Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 6226–6228, October 1980
Neurobiology

Elongating nerve fibers are guided by a pathway of material released from embryonic nonneuronal cells

(axonal guidance/neurotropism/cell–substratum adhesion/substratum-associated material/nerve cell culture)

FRANK COLLINS AND JAMES E. GARRETT, JR.

Department of Anatomy, University of Utah College of Medicine, Salt Lake City, Utah 84112

Communicated by Donald Kennedy, July 7, 1980

ABSTRACT **Dissociated parasympathetic neurons rapidly initiate neurite outgrowth when exposed to culture medium previously conditioned by the growth of embryonic heart cells. The inducer of neurite outgrowth in conditioned medium is a substratum-conditioning factor; that is, it does not act in a soluble form, but acts only when bound to the nerve cell culture substratum. When a sharp border is created between a region of the substratum coated with this factor and a region coated with unconditioned medium, neurites fail to cross this border; rather, they change their direction of outgrowth so as to remain on the conditioned substratum. Thus, long after the initiation of outgrowth has been induced, elongating neurites continue to respond to the substratum-conditioning factor in a manner that allows their outgrowth to be channeled along a pathway of this neurotropic substratum-associated material.**

Dissociated chicken parasympathetic ciliary ganglia neurons readily attach to a polyornithine-coated substratum in standard culture media, but do not extend neurites. However, when the medium on such cultures is replaced with medium previously conditioned by the growth of embryonic heart cells, extensive neurite outgrowth occurs (1). The response of neurons to the heart cell conditioned medium (HCM) is remarkably rapid: within 30 min of its addition more than 80% of the neurons have begun to extend neurites (2). When HCM is added, the first visible change in the neurons is the attachment to the substratum of the formerly nonadhering neuronal filopodia (2), suggesting that HCM may induce neurite outgrowth by promoting filopodial adhesion. This result is consistent with previous work demonstrating that conditions that increase cell–substratum adhesion promote neurite outgrowth from other types of neuron or from neuron-like tumor cells (3–6).

The material in HCM that induces neurite outgrowth does not act in a soluble form, but is active only when bound to the polyornithine substratum used to culture parasympathetic neurons (7). When a polyornithine-coated dish is exposed to HCM, the ability to induce neurite outgrowth is transferred directly to the dish surface, so that, even after repeated washing, such a pretreated dish is able to induce neurite outgrowth from dissociated neurons plated on it in unconditioned medium (7). If a series of dishes is prepared, each containing the same total amount of HCM, but differing in the fraction of it that has become bound to the substratum, the extent of neurite outgrowth on such dishes is proportional to the amount of substratum-bound material, but is unrelated to the amount in solution (7).

Thus, a substratum composed of the appropriate extracellular materials is sufficient to induce neurite outgrowth, even in the presence of unconditioned medium. This is also shown by the observation that neurite outgrowth is rapidly induced by the insoluble layer of extracellular microexudate secreted by embryonic heart cells directly onto the surface of culture dishes in which they have grown (8). Apparently, HCM and polyornithine are required only to produce an appropriately conditioned substratum—one that contains a filopodial adhesion factor. Other substrata that may also contain this factor, such as heart cell microexudate, are equally effective at inducing parasympathetic neurite outgrowth.

Although it is clear from these results that the *initiation* of neurite outgrowth in the present culture system depends on the substrate-associated material deposited by HCM, there is no evidence that, once initiation has occurred, elongating neurites are in any way influenced by the HCM-conditioned substratum. To determine if actively elongating neurites respond to the substrate-associated material from HCM, such neurites were presented with the opportunity of crossing from a region of the polyornithine substratum coated with HCM onto a region coated with unconditioned medium. Neurites failed to cross this border, demonstrating that they are capable of recognizing an HCM-conditioned substratum and of remaining in contact with it—altering their direction of outgrowth, if necessary, to maintain this contact. This system illustrates one way in which nonneuronal cells might direct the course of neurite outgrowth during embryonic development, by releasing material that becomes localized in the extracellular matrix–substratum over which elongating neurites move.

METHODS AND MATERIALS

Parasympathetic ciliary ganglia from stage 34 White Leghorn chicken embryos were dissociated into single cells with trypsin (2). HCM was prepared by incubating a confluent monolayer of stage 34 chicken embryo heart cells for 72 hr in unconditioned medium (Ham's F12 containing 10% fetal calf serum; both from GIBCO); the HCM was filtered through a 0.2-μm pore diameter filter before use (2). For culturing neurons, Falcon plastic 35-mm tissue culture dishes were coated overnight with polyornithine at 1 mg/ml (2). Cultures were incubated at 37°C in a humidified atmosphere of 3% CO_2/97% air.

A narrow rectangular strip of the surface of a polyornithine-coated dish was exposed to HCM as described below. The advantage of this method for treating a part of the substratum with HCM over other methods that we tried was that it allowed the substratum to be treated with a sufficient quantity of HCM to promote extensive neurite outgrowth, and also that it produced quite straight borders that could be precisely located. A strip of Whatman filter paper was wet with sterile, distilled water and laid flat on the surface of a polyornithine-coated dish, then allowed to dry. The dish was propped up at a 45° angle and excess HCM was placed in contact with the lower end of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: HCM, heart cell conditioned medium.

the paper strip. The HCM was drawn up the filter paper by capillary action and was seen to extend as a straight liquid border approximately 25 μm to either side of the edge of the paper. To prevent drying through evaporation, the lid was sealed onto the dish with petroleum jelly and excess HCM was always present at one end of the strip. After 4–16 hr at 4°C, the dish was placed on the stage of an inverted microscope and oriented in a special holder so that the location of any point on the dish could be noted by using micrometer scales, and that point could subsequently be relocated. In this way the location of points along the liquid HCM border was noted and the width of the HCM-coated region (i.e., the side-to-side distance between liquid borders) was measured at numerous defined locations along its length. The variability of repeated measurements of the width at any one point was less than 20 μm.

The dish was then flooded with Hanks' balanced salt solution (HBSS), the now-floating filter paper strip was gently removed, and the dish was washed several more times with HBSS. Dissociated ciliary ganglia neurons were then plated onto the dish in unconditioned growth medium at cell densities ranging from 2×10^3 to 8×10^4 per dish. A 1:100 final dilution of the chicken eye embryo extract described by Adler *et al.* (9) was added to the medium to promote neuronal survival (8). Living cultures were examined periodically to follow the course of neurite outgrowth, then fixed after 72–96 hr in culture and examined with phase-contrast optics. Appropriate controls are discussed in *Results*.

RESULTS

When dissociated ciliary ganglia neurons were plated in unconditioned medium onto polyornithine-coated dishes in which a narrow strip of the substratum had been pretreated with HCM, nerve cell bodies attached in equal numbers to the HCM-coated substratum and to the immediately adjacent, untreated substratum. However, only those nerve cells that attached to the HCM-coated substratum were able to extend neurites, as previously reported (7).

These neurites, after several days in culture, had formed a dense network that faithfully reproduced the shape of the region coated with HCM. This network had sharp borders, defined by the neurites themselves, and along which neurites ran for considerable distances without crossing (Fig. 1). These neurite-defined borders were located in the same place on the dish as the borders of the HCM-coated substratum; and the distance between the neurite-defined borders was the same as the width of the region coated with HCM (Table 1). Thus, after several days in culture, during which time neurites had grown to lengths of several millimeters, they had not expanded beyond the original HCM borders.

Table 1. Comparison between the widths of the regions coated with HCM and covered by neurites

Distance along strip, mm	Distance between HCM borders, mm	Distance between neurite borders, mm
1.50	3.72	3.71
2.50	3.94	3.94
3.50	4.12	4.08
4.50	4.30	4.28
5.50	4.55	4.55

The width of the region coated with HCM was measured before addition of neurons. The width of the region covered by neurites was measured after 78 hr in culture, at various points along the HCM-coated strip. The variability in repeated measurements of the same width was less than 20 μm.

In cultures containing fewer neurons, it was possible to follow the outgrowth of individual neurites. These approached the border at all angles, then turned sharply to run along the border for at least 50 μm before moving back onto the HCM-coated region proper. With rare exceptions, each neurite turned in the direction that made an obtuse angle to the direction with which it had approached the border.

Occasionally, neurites that had crossed the border were observed (Fig. 1, arrows). Some of these neurites had elongated for greater than 200 μm on the side not pretreated with HCM. Where it was possible to count individual nerve fibers, the frequency of fibers that had crossed the border, compared to those that ran along it without crossing, ranged from 0.2% to 0.5%.

Although there was nothing visible at the HCM borders that might have blocked the passage of neurites, it is well known that physical deformations of the substratum can channel neurite outgrowth (10). Therefore, to interpret the preceding results correctly, it is crucial to determine if neurites are responding to the HCM-coated substratum or to a mechanical or other barrier to their passage inadvertently produced by the method used to create the strip of HCM-coated substratum. Accordingly, strips of HCM-coated substratum were prepared in the usual way, but neurons were cultured on them in HCM (with or without eye extract) rather than in unconditioned medium. HCM deposits its inducer of neurite outgrowth onto the pre-

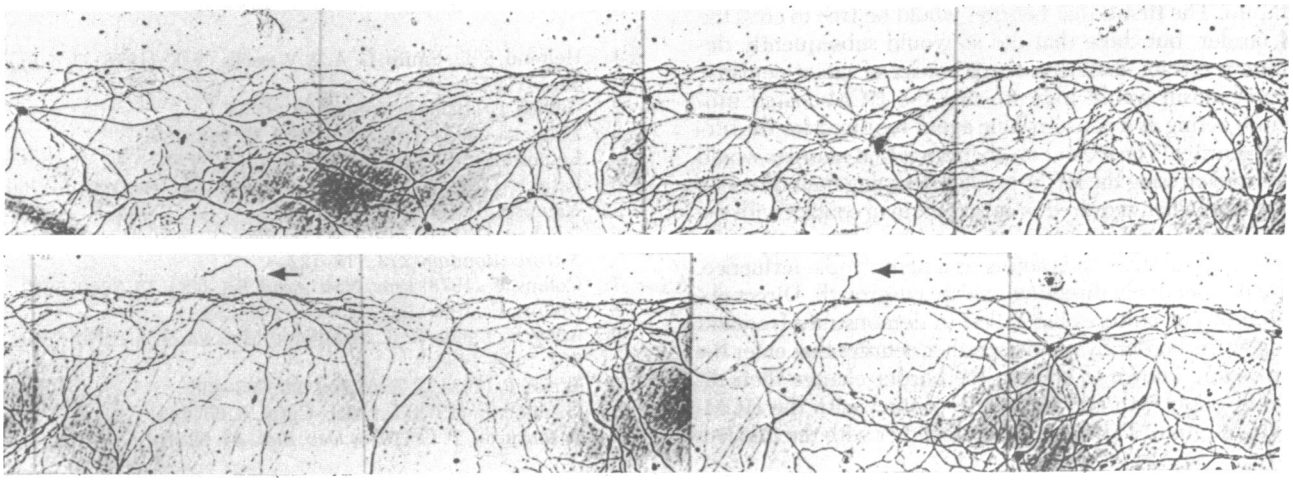

FIG. 1. Phase-contrast micrographs of neurite outgrowth at the location of the border between a region of the substratum coated with HCM (below) and a region coated with unconditioned medium (above) after 93 hr in culture. The arrows indicate neurites that have crossed onto the region coated with unconditioned medium. (×70.)

viously unexposed parts of such dishes, as shown by the fact that neurons on all parts of the dish extend neurites, not just those on the region of the former HCM-coated strip. The presence of material from HCM on all parts of the substratum would eliminate the former distinction between HCM-coated and untreated regions, but should not have eliminated any other, physical type of barrier to neurite outgrowth. In these cultures, neurites freely crossed over the site of the previously established HCM border and no trace of the border was seen in their pattern of outgrowth, indicating that the only barrier to the passage of neurites in the previous experiments was a change in the biochemical composition of the substratum.

It is important to note that the border is not simply one between "coated" and "naked" regions of the substratum. The unconditioned medium in which neurons are normally cultured contains serum, and it has been repeatedly demonstrated that serum-derived molecules adhere to culture substrata and constitute a layer of substrate-associated material (11). We have also observed a rapid buildup on the substratum of material that is reactive in standard protein assays when polyornithine dishes are exposed to unconditioned medium. Therefore, even those areas of the polyornithine dish that were not previously treated with HCM will receive a coating of material from unconditioned medium. Nevertheless, a polyornithine dish containing a strip of HCM-coated substratum can be exposed to unconditioned medium for 16 hr before neurons are added, without preventing neurites from following the HCM border. Thus, neurites seem to prefer to be in contact specifically with the material deposited onto the substratum by HCM.

DISCUSSION

The inducer of parasympathetic neurite outgrowth that is present in HCM does not act in a soluble form; rather, it must bind to the polyornithine culture substratum in order to be active (7). It has been shown previously that such an HCM-coated substratum is required for the initiation of neurite outgrowth (2, 7). The present work demonstrates that, long after initiation has occurred, neurites continue to respond to the material deposited onto the substratum by HCM. This continued response is evidenced by the great majority of neurites from ciliary ganglia neurons preferring to remain on an HCM-coated substratum when presented with the choice of crossing from that substratum onto one coated with unconditioned medium.

There are two alternative mechanisms by which the final distribution of neurites could be limited to the HCM-coated substratum. The first is that neurites would be free to cross the HCM border, but those that did so would subsequently degenerate or retract, leaving a sharp border of intact neurites. This mechanism would indicate that the HCM-coated substratum is acting as a neurotrophic agent required for the survival of neurites. The second mechanism is that neurites would refrain from crossing the HCM-border, changing their direction of elongation when required so as to remain in contact with the HCM. This second mechanism would indicate that the HCM-coated substratum is acting as a neurotropic influence, capable of selectively directing neurite outgrowth. Direct observation of our cultures has failed to demonstrate frequent crossing of the border by neurites; rather neurites that enter the 20-μm region known to contain the border change their direction of outgrowth and remain in contact with the HCM-coated substratum. This observation, together with the previous demonstration that the HCM-coated substratum is incapable of promoting the survival of ciliary ganglia neurons (7), indicates that the HCM-coated substratum exerts a neurotropic or directive influence on neurite outgrowth. The observation that a small but definite proportion of the neurites is able to cross the border and elongate on the other side suggests that the choice not to cross the border is subject to a low frequency of error.

The failure of neurites to cross the HCM border is apparently due to their ability to distinguish a substratum coated with HCM from one coated with unconditioned medium, and it is not a result of their failure to penetrate a physical or other barrier accidentally established by the method we used to create the original border. This is shown by the observation that neurites freely cross the border when material from HCM rather than from unconditioned medium is deposited on the formerly untreated portions of the substratum. Thus, in our experiments neurites are responding to the chemical composition of the substratum, preferring to remain in contact specifically with those components deposited by HCM.

Letourneau (12) has shown that the growth cone at the neurite's leading tip tends to remain on the more adhesive substratum when given a choice between substrata of different adhesivities. One possible explanation, therefore, for the preference of neurites to remain on an HCM-coated substratum is that the latter provides a more adhesive substratum for the growth cone than does a substratum coated with unconditioned medium. This explanation is consistent with the previous observation (2) that the first visible action of HCM in inducing neurite outgrowth is to promote the adhesion of neuronal filopodia (which are characteristic features of the growth cone) to the substratum. These results may be conveniently summarized by suggesting that HCM contains an adhesion factor for neuronal filopodia that, when bound to the substratum, induces and can also direct neurite outgrowth.

Our observations demonstrate that parasympathetic neurites remain within the confines of a substratum composed of material released from embryonic, nonneuronal cells. It is thus possible that during development the outgrowth of nerve fibers could be directed along pathways of the appropriate extracellular materials, which are released and deposited onto the extracellular matrix–substratum by neighboring embryonic cells.

The authors thank Drs. Richard Mullen and Gary Schoenwolf for their comments on the manuscript. This work was supported by National Institutes of Health Grant #NS-15130 to F.C.

1. Helfand, S. L., Smith, G. A. & Wessells, N. K. (1976) *Dev. Biol.* **50**, 541–547.
2. Collins, F. (1978) *Dev. Biol.* **65**, 50–57.
3. Luduena, M. A. (1973) *Dev. Biol.* **33**, 268–286.
4. Letourneau, P. C. (1975) *Dev. Biol.* **44**, 77–91.
5. Patrick, J., Heinemann, S. & Schubert, D. (1978) *Annu. Rev. Neurosci.* **1**, 417–443.
6. Schubert, D., LaCorbiere, M., Whitlock, C. & Stallcup, W. (1978) *Nature (London)* **273**, 718–722.
7. Collins, F. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5210–5213.
8. Collins, F. (1980) *Dev. Biol.* **79**, 247–252.
9. Adler, R., Landa, K. B., Manthorpe, M. & Varon, S. (1979) *Science* **204**, 1434–1436.
10. Weiss, P. (1934) *J. Exp. Zool.* **68**, 393–438.
11. Grinnell, F. (1978) *Int. Rev. Cytol.* **8**, 65–144.
12. Letourneau, P. C. (1975) *Dev. Biol.* **44**, 92–101.