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STRUCTURAL AND ELECTROPHYSIOLOGICAL PROPERTIES OF NYMPHAL AND ADULT INSECT MEDIAL NEUROSECRETORY CELLS: AN *IN VITRO* ANALYSIS

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SUMMARY

Medial neurosecretory cells (MNSC) from protocerebral nuclei of nymphal and adult cockroaches were cultured in a chemically defined medium in combination with embryonic organs or ovaries from the same species, for periods ranging between 2 and 12 weeks. Under these conditions, neurosecretory cells show vigorous outgrowth of large diameter axons, which differs in many respects from that previously observed from other neuronal embryonic, nymphal and adult explants cultured in the same medium. MNSC explants exhibit spontaneous unit electrical activity which is characterized by a low rate of discharge (1/sec) and regular interspike intervals. This activity contrasts with the spontaneous bursting discharges observed in long-term cultures of the neurosecretory cell population of the corpora cardiaca from nymphal and adult cockroaches.

INTRODUCTION

The outstanding feature of neurosecretory cells (NSC) in both vertebrates and invertebrates is that they are a class of neurons in which the ability to secrete has become extensively developed and of primary importance[2,4,19,27]. Leaving unanswered the question of whether the neurosecretory cell is of glandular or neuronal origin, it can be stated that its ability to produce and convey information differs to a considerable degree from that of conventional neurons. While the neurosecretory cell is receptive to nerve messages which it receives from other cells and to blood-borne chemical messengers, it acts upon its target cells primarily, if not exclusively, by releasing humoral macromolecular factors (in most instances identified as peptides) in the blood in an endocrine-like fashion or, less frequently, by a direct contact with endocrine or

non-endocrine tissues. The target cells, in turn, respond to the message according to rules characteristic of the response to hormones rather than to nerve impulses.

Insects have made a substantial contribution to our understanding of neurosecretory systems. Landmark investigations are the discovery by Kopeć[20] of a brain hormonal effect in a caterpillar and the research by Wigglesworth[32] which provided evidence for the release of a hormone regulating molting by the pars intercerebralis in the insect *Rhodnius prolixus*[3,17,19,28]. In the present report we shall consider in detail the NSC of the paired nuclei of the pars intercerebralis medialis of the nymphal and adult cockroach (*Periplaneta americana*). These are among the most intensively investigated insect NSC because of their relatively large size and their easy accessibility, being located near the dorsal surface of the protocerebral hemispheres. In addition, the median NSC (MNSC) are of great biological significance to the insect because they act either alone or in concert with other neurosecretory or endocrine cell populations to influence a variety of physiological activities ranging from reproduction[14] and molting[29,33] to the regulation of protein synthesis[10,12,18].

As part of a program directed to the *in vitro* analysis of the insect nervous system, we explored the possibility of culturing neurosecretory cells from nymphal and adult cockroaches. Results of previous investigations, directed to the analysis of corpora cardiaca (CC) from these donors, showed that this neuroglandular organ adapts remarkably well to the conditions of culture[30]. We now extend our *in vitro* approach to include one of the most conspicuous neurosecretory brain centers, that of the medial group of the pars intercerebralis of adult and nymphal cockroaches. The results to be reported here show that the medial neurosecretory cells (MNSC) vigorously display their structural and functional properties when freed from the influences present in their environment *in situ*. Indeed, we have shown that MNSC survive in excellent condition, produce nerve fibers of unusually large size, and fire nerve impulses for periods up to several months *in vitro*.

The present investigation consists of two parts: a structural analysis carried out with Nomarski phase differential optics and the electron microscope; and an electrophysiological study of the MNSC conducted with extracellular electrodes. The firing patterns of the MNSC were compared with those from another population of cultured cockroach NSC, the corpora cardiaca[30]. A preliminary report on some of the morphological aspects of this work has recently appeared[31].

MATERIALS AND METHODS

Preparation of cultures

Median protocerebral neurosecretory cells were dissected from nymphal cockroaches (*Periplaneta americana*) 2 months (5th instar) and older and from adults of both sexes. As indicated in Table I, some of these explants were cultured alone or in combination with different organs and tissues dissected out from cockroach embryos or with ovaries from larval or adult specimens.

The first step in the preparation of cultures was the anesthetization of cockroaches with either ether or CO_2. The anesthetized insects were then taped with their

ventral side up to a Maximow depression dish with Scotch transparent tape. The dorsal surface of the head capsule was sterilized with a cotton swab moistened with 95% ethanol. A rectangular opening was then made in the dorsal head capsule with fine dissecting knives in order to gain access to the brain. The brain was removed through this opening and placed in a Maximow dish filled with a sterile saline solution (0.9%). Several brains were collected in this manner and rinsed rapidly many times in this solution before they were placed in the culture medium described below. The sheath covering the brain was then dissected away with fine forceps in order to expose the medial cell clusters of the pars intercerebralis. The location and description of these cells is given in a subsequent section. The two medial cell groups, which are about 1.5 mm across, were then dissected out and transferred to a small culture dish containing a chemically defined medium which has been previously described[6,22]. Using fine dissecting needles, the MNSC clusters were gently pressed down until they adhered to the cover glass which was placed at the bottom of each culture vessel. In those cases where single cells or small cell aggregates were desired, the medial cell group was teased apart in the small culture dishes and the cells were allowed to settle onto the cover glass. When other insect explants were used, they were dissected free and prepared in a manner similar to that described above. After preparation, all cultures were transferred to desiccators and flushed with 5% CO_2 in 95% air and incubated at 29 °C for periods ranging from 2 to 12 weeks.

Techniques involved in the preparation of embryonic organs and tissues as well as of corpora cardiaca, have been described elsewhere[6,30].

Histology

Cultures were examined daily with an inverted microscope. Photomicrographs of living cells were taken with a Zeiss Nomarski microscope. Several histological procedures were used. For light microscope studies, cultures were fixed and stained *in toto*, using the performic acid–Victoria blue technique[13]. In other cases, explants were removed from the coverslip, fixed in Bouin's solution and stained with Gomori's chrome alum hematoxylin phloxine technique[15]. For ultrastructural study, cultures were detached from the coverslips and fixed in 4% glutaraldehyde in collidine buffer for 4 h. They were post-fixed in 1% osmium tetroxide in collidine buffer for 1 h, dehydrated and embedded in epon. Thick (1 μm) sections were cut with glass knives for light microscopic study and to determine the orientation of the specimen. Thin sections were cut with a diamond knife, stained in uranyl acetate and lead citrate and studied with an RCA EMU 3G electron microscope.

Electrophysiological studies

Seventy-eight cultures of MNSC taken from nymphal and adult cockroaches were examined for unit electrical activity. These cultures, which were maintained *in vitro* for 3–8 weeks, contained over 216 individual clusters of NSC. Fifty-four 4–8-week cultures containing over 150 nymphal CC were also used in the present experiments. For purposes of recording, the nutrient culture medium was replaced with a phosphate buffered physiological salt solution with a pH of 7.2 (see ref. 37)

about 15 min before an experiment was begun. Electrophysiological observations were carried out at room temperature, which was approximately 25 °C.

Extracellular electrical recordings were made with glass micropipettes with 2–5 μm tips which were filled with the same salt solution in which the cultures were immersed. The recording electrode was coupled to a PAR 113 AC preamplifier by means of a platinum wire. Recordings were single ended, with a large platinum wire inserted into the solution surrounding the culture preparation acting as the ground electrode. The band pass of the system was usually 0.03–10 kHz. All experiments were carried out on the stage of a Wild dissecting scope. Electrodes were guided into place under visual control with Leitz micromanipulators.

Permanent records of relatively short-term electrical events ($<$ 1 min) were obtained by photographing the oscilloscope screen. Longer term events were recorded with a Grass Model 5D polygraph, which served as an event marker. The input to the polygraph was filtered to give a band-pass of 300 Hz–10 kHz, integrated by a specially built circuit, and fed directly into the DC power amplifier of the Grass unit.

RESULTS

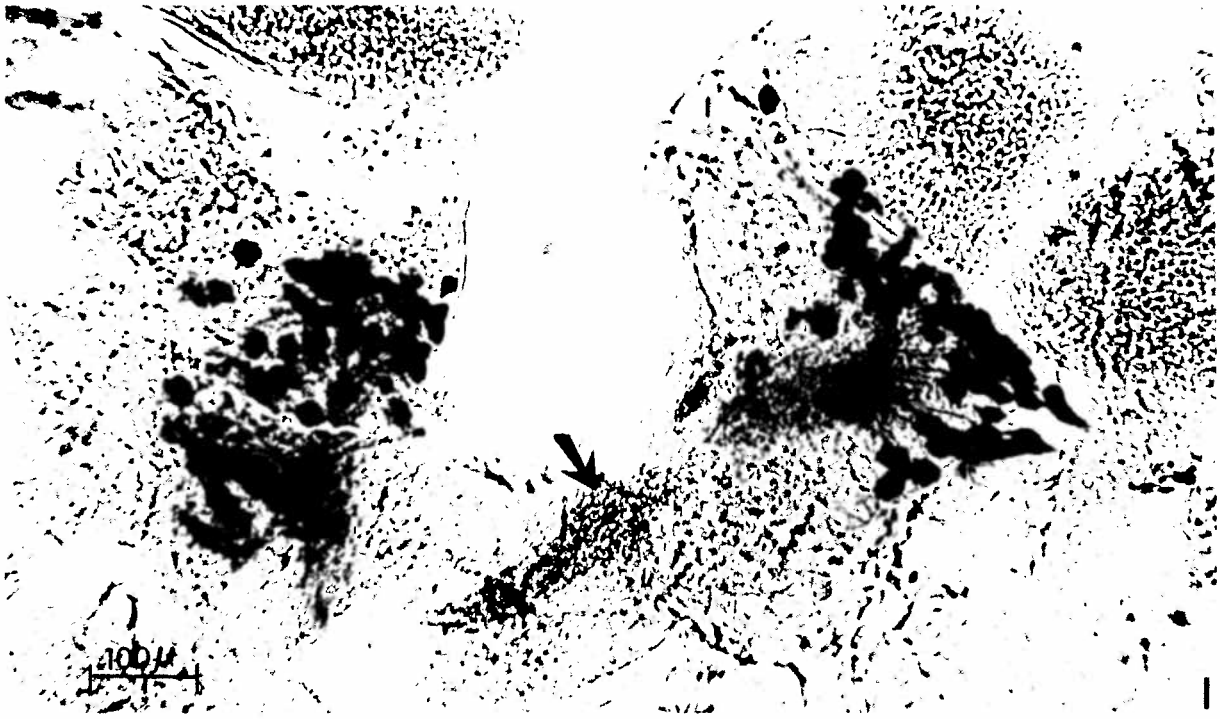
Median neurosecretory cells in situ

The location of the MNSC groups near the dorsal surface of the protocerebrum is shown in the Victoria blue stained whole mount preparation of Fig. 1. Dissection reveals that the axons of these cells gather into two large bundles, one from each cell cluster, and course toward the contralateral side, forming a chiasma with the fibers from the opposite cluster. These fiber bundles then run parallel to each other, traverse the proto- and deutocerebrum and emerge from the tritocerebrum as two nerves known as nervi corporis cardiaci I (see ref. 11). The two nerves enter into the paired corpora cardiaca. The complex interrelationship between the protocerebral NSC and the cells of CC have been discussed in a previous article[30].

The appearance, numbers, size and staining characteristics of the MNSC cells will be described. The number of cells refers to the total observed in both hemispheres. In this regard, it should be noted that there were usually small differences in the number of cells of a single type in each hemisphere of a given specimen.

A group of medium sized cells (10–17 μm) assumed a deep blue color when stained with the performic acid–Victoria blue technique, which indicates that they have a high content of cysteine- and cystine-rich peptides[11,13]. There were an average of 130 (range 103–153) of these cells counted in each of 9 Victoria blue whole mount preparations of the protocerebra of adult male cockroaches.

Many cells in the medial group did not stain darkly with Victoria blue, which indicates that they do not possess large amounts of cysteine- and cystine-rich peptides[11,13]. The following descriptions of these remaining cell types are based upon observations of 8 μm sections of the protocerebra of 3 or 4 adult male cockroaches stained with Gomori's chrome alum hematoxylin phloxine[15]. This technique is commonly used to stain insect neurosecretory material[34]. Many ovoid to round cells measuring 17–23 μm are intermingled with the smaller cells described above. The



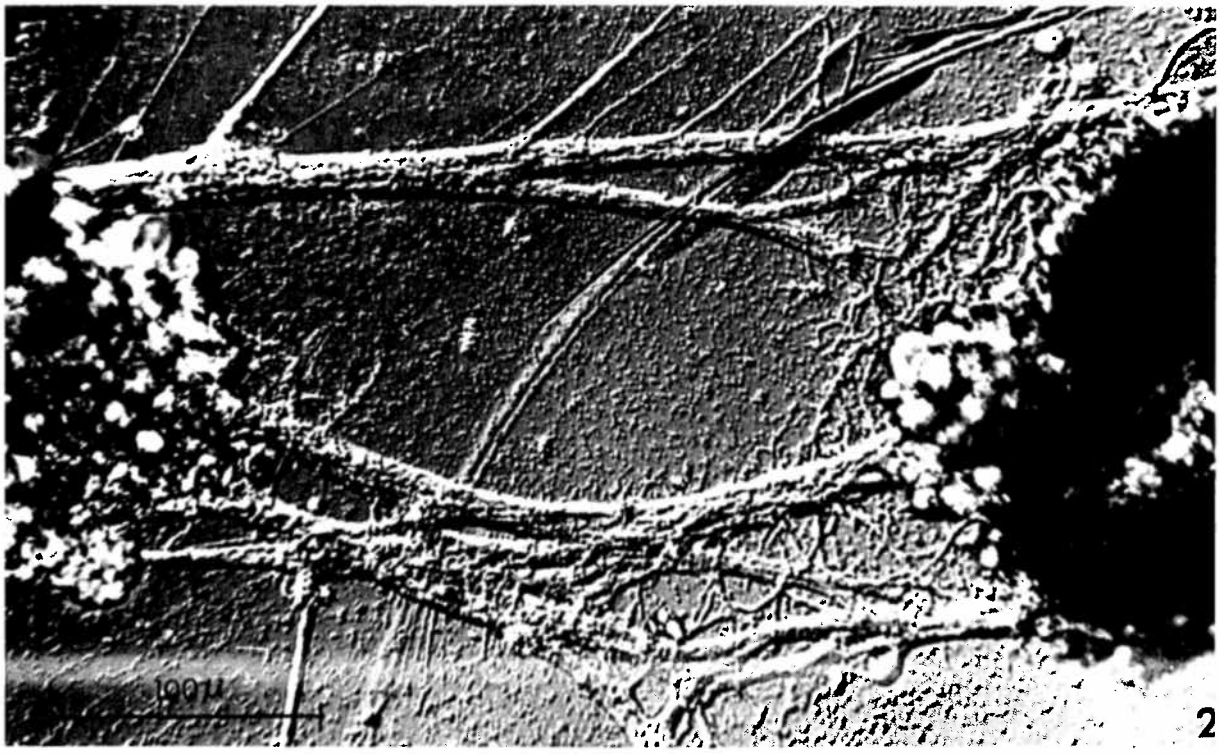


Fig. 1. Median neurosecretory cells which stain darkly with Victoria blue are shown in a whole mount preparation of the pars intercerebralis of an adult male cockroach. The arrow points to a fiber bundle emerging from right MNSC nucleus as it crosses the midline. The fiber bundle from the left nucleus is less well defined.

Fig. 2. Nomarski microphoto showing formation of large fiber bundles which interconnect to MNSC aggregates of a male adult specimen cultured *in vitro* for 2 months.

secretory granules of these latter cells are stained red by the phloxine component of the Gomori stain. There are an average of 76 (range 50–109) of these cells. A third class of cell, which is much larger (25–30 μm) and present in smaller numbers (14–18) than the previously described cells, is also found in the medial group. No Gomori stained granules were present in these cells in the 3 sectioned adult male brains examined. Two giant neurons measuring 34–38 μm are also present among the medial group. One is located on each side of the median furrow of the protocerebrum. However, a total of 3 such cells were noted in one of the 4 adult male preparations examined. These large cells contained no granules which were stained with the Gomori technique. It should be noted that the cells which stained darkly with Victoria blue contained numerous granules which stained blue-black with the Gomori stain. None of the other cell varieties noted above had any granules of this type.

The identification with the electron microscope of electron dense granules (100–300 nm) within a neuron provides another, and perhaps more definite, means with which to establish whether a cell is neurosecretory in nature[33]. As a complement to our light microscope studies, we carried out a search for electron dense granules in uranyl acetate–lead citrate stained preparations of adult male cockroaches with the electron microscope. The results indicate that both the perikarya and fiber processes of the small (10–17 μm) cells are filled with electron dense granules. The somewhat larger (17–23 μm) cells contained a lower and more variable number of granules. The relatively large (25–30 μm) cells contained only a few electron dense granules, which were located in the perikarya. We have not yet investigated the few very large medial cells with the electron microscope.

The description of cockroach protocerebral MNSC *in vivo* which is given above should not be considered exhaustive. Our primary objective was to specify in a preliminary manner the cells which were placed in culture.

Median neurosecretory cells in vitro: effect of auxiliary tissues

The types of culture preparations used in the morphological study of MNSC *in vitro*, as well as the lengths of the incubation period, are given in Table I.

Our most frequently used preparations consisted of entire clusters of MNSC. The area excised from each hemisphere of the protocerebrum and placed in culture extends slightly beyond the cluster of cells stained in the Victoria blue preparation of Fig. 1. The two freshly excised fragments of brain tissue obtained from each nymph were roughly cuboidal. The explants round up during the first few days of culture.

All of the cultures of cell aggregates and single neurons described below were incubated in the presence of embryonic organs (brain, thoracic ganglia and foregut) or ovarian follicles from nymphal and adult specimens (Table I). In all instances these organs were positioned at a distance from, and intermingled with, NSC clusters or individual cells. Previous studies have indicated that the presence of such tissues acts to 'condition' the medium and increase the amount of fiber outgrowth and survival of explants of different parts of the embryonic insect central nervous system *in vitro*[7,8,21–23]. The present results agree with these findings. Neurosecretory cells which were cultured in the presence of the auxiliary tissues mentioned above showed

TABLE I

CULTURES USED IN MORPHOLOGICAL STUDY

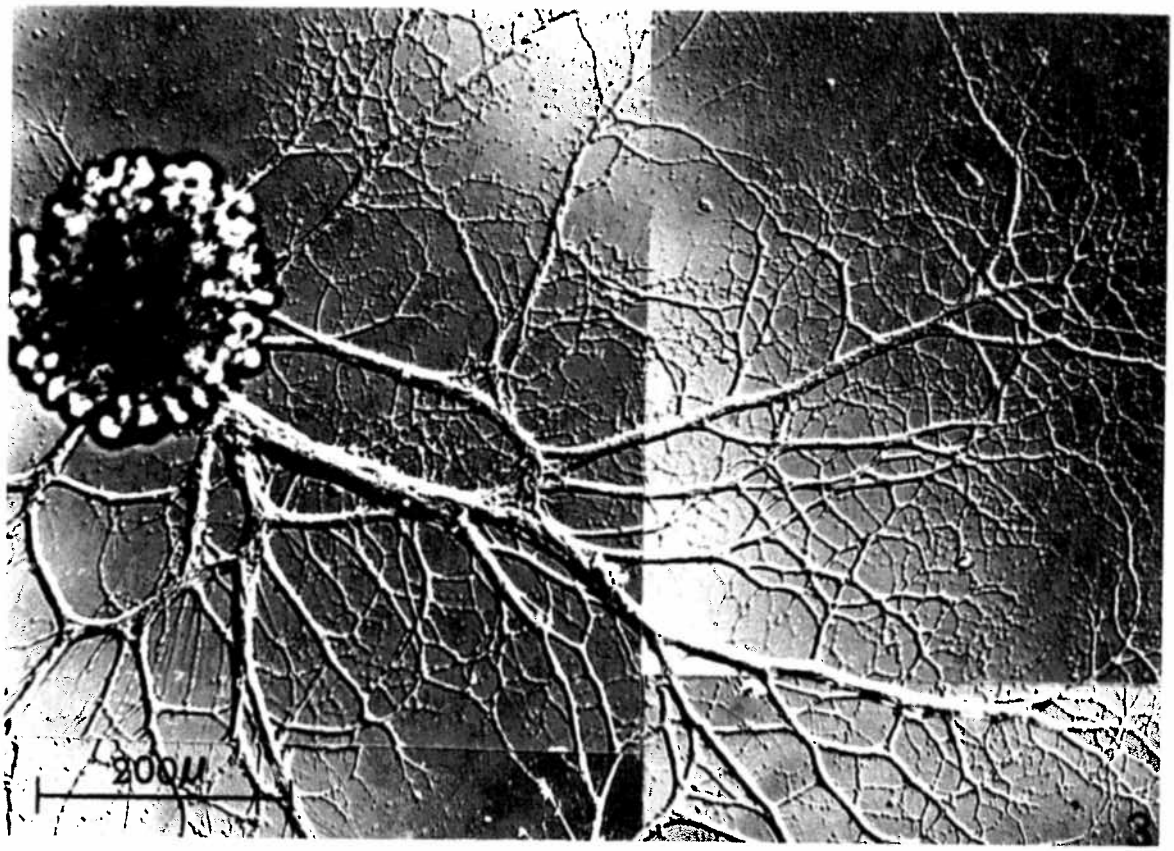
Organs explanted	*Duration*		*Total no. of cultures*
	1–5 weeks	*6–12 weeks*	
Neurosecretory cell aggregates* cultured alone			
(a) Nymphal specimens	47	21	68
(b) Adult specimens	19	6	25
Neurosecretory cell aggregates* combined with ovarian follicles			
(a) Nymphal specimens	105	56	161
(b) Adult specimens	48	25	73
Neurosecretory cell aggregates* combined with embryonic brain and foregut segments			
(a) Nymphal specimens	56	16	72
(b) Adult specimens	12	6	18
Dissociated neurosecretory cells* combined with ovarian follicles			
(a) Nymphal specimens	14	12	26
(b) Adult specimens	17	5	22
Proto-, deuto-, and tritocerebral parts of the brain combined with embryonic brain and foregut segments or ovarian follicles			
(a) Nymphal specimens	15	19	34
(b) Adult specimens	16	3	19

* Pars intercerebralis.

excellent survival and fiber outgrowth *in vitro*. In contrast, more than 95% of the cultures which were incubated in the absence of embryonic nervous system parts and foregut or adult ovarian follicles failed to exhibit significant fiber outgrowth. The limited outgrowth which was observed in a few cases consisted of only a few slender fibers which emerged during the first two weeks of incubation and were subsequently resorbed during the third and fourth weeks. The majority of the MNSC explants which were cultured in the absence of other tissues detached from the glass growth substrate and showed other signs of deterioration within the first few weeks of incubation.

Survival and nerve fiber outgrowth from groups of neurosecretory cells

The MNSC explants show excellent survival *in vitro* for periods ranging up to 3 months or longer. In addition, the majority of explants show a vigorous outgrowth of large caliber nerve fibers which start to appear toward the end of the first week *in vitro*. Fig. 1 shows the MNSC *in situ* stained with Victoria blue. Fig. 2 shows the two similar nuclei after two months of culture in the presence of multiple embryonic explants and photographed with Nomarski optics. Fibers grow out from paired explants and gather in large and compact bundles which interconnect with each other in a configuration reminiscent of that which forms *in vivo*. Another large cluster of MNSC dissected out from a 10th instar nymph cultured *in vitro* for 35 days is shown in Fig. 3. Also in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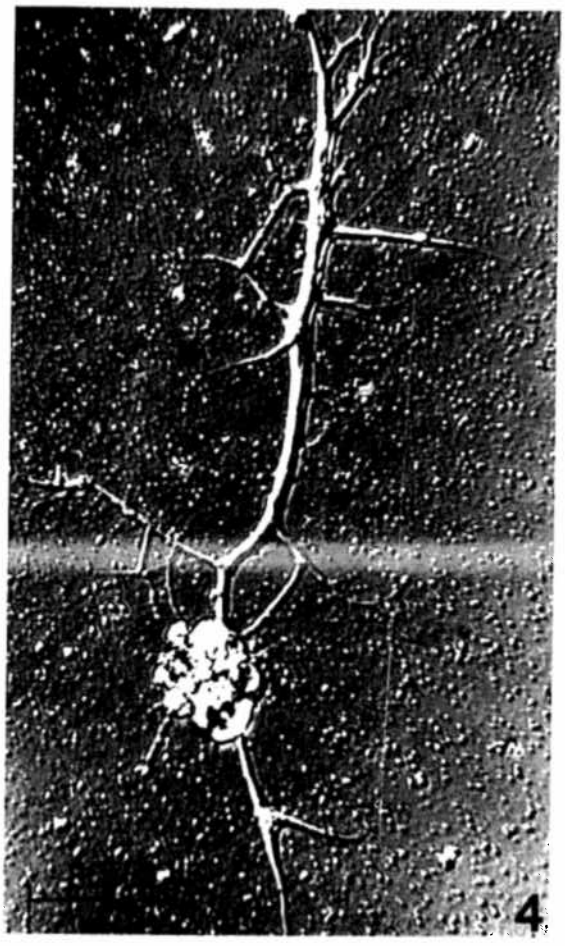
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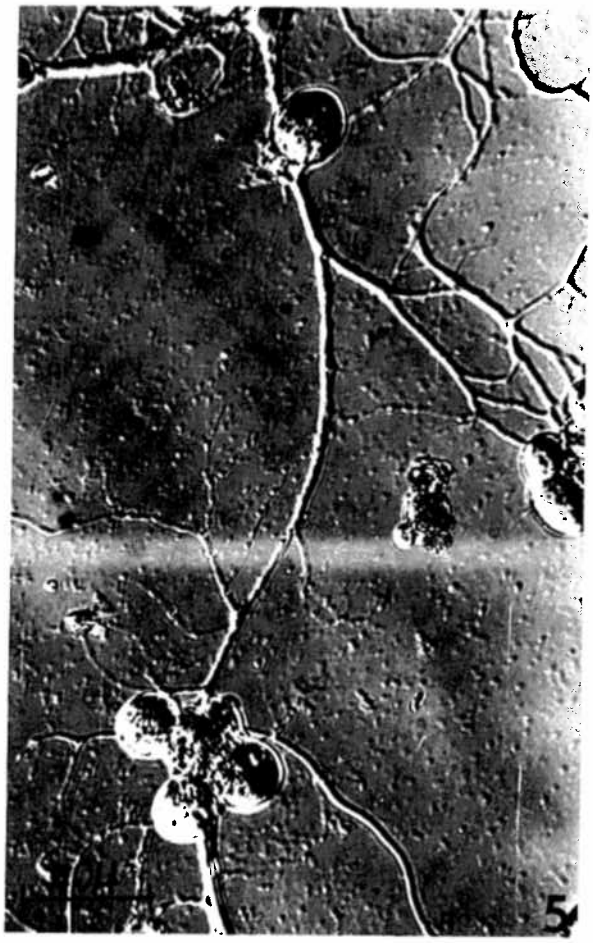
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this case the explant consists of the entire NSC population of one of the two paired medial protocerebral nuclei. Axons from individual cells collect in a few large bundles which branch out repeatedly, interconnecting with each other or ending in a fiber plexus in the absence of adjacent end organs. In these, as in other large or small explants, one of the outstanding features is the lack of glial or satellite cells among nerve fibers.

Small explants consisting of only a few MNSC also survive and grow neurite processes *in vitro*. Nomarski optics reveal that the spherical neuronal cell bodies which make up these clusters produce cylindrical fiber processes. These fiber processes often make connections with other small neighboring MNSC clusters (Fig. 5). In the interesting case shown in Fig. 4, a small MNSC cell aggregate produced numerous short, thorn-like collaterals which branch out at right angles from the club-like stem fiber.

Survival and axonal growth from single neurosecretory cells

In most culture vessels, individual nerve cells dissociated from the MNSC clusters attached to the surface of the coverslip laid in each dish and produced 1 or 2 fibers which grew out to a considerable distance from the cell body. Two of these cells are shown in Figs. 6 and 7. The cell depicted in Fig. 6 is of particular interest. Note the multiple dendrite-like processes which emerge from the cell body while from the opposite side, a large tubular fiber process emerges which has no collaterals and ends bluntly. Fig. 7 shows the most common axonal growth pattern from individual cells. These explants, consisting of only one neuron, show even more convincingly than the smaller cell aggregates the capacity of differentiated cells from MNS nuclei to survive in excellent condition and produce axons in the absence of contact with glial or other supporting cells.

Survival and growth pattern from non-neurosecretory explants

As shown in Table I, a total of 53 explants dissected out from nymphal and adult specimens were cultured under the same conditions as MNSC. The size of the explants was comparable to that of MNSC clusters described above. This study was carried out to provide control data on growth patterns of non-neurosecretory cerebral neurons with which we could compare the results from NSC population. All explants, even when cultured in the presence of embryonic tissues or ovarian follicles, showed poor, if any, axonal outgrowth. The fibers were much thinner and fewer in number than those produced by NSC, and underwent absorption in long-term cultures. Extensive

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Fig. 3. An aggregate of MNSC from a 10th instar nymph cultured *in vitro* for 35 days and photographed with the Nomarski microscope. Note the large tubular fiber bundles that originate from the cell complex and branch out into smaller fibers.

Fig. 4. A large axon with thorn-like collaterals, which emerges from a 5-cell aggregate of MNSC incubated for 3 weeks *in vitro*. The axon increases in diameter towards its distal end. MNSC obtained from an adult male cockroach.

Fig. 5. Small clusters of MNSC from a 10th instar nymph maintained *in vitro* for 2 weeks. Adjacent cell clusters in close proximity to one another become interconnected by outgrowing fibers. Nomarski microphoto.

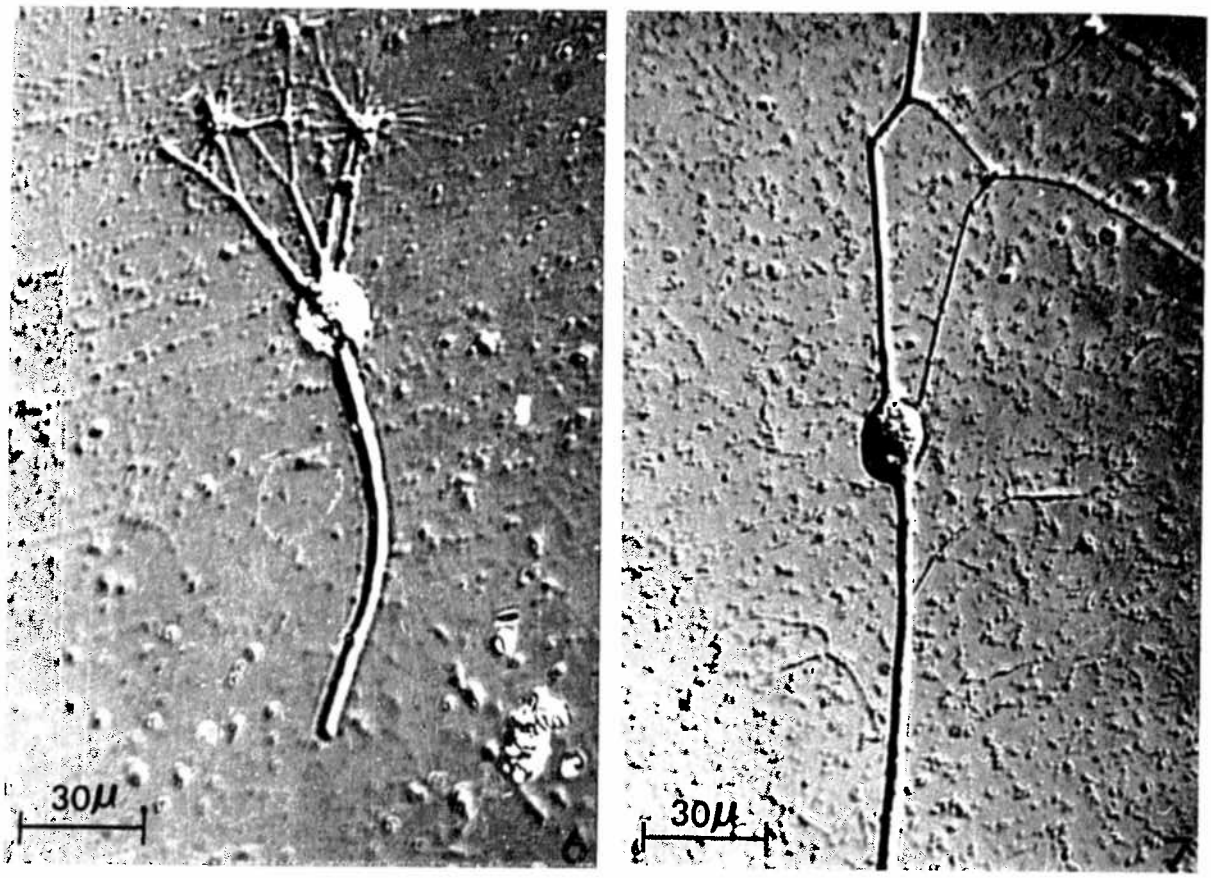
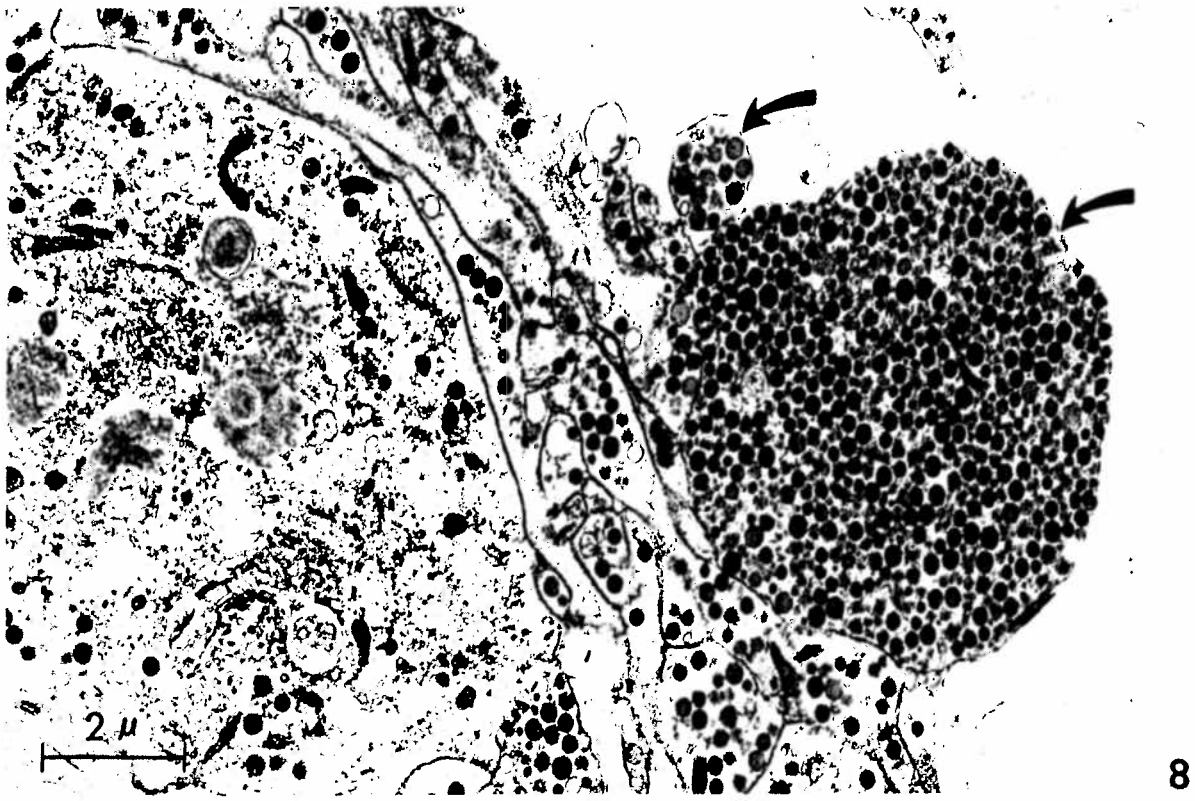


Fig. 6. Nomarski microphoto of single living MNSC from a 7th instar nymph cultured *in vitro* for 33 days. Notice the stout axon-like process that grows out from one pole of the cell and the dendrite-like arborizations growing out from the cell's opposite pole.
Fig. 7. A MNSC taken from a 5th instar nymph and maintained *in vitro* for 20 days. Bipolar outgrowth of substantial fiber processes is evident in this Nomarski microphoto.

studies of the explants at the Nomarski microscope showed progressive cell deterioration after a few weeks *in vitro*. Unit electrical activity, as will be described later, was totally absent, thus giving additional evidence for the poor condition of these cultures.

Histochemical and ultrastructural analysis of the MNSC cultures

Histochemical studies indicated that there is considerable variation in the amount of stainable NS granules found in different MNSC cultures from nymphal and adult specimens. Also there is considerable variability within a single MNSC cluster. For example, 5 or 6 cells in a cluster may show numerous granules in a stained preparation, while the remainder are devoid of granules. Granules which stained with Victoria blue in whole mount preparations were present in the soma of some cells which were maintained *in vitro* for periods ranging up to 6–8 weeks. Other cells had few, if any, such granules. Secretory material which stained blue-black with Gomori's technique was also identified in some serial sections of MNSC clusters which were cultured for periods up to 8 weeks. The Gomori and Victoria blue procedures revealed very few granules in the nerve fibers growing out from the cell bodies.



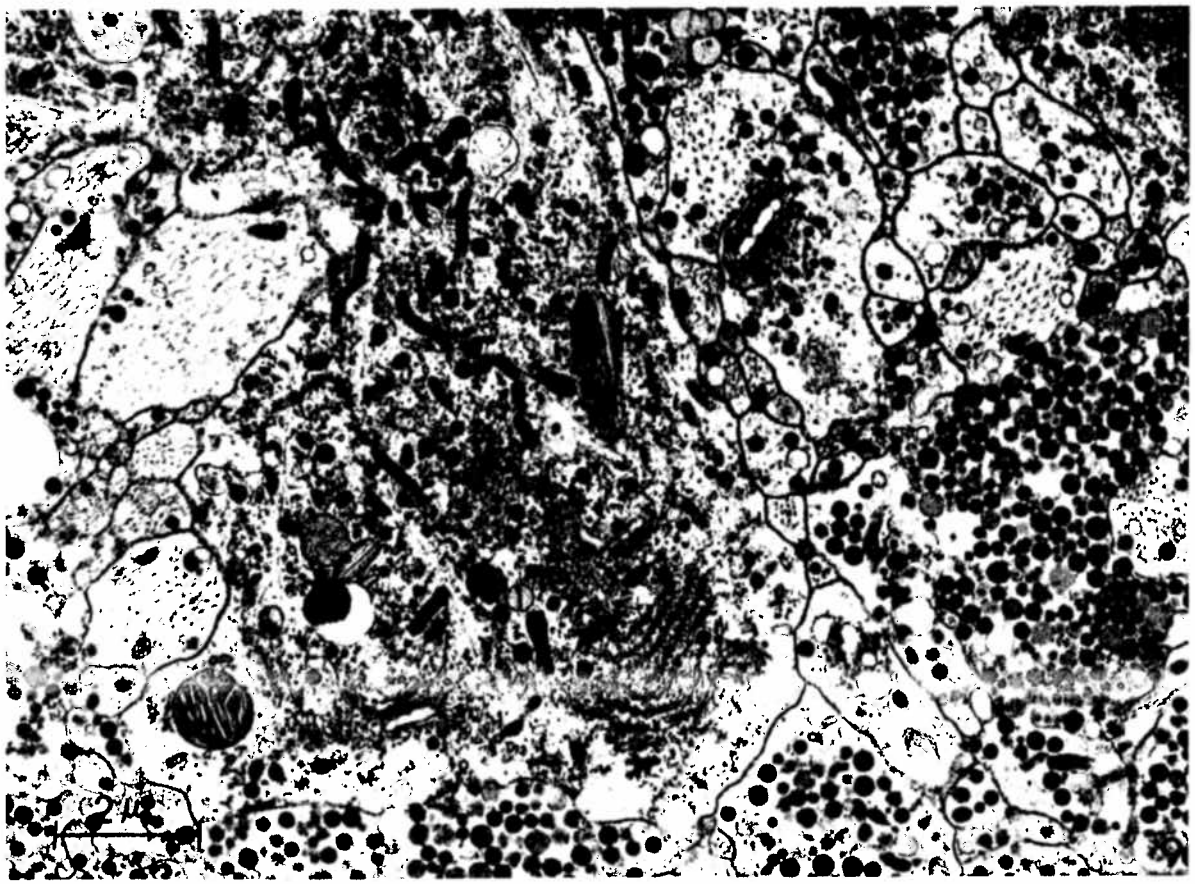


Fig. 8. Electron micrograph of axons (arrows) growing out of an MNSC cluster taken from a 10th instar nymph maintained for 1 month *in vitro*. Cross sections of axons are filled with electron dense membrane bound NS granules in much the same manner as they occur within the explant.
Fig. 9. Electron micrograph of the same culture shown in Fig. 8. Profiles of cell bodies and axons within the explant display varying amounts of electron dense NS granules, mitochondria, and other cell organelles.

all argue strongly that we are reporting spontaneous bioelectrical phenomena which arise as a result of endogenous processes of the MNSC.

Spontaneous unit electrical activity was recorded from clusters of MNSC from nymphal and adult cockroaches which were maintained *in vitro* for 3–8 weeks (Figs. 2 and 3). Ninety-seven of the 216 cell clusters examined in 78 cultures were electrically active. Of this electrically active group, many records were of multiple units or low amplitude units which were not analyzed further. The following description is based upon 37 records of single unit activity obtained from 23 different MNSC clusters. This is an adequate sample, considering the highly homogenous firing patterns of the MNSC. Preliminary attempts to record extracellular unit electrical activity from isolated MNSC (Figs. 6 and 7) have been unsuccessful.

Unit electrical activity in the MNSC clusters is characterized by a low rate of firing and a continuous pattern of discharge (Fig. 10a–d). The 37 units observed had a mean frequency of discharge of 1 spike/sec (range 0.2/sec–2.3/sec). The majority of MNSC units had relatively regular interspike intervals (Fig. 10a, b). Fig. 10c–e shows some of the most 'irregular' patterns of interspike intervals obtained. Only 1 unit having a burst firing pattern was observed (Fig. 10e). The duration of spike potentials from 10 units was measured. Spike durations were highly variable and long, ranging from 2 to 7 msec. An attempt to compare spike durations and firing patterns of cultured non-NS protocerebral cell clusters with those obtained from the MNSC proved unsuccessful. The non-NSC failed to survive and produce fiber outgrowth *in vitro*.

Unit electrical activity was recorded from clusters of MNSC both with and without outgrowing nerve fibers. This suggests that the presence of fibers is not the sole index of a healthy culture preparation. However, all successful recordings were obtained from clusters having cells which were well defined when viewed through the light microscope. Cells in such clusters looked like miniature clusters of grapes (Figs. 2 and 3).

When multiple units were detected, there was no apparent coupling of activity between individual units. This finding is in contrast to the concerted multi-unit burst discharges which have been observed in cultures of abdominal and thoracic ganglia[26]. Also, it seemed to be more difficult to detect units in the protocerebral MNSC than in cultures of the abdominal and thoracic ganglia examined in this previous study. At the present time, we are not able to determine whether this difference in unit detectability is due to a low number of spontaneously active cells in each MNSC cluster or whether the naturally low firing rate of the MNSC biases against their detection.

Electrophysiological properties of corpus cardiacum neurosecretory cells in vitro

The discharge pattern of units in long term (1–2 month) cultures of nymphal corpora cardiaca (CC) were investigated in order to provide data from another NSC population with which we can compare that obtained from the protocerebral MNSC. A detailed structural analysis of cultured CC NSC is presented elsewhere by Seshan and Levi-Montalcini[30].

Whole CC were cultured in a manner similar to that reported for the MNSC[30]. Several CC were placed in a single small culture vessel. Most vessels also contained

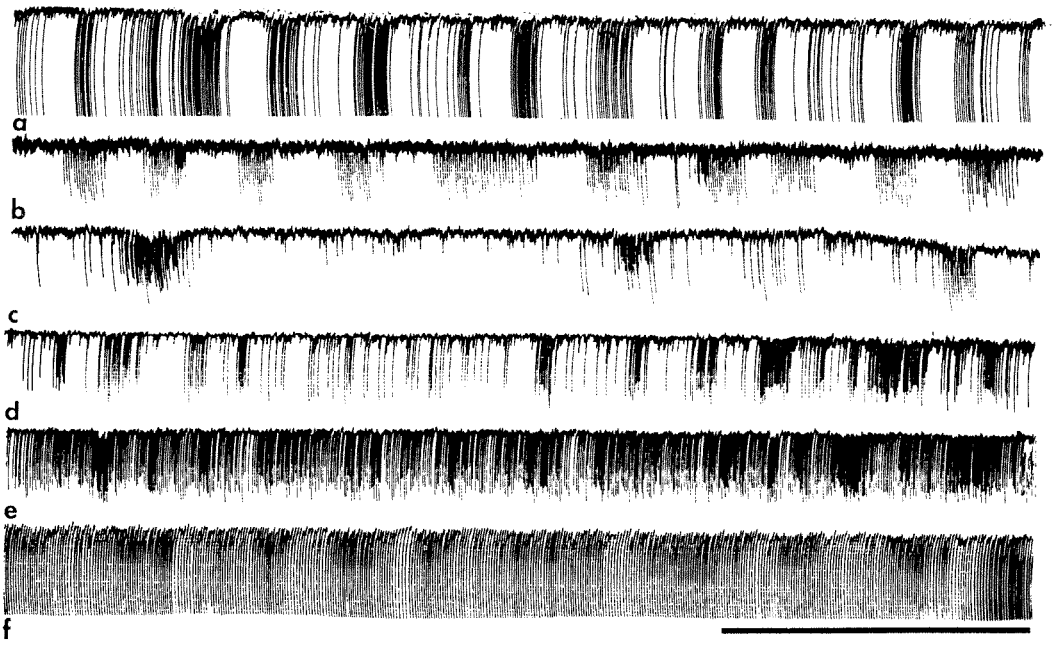


Fig. 11. Discharge patterns of spontaneous extracellular unit activity in cultures of corpora cardiaca NSC. a–d: bursting discharges observed in 4–8 week cultures of CC from 7–10th instar cockroach nymphs. e: a continuously active unit with a fluctuating rate of firing in a 6 week culture obtained from a 7th instar nymph. f: a rarely observed CC unit with regular interspike intervals, which was observed in a 6 week old culture obtained from a 7th instar nymph. The time scale is 1 min except in *c* and *f*, where it is 2 min.

corpora allata and/or ovarian follicles. Electrical recordings were made using the same equipment and procedures used for the study of the MNSC.

Over 140 CC in 54 culture vessels were examined for unit electrical activity. Of this group, about one-third of the CC were electrically active. Most units fired in a bursting pattern and had low rates of firing (Fig. 11a, b, c). The mean firing rate of 29 single units was 1.88 spikes/sec (range 0.28–4.37/sec). The interburst interval of units with a clearly defined bursting pattern averaged approximately 13 sec (range 2–25 sec). Many bursting units discharged at regular intervals (Fig. 11a–c). Other bursting units showed a more irregular pattern of firing (Fig. 11d). Even those units which had a relatively continuous pattern of firing showed periodic fluctuations in interspike intervals (Fig. 11e). Only one of the 29 observed CC units showed a continuous pattern of firing with regular interspike intervals (Fig. 11f).

DISCUSSION

In planning and performing the experiments reported in this article, our aim was to explore the possibility of culturing NS brain cells from nymphal and adult cockroaches in a chemically defined medium, according to a technique devised in our laboratory and used to culture other embryonic and fully differentiated nerve cells. It was hoped that this work would provide a baseline for further investigations under conditions more amenable to experimental analysis than those provided by the living

organism. The success met in this attempt, to our knowledge the first of this kind, shows that fully differentiated NSC adapt remarkably well to the conditions of culture, in spite of the fact that the medium and the environment are vastly different from those of the living organism.

Fully differentiated MNSC, whether they have been dissected out from advanced nymphal specimens or adult individuals as single cells or as small or large aggregates, produce axons which grow out profusely into the medium. In this respect, the nerve fiber outgrowth is similar to that from embryonic ganglia or brain dissected out from young embryos of the same species[1,6,7,22,30]. It differs from it, however, in two respects: the MNSC fibers are considerably larger in diameter than those from embryonic ganglia, and the MNSC fibers lack the glial and other satellite cells which are seen in large numbers around the embryonic explants.

MNSC fiber outgrowth differs considerably from that observed in explants of corpora cardiaca from nymphal and adult specimens. The pattern of nerve fiber outgrowth from corpora cardiaca, as described in detail in previous articles[24,30], is characterized by the slow growth of nerve fibers which branch out, mostly as individual filaments, from the entire surface of the gland and trace a sinuous, undulating path into the medium. In many instances these axons possess multiple enlargements which give the fibers a beaded appearance. These CC axons were markedly different from those of the MNSC, which were larger in diameter, tubular in appearance, without 'beads', and did not follow an undulating path of outgrowth. These differences between the pattern of nerve fiber outgrowth from the NSC of the pars intercerebralis and those of the corpora cardiaca are particularly interesting because the two structures share common structural and functional properties[34]. Thus the *in vitro* analysis has revealed population differences which may have passed unnoticed in the living organism.

One of the important issues involved in interpreting our results concerns whether the present morphological and electrophysiological findings were obtained from NSC of the medial group or from non-NSC which are present in the vicinity. Several factors suggest that we are observing an NSC population. The piece of protocerebral tissue which is initially explanted contains a very high proportion of NSC, thus maximizing the probability that these will be the cells observed *in vitro*. The fact that cells from the MNSC region survived much better than did adjacent non-NSC brain regions suggests that excellent survival *in vitro* may be a property of cockroach NSC unique to this region. These data further suggest that non-NSC within the explanted cell cluster may die off, leaving an even more highly concentrated MNSC population. Differential fiber outgrowth supplies other evidence. The fibers which were observed to grow out of the MNSC clusters are very different from the other cultured embryonic, nymphal and adult neuronal explants which we have prepared, which include frontal, ingluvial, thoracic, abdominal, subesophageal and cercal ganglia[1,6,30]. The fibers from the latter preparations were thin, with many branches, differing greatly from the large caliber tube-like processes arising from the protocerebral group. The presence of electron-dense NS granules in the soma and axons of cultured MNSC clusters and, to a lesser extent, the presence of granules in Victoria blue and Gomori stained prepara-

tions examined with the light microscope, also indicate that many, if not most, of the MNSC survive *in vitro*. We may, in addition, cite the electrophysiological evidence that the unit electrical activity of cultured MNSC has long spike durations, a characteristic of NSC in general[5,9,36]. Therefore, it seems most likely that we are recording from a population of NSC.

The finding that clusters of MNSC show spontaneous unit electrical discharges *in vitro* is in agreement with the findings of other investigators who have described such activity *in situ*[9,16,35]. The long duration action potentials observed in our culture preparations are also common to NSC *in situ*[5,9,16,35]. Previous investigators described 'spontaneous' activity in their *in situ* preparations; however, they were unable to determine whether the discharges were modulated in some way by their surround. A unique contribution of the present *in vitro* study is that we have shown that the MNSC are capable of producing action potentials when freed from the environment of the protocerebrum.

Another contribution of the present electrophysiological study was the finding that the CC NSC are capable of spontaneous discharge when cultured in a chemically defined environment free of all sources of neuronal and humoral influences arising outside of the CC itself. In this regard, it is interesting that neither Wilkens and Mote[35] nor Normann[25] reported significant spontaneous unit activity in *in vivo* preparations from *Sarcophaga bullata* and *Calliphora erythrocephala*, respectively. If we assume that some major physiological transformation of CC cells has not taken place during culture, our results suggest that CC unit activity may be inhibited *in vivo*, perhaps by its major source of input, the MNSC. Thus, some indirect support is given to Wilkens and Mote[35], who contend that the CC NSC are endogenously active and that they are regulated by inhibitory influences.

It is of particular interest that the firing patterns of the CC NSC were strikingly different from those reported for the MNSC. The CC units usually fired in bursts, while the MNSC units typically showed a continuous firing pattern, often with regular interspike intervals. The observation that two populations of NSC show such physiological differences, in addition to the previously described morphological differences, after many weeks *in vitro* offers compelling evidence that despecification or homogenization of various neuronal cell types does not take place under the conditions of culture.

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