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Neuronal Properties of Nymphal and Adult Insect Neurosecretory Cells in vitro

Abstract. *Neurosecretory brain cells from late nymphal and adult cockroaches were grown for 1 to 3 months in a chemically defined medium in combined cultures with embryonic organs or oocytes from nymphal and adult specimens. Under these conditions, neurosecretory cells show vigorous axonal growth. Electron dense granules in cell perikarya and axons of 3-month-old cultures are suggestive of neurosecretory activity in vitro.*

The development in our laboratory of a chemically defined medium which supports long-term survival and nerve fiber outgrowth from intact embryonic brain ganglia (*1*), dissociated embryonic nerve cells (*2*), and nymphal ganglia of the stomatogastric system of the cockroach *Periplaneta americana* (*3*) suggested use of the same medium and techniques to explore the insect neurosecretory (NS) system. We have already reported successful results with nymphal and adult corpora cardiaca cultured in vitro for periods ranging between 4 and 8 weeks (*4*). It was the object of the present study to extend this analysis to the NS cells located in the brain. The selection of the two large paired protocerebral NS groups was dictated by considerations of the key role they play in the life of the insect as well as by technical reasons. They are an ideal object for experimentation because of their ready visualization in the intact living brain in the dorsomedial aspect of the protocerebrum and because of their fairly large size, which makes them easily distinguishable from adjacent nonneurosecretory cells. The extensive literature available on the structural, ultrastructural, and electrophysiological properties of these cells in vivo (*5*) was an additional reason for their selection for studies in vitro.

On removal of the head capsule under aseptic conditions, the two groups of medial NS cells were identified in the intact brain under a stereomicroscope by their position, as well as by the pale bluish color due to light scattering (Tyndall effect) by the NS granules which fill most of the perikarya. Histological and electron microscopic studies performed immediately after dissection of the two groups in several nymphal and adult specimens showed that each group consists of about 200 neurons and an undetermined number of glial cells. The three cell types, known as A, B, and C cells, were identified by their size, color affinity, and content of NS granules. The medium-size A cells are characterized by their high staining affinity for performic acid Victoria blue dye, which selectively stains cysteine- and cystine-rich peptides. The intense blue shade of the cells and of their axons in brain whole mounts, as well as in histological sections, makes it possible to identify these neurons under the light microscope (*6*). Cell counts performed in ten adult specimens stained with this technique give an average of 70 cells of type A per group. In the electron microscope, the perikarya and their axons are filled with electron dense granules. The B cells, of about the same size as the A cells, do not stain with Victoria blue but take a red color with Gomori's chrome hematoxylin phloxine technique, which is routinely used for staining NS material. In the electron microscope, they show a variable number of electron dense granules. They comprise about one-third of the entire cell population. The C cells are much larger than the A and B cells; only a few small electron dense granules are seen in the electron microscope in their perikarya. About ten of these large C cells are present in each group. Scattered between the A, B, and C cells are neurons with few or no electron dense granules. Whether they represent a nonneurosecretory cell type or NS cells in a quiescent stage is at present unsettled. Electron microscopic studies of a large number of other brain cells in short- and long-term cultures show no evidence of electron dense granules in the perikarya and axons.

Immediately after dissection from the brain, the two groups of NS cells were transferred into the culture medium consisting of four parts of Eagle solution and five parts of Schneider's insect medium in small glass vessels, according to the technique previously described (*1–4*). The explants were dissociated in situ into cell clusters of various sizes (Fig. 1a) or into individual cells with the help of microneedles; in a few instances the groups were explanted in toto. Intact groups, cell clusters, or individual cells were gently pressed on the surface of a cover slip laid on the bottom of the culture dish until they firmly adhered to it. The cultures were divided into three groups. In the first, NS explants were not combined with other tissues or organs; in the second, they were combined with brain, ganglia, and foregut segments from 16-day embryos of the same species; in the third, they were combined with ovariole segments dissected out from late nymphal or adult specimens. The several tissues were positioned at a distance of 0.5 to 1 mm from each other in a variety of geometrical arrangements. In the second and third groups, embryonic tissues or ovarioles were also pressed on the cover slip until they adhered to its surface. The culture vessels were then placed in desiccators and incubated at 29°C. The results reported below are based on observations of 400 cultures examined daily at the inverted microscope. Photomicrographs were taken by using the Nomarski microscope. When the cultures were discontinued between the end of the second week and the third month, they were fixed and stained in toto with performic acid Victoria blue or used for electron microscopic studies.

Up to the end of the first week, no nerve fiber outgrowth was evident from NS explants in any of the three groups. From the beginning of the second week, differences appeared between NS explants of the three groups and became more pronounced in subsequent periods. Small or large cell clusters or intact cell aggregates of the first experimental group showed signs of deterioration, and no nerve fiber outgrowth took

place up to the end of the first month of incubation (Fig. 1b). The cultures were then discontinued. Histological studies indicated that all explants had undergone disintegration.

Neurosecretory explants of the second and third groups produced nerve fibers which increased progressively in number from the end of the second week to the second or third month in vitro. Although the pattern of nerve fiber outgrowth as well as the length and density of fibers differed markedly among cultures, and also among explants in the same vessel, more vigorous and extensive nerve fiber production took place from NS cell aggregates or individual units in the third than in the second group. The results are based mainly on the study of the third group, in view of the interest which attaches to the unexpected finding of a trophic effect exerted by oocytes on NS cells, which are known to play an important role in growth and maturation of the same germ cells.

Figure 1 shows nerve fiber outgrowth from a six-cell NS cluster (Fig. 1c), a single cell (Fig. 1d), and an intact NS aggregate (Fig. 1e), that were cultured in vitro for various lengths of time, as indicated; Fig. 1f shows detail of a thin fiber bundle produced by a small group of NS cells cultured in vitro for 2 months. In all cultures illustrated, multiple explants of the proximal segments of ovarioles from late nymphal or adult specimens were present; each segment included 10 to 12 oocytes.

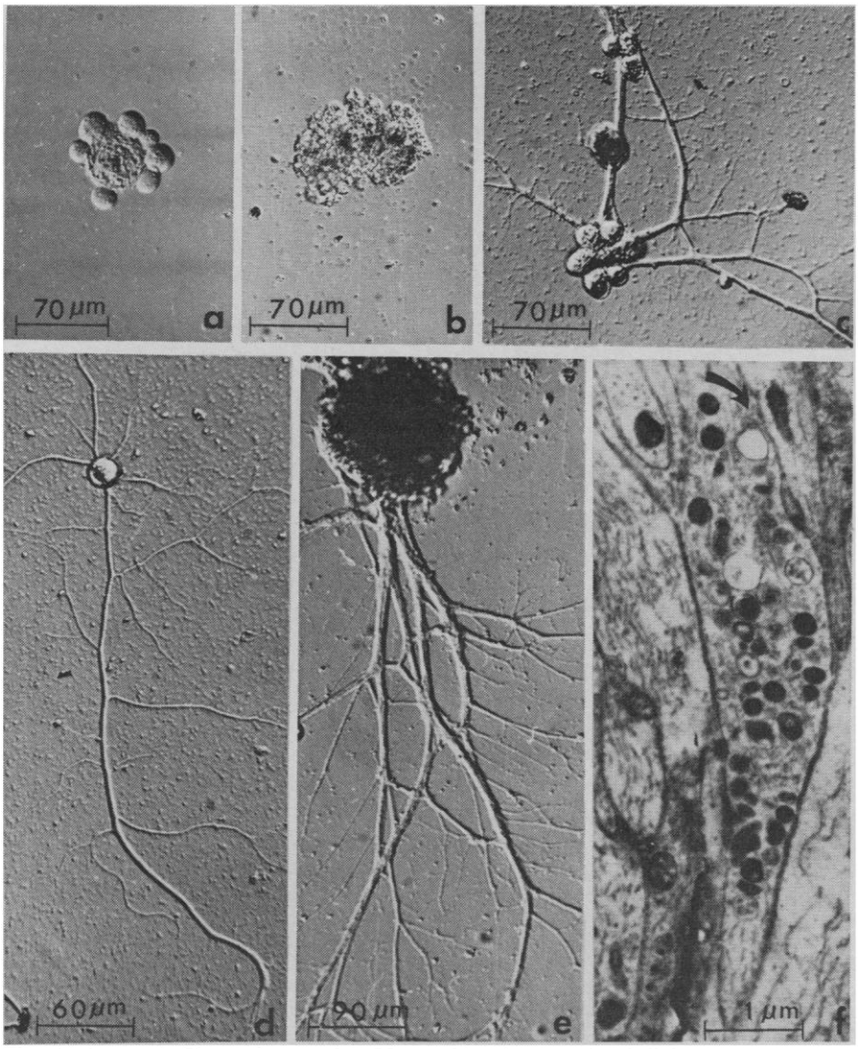

Fig. 1. (a to e) Photomicrographs (Nomarski microscope) of living neurosecretory cells from nymphal and adult cockroaches cultured in a chemically defined medium. (a) Seven small and medium cells and a large cell, possibly type C, from a sixth instar nymph immediately after dissection. (b and c) Compare neurosecretory cell clusters after 1 month in vitro from group 1 (b) and group 3 (c), the latter from a fifth instar nymph. (d) Neurosecretory cell from a tenth instar nymph after 3 weeks in vitro. (e) Neurosecretory aggregate from a sixth instar nymph after 1 month in vitro. (f) Electron micrograph of an axon (arrow) of a neurosecretory cell from a male adult specimen after 2 months in vitro. Note the large number of electron dense granules.

Nerve fiber outgrowth from large or small NS aggregates, as well as from single NS cells, compares in general features to that of fibers from nonneurosecretory neurons of the same species, as previously described (*1–4*). Hence, NS cells behave in vitro like conventional neurons in their ability to produce axons which, in turn, branch out individually or assemble in bundles that connect with adjacent NS or nonneurosecretory explants. In the experiments described here, NS explants positioned at some distance from each other invariably became interconnected by fiber bundles, as is the case with ganglionic and brain explants from the same species. Differences in the in vitro outgrowth of NS and conventional axons became apparent, however, at closer inspection. Axons emerging from NS explants are larger and have a more regular tubular contour than conventional nerve fibers. They give origin to short thornlike collaterals, which grow out at right angles from the stem fiber and may or may not join similar side branches from adjacent axons. Collaterals from nonneurosecretory fibers are, as a rule, much thinner and follow an undulating course. In two other ways, NS fibers differ from those produced by nonneurosecretory neurons: they enlarge rather than taper toward their distal end, and they are never enveloped by glial cells, which are always present in the migratory zone around embryonic brain and other ganglionic explants.

In order to see whether NS cells cultured in vitro for long periods still maintain the capacity for manufacturing and transporting the NS product, cells and axons from 3-month-old cultures were studied by light and electron microscopy. Whole mounts of cultures stained with performic acid Victoria blue show only a faint blue color in a few fibers, suggestive of the presence of NS material. More convincing evidence for the persistence of NS activity in vitro came from electron microscopic studies: electron dense granules in considerably large number were seen in most of the fibers branching out from intact NS groups and large or small cell aggregates. Electrophysiological studies show the existence of spontaneous unit electrical activity (*7*). This activity is characterized by a low rate of discharge (four per second or

less) and a continuous pattern of firing.

In conclusion, we believe that this in vitro technique may give valuable information on neuronal properties of NS cells and provide model systems for studying the nature of the synthetic, transport, release, and uptake processes associated with the NS phenomenon.

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Neuromuscular Junction in Myasthenia Gravis: Decreased Acetylcholine Receptors

Abstract. *The number of acetylcholine receptors was determined in the neuromuscular junctions of eight patients with typical myasthenia gravis and in five controls, by means of ^{125}I-labeled α-bungarotoxin binding. The junctional acetylcholine receptors were reduced in the myasthenic muscles as compared with the controls. This reduction in receptors may account for the defect in neuromuscular transmission in myasthenia gravis.*

Myasthenia gravis is a neuromuscular disorder manifested by muscular weakness and fatigability. Although the abnormality is thought to involve the neuromuscular junction, the precise nature of the defect is still uncertain. There has been considerable debate as to whether the nerve terminal or the postsynaptic region of muscle, or both, are affected by the disease process (*1*). We have examined the number and distribution of acetylcholine (ACh) receptors in a series of patients with myasthenia gravis and in a control group of patients.

Eight consecutive patients with typical myasthenia gravis, all of whom gave informed consent, were included in the study. In each case, the diagnosis was established by a typical history and physical findings of muscular weakness and fatigue. The diagnosis was confirmed by a decline of more than 15 percent in the amplitude of the third or fourth muscle action potential in response to a train of supramaximal stimuli applied at a rate of two to five per second (*2*). All eight patients showed improvement in muscle strength after injection of edrophonium hydrochloride (Tensilon) and after oral administration of pyridostigmine bromide (Mestinon). Six of the eight patients had been treated with anticholinesterase medication for a year or more, while the other two were not under treatment at the time of biopsy. One additional patient who manifested typical symptoms of myasthenia gravis 2 years earlier, but whose status had changed in the interim, was also included. Although still weak, he no longer had the clinical fatigability or the electrodiagnostic and pharmacological characteristics of myasthenia gravis. The control group consisted of a series of patients with a variety of problems ranging from aching pains to various neuromuscular disorders. Two of the control patients, H.H. and D.L., were considered normal.

Motor point biopsies were obtained by a modification of the method of Coers and Woolf (*3*). Anticholinesterase medication was withheld in all cases for a minimum of 10 hours prior to the biopsy, and when necessary, constant nursing care was given during this period. At open biopsy, the "motor point" was identified as the locus at which a visible twitch response could

Table 1. Acetylcholine receptor sites in control and myasthenic patients; S.E.M., standard error of the mean.

Patient			Diagnosis	Medication*	Tensilon response	Change in evoked potentials (%)	Receptor sites per junction† (Mean ± S.E.M.)	Relative grain density‡
Name	Age	Sex						
D.L.	50	M	Cerebral ischemia	Yes			$4.09 \times 10^7 \pm 0.50$	3.9
J.C.	19	M	Scapuloperoneal dystrophy	No			$3.63 \times 10^7 \pm 0.27$	
H.H.	55	M	Muscle aching	No			$5.72 \times 10^7 \pm 0.51$	3.6
J.R.	57	M	Lambert-Eaton syndrome	Yes		+ 200	$3.45 \times 10^7 \pm 0.35$	
J.A.R.	18	F	Facioscapulohumeral dystrophy	No			$2.19 \times 10^7 \pm 0.21$	
A.N.	38	F	Myasthenia gravis	None for 4 months	+	− 37	$1.15 \times 10^7 \pm 0.29$	1.0
T.T.	20	F	Myasthenia gravis	Yes	+	− 33	$0.41 \times 10^7 \pm 0.10$	1.4
T.H.	63	M	Myasthenia gravis	Yes	+	− 50	$0.54 \times 10^7 \pm 0.08$	1.2
V.F.	60	F	Myasthenia gravis	Yes	+	− 21	$0.68 \times 10^7 \pm 0.23$	
A.F.	54	F	Myasthenia gravis	Yes	+	− 15		1.5
F.S.	29	F	Myasthenia gravis	Yes	+	− 50		1.9
M.M.	41	F	Myasthenia gravis	No	+	− 30		1.8
E.D.	26	F	Myasthenia gravis	Yes	+	− 30		2.0
P.B.	76	M	Myasthenia gravis, in remission	Yes	−§ +¶	0‖ − 50	$3.56 \times 10^7 \pm 0.21$	

* Cholinesterase inhibitors were given as described in the text. † Number of α-bungarotoxin-binding sites determined with ^{125}I-labeled α-bungarotoxin. ‡ At neuromuscular junction from autoradiograms. The method of grading is described in the text. § April 1973. ‖ March 1973. ¶ June 1972.