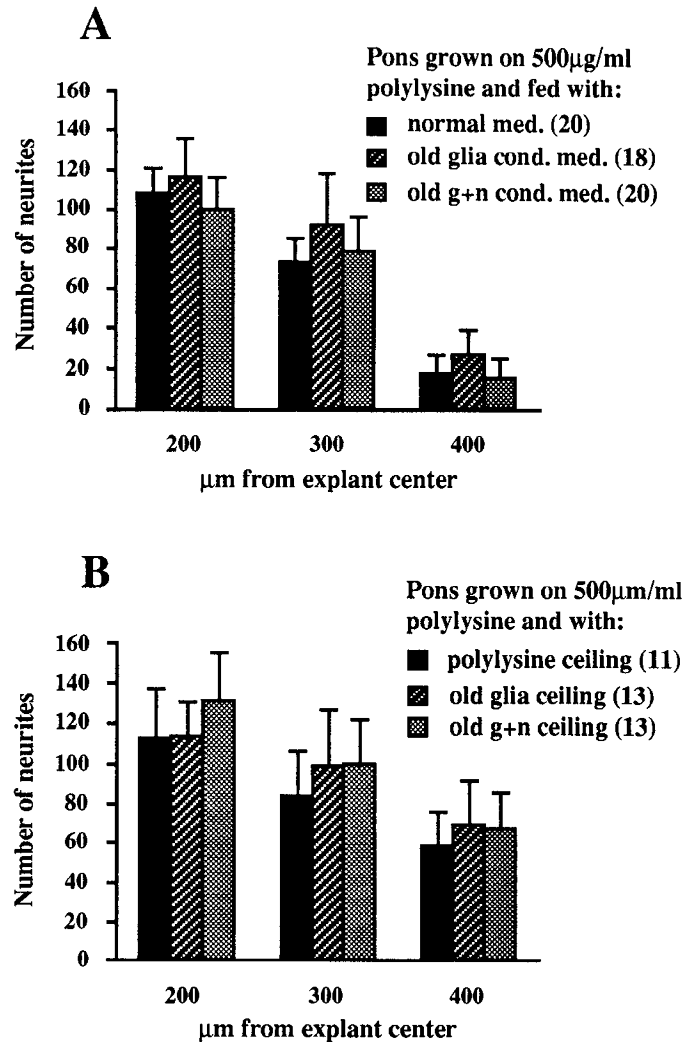
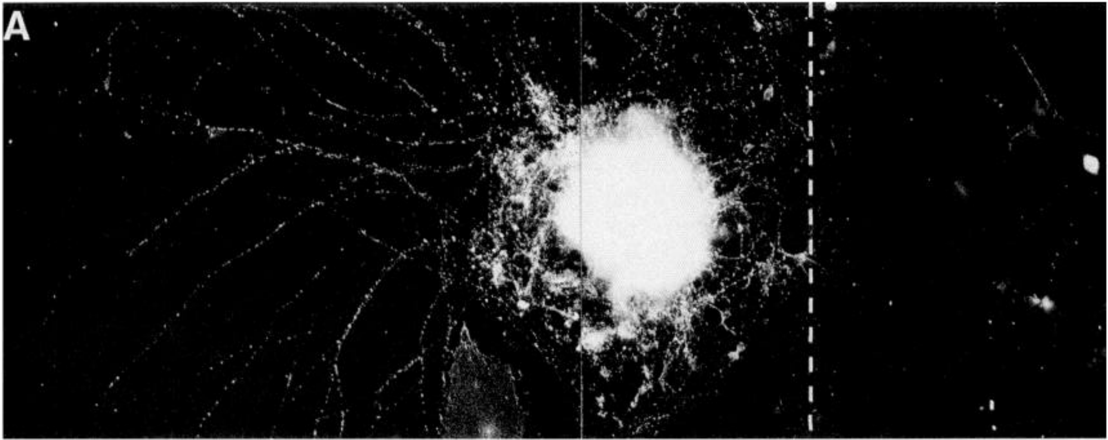


Figure 7. Aged astroglia do not release diffusible factors that are inhibitory. *A*, Conditioned medium from aged astroglia alone (23 DIV) or aged astroglia (24 DIV) to which neurons have been added neither inhibit nor promote neurite growth. *B*, Aged astroglia (24 DIV) or aged astroglia (24 DIV) with granule neurons placed as a ceiling do not release diffusible factors. If factors are released from astroglia, they are not inhibitory. The *numbers in parentheses* represent numbers of explants measured to obtain data for each bar. Error bars are SEM.

A property of all astroglial cells that are inhibitory to neurite growth is that they proliferate and have a polygonal morphology. Morphology alone, however, does not directly correlate with growth inhibition and support, since conditions that will arrest proliferation and lead to a restoration of growth support, such as treatment with TGF-β1, do not necessarily induce morphological differentiation. *In vivo*, the shape or morphological form of astroglia in young brain differs from that in mature brain. Radial forms of astroglia are present during the major periods of axon growth (Bovolenta et al., 1987; Hatten et al., 1990; Silverman et al., 1991). The results of Baehr and Bunge (1990) on adult retinal ganglion cell axon regeneration also support the hypothesis that the functional state and not the morphological shape of the astroglial cell is critical. Together, these findings suggest that differences in astroglial states are likely to be re-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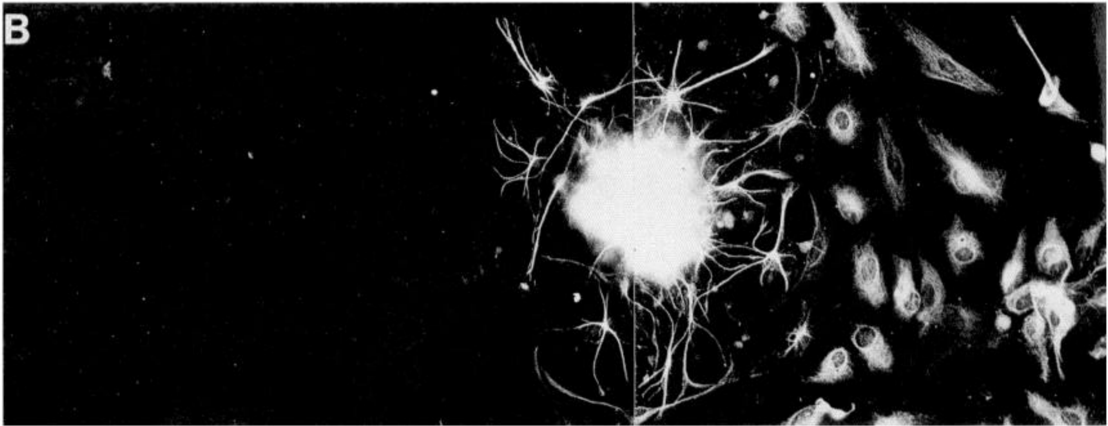
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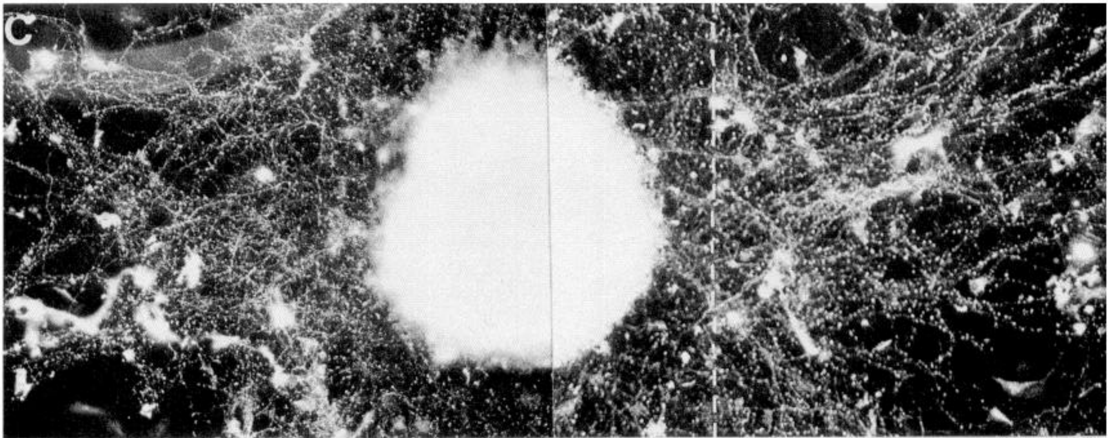
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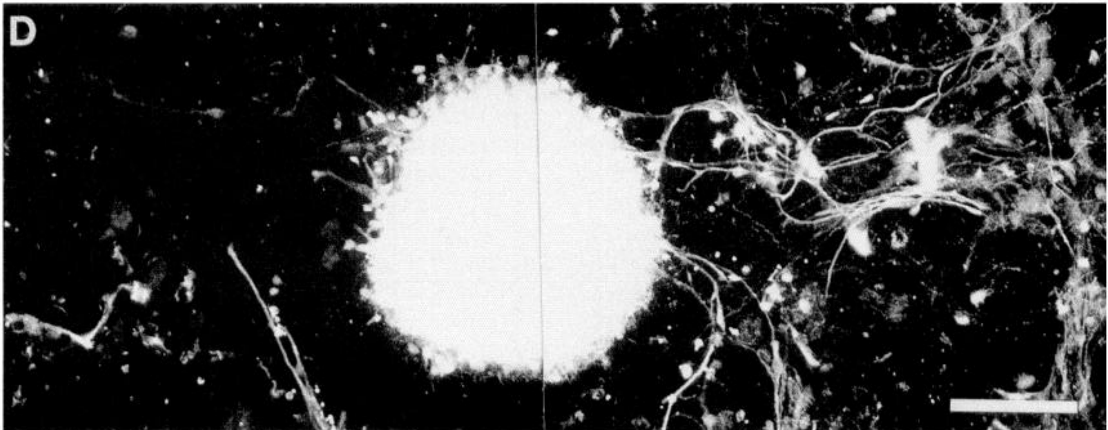
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flected at the molecular level, at the astroglial cell surface, and/or in the extracellular matrix, and that changes in morphology are not by themselves necessary to support neurite growth.

In the PNS, as in the CNS, the state of differentiation of glia, in this case Schwann cells, can differentially affect neurite outgrowth, particularly during regeneration (Baehr and Bunge, 1989). When adult rat retinal ganglion cells are cocultured with quiescent Schwann cells, neurite outgrowth is limited. However, if Schwann cells are activated by coculturing with purified dorsal root ganglion neurons or by adding serum and ascorbate, retinal ganglion cell survival and axonal regrowth was improved. Although the optimal state of Schwann cell differentiation for neurite outgrowth is active cell division, in contrast to astroglia in the CNS, these *in vitro* experiments again argue against a fixed program of astroglial maturation, and implicate the importance of neuron–astroglial interactions that influence astroglia state at any point in the lifetime of the animal. Further, they suggest that neuron–astroglia interactions are important for astroglial differentiation, and that the functional state of astroglia is critical for neurite outgrowth.

Molecular mechanisms of neurite growth support and inhibition by astroglia

Neuron–astroglia interactions are believed to be mediated by "adhesion molecules," a heterogeneous group of glycoproteins found either in the extracellular matrix, or anchored to the cell membrane. Most of these molecules promote neurite growth. Others have proposed that aged astroglia might lack membrane components found in young astroglia that facilitate neurite outgrowth (Tomaselli et al., 1988; Smith et al., 1990) or have additional membrane components that interfere with growth support. Our experiments favor the latter possibility, that aged astroglia actively produce growth-inhibiting molecules, rather than lack growth-supporting molecules. The inhibitory molecules require living cells, and are inactivated by fixation, heat, or lysis.

The growth-inhibiting molecules expressed by astrocytes include the extracellular matrix molecule tenascin (Faissner and Kruse, 1990), chondroitin-6-sulfate proteoglycan (McKeon et al., 1991), and keratan sulfate proteoglycans (Snow et al., 1990). Astrocytes that are unsupportive of neurite growth express high levels of tenascin, display an increased mitotic index and a cell surface with a "rocky" appearance (Grierson et al., 1990). If astrocytes are maintained in serum-free medium, astrocytes become growth supporting, developing a stellate morphology and having a reduced rate of proliferation. Correlated with these changes are a smooth astroglial cell surface and decreased expression of tenascin (Grierson et al., 1990).

Smith et al. (1990) found that while N-CAM or L1/G4 receptor systems are used by neurons growing over freshly cultured astrocytes, neurons growing over the surface of astrocytes aged in culture do not use these systems. This suggests that the normal ligand for N-CAM is not present on the aged astrocytes, or, as our results implicate, that the effects of the inhibitory molecules override any extant growth-supporting system. Although we do not have direct evidence, it is possible that the normal ligand for N-CAM is reestablished by reversing aged astroglia via adding neurons or growth factors. Other reports (Saad et al., 1991), however, indicate that growth factors cannot induce expression of neural adhesion molecules L1 and N-CAM on mature astrocytes.

Another component that might be missing on aged astroglia, or counteracted by inhibitory molecules expressed by proliferating astroglia, is laminin. Astroglia synthesize and release a variant form of laminin (Liesi and Risteli, 1989; Matthiessen et al., 1989). During neurite extension *in vivo*, a punctate form of laminin has been detected in retinal ganglion cells and optic neurites. As development proceeds, laminin deposits either disappear or are redistributed, and retinal ganglion axons selectively avoid regions that were previously laminin rich (Liesi and Silver, 1988; see also Ard and Bunge, 1988).

In contrast to the growth-inhibiting molecules, the activity of the growth-supporting molecules does not require living cells, nor is it subject to destruction by these various treatments. In agreement with this, Qian et al. (1992) have reported that neurite-promoting diffusible factors are produced by astroglia. While these latter experiments did not distinguish between freely diffusible molecules or those that are trapped by matrix, these factors appear to act in a region-specific manner; that is, only astrocytes from target regions can regulate the extension of specific afferents to those targets.

Our findings also suggest that if both growth-inhibiting and growth-supporting systems coexist, growth inhibition is more potent than growth support. This was demonstrated by placing ceilings of astroglia and neurons, which putatively release only growth-supporting molecules, over cultures of pontine explants on aged astroglia (not shown). This combination did not lead to improvement of neurite outgrowth, implying that the growth-supporting system could not overcome the growth-inhibiting effects of aged astroglia.

In the present study, a principal correlate of the increased support of neurite growth in aged astrocytes after addition of neurons, TGF-β1, or removal of serum was the cessation of proliferation. One explanation for the enhancement of growth-supporting properties in nonmitotic astroglia comes from studies of a variety of cell types, which show that postmitotic cells produce and assemble higher levels of extracellular matrix molecules (Vollberg et al., 1991; Baghdassarian et al., 1993). While

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Figure 8. The inhibitory effects of aged astroglia are contact mediated. Neurite outgrowth in "wall" cultures: explants were placed on the border (*dotted lines*) of a half-dish of polylysine (*A–D, left*) and aged astroglia (21 DIV) on polylysine (*A, B, right*) or aged astroglia on polylysine to which neurons were added 2 d before (*C, D, right*). *A*, Pontine neurites, labeled with AbNF2, grow well on 500 μg/ml polylysine (*left*), but poorly on aged astroglia (*right*). *B*, Same field labeled with AbGFP shows flat polygonal aged astroglia at the right side of the explant. Stellate astroglia positioned in and around the explant derive from the pontine explant itself, in culture for 2 d. *C*, Pontine neurites, labeled with AbNF2, grow well on both polylysine (*left*) and aged astroglia to which neurons were added (*right*). *D*, Same field as *C*, labeled with AbGFP. Astroglia appear stellate, as neurons were added 2 d before. Some astroglia moved across the border of the wall (*lower left*). Neurites grow better on polylysine than on a field of old astroglia (compare both sides of explant in *A*), and better on aged astroglia plus neurons than on aged astroglia alone (compare right side of explants in *A* and *C*). The apparent enhancement of explant outgrowth on polylysine alone in *C* as compared to *A* results from, first, the larger size of the explant in *C* and therefore a greater number of neurites extending from the explant. Second, aged glia to which neurons have been added release growth-promoting molecules that may diffuse and/or bind to the substrate (L.-C. Wang, unpublished results). These factors/molecules might therefore have seeped around to the side of the explant on polylysine alone and have further promoted growth. Scale bar, 100 μm.

we did not carry out extensive analysis of which molecules are more abundant after these treatments, the possibility exists that axons grow better on aged astrocytes brought out of the cell cycle because different or higher levels of matrix molecules are produced.

A different type of molecular mechanism involves Thy-1 and neuron–glial interactions. Two recent studies show that Thy-1 on the surface of neurites may play a role in stabilizing growth of neurites in general and may be important for mediating reduced growth on mature astroglia (Mahanthappa and Patterson, 1992; Tiveron et al., 1992). On astroglia, while Thy-1^- cells extend neurites, Thy-1^+ cells fail to send out processes. Removing cell surface Thy-1 by addition of anti–Thy-1 antibody or perturbation of Thy-1 function with phosphotidylinositol-specific phospholipase C or mutagenesis resulted in the enhancement of neurite initiation. These results imply that Thy-1 on the neural cell selectively interacts with the astroglia to limit neurite outgrowth. Whether the receptor for Thy-1 is present on mature astroglia is not known, but might explain the differences in growth on the two different states of astroglia.

Implications for injury

Our results point to a requirement for the proper astroglial state to support neurite outgrowth. This regulation is of obvious importance after injury, when astroglial proliferation and subsequent neuronal death can ensue (see Hatten et al., 1991). The lack of axonal regrowth near an astroglial scar could result from reversion of astrocytes to a state in which they are inhibitory to axon growth (Rudge and Silver, 1990; Geisert and Stewart, 1991). The presence of proteoglycans and tenascin on astroglia within a scar in adult brain (McKeon et al., 1991) supports this hypothesis.

New results suggest that TGF-β1 is released after injury, serving to decrease astroglial proliferation (Lindholm et al., 1992) and in our experiments to lead to neurite growth promotion *in vitro*. However, despite the dampening of astroglial proliferation, neurons encounter obstacles to regeneration, including in mature brain, the absence of developmentally regulated programs in neurons that impair their growth on astrocytes (Baehr and Bunge, 1990; Fawcett, 1992). The present evidence implicates the importance of epigenetic factors in the transition of an astrocyte that is unsupportive of neuronal function to one that can quickly become supportive of neuronal function. Future therapies might therefore involve reversion of astrocytes with growth-inhibitory properties back to a state in which growth-supporting properties predominate.

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