Serotonin localization in the gills of the freshwater mussel, *Ligumia subrostrata*

T. H. DIETZ, W. L. STEFFENS,[1] W. T. KAYS, AND H. SILVERMAN
Department of Zoology and Physiology, Louisiana State University, Baton Rouge, LA, U.S.A. 70803

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Exogenous serotonin stimulates sodium influx in Unionid mussels and in isolated gill tissue, which suggests that sodium transport in mussels is regulated by serotonin. The present study was an attempt to localize endogenous serotonin within the gill of *Ligumia subrostrata*. Examination of the gills at the light and electron microscopic levels revealed extensive branchial nerve tracts lying anterior–posterior between the adjacent gill filaments of each lamellar surface. In addition, there was a smaller nerve tract lying along the base of the water channel epithelium. These nerve tracts have a distinctive organization (containing nerve fibers and peripheral glial interstitial cells with gliosomes) and are capable of incorporating the vital dye procion yellow. Serotonin fluorescence following exposure to formaldehyde vapor was limited to the area of the nerve tracts. The identification of serotonergic neurons was further confirmed by light microscopic autoradiographs displaying silver grains localized principally over nerve tracts in gills exposed to tritiated serotonin. These morphologic data are consistent with the hypothesis, generated by previous physiologic data, that Na transport in freshwater Unionids is regulated by a neural serotonergic (cAMP-mediated) mechanism.

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L'addition de sérotonine exogène au milieu stimule l'influx de sodium chez les Unionidae et dans les tissus branchiaux isolés, ce qui permet de croire que le transport du sodium est sous le contrôle de la sérotonine chez les moules. Nous avons essayé, au cours de cette étude, de localiser la sérotonine endogène dans les branchies de *Ligumia subrostrata*. L'examen des branchies aux microscopes photonique et électronique a révélé la présence d'un important réseau de nerfs branchiaux dirigés antéro-postérieurement entre les filaments branchiaux adjacents de chaque surface lamellaire. De plus, un tractus nerveux plus petit longe la base de l'épithélium du canal où circule l'eau. Ces tractus nerveux ont une organisation caractéristique (ils contiennent des fibres nerveuses et des cellules gliales interstitielles périphériques contenant des gliosomes) et se colorent au jaune procion. A la suite d'une exposition à des vapeurs de formaldéhyde, la fluorescence caractéristique de la sérotonine n'apparaît que dans les tractus nerveux. La présence de neurones sérotonergiques a d'ailleurs été confirmée par l'examen d'autoradiographies au microscope photonique: des granules argentés sont apparus principalement le long des tractus nerveux des branchies exposées à la sérotonine tritiée. Ces résultats confirment l'hypothèse, élaborée déjà à la suite d'études physiologiques, selon laquelle le transport du Na chez les Unionidae d'eau douce est contrôlé par un mécanisme nerveux sérotonergique (par l'intermédiaire du cAMP).

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Introduction

Serotonin and other biogenic amines are effective stimulators of Na transport when injected into freshwater mussels (Dietz et al. 1982; Scheide and Dietz 1983). However, only serotonin causes a dose-dependent stimulation of sodium influx in isolated gills (Dietz and Graves 1981; Dietz et al. 1982). We have noted that dibutyryl cAMP and theophylline also stimulate Na transport in mussels (Graves and Dietz 1982; Dietz and Graves 1981; Dietz et al. 1982). Recently, we have reported the presence of a serotonin- and dopamine-stimulated adenylate cyclase in the gill tissue of freshwater bivalves (Scheide and Dietz 1983, 1984). We have previously reported a substantial quantity of serotonin in the gill tissue of freshwater mussels (Dietz et al. 1982), and it is conceivable that serotonin is a regulatory neurotransmitter in freshwater bivalves.

Serotonergic motor neurons apparently control gill ciliary activity and synchrony in marine bivalves (Wright 1979; Catapane et al. 1979). Previous studies have described the innervation pattern in the gills of the marine mollusc, *Mytilus edulis* (Paparo 1972; Aiello 1979; Catapane 1982). Both serotonin and catecholamines have been localized in these nerves by the formaldehyde-induced fluorescence method of Falck (1962) or an aluminum-enhanced modification of that method (Catapane 1982). When high performance liquid chromatography (HPLC) separation techniques are used, serotonin and dopamine are observed in the gills of *M. californianus* in approximately equal amounts (Smith and Sleet 1982).

In this report we describe the distribution and localization of serotonin in extensive nerve tracts of freshwater mussel demibranchs. These data provide morphological evidence for the existence of serotonin neurons in the region of the epithelial cells responsible for transepithelial sodium transport.

Methods

Male *Ligumia subrostrata* were collected from ponds near Baton Rouge, LA, and stored in aerated artificial pondwater (0.5 m*M* NaCl, 0.4 m*M* $CaCl_2$, 0.2 m*M* $NaHCO_3$, 0.05 m*M* KCl) for a week before use. The animals were maintained at room temperature (22–25°C) on laboratory photoperiod.

Gills from mussels acclimated to pondwater were prepared for ultrastructural observation in two ways. One method involved fixing small pieces of tissue with 2.5% glutaraldehyde in 20 m*M* sodium cacodylate buffer. For the second method, we used no buffer but neutralized the fixative to pH 7.8 with NH_4OH (0.1 m*M* EDTA was added to the fixative to chelate the endogenous calcium). The fixative was prepared immediately before use. The gill was postfixed with 1% osmium tetroxide, dehydrated in EtOH and embedded in L.R. White resin. Ultrathin sections were examined by conventional transmission electron microscopy (TEM) using a JEOL 100CX operating at 80 keV. Sections of 1 μm thickness were examined by scanning transmission electron microscopy (STEM) using the same instrument operating at 100 keV.

The nerve tracts in each mussel gill (demibranch) were localized

[1]Current address: Department of Anatomy and Radiology, University of Georgia, Athens, GA, U.S.A.

using the vital stain procion yellow M-4RF (Holl 1981). The mussels were injected with 0.2–0.6 mL/100 g body weight of 49 m*M* procion yellow and returned to pondwater for 1 h. The gills were removed and fixed in 10% formalin saturated with $CaCO_3$, pH 6.5. The tissue was refrigerated (2°C) and the formalin was replaced after 3 h. Control animals were bathed in 49 m*M* procion yellow in pondwater for 1 h before the gills were removed and the tissue was fixed in formalin. The gills were embedded in Paraplast, and 20-μm sections were viewed microscopically. In addition, unfixed whole mounts of the gills were viewed with a fluorescence microscope. A Leitz Ortholux II microscope was used for fluorescence studies. The microscope was equipped with a mercury-vapor lamp for epi-illumination and with a Leitz H2 filter block (390–490 nm; mirror 510 nm; suppression 515 nm).

Total serotonin content of the gill tissue and blood of mussels were determined using the ninhydrin fluorescence method of Snyder et al. (1965) as previously described (Dietz et al. 1982). By adding radioactive serotonin to a series of samples we determined that our recovery of serotonin was 80 ± 4%.

To localize serotonin, gill tissue was removed from mussels acclimated to pondwater and frozen in freon 12 followed by liquid nitrogen. The frozen tissue was freeze-dried overnight and then stored at −20°C over silica gel. Formaldehyde-induced monoamine fluorescence was developed according to the method of Falck (1962). The gills were placed in a sealed vessel containing formaldehyde gas (produced by heating paraformaldehyde to 80°C) for 2 h. The vessel contained dilute sulfuric acid to give 65% relative humidity at 80°C. The treated tissue was embedded in Paraplast, sectioned at 20 μm, mounted on slides, and covered with nonfluorescing immersion oil and a cover slip. The sections were viewed with a Leitz Ortholux II microscope as described above.

Illumination of the gill tissue with ultraviolet light (excitation 390–490 nm) caused the endogenous serotonin condensed with formaldehyde to fluoresce with a characteristic yellow color. Reserpine treatment causes the depletion of endogenous serotonin from nervous tissue. Mussels acclimated to pondwater were injected in the foot with 0.4 mg reserpine (in 2 μL chloroform) 2 days before removal of the gills for fluorescence observation.

Serotonin accumulation by nerves in the gills was determined by autoradiography. Gills from mussels acclimated to pondwater were removed and incubated in pondwater containing tracer amounts (10^{-12} *M*) of [^{3}H]serotonin (2 μCi/mL; 1 μCi = 37 kBq). To half of the gills additional serotonin was added (0.1 m*M* final concentration) to decrease the specific activity of the serotonin. After a 17-min exposure to the isotope, the gills were rinsed in 100 mL pondwater for 45 min to remove adsorbed isotope, and then fixed with Bouin's; the fixative was changed after 2 h. The gills were embedded in Paraplast, sectioned at 7 μm, and coated with Ilford L-4 emulsion. After a 4-week exposure (2°C), the slides were developed.

Results

The comparative morphology of lamellibranch gills has been detailed in the descriptive work of Ridewood (1903). Briefly, individual gill filaments making up the lamella of a demibranch are covered by a heterogeneous epithelium (Fig. 1A). Frontal cells (on the apical surface of a filament) are ciliated and have a microvillar surface. Four rows of lateral cells with longer cilia are located on the lateral surface of the gill filament. These cells mark the termination of the ciliated epithelium lining the filament. The lateral ciliated cells also tend to be more electron dense than the other epithelial cells of the gill filament (Fig. 1B). The gill filaments are supported by a pair of calcium-rich chitinous rods oriented dorso-ventrally in a discontinuous fashion (Ridewood 1903). The rods taper at their discontinuities, which repeat every 140–150 μm. These rods are surrounded by a periodic acid – Schiff (PAS) positive glycoprotein-like material (Silverman et al. 1983).

Innervation of these gills is less clearly known and is ascribed to the major branchial nerves located in the suprabranchial region. These major nerves radiate branches ventrally into the gill demibranchs. Each epithelial lamella of a demibranch is supplied by its own group of major dorsal–ventral branches which occur at 1-mm intervals of approximately 20 filaments. The dorsal–ventral nerve branch sends anterior–posterior nerve bundles between adjacent gill filaments every 140–150 μm (Fig. 2). These anterior–posterior branchial nerve bundles pass between the discontinuities in the paired chitinous rods described above. Nerve fibers from these anterior–posterior bundles course into the gill filaments and innervate the heterogeneous epithelium of the lamella (Fig. 1A).

The neural organization described above has been established and confirmed using procion yellow vital staining and electron microscopy. Gross observation of gill whole mounts by fluorescence microscopy after injection of procion yellow into the blood indicates 140–150 μm periodicity of fluorescence lying between discontinuities in chitinous rods (Fig. 3). Anterior–posterior cross section of the gill indicates that the procion yellow fluorescence is located in specific tracts at the base of the gill filaments (Fig. 4). Fluorescence can be traced from the interfilament tracts into individual filaments ending most often near the lateral ciliated epithelial cells (Fig. 4). Numerous cell bodies within the gill tissue accumulate procion yellow and unpublished data suggest that many of these structures contain dopamine. There is little or no staining of any other structures in the demibranch.

Observation of these same regions by electron microscopy reveals longitudinal tracts of nerves (Fig. 5) containing multiple nerve fibers. The nerve tracts are associated with several specific cell types. Glial interstitial cells (Hemming et al. 1983; Vitellaro-Zuccarello et al. 1983) containing large electron-dense oval inclusions, or gliosomes, (0.5–1.2 μm) are frequent and are intimately associated with the peripheral edges of the nerve fascicles (Fig. 5). Obliquely striated muscles also occur flanking the periphery of the nerve bundles. Associated with the anterior–posterior nerve tracts in these gills are large accumulations (25% of gill dry weight; Silverman et al. 1983; Steffens et al. 1985) of extracellular calcium concretions (Fig. 5*b*). Most of the concretions have been removed in this study by the acidic conditions of fixation (osmic acid or Bouin's), to allow observation of the neural network.

Widely distributed along the nerve fibers are numerous varicosities containing accumulations of 170-nm membrane-bound electron-dense vesicles. Neurofilaments and neurotubules are common in individual nerve fibers, and infrequently, mitochondria also are observed. These neurons often are seen in cross section, underlying the lamellar epithelium, particularly in the area of the lateral long-ciliated epithelial cells (Fig. 6). Although a few synapses were seen it was difficult to demonstrate more than the near presence of neuron cross sections under most epithelial cells. However, these epithelial cells do extend basal processes which surround nerve fiber bundles passing under the epithelial surface. Smaller nerve fiber tracts also were identified in the proximity of the water channel epithelium. Water channel epithelial cells also show extended basal processes surrounding neuron bundles, and occasionally synaptic connections were observed with the small but characteristic pre- and post-junctional electron-dense regions (Fig. 7).

Also of interest were apparent intramural ganglionic cell bodies located in the gill tissue. These were readily apparent in

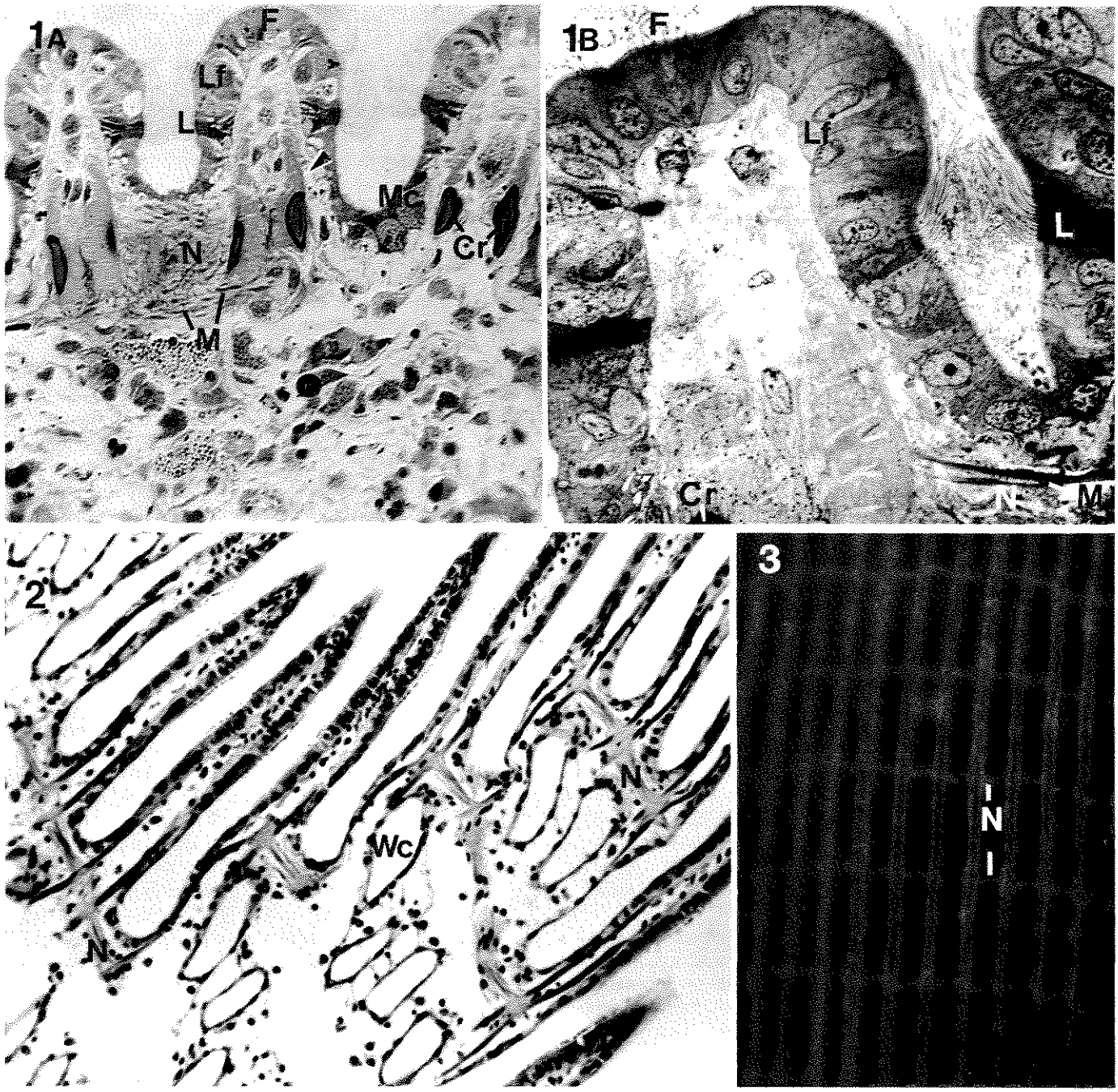


FIG. 1. (A) Cross section of a lateral demibranch of *L. subrostrata* embedded in L.R. White plastic and stained with paragon. Three types of ciliated epithelial cells on the gill filament are evident: frontal (F), latero-frontal (Lf), and lateral (L). The epithelial cells at the base of the filament have microvilli (but not cilia) with mucous cells (Mc) interspersed. Each filament contains a PAS-positive matrix (arrow) surrounding the area of the chitinous rod (Cr). In the region of the Cr discontinuity the fibrous material between gill filaments contains extensive neuronal (N) processes and darker staining striated muscle (M). 300×. (B) A scanning transmission electron micrograph (STEM) of a gill filament demonstrating the heterogeneous epithelium; the labels are the same as for Fig. 1A. 1000×. FIG. 2. Tangential section of a lateral demibranch fixed in Bouin's and stained with hematoxylin and eosin. The labels are the same as for Fig. 1A. The water canal (Wc) is the conduit for pondwater entering the central water channel 170×. Fig. 3. Fluorescent micrograph of a gill whole mount from *L. subrostrata* 1 h after procion yellow had been injected into the heart. A portion of the gill was photographed showing the anterior–posterior nerve tracts (N) connecting adjacent gill filaments at the point of chitinous rod discontinuity (110×).

procion yellow preparations (cf. Fig. 4) and some were confirmed by their positive reaction to serotonin fluorescence as described below. The presence of these cell bodies in molluscs has been reported previously (Baumgarten et al. 1973; Weber and Grosmann 1977; Vitellaro-Zuccarello et al. 1983).

Gill tissue of freshwater mussels contains large amounts of serotonin as detected by ninhydrin fluorescence (Snyder et al. 1965). The quantity of serotonin is 1.8 ± 0.2 μg/g wet tissue (n = 7) in gills from animals acclimated to pondwater and there is no difference in the serotonin content in gills from salt-depleted mussels (1.8 ± 0.1 μg/g wet gill tissue; n = 12). Ninhydrin fluorescence of extracted blood indicates that the

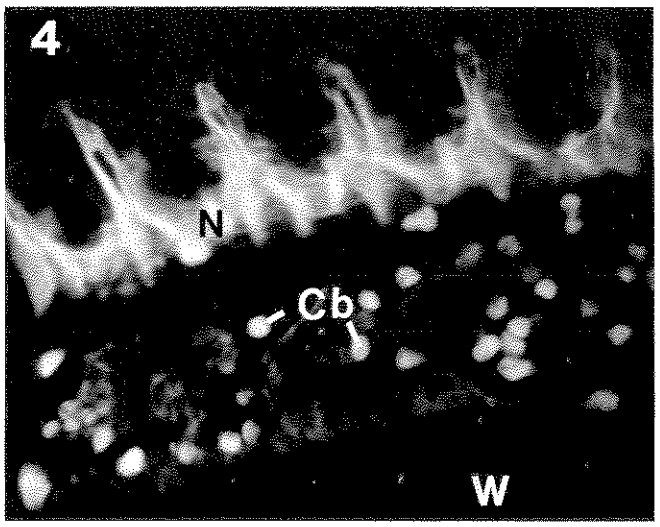


FIG. 4. Fluorescent micrograph of a gill cross section from *L. subrostrata* injected with procion yellow. The bright fluorescent nerve tracts (N) correspond to Fig. 1A and cell bodies (Cb) are evident in the tissue between the water channel (W) and gill filaments. 140×.

serotonin concentration is at the limit of detection: 0.8 ± 0.1 μmol/L (n = 12). Injection of seven mussels with reserpine at 0.4 mg/g dry tissue 48 h before sampling caused a significant reduction in gill serotonin content (1.0 ± 0.1 μg/g wet gill) with no effect on ninhydrin fluorescence of extracted blood.

Treatment of freeze-dried gills with formaldehyde induced a characteristic fluorescence associated with the biogenic amines (Fig. 8). Regions of intense yellow fluorescence, characteristic of serotonin, were evident in the gill sections containing the fibrous nerve tracts between adjacent gill filaments in the area of chitinous-rod discontinuity. The pattern is comparable to that observed using procion yellow (cf. Fig. 4) except the cell bodies are less numerous. Prior treatment of the mussels with reserpine virtually eliminated the yellow fluorescence in the gills. Autofluorescence of gills not exposed to formaldehyde vapors was a uniform pale green.

Isolated gills of mussels will accumulate serotonin. Autoradiographs of sections of gills incubated with picomolar [^{3}H]serotonin prominently display silver grains in the nerve tracts near the water channels, between the gill filaments, and also extending into gill filaments between the chitinous rod and the epithelial surface (Fig. 9). Gills exposed to the same level of radioactive serotonin in 0.1 mM unlabeled serotonin have a reduced level of silver grains in the region of the nerve tracts (not shown).

Discussion

This study has demonstrated that the freshwater mussel demibranch is extensively innervated by a precisely patterned neural network. The parallel pattern between the vital stain for neuronal processes (procion yellow) and the formaldehyde-induced fluorescence of serotonin indicates that a substantial number of the branchial nerves are serotonergic. The serotonin fluorescence, a qualitative index, is confirmed by the large amount of serotonin measured by the ninhydrin fluorometric assay.

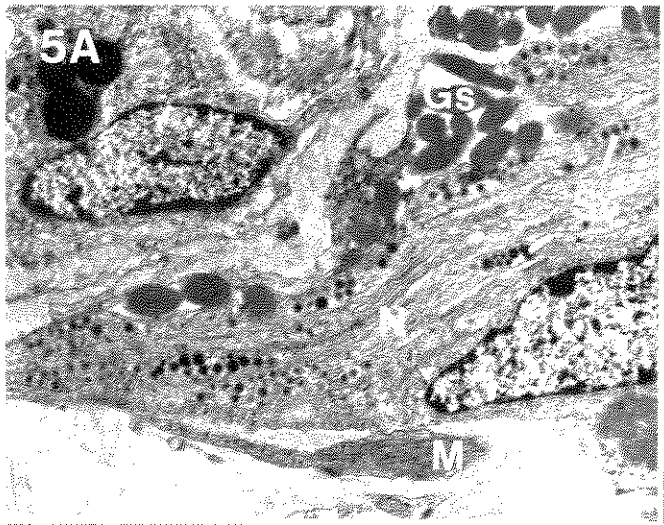
Neurons present in the serotonin-positive (by fluorescence) nerve tracts contain membrane-bound vesicles (170 nm), particularly at neuronal varicosities. Although the type of transmitter substance contained in these vesicles has not been directly confirmed, the uptake of serotonin demonstrated by autoradiography and the serotonin-dependent fluorescence pattern support the hypothesis. Finally, the ultrastructural appearance of the vesicles is similar to the granular vesicles observed in peripheral neurons of *Mytilus* containing serotonin (Aiello 1979; Vitellaro-Zuccarello et al. 1983).

Most regions of the epithelial surface cells have intimate contact with nerve bundles containing granular vesicles (Fig. 6). It is common to observe epithelial cells with extended processes, which surround bundles of neurons, on their basal borders. However, membrane specializations typical of synaptic connections were small and rarely observed (cf. Fig. 7). The relative absence in molluscs of the vertebrate type of synaptic connections has been noted (Satir and Gilula 1970; Vitellaro-Zuccarello et al. 1983). Gill tissue motor neurons have important functions in regulating gill ciliary activity and synchrony. Serotonin (10^{-5} M) stimulates ciliary activity in a variety of bivalves including *L. subrostrata* (Wright 1979; Paparo et al. 1983; A Paparo, personal communication).

We have reported previously that serotonin is the only biogenic amine, of several tested, that is an effective stimulator of sodium transport in isolated mussel gills (Dietz and Graves 1981; Dietz et al. 1982). The mechanism of serotonin stimulation of Na transport in mussel gills apparently is mediated through adenylate cyclase (Scheide and Dietz 1983, 1984). Sodium transport is stimulated when the isolated gills are bathed in dibutyryl cyclic AMP (cAMP), serotonin, or theophylline. The control system is specific for sodium since Cl transport is unaffected (Dietz and Graves 1981). Although sodium transport is stimulated in mussels after they have been salt depleted by prolonged exposure to distilled water, we did not observe any change in the serotonin content of the gills as measured by ninhydrin fluorescence. However, studies of content ignore the dynamics of transmitter release and reaccumulation and (or) synthesis by neurons.

Dopamine also stimulates ciliary movement in isolated gills of *L. subrostrata* (A. Paparo, personal communication). Dopamine probably is an abundant transmitter substance in the gill of this species. We have reported a dopamine-stimulated ade-

FIG. 5. Transmission electron micrographs of the tissue beneath the gill filaments in the region of the nerve tracts (N). The neuronal processes contain dense vesicles (Sv) and are bordered by glial interstitial cells containing gliosomes (Gs). (A) An obliquely striated muscle fiber (M). 10 200 ×. (B) A calcium concretion (C) and a cell body (Cb) containing dense vesicles. 8900×. FIG 6. An electron micrograph of the lateral ciliated cells (L) showing their more electron-dense nature which results from the numerous secretory vesicles, mitochondria, and basal-lateral membrane infolding. The base of the epithelial cells partially surrounds cross sections of nerve tracts (N). 4170×. FIG 7. An electron micrograph of a water channel epithelial cell basal extension surrounding a bundle of axonal processes containing synaptic vesicles (Sv). The arrows indicate membrane densities that suggest a synaptic connection. 24 700 ×. FIG 8. Formaldehyde-induced fluorescent micrograph of the gill of *L. subrostrata*. The characteristic bright yellow fluorescence of serotonin was evident in the nerve tract (N) region between gill filaments. Yellow fluorescence also was evident in scattered cell bodies (Cb) below the filaments. The gill epithelial cells typically had a light green autofluorescence (arrows). 220×. FIG. 9. Bright-field autoradiograph of unstained gill tissue labeled with [^{3}H]serotonin (10^{-12} M). The silver grains are concentrated in the nerve tract (N) region. Silver grains also are deposited beneath the water channel (W) epithelial cells where nerve fibers are observed (arrows). 490×.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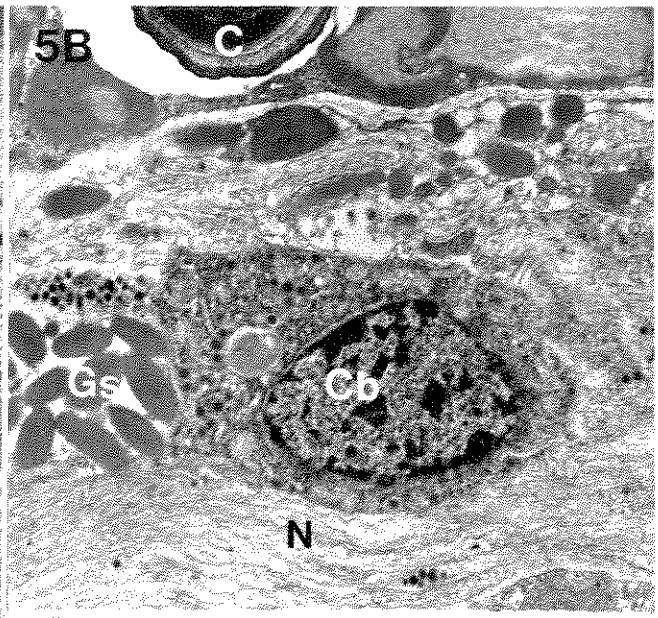
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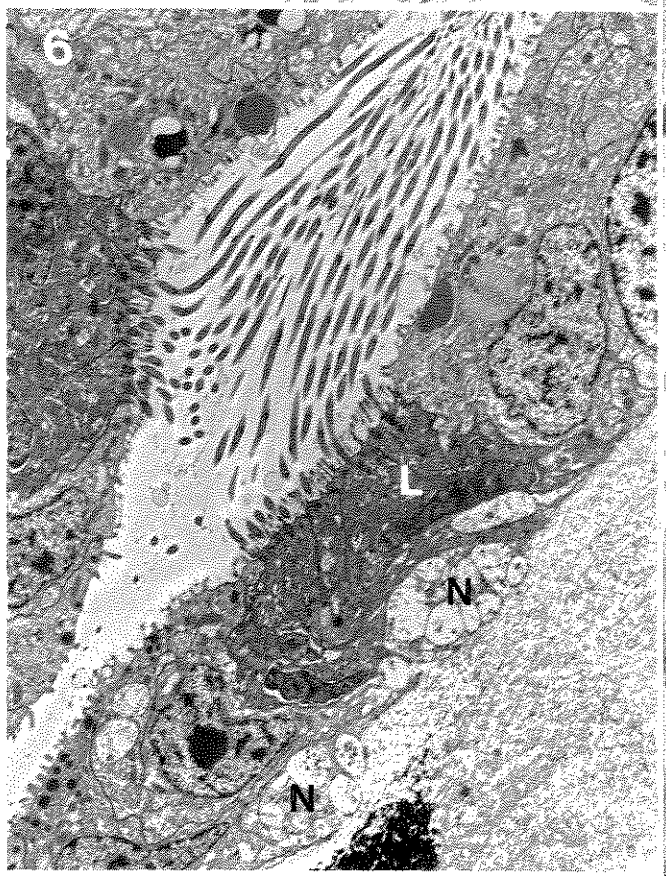
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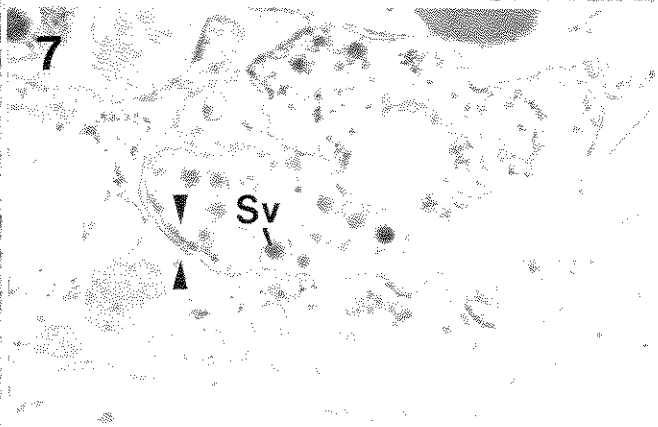
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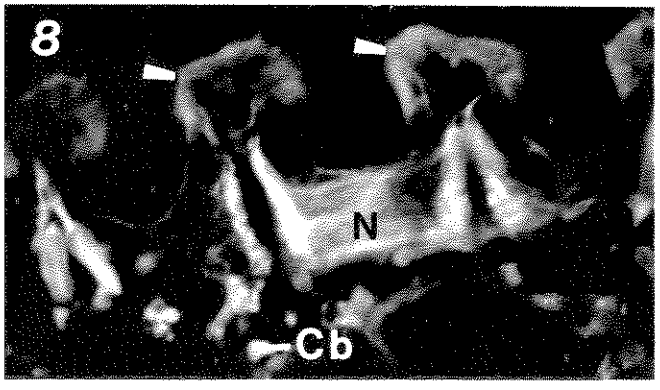
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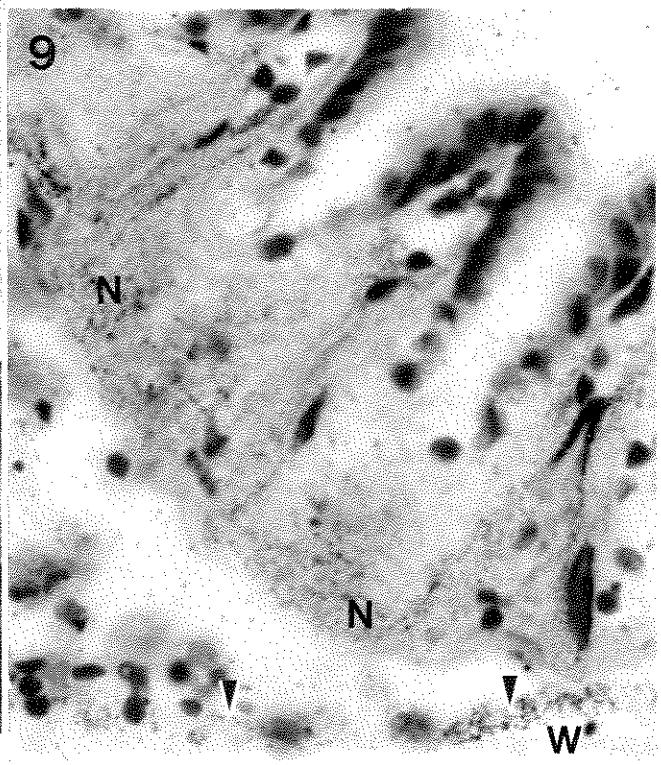
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nylate cyclase in the gill homogenate (Scheide and Dietz 1983). Dopamine may be involved with control of ciliary activity and perhaps in the regulation of the muscles contained in the gill tissue. However, dopamine has no effect on Na transport in the isolated gill (Dietz et al. 1982).

Blood sodium concentration and Na influx in mussels has been observed to undergo circadian changes (Graves and Dietz 1980; McCorkle-Shirley 1982). In addition, the mussels exhibit circadian rhythms in valve-gaping activities and foot movement (McCorkle et al. 1979). We have noted that serotonin exposure stimulates valve movements and sodium transport in these bivalves. The rapid changes in behavior and salt metabolism are consistent with a rapidly acting serotonin-based neuroendocrine reflex. The morphological results presented here are consistent with this hypothesis.

The freshwater mussel, *Ligumia subrostrata*, is well-endowed with serotonin (4.5 nmol/g wet gill), the concentration of which is similar to the serotonin content in *Unio pictorum* (3 nmol/g wet gill) (Hiripi 1968). Confinement of serotonin-specific fluorescence and autoradiographic grains to a precise pattern matching nerve tract regions indicates that serotonin is available in the gill for regulating sodium transport. The freshwater bivalves appear to depend on direct neural control of epithelial Na transport. Although salt depletion stimulates Na transport in these mussels, we observed no change in gill tissue serotonin content or ninhydrin fluoresence in extracted blood associated with salt depletion.

Of interest is the actual site of epithelial Na transport. We have reported previously that the gills of freshwater mussels are the principal site of Na transport (Dietz and Findley 1980; Dietz and Graves 1981; Dietz et al. 1982). In this study, the serotonin-specific fluorescence is predominantly observed near the epithelial cells of the gill filaments. In addition, serotonin-containing neural processes are to be found near the epithelial cells of the water channels.

The calcium concretions are principally located closer to the water channels than to lamellar epithelial cells, and partially surround the anterior–posterior nerve tracts. These calcium concretions are numerous and amount to 25–50% of the gill dry tissue weight (Silverman et al. 1983, 1985; Steffens et al. 1985). The concretions are apparent in fresh gills or in tissue fixed under neutral or alkaline conditions. Mildly acidic conditions dissolve the concretions and for this reason their numbers were reduced in this study.

Seasonal changes in tissue serotonin content have been noted in molluscs and these changes correlate with blood sodium concentration (Lagerspetz and Tirri 1968; Stefano and Catapane 1977; Salanki et al. 1974; Nemcsok and Szasz 1975). Recently, we have reported an elevation of blood sodium in *Anodonta grandis* entering the reproductive season and containing developing glochidia in the gills (Silverman et al. 1985). Coincident with the developing embryos is the loss of calcium concretions from the gills and a reduction in blood Ca (Silverman et al. 1985). These data suggest that calcium liberation for larval nutrition may be linked with sodium transport via a Na–Ca exchange mechanism which may be controlled by serotonin. It is conceivable, although it has not been investigated to date, that a major site of Na or Na–Ca transport is the epithelium of the water channels in the Unionid bivalve gills.

Acknowledgements

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