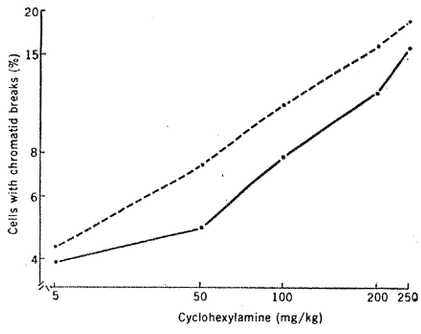

Fig. 1. The percentage of cells with breaks in relation to the concentration of cyclohexylamine. Hatched line, spermatogonial cells; solid line, bone marrow cells.

After orchidectomy, the spermatogonial cells were prepared by removing the tunica albuginea and releasing the seminiferous tubules in a petri dish. Hypotonic solution (0.25 percent trypsin in 0.055*M* KCl) was added to the free tubules, and the tubules were teased apart with forceps. After the tissue was exposed to the hypotonic solution for 40 minutes, it was fixed by adding an equal volume of a mixture of ethanol and acetic acid (3:1).

The seminiferous tubules were softened with acetic acid (45 percent) on the slide, the cover slip was added, and the tubules were squashed. The spermatogonial cells were immediately analyzed with the phase-contrast microscope.

The slides of bone marrow and spermatogonial tissue from each animal were read, and the percentage of breaks (percentage of cells with one or more breaks) was recorded. For each dose, at least 625 metaphase spreads from the bone marrow and 500 metaphase spreads from the spermatogonial cells were analyzed.

The mean percent breakage for the spermatogonial cells was 4.4, 7.6, 11.2, 16.2, and 19.2, whereas the mean percent breakage for the bone marrow cells was 4.0, 5.12, 8.0, 12.16, and 16.28 for 5, 50, 100, 200, and 250 mg/kg of cyclohexylamine, respectively (Fig. 1). The control values for the spermatogonial and bone marrow cells were 1.8 and 2.72 percent, respectively. A dose-response relationship between the concentration of cyclohexylamine and the percentage of breaks is evident. Analysis of means on the transformed data with the *t*-test indicates that the number of spermatogonial cells with one or more breaks was significant at the 95 percent confidence limit, when compared to the control, at a concentration of 1 mg/kg administered in five equal doses over a 5-day period. The analysis of bone marrow cells found significance (99 percent confidence limit) at 10 mg/kg but not at 1 mg/kg.

Single chromatid breaks predominated, with infrequent exchange figures. The single chromatid breaks produced by CHA are the type of abnormality believed most likely to have mutagenic and carcinogenic significance. If multiple breaks occur, the result will, in the majority of cases, consist of loss of viability, which is less important in terms of mutagenicity or carcinogenicity (*5*).

Significance of cytogenetic effects varies with the biological system and the concentration of chemicals used. Stone *et al.* have reported on the effect of cyclamate in leukocyte cultures in vitro (*6*), and Sax and Sax have reported on the effect of cyclamates in plant cells (*7*). The present study demonstrates the effect of a metabolite of cyclamate, cyclohexylamine, in both somatic and spermatogonial cells of rats. The significant effect on the rat spermatogonial cell occurs at the comparatively low concentration of 5 mg/kg when CHA (1 mg/kg) is administered daily for 5 days. The present investigation places CHA in a rather limited group of agents, such as certain viruses (*8*), chemicals (*9*), and irradiation (*10*), which produce chromosome abnormalities in vivo. Our observations indicate potential mutagenic, carcinogenic, or teratogenic effects that have yet to be determined.

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14 May 1969

Studying Neural Organization in Aplysia with the Scanning Electron Microscope

Abstract. *Preliminary attempts have been made to employ the scanning electron microscope in the mapping of a simple nervous system, the abdominal ganglion of* Aplysia californica. *Early results are encouraging: neuronal fibers have been identified and traced over relatively long distances from their cell bodies to structures tentatively identified as synapses and to structures tentatively identified as electrotonic connections.*

Because the abdominal ganglion of the mollusk *Aplysia californica* currently is the object of intensive, concerted electrophysiological investigations, because the neuronal cell bodies of this ganglion are large and easily identified, and because its cells and its neuropile have been studied extensively with the light microscope and the transmission electron microscope (*1, 2*), this ganglion was chosen as the object of our first attempted application of scanning electron microscopy to mapping neural networks. Subsequent work has shown that relatively clean neuronal surfaces can be obtained in a vertebrate retina (*3, 4*). If the same success were achieved in the *Aplysia* neuropile, then mapping should be possible with the scanning electron microscope. The problems in *Aplysia* are similar to those in the retina. In both cases cleavage must be achieved along neuronal boundaries, and extraneous interstitial material such as remnants of glial cells (in the ganglion) or pigment epithelium (in the retina) must be extracted sufficiently to allow a clear view of the neuronal topography. Rather drastic preparation techniques were used in the case of the retina. It was demonstrated, however, that although these techniques caused considerable shrinkage, they conserved proportions, integrity, and topography of both gross and fine structures (*3*). The same fixation, extraction, dehydration, and drying techniques were applied to the *Aplysia* ganglion.

The abdominal ganglion of an *Aplysia californica* was removed from the animal and mounted dorsal side up in a small dissecting dish filled with filtered seawater. An H-shaped incision was made through the sheath in the following manner: one rostral-caudal cut from right connective to branchial nerve, one rostral-caudal cut from left

connective to genital nerve, one lateral cut across the commissure. The sheath was pulled gently away from the dorsal surface of the ganglion and pinned to the side. This process caused some of the cell bodies to move aside, exposing the neuropile beneath. Next, the seawater was replaced with a 2 percent solution of glutaraldehyde in filtered seawater. After 48 hours, the preparation was placed in a 16 percent solution of glycerin in filtered seawater and left for another 48 hours. Next the preparation was rinsed gently with distilled water, then dehydrated with a series of ethanol solutions. With the pins still holding the ganglion in place and holding back the sheath, the alcohol was removed and the ganglion was exposed to air and allowed to dry. Throughout the entire fixation and dehydration procedure, the dissecting dish was covered except when the solutions were being changed. After drying, the preparation was removed carefully from the dissecting dish and mounted dorsal side up on an aluminum stub, then placed in a small vacuum system and coated by evaporation with a layer of aluminum approximately 100 to 300 Å thick.

The coated preparation was placed in the specimen chamber of a scanning electron microscope (*5*). The ganglion was mapped with a montage of micrographs each taken at × 57 magnification. Nerves and neuronal cell bodies previously identified with the light microscope were located in the montage, and general orientation was achieved. At × 560 magnification, a fiber from a 110-μm cell in the right hemiganglion, a fiber from a 45-μm cell in the left hemiganglion, a small fiber of unknown origin, and an apparent fiber bundle of unknown origin all were traced to the same small area in the region of the commissure, where they intersected (see Fig. 1). Two unusual features were visible at the intersection, a pair of rather fuzzy spheres and a flare of the 8-μm bundle. At this level of magnification, the structural details of the "fuzzy spheres" and the "flare" could not be resolved, and morphological identification was impossible.

Under higher magnification (× 5000) the fiber from the left hemiganglion cell proved to be a small bundle. By means of stereoscopic micrographs this bundle was traced back toward its origin. At a point approximately 240 μm from its cell body, the 2-μm fiber from the left hemiganglion apparently branched to form the bundle, which comprised six to ten 0.5-μm fibers. At the intersection, several of these small fibers from the left joined the 8-μm bundle and proceeded in the direction of the right hemiganglion. At least two of the small fibers from the left passed under the 8-μm bundle and were lost from view as they headed toward the fuzzy spheres. At high magnification the flare in the 8-μm bundle proved to be a mesh of more than 50 fibers ranging in size from 0.3 to 0.5 μm. Some of these small fibers disappeared into the densely packed material beneath the flare; some continued past the flare and converged on the fuzzy spheres.

At higher magnification the "fuzzy spheres" exhibited considerable structure (Figs. 2 and 3). Each sphere exhibited a laminar substrate toward which fibers converged from every direction and upon which fibers terminated with flattened knobs. The layers of the substrate were as thin as 0.3 μm (see Fig. 3). The converging fibers ranged from 0.3 to 0.5 μm in diameter, and the knobs ranged from 0.7 to 1.1 μm in diameter. Most of the knobs seemed to have five or six spots which were most firmly attached to the substrate (see knob in center of Fig. 3). These points of attachment were approximately 0.2 μm in diameter and seemed to be distributed around the perimeter of the knob. They were especially evident where fibers obviously had been under tension during the preparation and the knobs were pulled slightly away from the substrate. Occasionally, two adjacent knobs seemed to be attached to each other by the same kind of spot. Also occasionally, a knob was seen with a small fiber wrapped completely around its periphery, with the configuration of an Ω. On the exposed (dorsal) side of the structure, many of the converging fibers were broken a short distance from the substrate. The preparation was tilted to expose the fibers converging from the side and from beneath. Most of the fibers from the side and almost all of those from beneath were unbroken. Evidently many of the fibers on the dorsal side had emanated from tissue that was pulled aside during the preparation, and the fibers thus

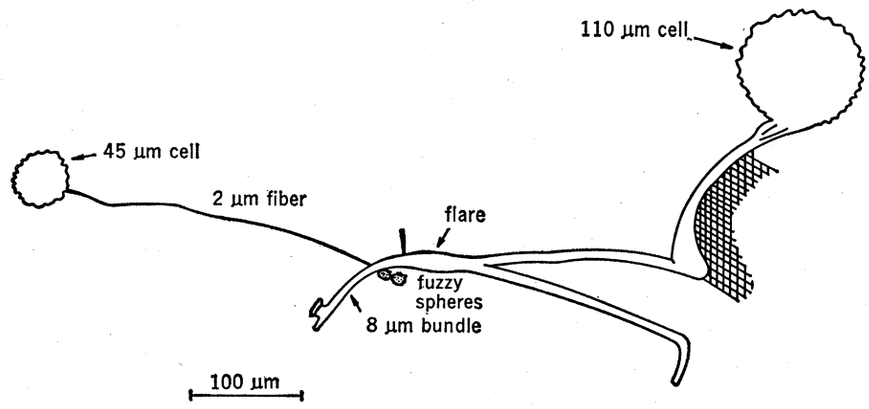


Fig. 1. Diagram summarizing the topography of a region near the commissure as inferred from stereoscopic micrographs of various magnifications. A 2-μm fiber was found emanating from a 45-μm cell body in the left hemiganglion, just medial from a large cell tentatively identified as L-11 (*1*). The fiber was traced for 290 μm into the region of the commissure, where it disappeared under an 8-μm fiber bundle. The 8-μm bundle was traced to the left of this intersection where it terminated in two broken ends, one with the characteristic collar of large broken fibers, the other with a blunt end characteristic of broken bundles of very small fibers (*7*). To the right of its intersection with the 2-μm fiber, the 8-μm bundle proceeded for 36 μm past two small, fuzzy, spherical structures. At this point it intersected a second 2-μm fiber, whose origin was obscured by interstitial materials. Almost immediately to the right of this intersection, the 8-μm fiber flared to approximately 11 μm, becoming slightly irregular in shape; 62 μm farther to the right it bifurcated, each branch appearing slightly flattened or ribbon-like, a shape that seems to be characteristic of fiber bundles in these preparations and that may be an artifact resulting from dehydration (*7*). One branch, apparently broken during preparation, extended to the right and caudally for 290 μm and tapered to a blunt end characteristic of bundles. The other branch proceeded to the right for 190 μm and became attached to densely packed material. Part or all of the branch then turned and proceeded rostrally for 100 μm, where it became completely detached from the substrate and had the appearance of a single 9-μm fiber. This fiber was traced to its emanation from a 110-μm cell body near the caudal extremity of the right hemiganglion. The junction between the cell body and the fiber exhibited evidence of the trophospongium reported by Coggeshall (*2*).

were broken. Apparently the attachments of the knobs to the laminar substrate were stronger than the fibers themselves; very few loose knobs were found on the ends of unbroken fibers, but many broken fibers were found connected to firmly attached knobs. In many cases the fibers exhibited a bifurcation and two broken ends. Thus it would appear that pairs of fibers had merged to form single fibers which then terminated with knobs. Examination of the fibers from beneath, however, revealed multiple anastomoses among intact fibers impinging on the laminar substrate (Fig. 4). This could account for the apparent merging of fibers on the dorsal side. Three abdominal-ganglion preparations of this type have been studied exhaustively under the scanning electron microscope. Two of the preparations exhibited clusters of knobs attached to laminar substrates. One cluster was found in the neuropile of each left rostral quarter ganglion, and two clusters (those described in the previous paragraph) were found near the commissure. The sizes and shapes of the knobs and the sizes of the fibers were the same in all of the four clusters. One of the clusters in the left rostral quarter ganglion exhibited many intact fibers terminating on the dorsal side but originating in the densely packed material on the ventral side of the cluster (Fig. 5). In this cluster, many knobs seemed to be attached to fibers that in turn terminated in knobs.

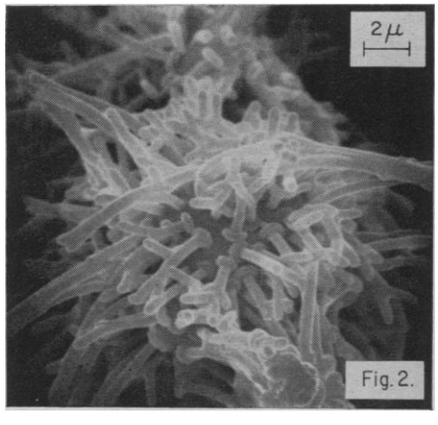
We *tentatively* conclude that the fibers in Figs. 2 through 5 are neuronal processes, that the knobs are synaptic knobs, and that the weblike structure in Fig. 4 represents cytoplasmic continuity and thus electrotonic connection among fibers. Even if the tentative conclusions are correct, there is no evidence as to whether or not the direct connections between knobs or the fibers wrapped around knobs are functionally significant (*6*), and there is no evidence as to whether the electrotonic connections are among fibers of the same cell or different cells.

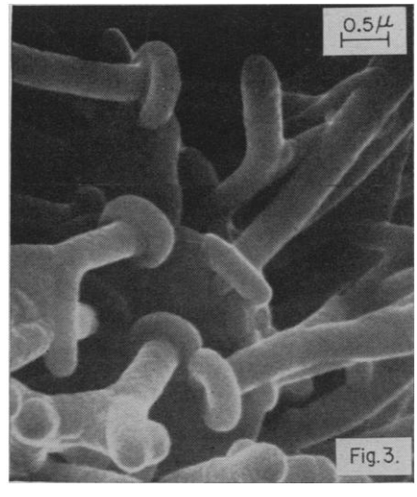
Viewing Figs. 2 through 5 and assuming for the moment that these conclusions are correct, one reasonably might inquire about the absence of space-filling material such as glial cells between the fibers. Two explanations seem plausible: (i) the glia, which have extraordinarily high water content, may have been extracted or preferentially reduced by the dehydration process; (ii) the glia may not be attached strongly to small fibers and may be removed in the process of dissection. Because preferential reduction should result in residual debris, which generally is found to be lacking around exposed fibers less than 1 μm in diameter in these preparations (*7*), the second explanation seems to be the more reasonable one. On cell bodies, where glia are known to be attached strongly, considerable debris is found (*7*). Some debris also is found on fibers larger than 1 μm.

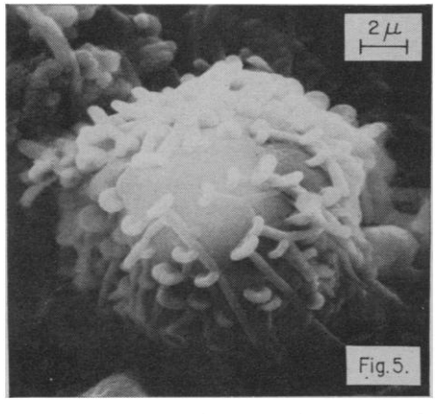
Because subsurface structures are not visible with the scanning electron microscope (*8*), the presence or absence of the vesicles and other structures found in *Aplysia* synapses (*2*) could not be determined. Furthermore, size and electron density of granules could not be used as clues to the identity of fibers (*2*). Our tentative conclusions therefore await substantiation by transmission electron microscopy.

If these tentative conclusions are correct, however, then the following picture emerges from the scanning-electron-micrographic evidence: in the abdominal ganglion of *Aplysia californica*, very small axonal branches exhibiting extensive anastomotic webs converge and terminate synaptically upon one another as well as upon laminar substrates. Furthermore, it is quite conceivable that the "laminar substrates" are in fact converging anastomotic webs whose meshes are filled with remnants of interstitial material, and that each cluster is composed entirely of fibers that are at once postsynaptic and presynaptic. On the other hand, the lamina (see Fig. 3) may be specialized receptive structures of purely postsynaptic neurons.

Although identifications of some microstructures observed during this study must await confirmation, the study itself has proved that the scanning electron microscope can be used to track fibers over long distances and to locate along those fibers structures







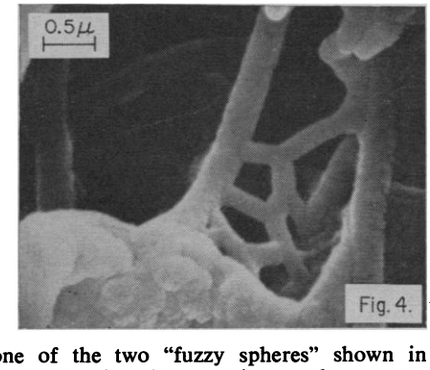


Fig. 2. Scanning electron micrograph of one of the two "fuzzy spheres" shown in Fig. 1, taken 30° from the vertical. Fig. 3. Scanning-electron-micrograph montage showing the knobs and the laminar substrate to which they attach, taken 43° from the vertical. Fig. 4. Scanning electron micrograph of fibers exhibiting multiple anastomoses, taken 34° from the vertical. Fig. 5. Scanning electron micrograph of a cluster of fibers and knobs in the left rostral quarter ganglion of another abdominal-ganglion preparation, taken 20° from the vertical.

that very well may be related to intraneuronal communication. Once located and tentatively identified these structures presumably can be isolated in ultrasections and examined with a transmission electron microscope. The scanning electron microscope in conjunction with the transmission microscope thus may become an extremely efficient tool in mapping the nervous system (*9*). This seems significant to us, since the lack of efficient mapping techniques is undoubtedly one of the greatest obstacles in neuroscience today.

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6. Employing transmission electron microscopy, Frazier *et al.* (*1*, pp. 1330–1331) observed vesicle-filled profiles contacting each other in addition to contacting the giant axon of cell R-2. They too were uncertain as to whether these contacts represent functional connections. They point out that if they are functional, they might account for the presynaptic interactions which were postulated by E. R. Kandel and L. Tauc [*J. Physiol.* **181**, 1 (1965)] for heterosynaptic facilitation in R-2. The connected knobs observed with the scanning electron microscope definitely are not attached to the giant axon of R-2, so they are not the same structures as those reported by Frazier *et al.* Furthermore, if all the fibers of the clusters are at once postsynaptic and presynaptic the consequences of the connections might be considerably more complicated than heterosynaptic facilitation.
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Puromycin: Effect on Memory of Mice When Injected with Various Cations

Abstract. *Puromycin dihydrochloride neutralized with bases of potassium, lithium, calcium, or magnesium fails to block expression of memory of maze learning in mice, unlike puromycin neutralized with NaOH. This failure may be due to cationic binding at anionic membrane sites with a resultant exclusion of sufficient peptidyl-puromycin to make it ineffective in blocking memory.*

Memory of maze learning in mice is blocked by puromycin dihydrochloride neutralized with NaOH and injected intracerebrally one or more days after the training experience (*1*); small intracerebral injections of saline made at least up to 2 months later remove the block and restore memory (*2*). Thus it is apparent that these injections of puromycin interfere with retrieval without substantially altering the process which maintains the basic memory trace. Several observations support our working hypothesis that this reversible suppression of the expression of memory is due to the interaction of peptidyl-puromycin with neuronal membranes, particularly synaptic membranes (*3*). Our report is a first step in testing the possibility that certain cations, through binding at anionic membrane sites, might interfere with the postulated interaction of peptidyl-puromycin and thus modify the effects of puromycin on memory. We have been concerned with expression of memory as affected by intracerebral injections of puromycin neutralized with a base of potassium, lithium, calcium, or magnesium and a comparison of the effects of these injections with those obtained with puromycin neutralized with NaOH.

The behavioral procedures have been described (*1*). Male and female Swiss Webster mice (5 to 10 months old) from our closed colony were used. The mice were trained in a Y maze with a grid floor through which intermittent shock, usually 40 volts, could be applied. The animal was placed in the stem of the Y maze. To avoid shock the mouse had to move into the correct arm within 5 seconds. If it entered the incorrect arm, it received shock until it moved to the correct arm. Mice with position preferences were trained to the opposite arm. Training was continued in one session of 10 to 20 minutes (usually about 15 trials) with an intertrial interval of a minute to a criterion of nine out of ten correct responses. Total errors were the sum of incorrect choices and of latencies greater than 5 seconds. The same procedure was used in tests for retention of memory of the training experience. These retention tests were given 5 to 10 days after intracerebral injection of the puromycin solutions, the longer intervals having been used for mice that recovered slowly from the effects of the injections. A final test of retention of relearning was given 2 weeks after the first retention test. Memory is evaluated in the retention tests in terms of the percentage savings of trials and errors. These percentages are calculated by subtracting the number of trials or errors to criterion in the retention tests from the number to criterion in training, dividing by the number in training, and multiplying by 100. Savings of 100 percent indicate perfect memory; zero savings, complete loss of memory.

In previous experiments with puromycin (Nutritional Biochemicals) we have neutralized the solution of the dihydrochloride to *p*H 6 with 1 equivalent of NaOH. In the present experiments, in addition to NaOH, we have used KOH, Li_2CO_3, $Ca(OH)_2$, or MgO for neutralization.

The injection technique has been described (*1*). All injections were bilateral and each had a volume of 12 μl. Bitemporal injections were used in mice treated 1 day after first learning (recent memory). For mice weighing 28 to 32 g each injection site received 90 μg of puromycin; for mice weighing 34 to 42 g and 43 to 48 g, each injection site received 120 and 150 μg, respectively. Combined bitemporal, biventricular, and bifrontal injections were used in the series of mice that were treated 9 days after first learning (longer-term memory). Each injection site received 30 μg of puromycin regardless of animal weight. The bitemporal injections with 120 μg of puromycin per injection contained a total of approximately 380 nmole of univalent cation or 190 nmole of divalent cation; those with 90 or 150 μg of puromycin per injection had proportionate amounts of cation. The combined bitemporal, biventricular, and