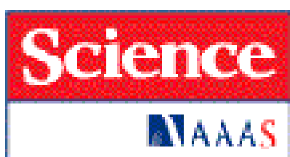

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Trophic Interactions Between Astroglial Cells and Hippocampal Neurons in Culture

Abstract. *Astroglial cells in primary culture release factors into the medium that promote the growth and prolong the survival of rat hippocampal neurons in vitro.*

It has long been suspected that glial cells play a trophic role in support of neurons (*1*, *2*). The most compelling evidence in favor of such a role comes from studies of cells in the peripheral nervous system. Nerve growth factor (NGF), which is essential for the development and maintenance of sympathetic neurons in vivo and in vitro, is produced by ganglionic glial cells (*3*), cell lines derived from gliomas (*4*), and a variety of nonnervous tissues (*5*). Other as yet unidentified factors (*6*, *7*), including some produced by cultured glial cells (*8*), also enhance the development of peripheral neurons in culture. No trophic factors with similar effects on neurons from the central nervous system have yet been described. It has been noted that central neurons "fare better" in culture when they contact nonneuronal cells (*2*), especially glial cells (*9*), but the basis for this effect is unknown.

A method for establishing low-density cultures of neurons from the rat hippocampus was described previously (*10*, *11*). Two features of this culture system make it particularly suitable for testing the influence of glial cells on neuronal development. Few nonneuronal cells are present in these cultures, so the effects of added glial cells can be readily assessed. In addition, the neurons do not reaggregate but remain dispersed, so their ability to survive and extend processes can be assayed simply by cell counting. It was noted previously that coculture of dissociated hippocampal neurons with explants of brain tissue increases the percentage of neurons that develop processes in vitro and enhances their survival (*10*). I now report that primary cultures of astroglial cells are similarly capable of enhancing the development of hippocampal neurons and that this effect is mediated by some substance or substances released by the glial cells into the culture medium.

Hippocampi were dissected from 19-day-old rat fetuses and trypsinized to obtain neurons, which were plated onto polylysine-treated cover slips (1.25×10^4 cells per square centimeter), and maintained in modified Eagle medium containing 25 percent human placental serum (*12*). Monolayer cultures of astroglia were prepared from 1- to 3-day-old rats according to a modification (*13*) of the method of Booher and Sensenbrenner (*14*). Astroglial cells cultured in this manner express many of the biochemical and physiological properties characteristic of astrocytes in vivo (*13*, *15*). At least 80 percent of the cells in these cultures are astrocytes, as judged by immunocytochemical localization of the astrocytic marker, glial fibrillary acidic protein (*16*). Some presumptive fibroblasts are also present, but neurons cannot be detected in these cultures.

Astrocytes growing on small cover slips were then introduced into the neuronal cultures. Alternatively, neurons were maintained in medium conditioned by prior incubation with glial cultures (*12*). Neuronal growth was assessed by counting the number of surviving neurons that developed processes. About 3 hours after plating, when adhesion was essentially complete (*10*), a count was made of the total number of attached cells in marked regions of each culture. At subsequent times these same regions were examined and the number of neurons with processes was counted and expressed as a percentage of the number of cells initially present. In addition, some regions were sequentially photographed so that the growth of individual neurons could be followed.

When the hippocampal cells were cultured by themselves, about 17 percent developed neurites during day 1 of culture (Fig. 1A). At this stage they resembled other central and peripheral neurons in culture (Fig. 2A) (*10*), with their processes tipped by prominent, motile growth cones. Despite their apparently normal initial growth, the hippocampal neurons survived only a short time under these conditions. Process elongation largely ceased by day 3. The cells appeared to become vacuolated, their processes degenerated, and they died (Fig. 2B). By day 6 only a few intact process-bearing neurons remained (Fig. 1A), and the total number of surviving neurons was less than 5 percent of the number plated (*10*).

When astrocytes growing on cover slips were added to hippocampal cell cultures, the number of neurons that developed processes was significantly increased ($P < .01$, Student's *t*-test) (Fig. 1A). In addition, their survival was prolonged considerably. Since the added astrocytes remained largely confined to the cover slip, it is unlikely that these effects resulted from contact between the neurons and the astrocytes. Rather, it seems that some substance was released by the astrocytes into the medium. To test this, neurons were maintained in medium that had been incubated for 2 days with astrocyte cultures. The results obtained with this conditioned medium were similar to those of the coculture experiments: about twice as many neurons developed processes when maintained in conditioned medium as when maintained in control medium. At first there were no obvious morphological differences between cells in each condition (Fig. 2, A and C), but after 1 week the differences were marked (Fig. 2, B and D). Cells in conditioned medium did not undergo the degenerative changes seen in the control cultures and, after 7 days, bore considerable resemblance to hippocampal pyramidal neurons, with apical and basilar dendritic trees (*11*). A dense plexus of

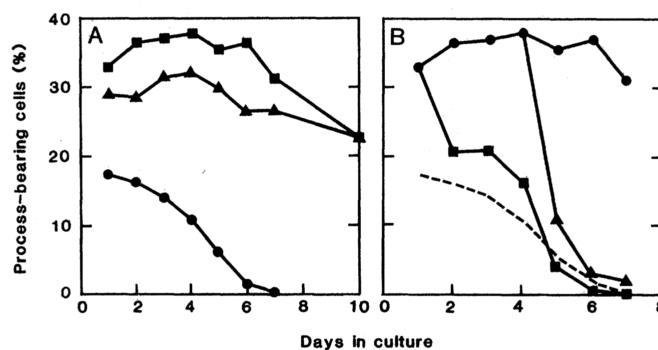

Fig. 1. (A) Influence of astroglial cells on the growth of hippocampal neurons. Neurons were cultured alone (●), in the presence of astrocytes (▲), or in medium conditioned by incubation with cultured astrocytes (■). The number of neurons with one or more processes greater than two cell diameters in length was counted after various periods and expressed as a percentage of the number of cells present 3 hours after plating. Neurons that had begun to degenerate were not included. (B) The effect on neuronal growth of withdrawing astrocyte-conditioned medium. Cultures of hippocampal neurons were established in conditioned medium. Some of the cultures (●) were maintained in that medium; others were transferred to control medium after 1 day (■) or 4 days (▲). The growth of cells established and maintained in control medium is shown for comparison (- - -).

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fine fibers ran between the cells. The number of process-bearing neurons remained relatively constant during the first week in the conditioned medium and gradually declined thereafter; few survived longer than 2 weeks (17). Peacock *et al.* (18) recently showed that when hippocampal neurons are allowed to develop on a monolayer of nonneuronal cells, some survive for up to 2 months.

The effects of the conditioned medium on hippocampal neurons were reversible. When conditioned medium was replaced by control medium, the number of process-bearing neurons rapidly declined to control levels (Fig. 1B). It seems that the growth-promoting agent produced by astroglial cultures must be continuously present in the medium in order to be effective. Unlike some of the factors that enhance neurite formation by peripheral neurons (7), it does not bind to the substrate in amounts sufficient to promote nerve growth when conditioned medium is replaced by control medium.

These results document a clear instance in which astrocytes produced trophic substances that acted on neurons from the central nervous system. Cultured astrocytes produce a broad spectrum of soluble macromolecules and release them into the medium (19); the molecules responsible for the effects described remain to be identified. Nerve growth factor is apparently not involved, since purified mouse NGF (5 or 100 ng/ml) does not enhance the growth of hippocampal neurons (20).

The ultimate significance of this example of glial-neuronal interaction hinges on the answers to several questions. Do other types of central neurons also respond to this factor? Are other cell types similarly capable of producing the factor, or is this an example of a specialized function of astroglia? Is the factor of importance for neuronal development in vivo? Answers to these questions must await identification of the molecules involved and their mode of action. Until these factors are isolated, medium conditioned by astroglia may be of practical value in maintaining other central neurons in culture when the presence of glial cells themselves is undesirable.

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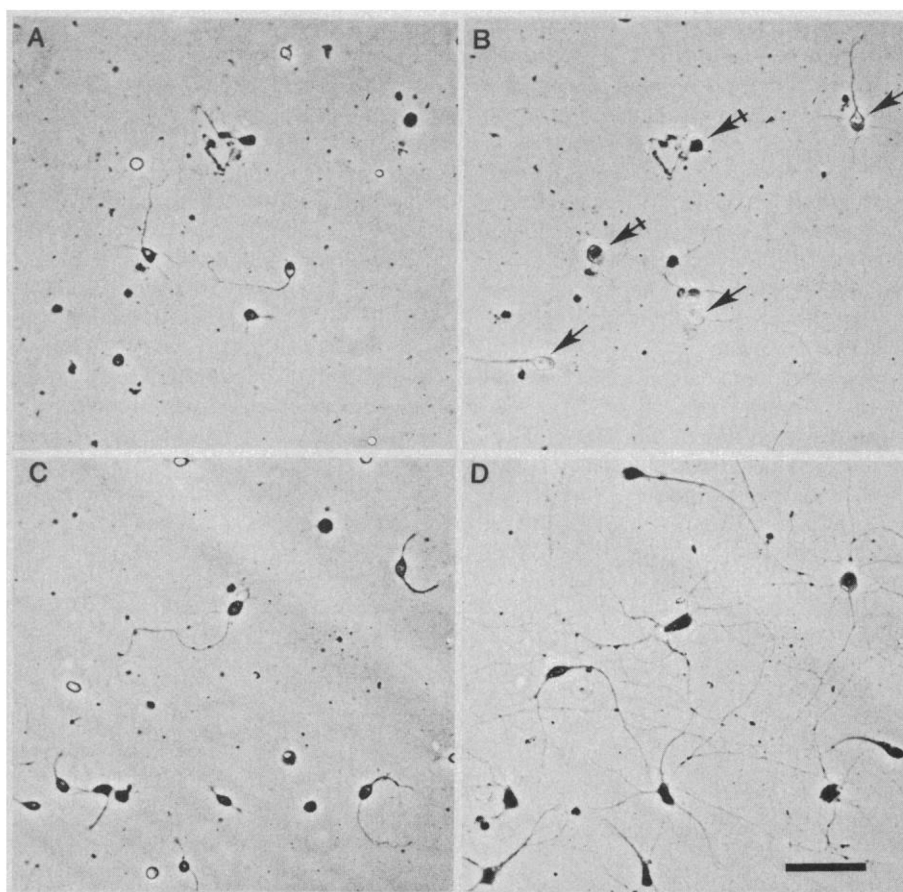

Fig. 2. Phase-contrast photomicrographs of cells maintained in control medium (A and B) or in astrocyte-conditioned medium (C and D). Selected regions of the cultures were photographed after 1 day (A and C); the same regions were photographed again after 7 days (B and D). The cells that developed processes within 1 day appeared similar under both culture conditions. After 7 days there were obvious morphological differences. Many cells that grew processes after 1 or 3 days in control medium degenerated completely after 7 days (B, barred arrows). Others appeared to be filled with phase-bright vacuoles (arrows). In contrast, cells in conditioned medium (D) showed no degenerative changes and developed an extensive network of fibers. Scale bar, 50 μm.

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12. Full details of the methods for culturing hippocampal neurons are presented in (10). When astroglia and neurons were to be cocultured, glia were grown to confluence on 7-mm-diameter plastic cover slips. One cover slip (containing about 5 × 10⁴ astrocytes) was added to each neuronal culture (containing about 2.5 × 10⁴ neurons) shortly after the neurons were plated. Conditioned medium was prepared by incubating 4 ml of medium with confluent astroglial cultures in 60-mm dishes for 2 days. Process-bearing neurons were counted in several randomly selected regions in each culture. On average, these areas initially contained about 400 cells. When neurons and astroglia were cocultured, the cell counts were made in regions some distance from the glia-bearing cover slips so that effects due to contact between neurons and glia could be excluded. The data presented are based on the means of 3 to 12 cultures maintained under each condition. Standard deviations averaged 13 percent of the means.
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