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Axonal Growth from Insect Neurons in Glia-Free Cultures*

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Abstract. Mechanical dissociation of nerve and glial cells from 16-day embryos of the cockroach *Periplaneta americana* and their subsequent culture in a CO_2-conditioned, chemically defined medium results in the survival of neurons but not of glial cells. The dissociated nerve cells remain alive and in excellent condition for many months and build a dense fibrillar network in presence of foregut explants from the same embryos. These explants are invaded by numerous and large fiber bundles emerging from the fibrillar network. The morphological characteristics of nerve cells and the structural and ultrastructural features of nerve bundles in long-term glia-free culture are described.

The trophic role of glial cells is perhaps one of the most generally accepted and least challenged principles of the structural and functional organization of the nervous system. Light and electron microscopic studies of the vertebrate and invertebrate nervous system have provided evidence for the ubiquitary presence of these cells in this system and around afferent and efferent nerve fibers.

The detailed topography and membrane relationship of nervous and nonnervous cells, and between the latter and nerve fibers, have been studied in great detail in the insect brain and ganglia where the well-known segregation between a cortical cellular ring and a central complex, the neurophile, which contains axons and branching processes, provides a most favorable condition for the structural and ultrastructural analysis of these cell-to-cell and cell-to-fiber relationships.[1-3] The concept of an essential role of glial cells received additional support from recent biochemical studies. Of particular interest in this connection is the hypothesis submitted by Treherne of glial involvement in the regulation of the extra-axonal sodium level, which would explain the ability of insect axons to function for extended periods in the absence of sodium ions in the bathing fluid.[4] The finding of glycogen deposits in the glial cells around the neuron cell bodies, and in the glial processes permeating the neuropile, further added to the notion of the outstanding role of these cells in a number of metabolic processes.[5, 6]

The study of neuron-glial cells and the glial-axon relationship *in vitro* became possible recently, thanks to the development of a chemically defined medium that is most favorable for the survival of insect brain and ganglia *in vitro*, the outgrowth of nerve fibers, and the migration of glial and nerve cells from these explants. Under these conditions glial cells migrate in large numbers from intact brain and ganglia and form a carpetlike structure of a very tenuous texture

around the explants. Superimposed on the thin cytoplasmic layer of these cells are nerve fibers and nerve cells easily distinguishable because of their round or oval shape, their large nucleus and nucleolus, and the opposito-polar filaments which leave the cells and grow for a considerable length away from the cell bodies.[7, 8] The results of these investigations showed that (*a*) the close relationship that characterizes nerve and glial cells in the living organism still subsists under the conditions of culture, and (*b*) the culture medium used in these experiments supports survival and growth of both nerve and glial cells.

In an attempt to further explore the relationship between these two cell types and the outgrowth of nerve fibers *in vitro*, we resorted to the technique of dissociating embryonic brain and ganglia from this same species in order to see whether these dispersed cells would reestablish contact with each other, and to examine the modality of nerve fiber outgrowth from dissociated nerve cells.

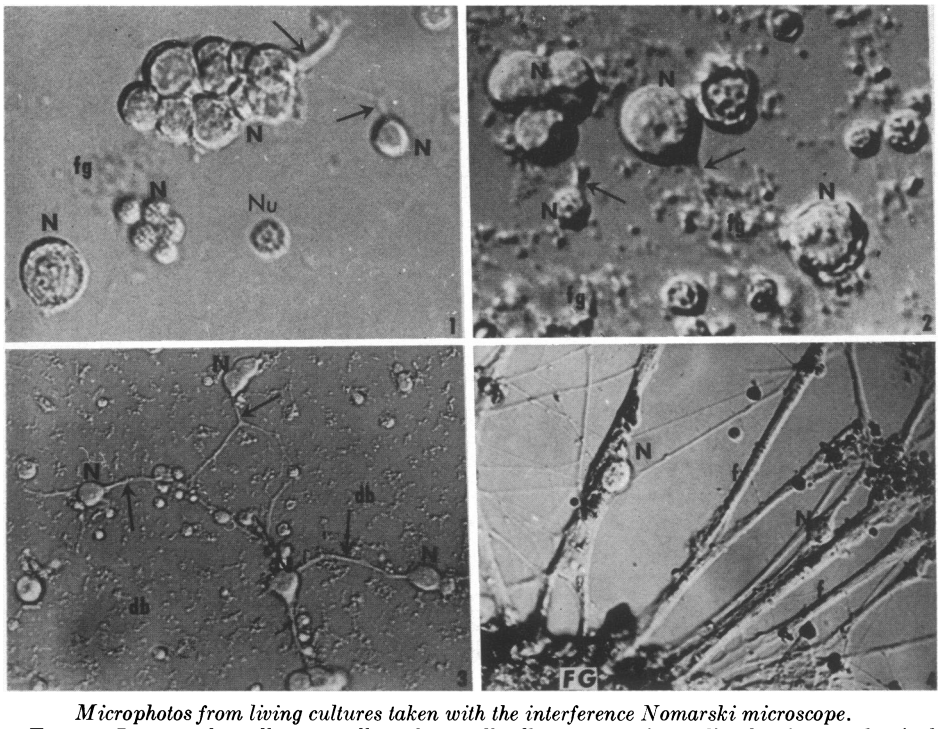
The unforeseeable outcome of these studies was to show that under these conditions nerve cells cultured for 4 months *in vitro* survive and produce a dense fibrillar network in the total absence of glial cells. The survival of neurons but not of glial cells, the production of nerve fibers and their connections with explants of the digestive tract from the same embryos, are the subject of the present report.

Materials and Methods. Cockroach embryos were obtained from oothecae that were collected daily and incubated at 29°C. At this temperature embryos hatch between the 29th and the 30th day. 16-day-old oothecae were sterilized with iodine and alcohol and slit in half along the upper ridge. The embryos were carefully extracted from the oothecae. Brain, subesophageal, and thoracic ganglia were dissected out and collected in the culture medium which consisted of four parts of Eagle's medium and five parts of the Schneider's insect solution.[7] A segment of the digestive tract which corresponds to the foregut was also dissected out, washed in many changes of the culture medium, and stored for subsequent use.

Brains and ganglia dissected from 30 embryos were collected in small test tubes containing 0.5 ml of culture medium. They were dissociated mechanically by suction of the fragments in Pasteur pipettes and by foreing out with some pressure the nervous tissue against the glass wall of the tubes. The operation was repeated many times until brains and ganglia, which at this stage are very soft, were dissociated in small cell clumps and individual cells. The clumps were discarded by leaving the suspension at room temperature for some minutes and then transferring the supernatant with smaller pipettes in another test tube. Two drops of this suspension were then placed in small culture vessels prepared in advance with 0.10 ml of the incubation medium and then placed in a sealed dessicator filled with 5% CO_2 in air as described in previous articles.[7, 8] In the present cultures, soon after the cell suspension was dropped into the culture chambers, two or three fragments of the foregut were added in each vessel. They were gently pressed on the cover slip placed in each culture chamber until they firmly adhered to it.

The results to be reported are based on the study of 200 cultures examined *in vivo* and then, in most instances, fixed 1–4 months later. Fresh cultures were examined at the inverted microscope and at the interference Nomarski microscope, while fixed cultures stained with toluidin or silver Cajal modified technique[8] were examined at the interference microscope and under oil immersion with a light microscope. Some cultures were fixed with glutaraldehyde 3%, postfixed in OsO_4, dehydrated, and embedded in Epon 812. Selected areas for observation were marked under the microscope and then these areas were carefully detached from the cover slip with a razor blade. The tissue was sectioned with LKB 8801 A, stained with uranyl acetate and lead citrate, and studied at the Siemens's Elmiskop IA electron microscope.

Results. (*a*) **Characteristics of 1- to 7-day cultures:** The cell suspension was examined at the inverted microscope immediately after dissociation, and then a drop covered with a cover slip was photographed at the interference microscope. Large and small round cells are present in the rather dense cell suspension. Intermingled with the intact cells are free nuclei and a large number of cell fragments (Fig. 1). After 24 hr of incubation at 29°C, all cells have become attached to the cover slip and surround the foregut explants which also adhere to it. At the interference microscope the cells show a large nucleus surrounded by a thin cortical cytoplasmic layer, a prominent nucleolus, and, in some instances, a short filament which grows out from one of the cell apical poles and ends abruptly at a short distance from its point of origin (Fig. 2). The space between intact cells is literally covered with small cell fragments scattered among the cells or adhering to their surface. At 48 hr a large number of intact cells exhibit a short filament which gives a pear-shaped form to the cells. Cell fragments have now lost all morphological characteristics. They appear as granules mainly aggregated in small lumps of different size, evenly scattered among the cells.



Microphotos from living cultures taken with the interference Nomarski microscope.

FIG. 1.—Large and small nerve cells and a small cell aggregate, immediately after mechanical dissociation of brains and ganglia, Nerve cells (*N*), Free nucleus (*Nu*), Cell fragments (*fg*). Arrows point to short filaments, probably the cell axons. ×500.

FIG. 2.—24-hr-old culture of dissociated cells. Round and pearshaped cells (*N*) with short filament (*arrows*). Cell fragments (*fg*) scattered among the intact cells. ×800.

FIG. 3.—Dissociated nerve cells at the periphery of 1-month-old culture. Nerve cells (*N*) come in reciprocal contact through their axons (*arrows*). Cell debris (*db*) among the nerve cells. ×400.

FIG. 4.—4-month-old culture. Large fiber bundles produced by dissociated nerve cells enter into the foregut explant (*FG*). Neuron (*N*). ×500.

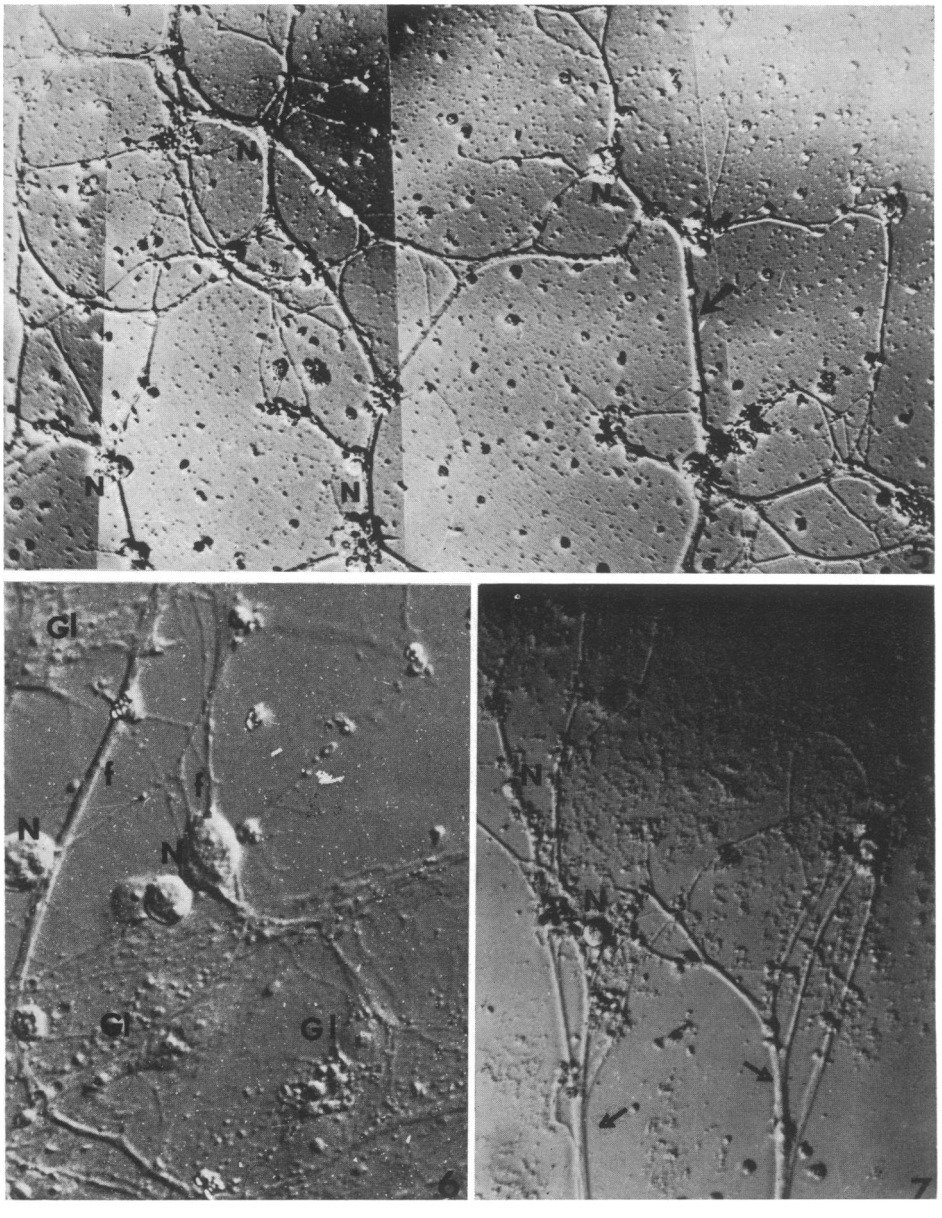
They persist as such until the third or fourth month, when the cultures were discontinued. Only a circular area around the foregut explants appears free from these remnants of cell debris. From the foregut explants a large number of spindle-shaped cells has migrated out and spread in single rows along divergent paths at some distance from the explant.

(*b*) **Characteristics of 1- to 4-week cultures:** Toward the end of the first week of culture, dissociated cells which happen to be in close proximity of the foregut explant differ markedly from the same cells at the periphery of the culture dish. The former have produced a long filament which joins filaments emerging from adjacent dissociated cells. They bypass the spindle-shaped cells migrated from the explant (without establishing any connection with them) and direct their course toward the explant. At the end of the fourth week, a large number of these filaments have entered into the explant and their distal part becomes lost to sight in the dense texture of this tissue. Dissociated cells at a distance from the explant have also produced filaments which come in contact with fibers outgrowing from adjacent cells, but these filaments are much shorter than those emerging from cells proximal to the explant and show no preferential orientation (Fig. 3).

(*c*) **Characteristics of 1- to 4-month cultures:** The most outstanding feature of these long-term cultures is the formation of fiber bundles of progressively larger size which take origin from the peripheral fibrillar network and interconnect this peripheral net with the explant by running in a direction perpendicular to both the fibrillar net and the explant (see Fig. 9). When two or three foregut explants are present on the same cover slip, each explant becomes the center of a similar network. The study of the entrance of these large fiber bundles into the explants is greatly facilitated in these long-term cultures by the progressive cellular depletion around the explant. The spindle-shaped cells migrated out from the explant during the first month of culture have died out and are not replaced by new migratory waves. At the end of the fourth month of culture very few of these cells are still present, and the fiber bundles entering into the explant from all sides come in sharp relief on the clear acellular background (Figs. 4 and 9).

The following account of long-term cultures is based on the observations at the inverted microscope, interference Nomarski microscope, and at the light microscope with an oil immersion objective of 3- to 4-month-old cultures. Similar pictures are observed in 1- to 2-month-old cultures although the fibrillar net is less intricate and the fiber bundles less numerous than in older cultures.

Cells which were mainly isolated from each other in younger cultures are now frequently seen as small cell groupings of 3 to 5 units. Since we have never seen mitotic figures among the dissociated cells, these small aggregates are most likely the result of the shifting of adjacent cells which come in contact with each other. They are now found in the nodal points of a dense and intricate network which extends in the form of a circular belt around the explant, at some distance from it (Figs. 5 and 9). Fibers growing out from these cells, either from one or from the two opposite poles, merge with neighboring fibers building the intricate fibrillar network represented in Figure 5. In no instance are glial or other satellite cells found among the round or pear-shaped cells which exhibit all structural



Microphotos from living cultures taken at the interference Nomarski microscope.

FIG. 5.—Peripheral segment of the fibrillar network from a 4-month-old culture. Nerve cells (*N*) at the nodal points of the net. Cellular debris present throughout all the field. ×300.

FIG. 6.—Glial and nerve cells in the migratory zone from a brain explant in a 9-day-old culture. Glial cells (*Gl*) are well apparent in spite of their tenuous flattened cytoplasm. Neuron (*N*), Nerve fiber (*f*). ×900.

FIG. 7.—Assembly of nerve fibers into large fiber bundles from peripheral fibrillar network in a 4-month-old culture. ×300.

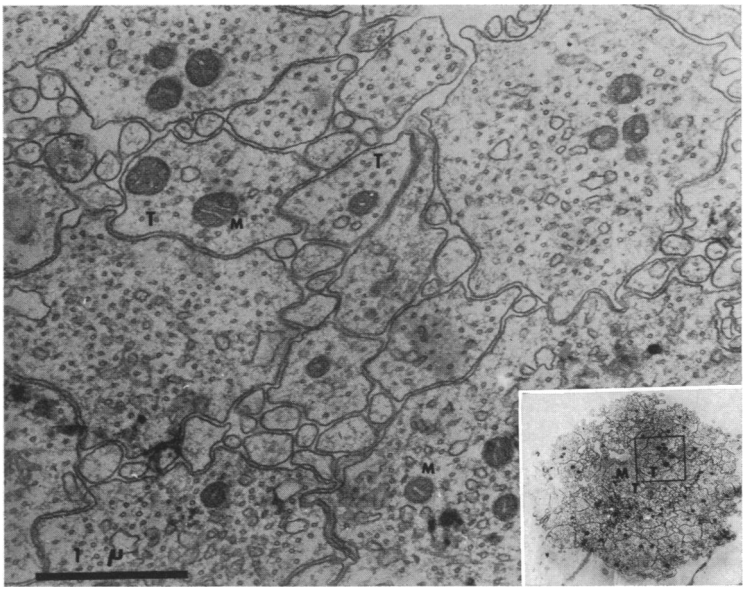


FIG. 8.—Electron micrograph of a cross section of one of the nerve bundles showed in Fig. 4. Neurotubules (*T*), mitochondria (*M*). Glial and mesaxons membranes are absent. Cross section of the whole nerve bundles in the insert. Square corresponds to the section enlarged in the same picture.

features characteristics of nerve cells. The possibility that glial cells are present but are overlooked in view of their tenuous texture and flattened surface will be considered in the *Discussion*.

Nerve fibers which build the dense fibrillar network crisscross each other in all directions, merging together and then departing again to join other fibers in an entirely random fashion (Fig. 5). From the inner surface of this fibrillar and cellular belt, fibers collect in progressively larger number and form the large bundles which direct their course toward the explant (Figs. 4 and 7). Electron microscopic studies to be reported in detail elsewhere[9] show that the fibers entering into the explant establish synaptic junction with the muscular layer of the foregut wall. Cross sections of the fiber bundles studied at the electron microscope give definite evidence that the individual fibers which form the bundles are indeed axons, as proved by the identification of well-recognizable neurotubules arranged at distance from each other (Fig. 8). The picture is in all respects comparable to that of axons dissected out from nerves of living specimens of this species.[10] In one way, however, the cross sections of these fiber bundles differ from

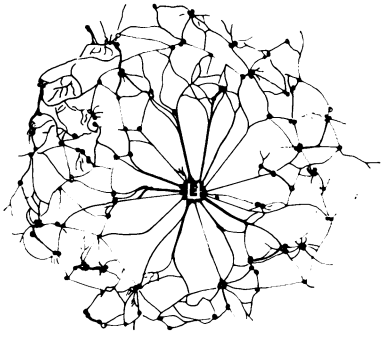


FIG. 9.—Drawing of a 4-month-old culture of dissociated nerve cells around a foregut explant (*E*).

those of nerve fibers dissected from cockroach specimens: in the latter, axons are surrounded by glial sheaths and by glial cells which are well recognizable in the nerve outer layer, while the same glial sheaths and cells are entirely absent from these *in vitro* "nerve bundles." Also absent are the mesaxons which arise from glial cells in normal nerve fibers and coil loosely around individual axons (Fig. 8).

Discussion. The results of this investigation show that nerve cells dissociated by mechanical procedures from ganglia and brains of embryos of *Periplaneta americana* survive in a chemically defined medium for months in the total absence of glial cells. The perfect status of preservation of nerve fibers examined at the ultrastructural level in 4-month-old cultures suggests that nerve cells and nerve fibers could survive for an even much longer time in this CO_2-conditioned, chemically defined medium.

Of the results presented above, four aspects are worth some consideration and comment. They are (1) a search for the most plausible cause for the disappearance of glial cells from the mixed glial-nerve cell population present in the tissues of origin, (2) the evidence that these cells are absent and that all cells which succeeded in surviving and growing in these cultures are nerve cells, (3) the remarkable organizing ability of dissociated nerve cells and nerve fibers in a peripheral outer ring connected through radial fibers with the explant, and (4) the excellent status of preservation of axons and their aggregation in fiber bundles in the absence of their omnipresent ancillary cells, the glial and perineurium cells.

(1) As reported in *Materials and Methods*, the dissociation of the soft nervous tissue dissected out from embryos of *Periplaneta americana* was obtained by a mechanical rather than a chemical procedure. The statement that this technique is fatal to glial but not to nerve cells is based on considerations of the different texture and morphology of the two cell types. Glial cells consist of a very thin and flattened cytoplasm with indented margins. They closely adhere to nerve fibers and nerve cells and even form extended fingerlike projections inside the neuropile, wrapping themselves tightly around individual axons. Nerve cells, on the contrary, are characterized by a round or pear-shaped contour, a dense texture, and loose connections with adjacent structures, particularly during embryonic stages. The mechanical disruption of brains and ganglia has, therefore, quite different effects on the two cell types. Glial cells suffer irreversible damages in the attempt to isolate the cell bodies from surrounding tissues, while nerve cells easily dissociate themselves from other cells or fibers.

(2) The evidence that all glial cells have been destroyed at the time of preparation of the cell suspension is based on the observation at the inverted microscope and at the Nomarski interference microscope, of a very large number of free nuclei and cell fragments among intact cells, as well as on the systematic screening at the interference microscope and at oil immersion of the cell population in short and long-term cultures. In no instance was it possible to detect any glial or perineurium cell. The possibility that these cells, even if present, might have escaped observation, in view of their very tenuous consistency, is most unlikely, since in previous experiments of cultures of intact brains and ganglia from the same species, glial and perineurium cells are well evident and in fact

form the large majority of the cells which migrate out from these explants (Fig. 6). Hence, these cells, if present in these cultures, should be easily recognized, especially in view of the fact that they usually closely adhere to nerve fibers and neighboring nerve cells. It is therefore concluded that all dissociated cells are neurons.

(3) The organization of dissociated nerve cells in an outer fibrillar and cellular ring, which gives rise from its inner margin to large fiber bundles radially directed toward the foregut explant, speaks in favor of some trophic and organizing influence exerted by these explants on dissociated nerve cells. This hypothesis is also supported by the observation that large bundles enter into the explants and form synaptic junctions in the muscular layer of this tubular segment. This trophic influence and a more detailed analysis of this effect are the object of another article.[9]

(4) Ultrastructural studies show that the axons assembled in nerve bundles are remarkably similar in their structural configuration at the subcellular level to axons of nerves dissected out from these insects. However, they differ, from them in the total lack of a perineurial sheath, glial cells, and mesaxon membranes inside the nerve bundle which are, as is well known, constant features of the insect nerves. The question whether these "defective" nerves might be endowed with normal function is now under investigation. This possibility is suggested but not proved by the formation of apparently normal synaptic junctions between these fiber bundles and the visceral muscle in the foregut wall.

As concluding remarks we wish to add that, while not questioning the obviously important role of glial cells in the insect nervous system, the results reported in this article give evidence for the remarkable survival capacity of nerve cells in glial-free cultures and for their ability to produce nerve fibers which in turn assemble in well-organized fiber bundles in the absence of their ancillary glial cells.

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