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DISTRIBUTION AND FUNCTION OF THE BRANCHIAL NERVE IN THE MUSSEL[1]

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The pumping of sea water by the mussel, *Mytilus edulis,* is accomplished primarily by the beating of cilia on the lateral epithelium of the gill filaments. The possibility that this activity is under the influence of the nervous system has been both suggested for its plausibility and dismissed for lack of evidence (Field, 1922; Gray, 1928; Lucas, 1931a, 1931b). More recently, however, work from this laboratory demonstrated that ciliary activity in the gill of *M. edulis* is depressed by transecting the branchial nerve (Aiello, 1960) and increased by electrical stimulation of the branchial nerve (Aiello and Guideri, 1964). In the latter paper it was mentioned that branches of the branchial nerve had been traced to the gill filaments. The present paper gives a detailed account of this work.

Material and Methods

Fresh mussels (*Mytilus edulis*) were purchased at a local fish market and kept in sea water at 5° C. until used. Isolated gill-nerve-ganglion preparations were made by removing one gill with its associated branchial nerve and visceral ganglion and a small piece of adductor muscle. Experiments were conducted in sea water at 22°–25° C., pH 7.7–7.9. Electrical stimulation (0.5 to 30 volts, 1 msec. biphasic pulse, 10 pulses per second for 5 to 300 seconds) was supplied by a Grass Model S4 stimulator and delivered through a pair of tungsten wires, one inserted into the adductor muscle and the other placed in the desired position on the ganglion, nerve or gill. The rate of beating of lateral cilia was determined by synchronization with stroboscopic light as described earlier (Aiello, 1960).

Histological sections were made from animals prepared *in toto* after removing the shell. The following preparations were made: fixed in 0.1% osmic acid in sea water and left unstained; fixed in 10% formalin in sea water and left unstained or stained with Harris hematoxylin and eosin; fixed in formalin-acetate and stained with dimethylaminobenzaldehyde (Glenner and Lillie, 1957); gold impregnation (Cole, 1946); silver impregnation (Rowell, 1963); silver impregnation after

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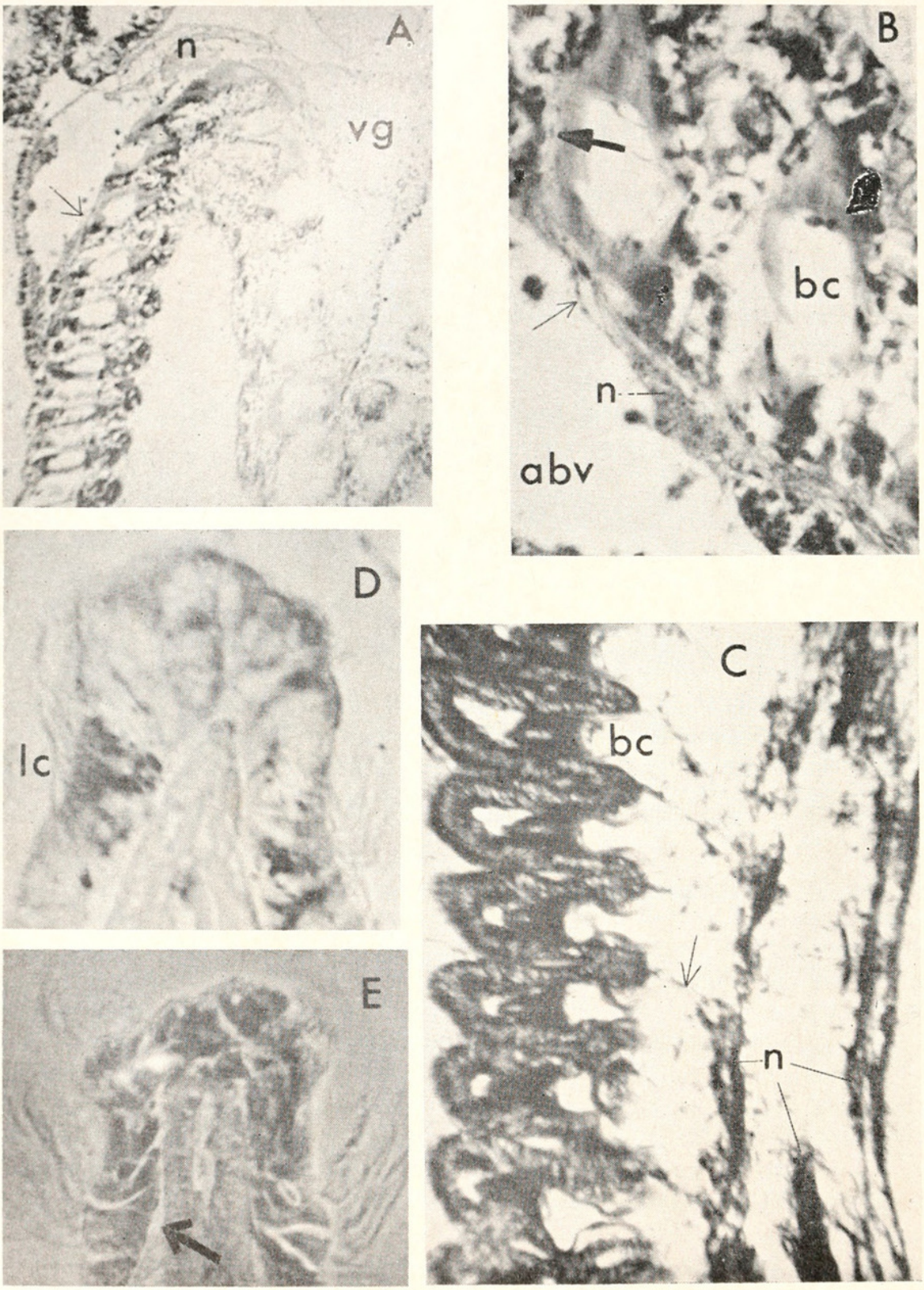

FIG. 1. Gill of *Mytilus edulis*. A. Cross-section of gill axis from whole animal fixed in osmium tetroxide; hematoxylin and eosin; 10 μ thick; 87 $\times$; shows visceral ganglion (vg) and branches of the branchial nerve (n); arrow indicates a nerve fiber entering a filament. B. Same preparation as A; 280 $\times$; small arrow indicates same nerve fiber as in A; large arrow indicates a continuation of the same fiber under the epithelium; nerve bundle (n) is inside the afferent branchial vein (abv) but separated from the blood channel (bc) by the abfrontal end of the filament. C. Horizontal section through the gill at about the level of the nerves indicated by n in B above; Cole gold chloride stain; 10 μ thick; 332 $\times$; one of a group of nerve bundles (n)

treatment with copper sulfate (Betchaku, 1960). Sections were cut at 5 to 40 μ thickness in the following planes relative to the intact animal and gill axis: cross, giving longitudinal sections of the gill filaments; horizontal (frontal), giving cross-sections of the gill filaments; and sagittal, giving frontal sections of the gill filaments.

Whole mounts and teased preparations were left unstained or treated with gold, hematoxylin and eosin, or 0.2% methylene blue in sea water. Whole living animals were also studied after injecting the visceral mass or the afferent branchial vein with 0.2% methylene blue in sea water or Pelikan brand India ink diluted with nine volumes of sea water.

Observations were made under bright field, phase contrast and epi-illumination, depending on the specimen.

Results

Distribution of the branchial nerve

Careful observation, aided by fine dissection, of the ventral aspect of the soft parts of the mussel under a dissecting microscope at 30 × magnification confirms in general the description of the branchial nerve given by Field (1922; p. 172): "The branchial nerve arises from the outer posterior side of the visceral ganglion and runs obliquely downward and backward to the base of the gills, which it follows to the posterior extremity. Throughout its course, but more so at its beginning, it gives off a great number of very fine fibrils that run in a mass anteriorly along the axis of the gills." In addition a great number of fibers arise directly from the visceral ganglion, and these can be seen clearly silhouetted against the underlying blood vessels when the latter are filled with India ink injected in the afferent branchial vein. The anteriorly directed fibers do not continue to run in a mass but distribute themselves as bundles which run in close association with the afferent branchial vein.

Although the visceral ganglion and branchial nerve lie on the median side of the gill axis, some branches of the branchial nerve pass through the supporting tissue above the blood vessel to supply the lateral side of the gill. This distribution is evident in sections of fixed material (Fig. 1 A) and in the published works of Lucas (1931b, his Plate I, Fig. 3) and Field (1922, his Figs. 147 and 153), although Field did not identify branches of the branchial nerve as such. In our material each individual bundle remained fairly intact until it reached a particular part of the gill axis, whereupon it distributed its fibers to a series of adjacent filaments. Such a point is indicated by the arrow in Figure 1 A. This nerve bundle lies within the afferent branchial vein and sends fibers into the filament between the chitinous rod and the ciliated epithelium. This is seen more clearly in Figure 1 B which is an enlargement of the area around the arrow in Figure 1 A. The heavy arrow in Figure 1 B points to a continuation of a nerve fiber under the

sends a few fibers, marked by the arrow, to a filament; the blood channel (bc) opens directly into the afferent branchial vein. D. Cross-section of a single filament; Betchaku copper-silver stain; 10 μ thick, 921 ×; lateral cells (lc) show intense staining; nerve fibers appear in cross-section as lighter-stained dots at base of lateral cells. E. Cross-section of single filament; Glenner and Lillie DMAB-fuchsin stain; 5 μ thick; 883 ×; arrow indicates two nerve fibers at the base of the lateral cells.

ciliated epithelium, which at this level is not differentiated into special tracts. The lining of the blood vessel seems to be discontinuous in this region and the nerve labeled *n* in Figure 1 B distributes itself to the adjacent filaments. Ventral to this region a continuous layer of connective tissue separates the other nerve bundle from the filaments. This bundle, which appears much darker and lies below and to the right of the one marked *n* in Figure 1 B, was followed anteriorly in serial sections for about 0.5 mm. and found to move up toward the gill axis and enter the filaments in a similar manner. A horizontal section through this part of the gill confirms these observations (Fig. 1 C). In all preparations nerve fibers were detected in cross-sections of gill filaments as groups of circular or oval structures lying at the base of the lateral cells (Fig. 1 E). They were sometimes associated with branching structures seen only with gold or silver staining (Fig. 1 D). Single fibers seem to be present under other parts of the epithelium but because they do not occur there in groups they could not be traced with certainty back to the branchial nerve. Fibers under the lateral cells appear to course the length of the filament; usually three or four individual fibers could be counted. Branching was most frequently observed immediately after the nerve first entered the filament. Although one would expect to see individual fibers as small dots in every cross-section, we could find silver-staining structures under the lateral cells in only about 20% of the sections. In longitudinal sections of the filaments (frontal sections of the gill) in which the cut happened to be made through the base of the lateral cells, one would expect to see the fiber as a thin black line underneath the lateral cells. Actually, fibers were easily identifiable as such only where they first entered the filaments. Further along in the filament we most frequently saw a string of granules. Our interpretation of this pattern is that they are vesicles of 0.25–0.4 μ diameter along a fiber whose diameter is about the same as the resolving power of the microscope (0.21 μ). The fact that this structure is not merely a basement membrane was especially clear in those sections in which the cells were torn off the chitinous rod, leaving the nerve fibers in a clear space.

Fine structure and staining affinities of the branchial nerve

Grossly, the branchial nerve and visceral ganglion appear opaque white. After osmium fixation, nerve fibers, connective tissue fibers, cilia and many other structures appear black but there is no evidence of a myelin sheath comparable to that of vertebrate myelinated nerve. After formalin fixation and staining with Harris hematoxylin and eosin, the nerve cell cytoplasm stains faint blue, the nucleus darker blue, the nucleolus lighter blue and the fibers stain hardly at all. In both preparations the nerve fiber appears to be surrounded by an unstained space and a limiting membrane. After staining for indoles with dimethylaminobenzaldehyde the ganglion and the nerve appear faint blue. A blue tinge is also evident in nerve fibers under the lateral epithelium cells which themselves are deeper blue or blue-gray, especially in their basal half. Large blue granules are also seen in areas having no identifiable innervation.

In unstained formalin sections the nerves are colorless. Under phase contrast a nerve fiber in cross-section appears as a dark spot in a clear circle with a limiting membrane. Methylene blue stained the nerve after about one hour but was not

found to be very useful because it stained most other cells more intensely. Individual fibers were not visibly stained. With gold the fibers in the nerve bundles appeared brown or black, depending on the depth of staining. In some sections stained with silver after copper treatment, the fibers under the lateral cells and associated structures lying in or between the basal half of these cells were stained black, in clear distinction to other structures which were stained various depths of brown. A second black-staining structure was situated around the nucleus of the lateral cell. It was not connected to any nerve and may have been Golgi material.

Conduction pathways

Electrical stimulation of various parts of the ganglion and of various nerve bundles in the gill axis caused cilio-excitation of particular filaments. In these experiments the electrode was placed on the surface of the ganglion or inserted into one of the bundles in the supporting tissue of the gill axis. The stimulus was applied and the voltage slowly increased until some part of the gill was found to have rapidly beating cilia. The stimulus was then discontinued and the ciliary beating rate decreased. This was repeated a few times to make sure that the applied stimulus was the cause of the cilio-excitation in that area of the gill. Using the lowest effective voltage, cilia on only a few filaments within 2 or 3 mm. of each other were excited. By placing the electrode on different parts of the ganglion it was possible to affect different small areas of the gill. Stimulation of branches of the branchial nerve affected only filaments directly below or anterior to the point of stimulation. When the reflected (ascending) lamella was separated from the main (descending) lamella at the ventral end but left connected by the interlamellar blood vessels, stimulation of the nerve to that filament resulted in excitation of the cilia on the main lamella but not those on the reflected lamella. This indicates that cilio-excitatory fibers do not pass over to the reflected lamella through this connection but instead continue down the main lamella and up the reflected lamella under the lateral epithelium. These interlamellar connections must have their own nerve supply which is distinct from the cilio-excitatory nerves, because during these experiments contraction of muscles lining the interlamellar blood vessels usually, but not always, occurred in those filaments showing cilio-excitation. Generally, 20 to 30 volts were required to elicit muscle contraction, whereas cilio-excitation could be obtained with 5 to 10 volts. That this muscle contraction is not due merely to current spread is indicated by the fact that the electrodes must be placed in just the right position at the base of the gill to get the effect. Stimulation of the visceral ganglion always caused a gross movement of the entire gill of a few millimeters, due to contraction of these muscles.

Further evidence that the sub-epithelial nerve is the only pathway for cilio-excitation is provided by the observation that damage to the surface of the lateral cells prevents their cilia from beating but does not prevent nerve stimulation from simultaneously activating the cilia on lateral cells both central and peripheral to the damaged area. However, damage which is deep enough to remove all cellular material, leaving a bare strip of chitinous rod, does prevent nerve stimulation from affecting lateral cilia peripheral to the damage. These cells are still stimulated by exogenous 5-hydroxytryptamine.

Discussion

Distribution and function of the branchial nerve

The data presented above give the morphological basis for the nervous control of ciliary activity and movements of the gill filaments. The latter system was described by Setna (1930) in *Pecten* sp., in which he found fibers in both the abfrontal and frontal region of the gill. Those in the abfrontal region innervated the muscles of the interlamellar connections. Those in the frontal region were not traced to terminations and the question of ciliary control was not discussed. In our work we could not trace fibers to the interlamellar connections but could occasionally activate them independently of cilia, suggesting a separate nerve. The function of these muscles is not known but it has been observed that in addition to moving the filament slightly, they cause accordion-like shortening of the interlamellar blood vessel. This causes the blood to shoot up the filament and, even though it washes back again during relaxation, the to-and-fro movement might increase the efficiency of an otherwise very sluggish branchial circulation. The same rhythmic movement occurs spontaneously in the intact animal.

Nervous stimulation also influences the frontal and latero-frontal cilia. These are concerned mainly with feeding, and would presumably be most active when the number of food particles impinging on the frontal surface of the gill was greatest. Because this factor is not directly related to the rate of lateral ciliary beating it would not necessarily be to the animal's advantage to activate all cilia concurrently. In our experience stimulation of the lateral cilia was accompanied by an apparent increase in the beating of the lateral and latero-frontal cilia, but since the rate of these cilia was not measured we cannot be sure of quantitative relationships. When the branchial nerve is cut, the lateral cilia stop first, followed by the latero-frontal cilia (Aiello, 1960). The frontal cilia continue to beat, but more slowly. There is, therefore, no evidence yet for the independent control of different tracts of ciliated epithelium on the same filament.

There is morphological and physiological evidence for independent control of filaments. About 10 adjacent filaments are innervated by fibers from one bundle. Possible advantages to the mussel are: the flow of water over particular respiratory plicae could be adjusted; some filaments could pump water while others rested; frontal cilia on those filaments being struck by food particles could be selectively accelerated. Whether or not the animal does these things is not known but it has been observed by ourselves and others (Babak, 1913; Lucas, 1931a) that lateral cilia on various filaments periodically stop for a few seconds and then start beating again. Particle transport is also influenced by mechanical factors (Gosselin and O'Hare, 1961) and one of these, interfilamentar spacing, is under nervous control in lamellibranchs. This was reported for *Ostrea virginica* (Jørgensen, 1955) but the problem has not been studied in *Mytilus* sp. Our experiment, in which low voltage stimulation of particular spots on the surface of the visceral ganglion caused cilio-excitation on individual filaments, indicates that the distribution of the postganglionic filbers permits such discrete control. Whether or not presynaptic arrangements permit such control in nature was not determined. The question of intra-ganglionic connections in *M. edulis* visceral ganglion was studied by Rawitz (1887), who concluded that fibers from the peripherally placed cell bodies

form a nerve net in the center of the ganglion. This arrangement would be likely to give mass discharges of cilio-excitation. Fortuyn (1920) came to a contrary conclusion, which is more compatible with our physiological data, namely, that the central mass of fibers constitutes a neuropile with few if any functional connections. He also described the localization within the ganglion of association neurons. Freidenfelt (1897) came to a similar conclusion regarding fibers in the central mass of the visceral ganglion of *Anodonta* sp. In addition, Freidenfelt described association neurons at the junction of the posterior pallial and branchial nerves near their emergence from the visceral ganglion, and pointed out their close association with the overlying osphradium. He postulated that the osphradium and the root of the branchial nerve constituted an independent sensory-motor center. He was referring to motor fibers to smooth muscle in the subfilamentar structure of *Anodonta* gill, but the same reasoning could apply to cilio-excitatory fibers to the epithelium in *M. edulis*. There is no evidence in the literature or in our data for sensory fibers in the gill filaments.

Innervation of the ciliated cell

We are uncertain of the detailed structure of the innervation apparatus. We only observe that the nerve fibers run between the base of the ciliated cells and the supporting chitinous rod. With silver stain, there seems to be a basket-like structure in the basal half of the lateral cell which appears to be either connected to or closely apposed to the underlying fiber. In view of the relationship between endogenous 5-hydroxytryptamine and cilio-excitation through branchial nerve stimulation (Aiello, 1965), it is interesting that the lower half of the lateral cell and the branchial nerve throughout its length stain faint blue with dimethylaminobenzaldehyde, thereby suggesting the presence of 5-hydroxytryptamine. Presumably, nerve impulses release 5-hydroxytryptamine from either the nerve fiber or the ciliated cell and this in turn stimulates ciliary activity.

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Summary

1. Using standard dissecting and histological techniques, branchial nerve fibers have been traced from the visceral ganglion to the ciliated epithelium of the gill in the mussel, *Mytilus edulis*.

2. The pattern of activation of cilia obtained by electrical stimulation of the visceral ganglion and branchial nerve indicates that individual filaments or small groups of adjacent filaments are independently innervated, allowing for discrete control of ciliary activity on different parts of the gill.

3. The relationship of this innervation to the known functions of the gill is discussed.

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