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Growth cone formation in cultures of sensory neurons

(filopodia/microtubules/collateral sprouting)

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ABSTRACT **Three experimental situations have been found in which cultured sensory neurons from embryonic chicken will form growth cones from positions along the length of the neurite. If the neurons are dissected with a remaining short axonal stump and plated into serum-free medium, they can form a morphologically normal growth cone from the stump within 15 min, even in the presence of cycloheximide or puromycin. When neurites growing in culture media with low levels of serum are cut at any point with microneedles, growth cones are produced quickly from the amputated stump, usually within 20 min. Treatment of growing neurons with low concentrations of colchicine, Colcemid, or podophyllotoxin results in the progressive appearance of lateral filopodia and regions of flattened cytoplasm that closely resemble growth cones except for their preterminal positions. These observations show that the potential to form growth cones is distributed throughout the neuron and suggest that this is normally repressed in some way by the neuronal microtubules.**

Wherever the axons or dendrites of vertebrate nerve cells have been observed to grow in culture, they have been found to possess a terminal appendage known as the growth cone. This is distinguishable from the rest of the neuron by its numerous filopodia and flattened veil-like regions and by its continual movement (1, 2). Its special functions may include the primary attachment of the cell to the substratum (3), an active part in micropinocytosis (4), guidance of the neurite (5), and the assembly of surface membrane during growth (6).

In the most extensively studied kind of nerve culture—that of embryonic sensory or sympathetic neurons growing in the presence of nerve growth factor (7)—neurite extension does not occur immediately after explantation (8). Furthermore, once initiation has occurred, growth cones are formed from existing ones rather than from other parts of the cell (3) and under the usual conditions they do not regenerate quickly upon experimental amputation (9).

These observations raise the possibility that the growth cone is a complex structure whose formation is the rate-limiting step in the initiation of neurite growth. We now describe experiments that bear upon this possibility and examine the formation of growth cones from cultured sensory neurons under a variety of conditions.

MATERIALS AND METHODS

Cultures were prepared from the dorsal root ganglia of 11- to 13-day chicken embryos by procedures that have been described (9). Cells were incubated at 37° in medium C, which is composed of Leibovitz L15 (GIBCo BioCult, Glasgow, Scotland) with 0.6% glucose, 2 mM L-glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 0.1 μg of mouse nerve growth factor per ml purified to the DEAE-cellulose fraction described by Varon *et al.* (10). With a few exceptions, which are noted, this medium was supplemented with 0.6% methylcellulose and 10% fetal calf serum. All cells were grown on glass coverslips which in some experiments were built into the culture dish to give good optical properties. Amputations were performed as described (9), with a glass microelectrode broken to a tip of about 10 μm diameter.

The following drugs were used in various experiments: cycloheximide, cytochalasin B, demecolcine (Colcemid), colchicine, puromycin (all from Sigma Chemical Co.) and podophyllotoxin (Aldrich Chemical Co.). Lumicolchicine was prepared from colchicine by the method of Wilson and Friedkin (11).

Growth Cones from Freshly Dissected Neurons. Ganglia from 11- to 13-day chicken embryos were dissected together with a short length of postganglionic nerve, and cleaned of associated tissue. They were incubated in 0.25% trypsin in a calcium- and magnesium-free balanced salt solution at 37° for precisely 25 min. They were quickly washed in balanced salt solution (GIBCo BioCult, Glasgow, Scotland) and put into 0.1% soybean trypsin inhibitor (Sigma Chemical Co.) in balanced salt solution. After incubation for 5 min at room temperature, they were again washed into balanced salt solution and dissociated by gentle trituration with a pasteur pipette of terminal bore 1.0–1.5 mm. The cells were then plated into medium lacking serum.

For the assays described in Table 1, the cells were pipetted onto plain glass coverslips in warm serum-free medium containing, where appropriate, a drug to be tested. They were incubated at 37° and then fixed with warm Vaughn–Peters aldehyde (12). After, usually, 16 hr in the aldehyde at room temperature the cultures were washed several times in distilled water, drained, and mounted onto glass slides with Aquamount (Gurr, High Wycombe, England). They were examined by phase-contrast microscopy and the following types of cell were counted: rounded neuronal cell bodies with no major extension, neuronal cell bodies with an axonal stump at least one soma diameter long (Fig. 1*a*), and neuronal cell bodies with an axonal stump bearing a terminal growth cone showing filopodia and flattened veil-like regions (Fig. 1 *b* and *c*). Because of the subjective nature of the assay, all counts were made blind, on slides that had been covertly numbered in a random sequence by a second person.

Protein Synthesis. Protein synthesis was measured in cultures that had been freshly plated in the above manner. The cells were put into dishes containing 3 ml of medium C lacking methionine and supplemented with 10 μCi of L-[^{35}S]methionine (780 Ci/mmol; Radiochemical Centre, Amersham, England). Where appropriate, cycloheximide, or puromycin was added. The cultures were incubated for 1 hr at 37°, rinsed in balanced salt solution, and then dissoved in 1 ml of 1% sodium dodecyl sulfate/1% 2-mercaptoethanol/1% bovine serum albumin and heated for 2 min at 100°. The samples were extensively dialyzed against 10 mM NaCl/0.1% sodium dodecyl sulfate and the nondialyzable radioactivity was measured in a scintillation counter. Protein synthesis in established cultures was measured in the same way except that the incubation in L-[^{35}S]methionine was for 3 hr.

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RESULTS

When chicken sensory ganglia are dispersed with trypsin, neurons are seen that retain a short stump which is, presumably, derived from the original axon (8). Under the usual conditions of culture these quickly retract into the cell body (8), but we found that in serum-free medium many remain extended through adhesion of the culture substratum. These were found to have formed terminal plaque-like specializations at their cut end which developed filopodia and broad flattened regions and began to move in the distinctive way of normal developing growth cones (Fig. 1).

If the conditions of dissociation and trypsinization were carefully controlled, up to 70–80% of the neurons recovered from the ganglion had stumps. If these were plated under defined conditions, the formation of growth cones could be assessed quantitatively (Table 1). The number of cells with stumps fell to a lower value soon after plating, presumably because some retraction into the soma still occurred. This reduction in number was inhibited by cytochalasin but accelerated by colchicine, in keeping with the effects these drugs had on the collapse of isolated segments of neurites (9). Growth cones were seen as early as 10 min after plating into serum-free medium and increased in number from that time. In one assay, carried out under the conditions described in Table 1, 1% of the axonal stumps had growth cones after 10 min, 9% at 20 min, 17% at 50 min, and 53% at 80 min. In the absence of nerve growth factor fewer growth cones were present—between 30 and 50% of those formed in control conditions.

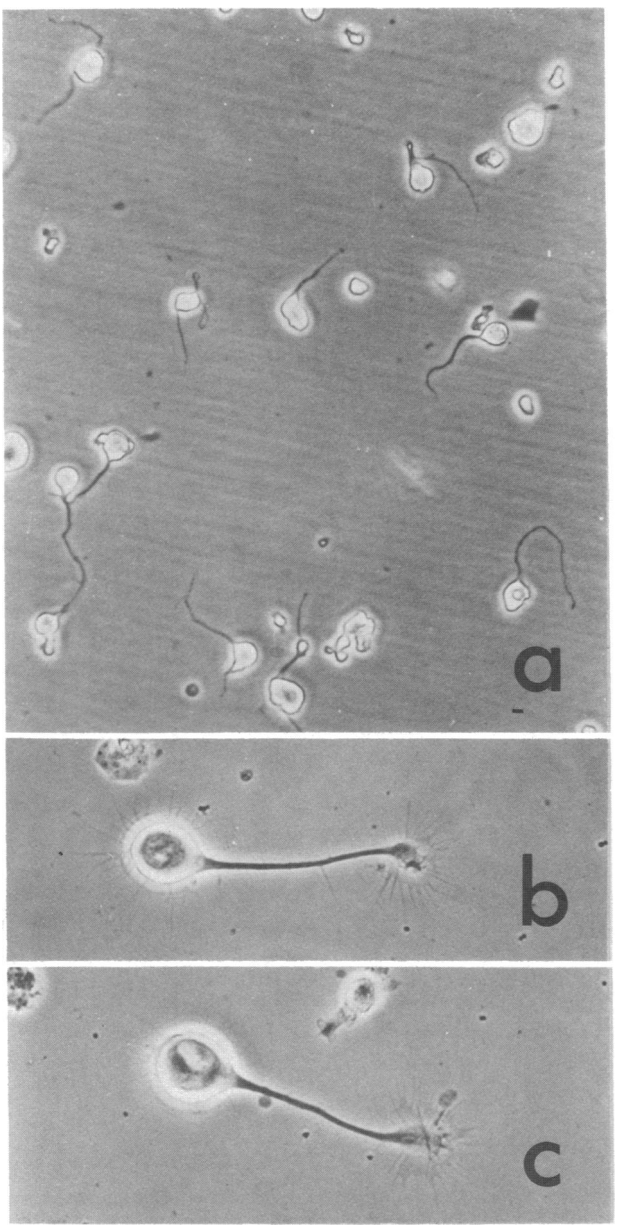

FIG. 1. Formation of growth cones from the axonal stumps of freshly dissected neurons. (*a*) Sensory neurons 5 min after they were plated onto a plastic surface. (×220.) (*b* and *c*) Growth cones formed from axonal stumps 20–30 min after they were plated. (×550.)

Table 1. Effect of drugs on growth cone formation from axonal stumps of freshly dissected sensory neurons

Assay medium	Cells with stumps	Cells with growth cones	% stumps with growth cones
Medium C	46	12	26
Balanced salt solution	75	18	24
C + puromycin (10 μg/ml)	75	17	23
C + cytochalasin B (1 μg/ml)	181	1	1
C + colchicine (5 μg/ml)	27	1	4

Assays were carried out as described in *Materials and Methods;* 400 neuronal cell bodies were counted under each experimental condition. All incubations were for 60 min at 37°, and the drugs were present at the indicated levels throughout the incubation. In a separate experiment (not shown), we found that cycloheximide at 10 μg/ml did not reduce the fraction of cells bearing growth cones under these conditions. Incorporation of radioactive methionine into nondialyzable material under these conditions was reduced to 1% of control values by cycloheximide and to 30% by puromycin.

Growth cones were found in normal numbers in cells that had been plated into simple buffers without amino acids or into medium that contained cycloheximide or puromycin at 10 μg/ml (Table 1). A number of individual cells were observed for 2–3 hr after changing into normal medium C with serum. Although these showed normal ruffling activity at their cut ends, their neurites did not commence growth during this time.

These observations seemed to contradict a result obtained earlier in which it was found that when neurites were cut, the existing growth cones could migrate and branch but that other regions of the fiber did not readily form new growth cones (9). A possible reason for this difference was thought to be the limited adhesion that the neurites have to the substratum under these conditions which might not provide an opportunity for the cut segment to initiate a growth cone. The initiation of neurite extension is promoted by an increase in the adhesivity of the culture substratum (13, 14), and so we repeated our earlier experiments on cultures that were in low serum medium (15). (It was also found to be an advantage to use the larger neurones from 13- to 14-day embryos.) Such cells formed a more secure attachment to the surface without showing a major change in their form or behavior. When they were cut, the stumps quickly formed "beads" or retraction bulbs. This was followed in the *proximal* bulb by the appearance of filopodia and, usually within 35 min but sometimes as soon as 10 min after the amputation, by the development of morphologically normal growth cones. Once again, the newly formed growth cones showed the same movements as established growth cones and, in contrast to the previous situation, the neurite that bore them recommenced growth from the time of their formation (Fig. 2).

In a series of 19 amputations, 13 resulted in the formation of a growth cone from the proximal stump within 35 min. This included fibers cut less than 50 μm from the cell soma as well

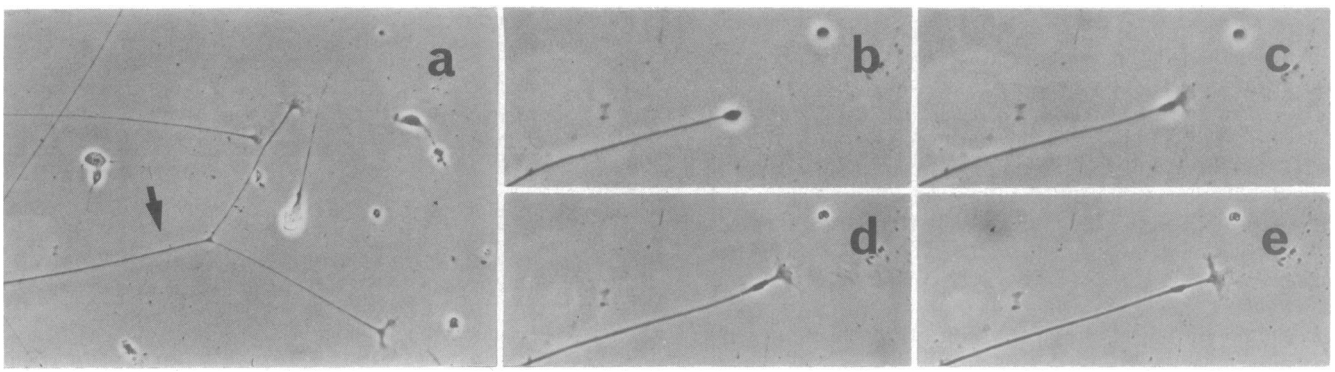

FIG. 2. Amputation of neurites in low serum medium. (*a*) The branched outgrowth was cut with a microelectrode at the point indicated by the arrow and the region distal to the cut was cleared away. (×200.) (*b–e*) Formation of a growth cone from the proximal stump 1 (*b*), 20 (*c*), 35 (*d*), and 40 (*e*) min after the operation. (All ×350.)

as those cut near the periphery. Although all of the 13 had formed growth cones within 35 min, in 7 cases these were apparent 15 min after the operation. On three other occasions, a single fiber was cut more than once, twice in two fibers and three times in another, and at every cut new growth cones formed quickly from the stump. Two isolated observations were made of fibers that were, fortuitously, only partially severed with the microelectrode. These gave rise to preterminal growth cones at the site of injury which, in one recorded case, then produced a collateral branch. Attempts to perform this operation at will, however, have not been successful.

The same regenerative ability was not shown at the other side of the cut. The distal stump formed a retraction bulb that did not regenerate to form a new growth cone, even though in some instances it was followed for over 5 hr. As observed in the previous experiments (9), the retraction bulb remained as a phase-dense structure throughout the life of the isolated segment.

The effects of antimitotic drugs on cultured neurons have been described (16, 17). They cause the arrest of growth and the eventual retraction of neurites while the activity of the growth cone continues. During experiments performed for other reasons we noticed that the morphological details of the retraction were more complex than had previously been thought. It appeared that the retraction of the neurite was accompanied by the formation of varicosities, filopodia, and flattened regions at discrete, but not regular, positions along the length of the neurite while the existing structure at the end of the fiber degenerated (Figs. 3 and 4). The flattened regions bore filopodia, had the same distinctive morphology and moved in a similar way as growth cones in normal neurites except for their position (Fig. 4). In the light microscope, in fact, it was sometimes difficult to distinguish them from the situation often encountered in a dense culture in which the growth cone of one fasciculating fiber is observed to grow along the length of another. However, in cultures plated at low density they were repeatedly seen on cells that were obviously single, and electron microscopy (unpublished results) confirms that they occur within the length of a single process.

Colchicine, Colcemid, and podophyllotoxin all produced similar effects, while the colchicine derivative, lumicolchicine, was inactive at 1 μg/ml or 10 μg/ml. The effects of Colcemid were seen in cultures that had been pretreated for 3 hr with 10 μg of cycloheximide or puromycin, treatments that reduced the incorporation of radioactive methionine into nondialyzable material to 3% and 7%, respectively, of the value in control cultures. The changes produced by those drugs were restricted to the terminal region of the neurite (Fig. 3) and, at least at the early stages, cells examined by light or scanning electron microscopy were found to have a normal morphological appearance on the soma and more proximal portions of the neurite. The course of the response to a 30-min exposure to 1 μg of Colcemid per ml is shown in Fig. 3; similar changes were observed at 0.1 μg/ml after 2–3 hr or at 10 μg/ml within 20 min. Low temperatures were less efficient in causing these changes than antimitotic drugs, but some cells showed preterminal growth cones upon returning to 37° after a period of 1 hr at room temperature (21–24°).

DISCUSSION

Taken together, these observations show that growth cones may be produced readily from any part of the neurite in culture. They appear, usually within 30 min, at the end of a short axonal stump, at the severed end of a fiber cut close to the soma or near the periphery, or at multiple points along the neurite treated with antimitotic drugs. It seems reasonable to conclude from this that a growth cone is incipient at any point along the neurite and, moreover, that growth cone formation is unlikely to be the rate-determining step in neurite formation. The 5- to 8-hr delay in axonal growth seen in neurons freshly explanted under the usual conditions cannot easily be explained on this basis. Since growth cones were found to form at normal rates in buffers lacking amino acids or where the synthesis of protein was severely impaired by cycloheximide or puromycin, the synthesis

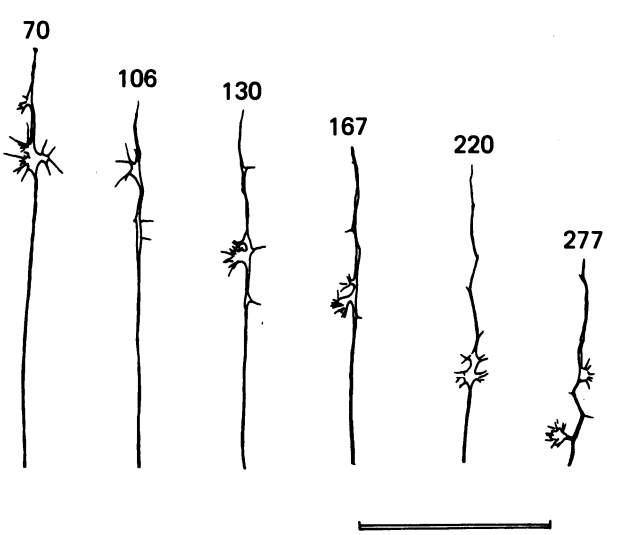

FIG. 3. Outline of a neurite retracting after Colcemid treatment. A culture of chicken sensory neurons was treated with Colcemid (1 μg/ml) for 30 min and then washed into normal medium C. The outline of the neurite was traced from photographs taken at the indicated times (min) after introduction of the drug. Calibration bar, 100 μm.

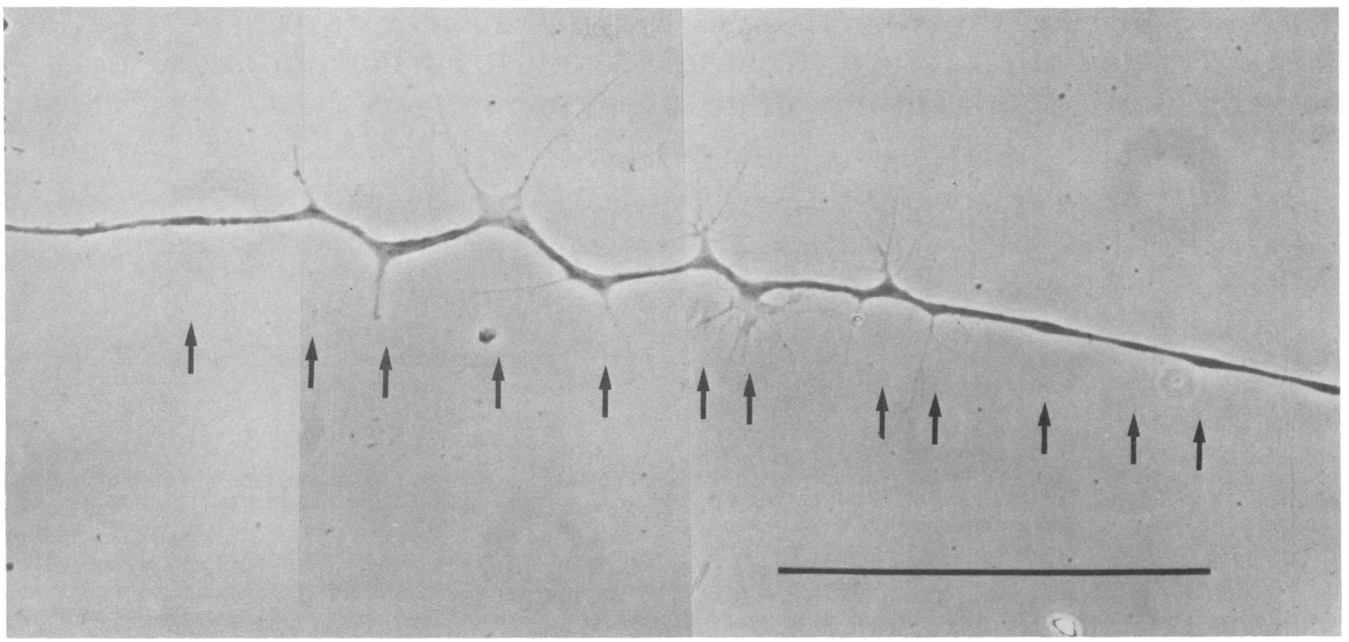

FIG. 4. Neurite after Colcemid treatment. Photograph of the same fiber shown in Fig. 3 taken 260 min after exposure to Colcemid (1 μg/ml). Positions of preterminal growth cones, lateral filopodia, and phase-dense dilations are indicated by arrows. Calibration bar, 50 μm.

of new proteins is probably not an essential prerequisite. If special macromolecules are required for the functions of the growth cones, they are presumably present in adequate amounts throughout the neuron.

The simplest interpretation of our results, however, is that the growth cone is an outwelling of ordinary neuronal cytoplasm. The distinctive properties of the growth cone would then be, largely, those of undifferentiated cytoplasm confronted with a culture substratum. Indeed, many similar features are shown by fibroblasts and other cells in culture as they settle onto a surface (18). Filopodia (or microspikes) and flattened areas extend outwards from such cells, and those that do not establish contact are withdrawn (19). A growth cone could be like such a cell that is trying to settle onto a surface but never achieves a stable flattened state.

If a growth cone is potentially able to form at any point along the neurite, why is it normally restricted in position to the growing end? Presumably, a form of repression or containment is at work, and our observations with cultures treated with antimitotic drugs suggest that microtubules are involved in this. A related observation has been made of the effects of Colcemid on cultured fibroblasts, in which the flattened ruffling areas normally restricted to the poles of the cells are seen to extend around the periphery (20). We do not know how these effects are caused but, for the neurite, there are two distinct possibilities. One is that it is the absence of microtubules, produced by depolymerization, that allows the growth cones to form; and it is relevant to note that in normally growing neurites the microtubules terminate at or near the base of the growth cone (21, 22). The other is that the primary target is the fast component of axonal flow (see, for example, ref. 23), and that it is the restriction of this transport system that causes filopodia and flattened regions to form closer to the cell body.

It is always dangerous to extrapolate from observations in tissue culture to the animal. However, many examples are known in which branches form from the preterminal regions of dendrites, for example, in the normal differentiation of cerebral dendrites (24) or in the terminal regions of motoneurons in response to the axotomy of neighboring nerves (25), and it could be that the present study is of relevance to those. If our results are applied to such phenomena, two predictions may be made. The formation of preterminal branches would be expected to involve local disruption, or inactivation, of microtubules; and branches would be expected to be initiated without the immediate need for protein synthesis.

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