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Branchial Musculature of a Venerid Clam: Pharmacology, Distribution, and Innervation

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Abstract. This study was meant to analyze the neural control of the branchial muscles of the clam *Mercenaria mercenaria.* Gills isolated from the animal contract in response to 5-hydroxytryptamine (5HT), dopamine (DA), and acetylcholine (ACh); but the ACh contraction occurred only if the gills had been pretreated with the cholinesterase inhibitor eserine. The 5HT antagonists cyproheptadine and mianserin blocked the contractile effects of all of the agonists. However, gills exposed to the 5HT antagonists and eserine relaxed in response to ACh. The DA antagonist SCH-83566 inhibited the effects of DA, but had no effect on contractions induced by 5HT and ACh. The ACh antagonist hexamethonium inhibited both the excitatory and inhibitory effects of ACh, but had no effect on contractions induced by 5HT and DA. 5HT and DA in gill tissue were visualized by using immunohistochemistry. Within each gill filament are dorsoventral neurons running adjacent to the epithelium and containing immunoreactive 5HT and DA. A complex network of 5HT-positive fibers is associated with the septa, blood vessels, and muscles, whereas DA-positive fibers are restricted to the septa. We propose that 5HT is the excitatory transmitter to the gill muscles, and that DA and ACh exert their excitatory effects by stimulating 5HT motor nerves. ACh may also be an inhibitory transmitter of the muscles.

Introduction

In most clams, the water current that supports respiration and feeding is driven through the gills by the beat of the lateral cilia. But the diameter and shape of the passages through the gill—and thus the flow of water—are controlled by contractions of the branchial musculature. These two fundamental activities of the branchial pump—ciliary and muscular—are regulated and coordinated by transmitters and modulators that are released at synapses by neurons that constitute an extensive network in the gill. The neural control of bivalve gill cilia has been extensively studied; this paper focuses on the musculature.

The gills of the venerid clam *Mercenaria mercenaria* are eulamellibranch and plicate (Kellogg, 1892). That is, the filaments are connected to adjacent filaments *via* tissue junctions, and the descending and ascending lamellae are connected to each other and thrown into a series of folds (the plicae) by interlamellar septa (Fig. 1). The dorsoventral spaces within the gill, defined by adjacent septa and the intervening plicae, are the water tubes. The plicae exist in two conformations (Fig. 1C): either their contours are smooth—the "primary folds" of Kellogg, or the "major plicae" of Eble (2001)—or smaller depressions appear at the apexes of the plicae, giving rise to "secondary folds" (Kellogg), or "minor plicae" (Eble). We have seen the plicae alternate between these two conformations. Dorsoventral blood vessels lie at the apex of each plica, within each septum, and within each filament (Kellogg, 1892; Eble, 2001). The blood channels of the branchial filaments are connected with the dorsoventral and septal blood vessels by a meshwork of horizontal blood vessels that are actually interlamellar abfrontal extensions of the filaments (see fig. 34 of the venerid *Tapes aureus* in Ridewood, 1903; fig. 4.20 in Eble, 2001; fig. 1 in Medler and Silverman, 2001). The horizontal meshwork of vessels (collectively called the "subfilamentar tissue"; see Ridewood, 1903) lines the water tubes (Fig. 1D).

That bivalve gills contain muscle fibers and are capable

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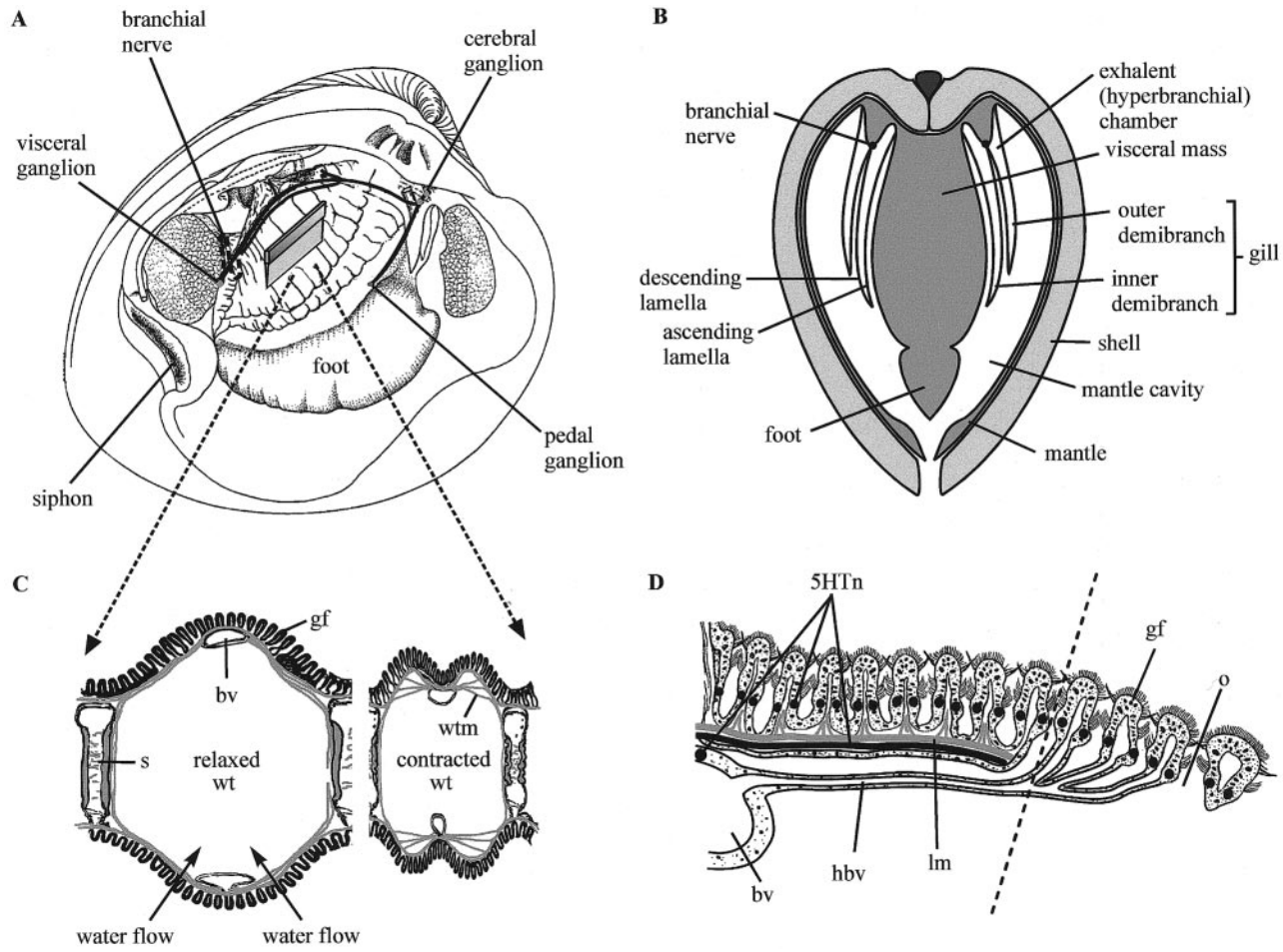


Figure 1. Diagramatic anatomy of *Mercenaria mercenaria:* adapted from various sources on the basis of our own observations. (A) A clam on the half shell. (B) Cross section of a clam. (C) Cross section of a water tube: with the musculature relaxed (left), and contracted (right). The water tube muscles are within the walls of the horizontal blood vessels; the vessels are not shown here. (D) Details of a relaxed demibranch (as in C, left). This cross section is slightly out of the horizontal plane; thus, the filaments to the left of the dashed line are at the level of the interfilamentar tissue junctions that contain the longitudinal muscles; whereas the filaments to the right of the line are at the level of the ostia and horizontal blood vessels. The walls of the horizontal blood vessels contain both the water-tube muscles and a dense network of serotonergic nerves; neither of these is shown. Abbreviations: bv = blood vessel; gf = gill filament; hbv = horizontal blood vessel; lm = longitudinal muscle; o = ostium; s = septum; wt = water tube; wtm = water tube muscle; 5HTn = serotonergic neuron.

of muscular activity has been known for over a hundred years since Kellogg (1892) published his observations on branchial anatomy and movement in a variety of bivalves, including *Mercenaria.* Longitudinal muscle fibers have been described in the interfilament tissue junctions and septa of *Mercenaria* (Kellogg, 1892; Ridewood, 1903; Eble, 2001) and many other bivalve gills. In addition to these longitudinal muscles, called "horizontal muscles" by Atkins (1943) and others, Medler and Silverman (1997) noted the presence of a diffuse network of muscle fibers in the water-tube epithelium of the non-plicate gills of *Dreissena polymorpha*. The plicate gills of *Mercenaria* lack a water-tube epithelium, but they contain a similar network of muscle fibers in the walls of the horizontal blood vessels (Fig. 1).

Neural elements occur within the filaments of both filibranch and eulamellibranch gills (*e.g.*, Setna, 1930; Aiello, 1990), but they also occur in the gills of eulamellibranchs (like *Mercenaria*) in association with the septa, blood vessels, and interfilamentar muscles; structures that, by definition, do not occur in filibranch gills. Indeed, neurons have been reported in association with the longitudinal muscles in a unionid mussel, *Ligumia subrostrata* (Dietz *et al.*, 1985), in *Mercenaria* (Gainey *et al.*, 1999a), and in an oyster, *Crassostrea virginica* (Nelson, 1960). Nerves have

also been observed in the interlamellar septa in *Solen marginatus* and *Ensis siliqua* (Atkins, 1937), *Mercenaria* (Gainey *et al.*, 1999a), and *Crassostrea* (Nelson, 1960, Galtsoff, 1964); and in the water-tube muscles and ostia of *Mercenaria* (Candelario-Martinez *et al.*, 1993).

An extensive literature indicates that the flow of water through bivalve gills varies continuously within wide limits in response to both physical and biological factors (summarized by Dame, 1996; Jorgensen, 1996; Bayne, 1998). But the lateral cilia in both *Mercenaria* and *Mytilus edulis* beat only within a relatively narrow range of frequencies (about 10–25 beats/s) (Aiello, 1960; Catapane, 1983; Gainey *et al.*, 1999a), so the stimulatory and inhibitory motor nerves to the cilia seem to be activating a simple on-off switch. Medler and Silverman (2001) found, in *Mercenaria*, that the geometry of the water tubes changed, and their diameters decreased, in response to 5-hydroxytryptamine (5HT; serotonin). Such changes would tend to modify flow (Grunbaum *et al.*, 1998), so changes in the tone of the branchial musculature might well be participating in the continuously variable regulation of water flow through the gill.

Although the branchial muscles have the potential to modulate water flow through the gills, and neural elements are clearly present, the pharmacology and neural control of these muscles has received relatively little attention. In brief, acetylcholine (ACh) contracts the gill muscles in both *Dreissena polymorpha* and *Corbicula fluminea* (Snow *et al.*, 1995; Medler and Silverman, 1997), whereas 5HT relaxes the gill muscles of *Ligumia subrostrata* (Gardiner *et al.*, 1991) and contracts those of *Mercenaria* (Gainey *et al.*, 1998; Medler and Silverman, 2001). In addition, the peptide FMRFamide contracts the gill muscles of *Dreissena* (Medler and Silverman, 1997). The relationships between the effects of possible neurotransmitters on gill muscles, the distribution of these agents in identifiable neural networks, and the interactions among the elements of the networks are at present unexplored.

We have been using the gill of the quahog *Mercenaria mercenaria* to study the neural control of branchial water flow. In a previous study, we found that 5HT and dopamine (DA), respectively, switch the activity of the lateral cilia on and off, and that YFAFPRQamide, an SCP-like peptide endogenous to *Mercenaria*, modulates the effects of DA (Gainey *et al.*, 1999a). Now we report on the pharmacology of the branchial muscles, focusing especially on the actions of 5HT, DA, ACh, and their antagonists. We have also investigated the distribution of the branchial muscles and their innervation by immunoreactive serotonergic and dopaminergic nerves, expecting the findings to be consistent with our pharmacological observations. Preliminary results of this study have been presented to the Society for Integrative and Comparative Biology (Gainey *et al.*, 1998, 2001).

Materials and Methods

Animals

Quahogs (*Mercenaria mercenaria* L.) that had been dug from various locales along the northeast Atlantic coast were purchased from Harbor Fish, Portland, Maine. The animals were held at 10 °C in natural seawater (30 ppt) on a 12-h light/dark cycle. Individuals were held a minimum of 3 days before use.

Gill preparation and apparatus

Gills were dissected away from the body wall and separated into demibranchs, and the branchial nerves removed (Fig. 1). Muscular contractions were recorded as changes in the length of the anterior-posterior axis of the isolated demibranchs.

Contractions of the branchial muscles were recorded in either of two ways: (1) Isolated demibranchs were suspended in organ baths and attached with thread to isometric force transducers (Grass FT03 and UFI 1030) equipped with springs; the resulting contractions were therefore semi-isotonic. The transducers were interfaced to Biopac DA 100 amplifiers and a Biopac MP100 analog-to-digital converter. (2) Ultratrasonic crystal transceivers (Sonometrics) were tied to the ends of demibranchs with thread. One end of the demibranch was pinned to a piece of rubber band that was glued with rubber cement to the bottom of a plastic petri dish (4.7-cm diameter); the petri dishes were placed on a cooling plate to maintain temperature. Under these conditions, the muscles were unrestrained and contracted against virtually no external load. The isotonic contractions were measured with a digital ultrasonic measurement system (Sonometrics TRX series 8). In both cases, the magnitude of the contractions was measured with AcqKnowledge version 3.5 (Biopac Systems).

All experiments were carried out at 10 °C in aerated artificial seawater (ASW; recipe in Welsh *et al.*, 1968). To retard the oxidation of dopamine (DA), the water was buffered with an ascorbic acid buffer as described by Malanga (1975); this buffered seawater was used in all of the experiments.

Production and analyses of dose-response curves

Our initial experiments were performed with force transducers; but prolonged contraction against the load of the springs used with these devices caused the gill muscles to fatigue. Consequently, we exposed each demibranch only once to a single concentration of agonist, and the dose-response curve was constructed from these individual responses. In later experiments with the Sonometrics digital ultrasound measurement system, no external force was applied to the muscles. No evidence of fatigue was observed,

so a single demibranch could be used to construct an entire dose-response curve. Because the response to serotonin (5HT) and DA has a seasonal component (Gainey, pers. obs.), the dose-response data reported here were collected between November and July.

All contractions and relaxations, measured in millimeters, were expressed as a percentage of the initial length of each demibranch. Regression lines were fitted with a logistic function of the form: response $= \alpha/1 + \exp(\beta_0 + \beta_1 *$ log(agonist)), where α is the asymptotic value of the maximal contraction, and β_0 and β_1 are intercept and slope parameters. Initially, all three parameters were estimated using nonlinear regression (Systat, v 9); later, α was fixed in the model, reducing the error estimates of the remaining parameters. The concentrations of agonist giving half-maximal responses (EC_{50}) were estimated according to the following formula: $EC_{50} = 10\text{^}(-\beta_0/\beta_1)$.

Effects of antagonists

Each of the four demibranchs from the same clam were suspended in an organ bath and attached to a force transducer. After 15 min of relaxation, each of the demibranchs was exposed to an agonist at a standard concentration: 5HT $= 2 \times 10^{-5}$ *M;* DA and acetylcholine (ACh) $= 5 \times 10^{-5}$ *M.* After the resulting contractions had stabilized, the baths were flushed, and an antagonist at 10^{-4} *M* was added to three of the four demibranchs. After 60 min, the standard dose of agonist was reapplied to all four demibranchs, with the antagonist still present on the three demibranchs. The total number of demibranchs treated with a specific antagonist is given in the data tables.

The effect of the antagonist was expressed as the ratio between the second and first agonist-induced contraction (contraction ratio). Analysis of the contraction ratios of untreated controls with a Kolmogorov-Smirnov one-sample test revealed that these data were not normally distributed ($P < 0.001$, two-tailed, $n = 139$). The contraction ratios were therefore normalized by a logarithmic transformation, and the normality of this transformation was checked as above (Kolmogorov-Smirnov; $P = 0.614$). The ln transformed ratios of the controls were tested against a mean of 0 (since ln 1 = 0) with a one-sample *t* test. This is mathematically equivalent to a paired *t* test because the contractions used to construct the ratios were from the same demibranch.

Since the contraction ratios of the controls for 5HT, DA, and ACh were all significantly greater than 1, the normalized contraction ratios of the antagonists were compared to the normalized contraction ratios of the appropriate agonist control using *post hoc* paired Tukey HSD tests after an initial one-way ANOVA. But some of the antagonist contraction ratios were 0, thus these ratios become undefined by a logarithmic transformation. To overcome this limitation, 0.1 was added to all contraction ratios prior to the logarithmic transformation. Although the statistical tests were performed on the ln-transformed data, tabular data are presented in the Results section untransformed for clarity. The *P* values reported for these tests are one-tailed probabilities; *P* values less than 0.05 were considered significant. In some of the experiments—*e.g.,* ACh after exposure to cyproheptadine or mianserin—the gills relaxed rather than contracted; these data are coded in the tables as negative values.

The concentration of antagonist that produced 50% inhibition (IC_{50}) was calculated using the experimental design described above, except that the demibranchs were exposed to lower concentrations of antagonists. Contraction ratios—*i.e.,* the ratios of the second to the first contractions—were regressed against the log of the concentration of antagonist. Because the contraction ratios were significantly greater than 1 for all of the controls, the IC_{50} was calculated by solving the regression equation for a contraction ratio that was 50% of the mean contraction ratio of the control.

Branchial anatomy

For relaxed specimens, isolated demibranchs were kept overnight, at 5 °C, in isotonic $MgCl_2$ in ASW (7.6% $MgCl_2$ in distilled water added to an equal volume of ASW). For contracted specimens, the isolated demibranchs were placed in 10^{-4} *M* 5HT immediately after dissection. To observe the inner face of the water tubes, we cut dorsoventrally along several septa with fine scissors, separating a section of the demibranch into two layers. One of these was removed, and the remainder of the demibranch was then pinned to the bottom of a small petri dish, which had been coated with Sylgard. Fixation—always carried out at 5 °C—varied with the object to be observed (*e.g.,* muscle, 5HT, DA) and is described below. Because mammalian antibodies were used for the immunohistochemistry, subsequent rinses and solutions were made with mammalian phosphate-buffered saline (PBS).

Crysostat sections were prepared as follows. After fixation and a 15-min rinse in PBS (0.1 *M* sodium phosphate, 140 m*M* NaCl; pH 7.3), the demibranchs were placed in a solution of 30% sucrose/PBS overnight at 5 °C. Pieces of demibranch were then placed in Tissue Tek OCT compound, frozen, and sectioned at 12 μm. Sections were placed on gel-coated slides and stored at −20 °C until used.

Thick sections were prepared as follows. After fixation and three 15-min rinses in PBS, pieces of demibranch were placed in a plastic mold and covered with 12% Type A pigskin gelatin in 0.1 *M* PBS that had been heated to 50 °C. After the gelatin had cooled, the tissue was sectioned at 100 μm with a vibratome. The sections were heated briefly at 50 °C on gel-coated slides to melt the excess gelatin.

We usually processed whole mounts and sections simultaneously and therefore followed a schedule designed for whole mounts. All of the steps in this protocol were carried out at 5 °C.

- After fixation, rinse four times (1 h for each rinse) in PBS (0.1 *M*, pH 7.3); or for DA, in 0.05 *M* PBS with 1% sodium metabisulfite.
- Incubate overnight in blocking solution (0.25% goat serum/1% Triton X-100/1% BSA/PBS); for DA, include 1% sodium metabisulfite.
- Incubate overnight in primary antibody diluted appropriately with PBS.
- Rinse four times in PBS (two 30-min rinses, one overnight rinse, one 30-min rinse).
- Incubate overnight in secondary antibody, phalloidin, or both, the reagents diluted appropriately in PBS.
- Rinse three times in PBS (1 h, overnight, 1 h).
- Mount the specimens under coverslips in 60% glycerol-1% *n*-propyl gallate/PBS.

Muscle. The branchial muscles were visualized with phalloidin conjugated to the fluorescent probe Alexa Fluor 488 (Molecular Probes, Eugene, Oregon), the conjugate used in a concentration of 1 unit/100 μl in 0.1 *M* PBS. For single-stained preparations, whole mounts were fixed for 1 h in 4% formaldehyde with 0.01 *M* PBS (pH 7.3; 530 m*M* NaCl), rinsed twice, and then stained overnight. To double-label immunochemically stained preparations, the phalloidin was added to the tissues at the same time as the secondary antibody.

5HT and YFAFPRQamide. Pieces and sections of demibranch were fixed overnight in 4% paraformaldehyde in 0.01 *M* PBS (pH 7.3; 530 m*M* NaCl); the fixative was prepared as described in Gainey *et al.* (1999a). For 5HT, the primary polyclonal antiserum was raised in rabbit to 5HT conjugated to BSA with paraformaldehdye (Diasorin, Stillwater, Minnesota). For YFAFPRQamide, the primary polyclonal antiserum was raised in rabbit to the peptide conjugated to thyroglobulin (custom synthesis, etc., by SynPep, Dublin, California). In both cases, the secondary antibody was raised in goat to rabbit IgG conjugated to Alexa Fluor 594 (Molecular Probes).

Dopamine. Pieces and sections of demibranch were fixed for 2 h in 5% glutaraldehyde/1% sodium metabisulfite/PBS (0.01 *M;* pH 7.3; 530 m*M* NaCl). The primary polyclonal antiserum was raised in rabbit to DA conjugated to BSA with glutaraldehyde (Diasorin). The secondary antibody was raised in goat to rabbit IgG and conjugated to Alexa Fluor 594 (Molecular Probes). For negative controls, the primary antibodies were omitted from a slide in each series of preparations.

Confocal images of 5HT distribution were made with a Leica LSCM SP2 microscope at the Whitney Laboratory, St. Augustine, Florida. Fluorescent images were made with a Nikon Eclipse TE200 microscope equipped with a Spot RT digital color camera (Diagnostic Instruments). Images were prepared for publication with Adobe Photoshop.

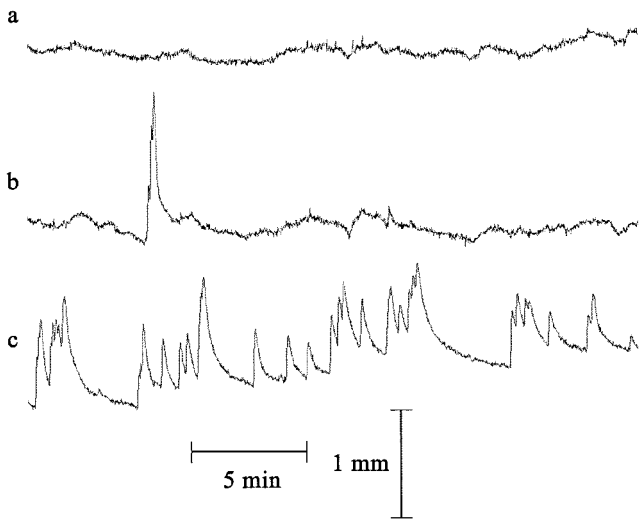


Figure 2. Traces of contractile activity recorded from three untreated demibranchs taken from a single clam. (A) Quiescent. (B) Occasional, spontaneous contraction. (C) Arrhythmic, spontaneous contractions. Contractions were recorded with force transducers.

Drugs

All chemicals were purchased from Sigma-Aldrich, St. Louis, Missouri, or ICN Pharmaceuticals, Costa Mesa, California. The specificities of the antagonists listed in the tables were obtained from the *Cell Signaling & Neuroscience* catalog (2000/2001 ed.) of Sigma/RBI.

Results

Activity of isolated gills

Most of the isolated demibranchs were quiescent in the organ baths (Fig. 2a), but occasionally gills would contract spontaneously and relax (Fig. 2b), and on rare occasions they would beat arrhythmically (Fig. 2c). All three demibranchs in Figure 2 were from the same clam; the fourth, not pictured, was also quiescent. Of the hundreds of preparations we have observed, only a handful showed the spontaneous, arrhythmic contractions seen in Figure 2c.

Pharmacology of branchial muscles

Agonists. 5HT, DA, and ACh contracted the gill muscles in a dose-dependent manner, but the response to ACh was observed only in gills pretreated for 15 min with 10^{-4} *M* eserine (Fig. 3). The responses to all three agonists were

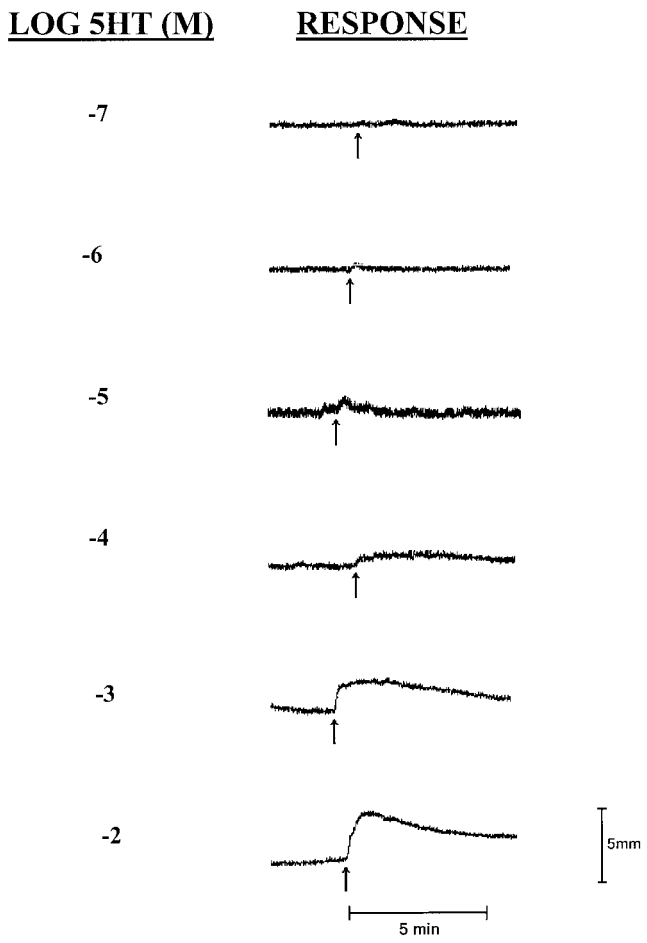


Figure 3. Traces of contractions in response to increasing concentrations of 5HT; successive doses were added at the arrows. Each response is from a separate demibranch; data were recorded with force transducers.

indistinguishable: the gills contracted tonically and, after 30 s to several minutes, reached their maxima.

Dose-response curves for the three agonists were prepared (Fig. 4), and their characteristics are listed in Table 1. The rankings of the EC_{50} values are ACh < 5HT < DA, and since the 95% confidence intervals do not overlap, the values are statistically different. The maximal contractions in response to 5HT and DA are equal, and both are significantly larger than the maximal contractions induced by ACh. The comparative data noted above were independent of the method used to record the contractions. However, if we consider each agonist separately, then its EC_{50} is significantly less, and its induced contractions were larger (except for DA) when the contractions were recorded with the digital ultrasound system rather than with force transducers (Table 1).

The following neurotransmitters, all applied to the tissue at 10^{-4} *M*, neither contracted nor relaxed the gills: ATP, GABA, histamine, and octopamine. Furthermore, the following three peptides—all found in *Mercenaria* and all applied at 10^{-6} *M*—neither contracted nor relaxed the gills: FMRFamide, AMSFYFPRMamide, and YFAFPRQamide.

Previously, we found that YFAFPRQamide modulates the effects of DA on the lateral cilia and those of 5HT on the frontal cilia (Gainey *et al.*, 1999a). Therefore, to determine whether the peptide would modulate the effects of 5HT or

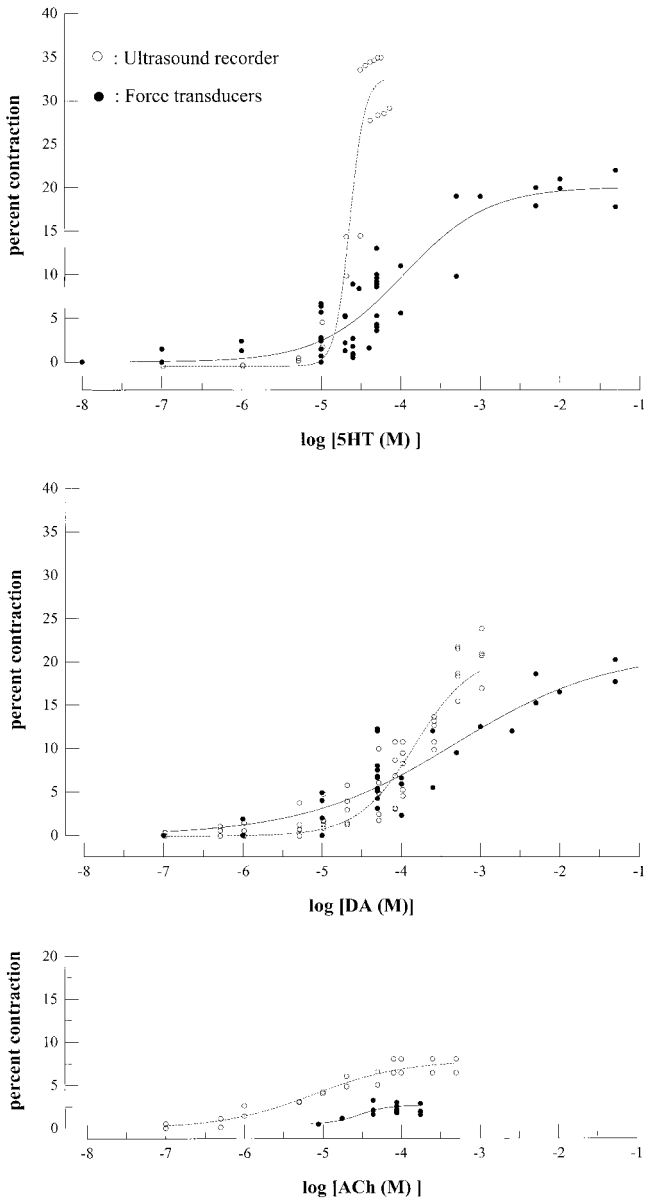


Figure 4. Dose-dependent muscle contractions (as percentages of the resting length) in response to 5HT, DA, and ACh. Solid circles and lines: data recorded with force transducers; each datum is the response of a separate demibranch. Open circles on dashed lines: data recorded with an ultrasound system from either two (5HT, ACh) or five (DA) demibranchs; each preparation was exposed to increasing concentrations of agonist. Demibranchs exposed to ACh were pretreated with 10^{-4} *M* eserine.

Table 1

Summary of dose-response effects for 5HT, DA, and ACh

Agonist	Type*	EC_{50} (M)†	(95% CI)‡	Cmax (%)§	(95% CI)‡
5HT	ft	1.1×10^{-4}	$(0.5–1.8 \times 10^{-4})$	20	(17–23)
	us	2.1×10^{-5}	$(1.9–2.5 \times 10^{-5})$	33	(28–38)
DA	ft	5.9×10^{-4}	$(2.3–9.5 \times 10^{-4})$	21	(14–29)
	us	1.4×10^{-4}	$(1.2–1.7 \times 10^{-4})$	32	(17–48)
ACh	ft	1.5×10^{-5}	$(0.34–3.3 \times 10^{-5})$	2	(1–3)
	us	8.6×10^{-6}	$(0.2–1.3 \times 10^{-5})$	9	(7–11)

* ft = force transducers used to measure contractions; us = ultrasound used to measure contractions.
† Concentration of agonist giving a half maximal response.
‡ 95% confidence intervals associated with the estimates.
§ Maximal predicted contraction.

DA on the muscle, we applied YFAFPRQamide to the demibranchs before exposing them to 2×10^{-5} *M* 5HT or DA. At concentrations ranging from 10^{-9} to 10^{-6} *M* (5HT) or 10^{-8} to 10^{-6} *M* (DA), and exposures ranging from 15 min to 1 h (5HT) or 1 h (DA), the peptide had no effect upon contractions induced by either 5HT or DA.

Antagonists. Because the three effective agonists contract the gill, and since the mechanical responses to 5HT, DA, and ACh are indistinguishable, we asked whether the muscles have receptors for each of the agonists, or whether one or more of the agonists are acting indirectly by stimulating the release of another agonist from motor nerves. To test these possibilities, antagonists were sought for each agonist, and these agents were cross-tested against the other agonists.

- ***Controls.*** In control experiments, each gill received two consecutive, equal doses of the same agonist. For each of these agonists, the second contraction in response to the same concentration was usually larger than the first, and the contraction ratios were significantly greater than 1 (Table 2). Moreover, when

Table 2

The effect of antagonists on the actions of 5HT, DA, and ACh

Antagonist	Type*	Agonist†	Mean contraction ratio ± SD (*n*)‡	*P*§
None (control)	—	5HT	2.20 ± 1.86 (72)	<0.001*p
None (control)	—	DA	2.18 ± 1.74 (46)	<0.001*p
None (control)	—	ACh	3.21 ± 3.65 (18)	0.001*p
Cyproheptadine	$5HT_2$	5HT	0.324 ± 0.495 (11)	<0.001*i
		DA	0.076 ± 0.162 (10)	<0.001*i
		ACh	−1.19 ± 2.10 (9)	*r
Mianserin	$5HT_2$	5HT	0.475 ± 0.193 (8)	0.002*i
		DA	0.494 ± 0.490 (8)	<0.001*i
		ACh	−0.449 ± 0.701 (9)	*r
SKF-83566	DA_1	DA	0.483 ± 0.300 (9)	0.003*i
		5HT	0.869 ± 0.233 (9)	0.32
		ACh	0.881 ± 0.530 (3)	0.22
Hexamethonium	ACh_n	ACh	0.215 ± 0.262 (5)	<0.001*i
		DA	1.05 ± 0.593 (6)	0.49
		5HT	2.14 ± 0.850 (8)	0.50

* The primary type of mammalian receptor blocked by the antagonist.
† Agonist concentrations: 5HT = 2×10^{-5} *M;* DA & ACh = 5×10^{-5} *M*. Antagonist concentrations were 10^{-4} *M*.
‡ Contraction ratio: height of contraction after exposure to the antagonist/height of contraction before exposure to the antagonist.
§ *P* values are one-tailed probabilities: *i = significant inhibition; *p = significant potentiation; *r = relaxation, the second contraction was coded as a negative value.

Table 3

Antagonists that had no significant effect upon the action of 5HT and DA

Antagonist	Type*	Agonist†	Mean contraction ratio ± SD (*n*)‡	*P*§
Ergonovine	DA/5HT	5HT	1.42 ± 1.38 (4)	0.5
NAN-190	$5HT_{1A}$	5HT	1.66 ± 1.52 (6)	0.49
Ketanserin	$5HT_2$	5HT	1.68 ± 1.15 (6)	0.5
Ritanserin	$5HT_2$	5HT	1.47 ± 1.67 (7)	0.49
MDL 72222	$5HT_3$	5HT	3.50 ± 1.73 (6)	0.43
Tropisetron	$5HT_3$	5HT	1.78 ± 0.680 (6)	0.5
Bulbocapnine	DA	DA	3.58 ± 6.29 (6)	0.50
Butaclamol	DA	DA	1.78 ± 1.60 (17)	0.33
Ergonovine	DA/5HT	DA	1.55 ± 1.24 (4)	0.50
Apomorphine	DA_2	DA	4.23 ± 4.93 (7)	0.49
Chlorpromazine	DA_2	DA	3.60 ± 1.06 (5)	0.45
Fluphenazine	DA_2	DA	2.60 ± 1.61 (6)	0.50
Pimozide	DA_2	DA	2.72 ± 1.67 (6)	0.50
Spiperone	DA_2	DA	1.04 ± 0.812 (16)	0.28
Sulpiride	$DA_{2/3}$	DA	0.580 ± 2.23 (8)	0.50

* The primary type of mammalian receptor blocked by the antagonist.
† Agonist concentrations: 5HT = 2×10^{-5} *M;* DA = 5×10^{-5} *M*. Antagonist concentrations were 10^{-4} *M*.
‡ Contraction ratio: height of contraction after exposure to the antagonist/height of contraction before exposure to the antagonist.
§ *P* values are one-tailed probabilities.

ANOVA was performed, none of the contraction ratios were significantly different from each other [$F(2, 132) = 1.315$; $P = 0.272$]. These control responses rendered the usual analytical techniques inapplicable, so the effects of antagonists were examined with the statistical tests described in the Methods section.

- ***5HT.*** The effects of 5HT were inhibited by the $5HT_2$ antagonists cyproheptadine and mianserin. The IC_{50} for cyproheptadine was 1.2×10^{-7} *M*, while that for mianserin was 1.6×10^{-5} *M*. These antagonists also blocked the effects of DA and, unexpectedly, caused ACh to relax the gill muscles (Table 2). The following 5HT antagonists were ineffective at 10^{-4} *M:* ergonovine, NAN-190, ketanserin, ritanserin, MDL 72222, and tropisetron (Table 3).
- ***DA.*** SKF-83566, a DA_1 antagonist, significantly inhibited the effects of DA but had no effect upon the activity of either 5HT or ACh (Table 2). The IC_{50} for SKF-83566 was 3.0×10^{-5} *M*. The following DA antagonists were ineffective at 10^{-4} *M:* bulbocapine, butaclamol, ergonovine, apomorphine, chlorpromazine, fluphenazine, pimozide, spiperone, and sulpiride (Table 3).
- ***ACh.*** Hexamethonium, an ACh_n antagonist, significantly inhibited the relatively small contractions induced by ACh, but hexamethonium had no effect upon the activity of either 5HT or DA (Table 2). The IC_{50} for hexamethonium was 1.0×10^{-5} *M*.

When gills were pretreated, not only with 10^{-4} *M* eserine, but also with 10^{-4} *M* cyproheptadine or mianserin, then ACh would produce a dose-dependent relaxation of the branchial muscles. For gills treated with cyproheptadine, the EC_{50} was 3.3×10^{-5} *M* (95% CI: 2.3×10^{-5} to 4.3×10^{-5} *M*), and the maximal relaxation was 6.2% (95% CI: 3.9% to 8.5%; Fig. 5). For gills treated with mianserin, the EC_{50} was

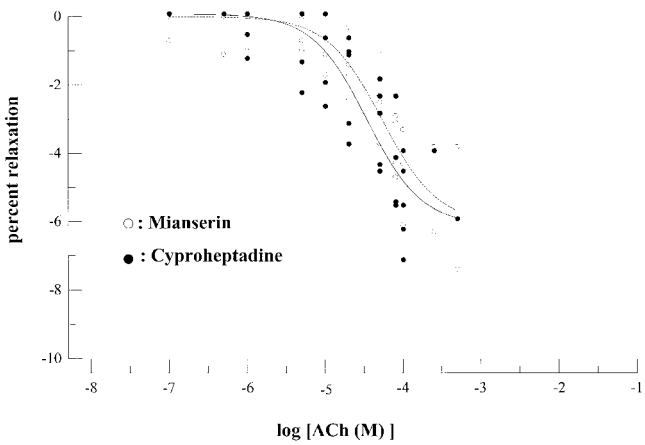


Figure 5. Dose-dependent muscle relaxation in response to ACh. Relaxations were expressed as a percentage of the resting length, and plotted as a negative value. Solid circles: data from five demibranchs pretreated with 10^{-4} *M* cyproheptadine. Open circles: data from four demibranchs pretreated with 10^{-4} *M* mianserin. In addition, all demibranchs were pretreated with 10^{-4} *M* eserine. Data were recorded with an ultrasound system. Ultrasound echoes in the organ baths prevented the measurements of some of the responses.

5.5×10^{-5} *M* (95% CI: 4.0×10^{-5} to 7.0×10^{-5} *M*); and the maximal relaxation was 6.1% (95% CI: 4.3% to 7.8%; Fig. 5). The ranges of the 95% confidence intervals for both the EC_{50} and the maximal relaxation overlap; that is, the parameters are not different.

Branchial anatomy

Distribution of muscles in the gill. The structure and disposition of the branchial musculature in the inner and outer demibranchs are indistinguishable, and three groups of these muscles have been observed: longitudinal muscles, dorsoventral muscles, and water-tube muscles. The well-defined longitudinal muscle bands are about 50 μm wide and about 125 μm apart. They run at right angles to the gill filaments, at their base, and are contained within the interfilament tissue junctions that hold adjacent filaments together. The longitudinal muscle bands run the length of a demibranch, passing through each septum, and between each dorsoventral blood vessel and the filaments lying over it (Fig. 6A, B, C).

The dorsoventral muscles run through the center of each gill filament. Where the gill filaments intersect with the interfilament tissue junctions, these dorsoventral muscles send branches into the underlying longitudinal muscles (Fig. 6B).

The water-tube muscles form a complex, but regular, lattice-like network associated with the horizontal blood vessels of the subfilamentar tissue (Fig. 6A). Because most of these muscles cross the septa—from one side of a water tube to the other—and appear to be continuous with the network of muscle fibers within the blood vessels, they form a mesh of circular muscles within the inner wall of each water tube. In cross section, at least some of these muscles also run diagonally from the abfrontal face of the gill filaments towards the septa and blood vessels (Fig. 6C).

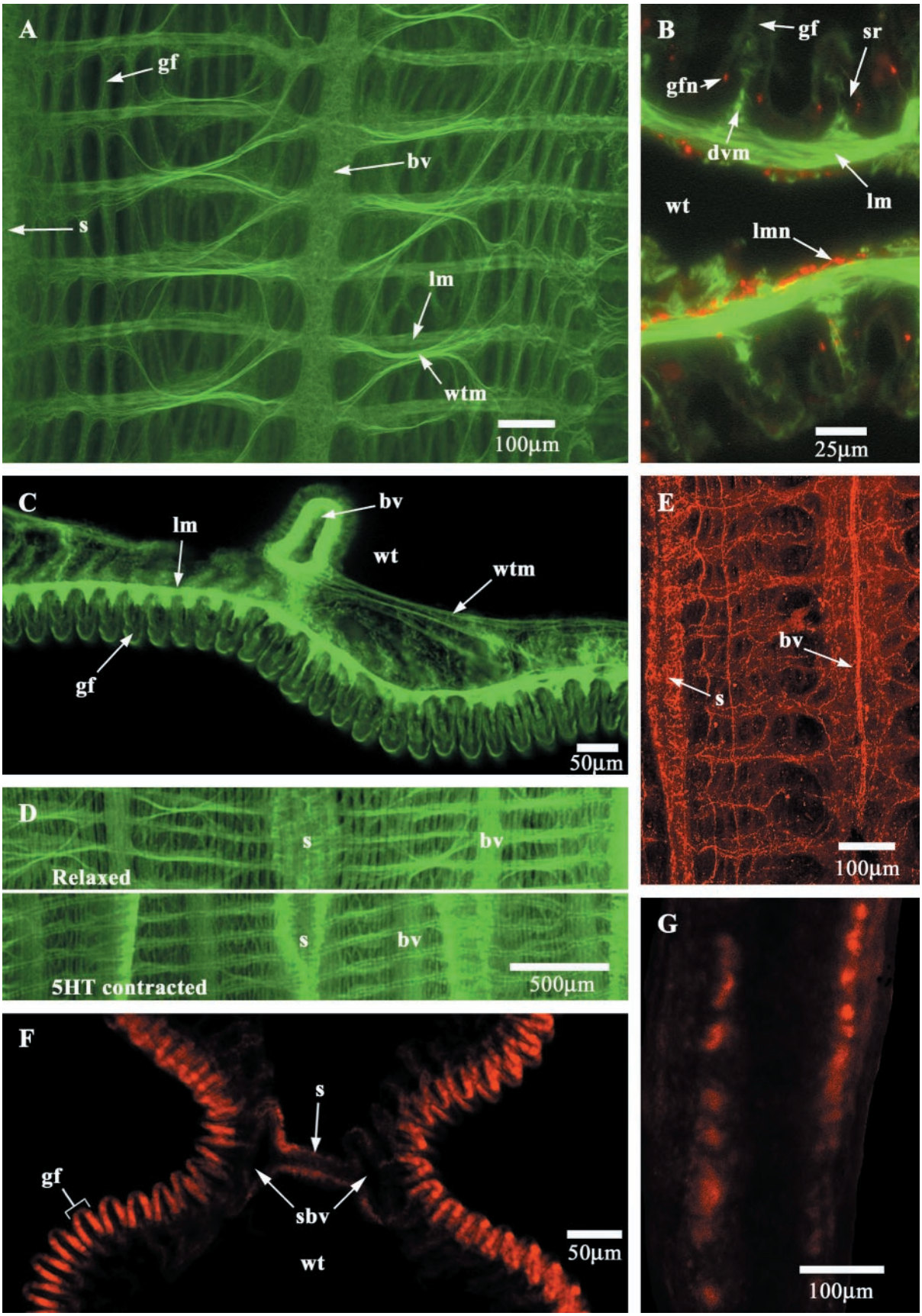
Finally, comparison of relaxed and contracted gills revealed the following: (1) the distance between adjacent septa decreased; (2) the vertical spacing between the longitudinal muscle bands decreased, as well as the vertical spacing between the water-tube muscles; and (3) the interfilament space decreased (Fig. 6D). As a result of muscle contraction, the outer, frontal, faces of the gills take on a zigzag appearance. In addition, in freshly dissected gills in which the water tube had been cut open, we observed that if the blood vessel was gently pinched, contraction of the water-tube muscles brought the blood vessel towards the center of the water tube. This resulted in the formation of secondary folds (plicae) and decreased the cross-sectional area of the water tube (Fig. 1C). The blood vessel also constricted in response to the mechanical stimulation.

Distribution of 5HT in the gill. Two distinct networks of immunoreactive serotonergic varicose nerve fibers are observable in the gill. Each gill filament contains two dorsoventral nerves that run under the epithelium near the lateral cilia and parallel to the dorsoventral muscle fibers (Fig. 6B); we observed no cross connections between the two fibers within a filament.

The second, more complex, network of serotonergic fibers is associated with the gill musculature; the unit of this network is a water tube. First, two large bundles of nerves run dorsoventrally within the septa, and another large bundle runs the length of each of the blood vessels. In addition to these large dorsoventral bundles, four finer ones run parallel to the septa, and two run parallel to the large nerve bundle in the blood vessel (Fig. 6E). These fine dorsoventral nerves are all associated with the subfilamentar tissue. Longitudinally disposed nerves run at right angles to and between the septa and the blood vessels in each water tube; these cross connectives are located within the interfilament tissue junctions adjacent and parallel to the longitudinal muscles (Fig. 6B). We also observed what appear to be fine branches of this longitudinal nerve running into the dorsoventral muscles of the filament (see the filament just above the measurement bar in Fig. 6B). Other longitudinal nerves are associated with the water-tube muscles (Fig. 6E). Finally, we observed connections between the dorsoventral nerves within the gill filaments and those associated with the longitudinal muscles. The primary antibody was omitted from the control sections (not shown), which had minimal background fluorescence comparable to the background in Figure 6E.

Distribution of DA in the gill. Immunoreactive dopamine was found only in the gill filaments and the septa. Within the gill filament epithelium, there are several pairs of dorsoventral cords (Fig. 6F). When viewed from the outer face of the gill, these cords have a granular, rather than a smooth, appearance. The dopaminergic fibers that are confined to the septal epithelium are a pair of bundles connecting the ascending and descending lamellae (Fig. 6F, G). Thus, each septum contains paired cross connectives that are stacked vertically in the gill and spaced at about the same distance as the longitudinal muscles. No dopaminergic fibers are associated with either the longitudinal or water-tube muscles. Moreover, control sections prepared without the primary antibody (not shown) had minimal background fluorescence, comparable to the background in Figure 6F.

Distribution of YFAFPRQamide in the gill. Immunoreactive YFAFPRQmide was concentrated in the outer half of the gill filament epithelium (Fig. 7). Weak fluorescence was found in the subfilamentar tissue (the abfrontal face of the filaments) and the blood vessel epithelium. The level of fluorescence in the musculature was comparable to background fluorescence in negative controls.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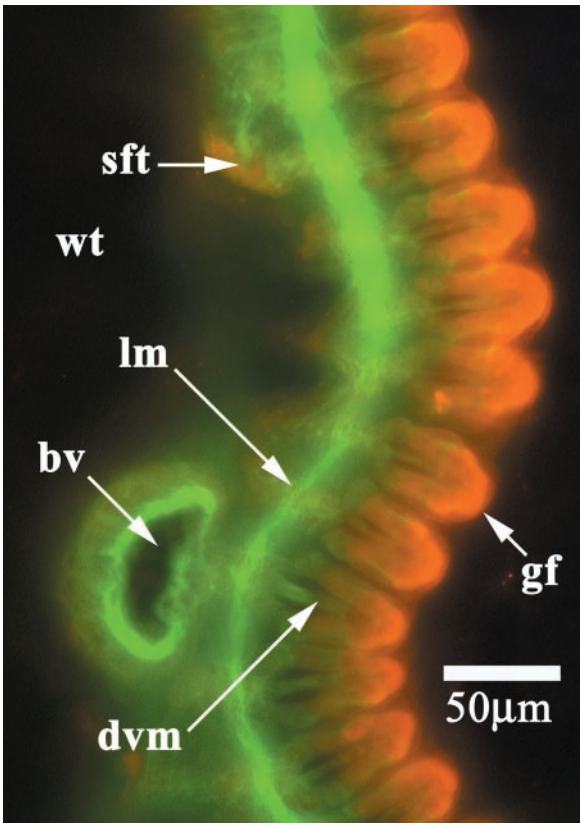

Figure 7. Thick (100 μm) cross section of a demibranch showing the distribution of muscle fibers and the SCP-like peptide YFAFPRQamide. The muscle fibers are stained with phalloidin and fluoresce green. Red fluorescence is associated with YFAFPRQamide and is concentrated in the tips of the filaments, but lesser amounts are also found on the abfrontal face of the filaments in the subfilamentar tissue. Abbreviations: bv = blood vessel; dvm = dorsoventral muscle; gf = gill filament; lm = longitudinal muscle; sft = subfilamentar tissue; wt = water tube.

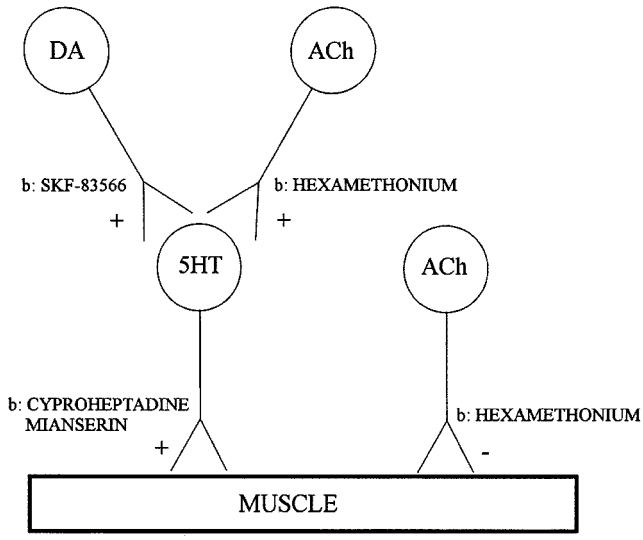

Figure 8. A diagrammatic summary of the most parsimonious model of the control of gill muscle. Abbreviations: b = block by specific antagonists; + = postsynaptic stimulation; − = muscle relaxation. Based upon our immunohistochemical observations, we hypothesize that the synapses between the DA and 5HT neurons are within the gill septa. See text for further details.

Discussion

We have shown that isolated demibranchs of *Mercenaria* contract in response to 5-hydroxytryptamine (5HT), dopamine (DA), and acetylcholine (ACh). Moreover, although two 5HT antagonists block the excitatory effects of all three agonists, the DA and ACh antagonists act specifically, blocking only the effects of their respective agonists. We have also demonstrated that immunoreactive 5HT is localized in varicose fibers associated with the longitudinal and water-tube muscles, whose contractions were recorded in the pharmacological experiments. The DA-containing fibers, in contrast, are not associated with these muscles. We hypothesize, therefore, that 5HT is the excitatory transmitter released at neuromuscular junctions in the gill, whereas DA and ACh act by stimulating serotonergic motor neurons (Fig. 8). Furthermore, because the networks of DA- and 5HT-containing neurons are distinct in their distribution and overlap most extensively in the septa, we suggest that these

Figure 6. Anatomical details of branchial muscles and nerves. The muscle fibers are stained with phalloidin and fluoresce green; the neurons are immunochemically stained for 5HT or DA and fluoresce red. (A) Inner face of a water tube showing the iterated arrangement of muscle fibers. (B) Cross section (12 μm) of a demibranch showing immunoreactive serotonergic neurons associated with branchial muscle. (C) Thick (100 μm) cross section of a demibranch showing muscle fibers associated with the gill filaments and blood vessel. (D) Inner face of two water tubes, top with muscles relaxed, bottom with muscles contracted in response to 10^{-4} *M* 5HT. (E) Inner face of a water tube showing the distribution of the dense serotonergic nerve net. (F) Thick (100 μm) cross section of a demibranch; DA immunoreactivity is restricted to the epithelium of the filaments and the septum. (G) DA immunoreactivity in a dorsoventral section through a septum on the inner face of a water tube (the same orientation as E). Note the faint staining of the filaments, which are well out of the plane of focus. Abbreviations: bv = blood vessel; dvm = dorsoventral muscle; gf = gill filament; gfn = gill filament nerve (serotonergic); lm = longitudinal muscle; lmn = longitudinal muscle nerve; s = septum; sbv = septum blood vessel; sr = skeletal rod; wt = water tube; wtm = water tube muscle.

dorsoventral structures are the sites of the proposed excitatory dopaminergic synapses on the 5HT motor neurons.

The dual effect of acetylcholine

Our pharmacological experiments have also revealed an additional action of ACh. When gills were exposed to one of the 5HT antagonists, as well as to the cholinesterase inhibitor eserine, the muscles would *relax* in response to ACh. Moreover, this effect was blocked by the nicotinic antagonist hexamethonium. These data imply that, in addition to exciting the muscle by releasing 5HT, ACh also inhibits the muscle directly (Fig. 8). This dual effect of ACh explains part of its weak stimulatory action; that is, the maximal contraction produced by ACh was only about 25% of the maximal response to either 5HT or DA. But because ACh stimulates the muscle in the absence of 5HT antagonists, we infer that this stimulatory action of ACh is more potent than its inhibitory effect. However, even accounting for the inhibitory effect of ACh, the excitatory effect is still only about 50% of the maximal effect of either 5HT or DA. We conclude that the weak ACh stimulation of the branchial muscles in *Mercenaria* reflects characteristics of the cholinergic innervation, the postsynaptic receptors, or the signal transduction mechanism in this species. The distribution of cholinergic fibers has, however, not yet been investigated.

Innervation and function of the branchial musculature

Our morphological studies indicate that the serotonergic and dopaminergic innervations of the *Mercenaria* gill are distinct and that the musculature lacks dopaminergic innervation. Furthermore, the motor innervation of the gill muscles and cilia seems to be organized in three divisions: the serotonergic neural network associated with the water-tube muscles; the serotonergic innervation of the longitudinal and dorsoventral muscles; and the innervation of the cilia on the surface of the gill filaments, including serotonergic, dopaminergic, and peptidergic elements. When these findings are considered together with our pharmacological data and behavioral observations, a picture of integrated gill function begins to emerge.

The water-tube muscles and their neural network. The water-tube muscles occur in the walls of the major dorsoventral blood vessels, the subfilamentar horizontal vessels, and in the septa. When relaxed gills are exposed to 5HT, the plicae narrow, and secondary folds appear (see Fig. 1C; also fig. 4 in Medler and Silverman, 2001). We have also observed that if a dorsoventral blood vessel is gently pinched, the adjacent water-tube muscles contract, bringing the vessel inwards towards the center of the water tube, producing the secondary plical fold. This response decreases the cross-sectional area and modifies the shape of the water tubes, thus reducing the flow of water through the gill. In addition, pinching the blood vessel causes it to constrict locally, suggesting that the water-tube muscles also regulate the diameter of the blood vessels and thus the flow of hemolymph through them.

The longitudinal and dorsoventral muscles and their innervation. The longitudinal muscles run along the inside of the interfilament tissue junctions, perpendicular to the gill filaments and to the dorsoventral muscles, which lie within the filaments. However, the two sets of muscles are closely apposed, and more important, the dorsoventrals appear to be composed of branches of the longitudinals (Fig. 6B, C). Thus, we speculate that this orthogonal net of muscle acts as a unit. The longitudinal muscle is accompanied by a serontonergic neural plexus, and we have observed serotonergic varicosities among the longitudinal muscle fibers. Moreover, the dorsoventral muscles appear to be innervated by branches of the neural plexus (Fig. 6B).

In response to 5HT, the overall length of the gill decreases, as does the spacing between individual filaments, and thus the diameter of the ostia; this is the action of the longitudinal muscle. In addition, however, the 5HT-treated gill decreases in height in a dose-dependent manner (Gainey, pers. obs.)—the action of the dorsoventral muscles. When gills are exposed to 10^{-4} *M* 5HT, the dorsoventral contraction of the gill is very strong and produces a zigzag formation of the filaments on the frontal face of the gill. This phenomenon has also been observed in contracted gills of *Corbicula fluminea* (Medler and Silverman, 2001, fig. 2).

Innervation of the branchial filaments. The gill filaments bear the functionally and morphologically distinct tracks of cilia for which the bivalves are well known. Among these effectors, the lateral cilia—which constitute the branchial pump and thus produce the water current—have been studied best. In *Mercenaria*, the beat of these cilia is stimulated by 5HT and inhibited by DA (Aiello, 1970; Gainey *et al.*, 1999a), results that are consistent with our identification of both serotonergic and dopaminergic fibers within the gill filaments. Furthermore, an identical pattern of structure and function has been demonstrated in a filibranch, the blue mussel *Mytilus edulis* (reviewed by Aiello, 1990), so we presume that these fibers, in fact, innervate the lateral cilia in *Mercenaria*. The frontal cilia, which move food particles along the gill, are slowed only weakly by 5HT (Gainey *et al.*, 1999a). But the function of the innervation is not clear, for the frontal cilia continue beating even when the clam is closed (Shirley Baker, University of Florida, pers. comm.).

An endogenous SCP-like peptide, YFAFPRQamide, modulates the effects of DA on the lateral cilia and those of 5HT on the frontal cilia (Gainey *et al.*, 1999a); but this peptide has no effect—direct or indirect—on the branchial musculature. YFAFPRQamide-related immunoreactivity occurs almost exclusively in the region under the epithelium

Table 4

Effects of classic neurotransmitters on the branchial muscles of bivalves

SUBCLASS			Effect‡			
Species	Method*	Habitat†	5HT	DA	ACh	Source
PTERIOMORPHIA						
Mytilus edulis	DVO	M	+			Jorgensen, 1975
	TR		+	+		Gainey, pers. obs
Crassostrea virginica	TR	M			+	Roop & Greenberg, 1967
PALEOHETERODONTA						
Anodonta grandis	Ostia	F	−			Gardiner *et al.*, 1991
Ligumia subrostrata	Ostia	F	−	0	0	Gardiner *et al.*, 1991
HETERODONTA						
Dreissena polymorpha	V/TR	F			+	Snow *et al.*, 1995
	V/Ostia		−		+	Medler & Silverman, 1997
Corbicula fluminea	IFD/Ostia	F			+	Medler & Silverman, 2001
Mercenaria mercenaria	TR	M	+	+	+/−	This study

* DVO, direct visual observation of isolated gills. IFD, interfilament distance, recorded on videotape. Ostia, change in diameter of ostia measured. TR, direct measurement with a transducer of movement or force development. V, measurement of length or area changes recorded on video tape.

† M, marine; F, fresh water. Habitat is defined in terms of salinity. Note that the species listed here as "marine" are all at least moderately euryhaline (5–15‰ to 30−40‰). The criterion for designation of habitat as "fresh water" (F) is the ability of animals to live and reproduce (or survive prolonged immersion) in fresh water.

‡ +, excitation [increased tone (or ostia increased in diameter); or increased rate, regularity, or amplitude of contractions]. −, inhibition [relaxation (or decreased diameter of ostia), or reduced rate, amplitude, or regularity of contractions]. 0, no response observed. The predominant responses of the tissues to each transmitter are listed.

bearing the gill cilia, and in nerves running out to that region (Gainey *et al.*, 1999a). We have certainly not identified all of the transmitters in the innervation of the filaments, but the morphological restriction of YFAFPRQamide to the filaments, and its physiological restriction to effects on cilia, suggests that innervation of the branchial filaments may be exclusively in the service of the cilia, and that the remaining two neural divisions regulate the muscles. These considerations also support our hypothesis that the proposed synapses of dopaminergic and cholinergic neurons onto serotonergic neurons will be found in the septa.

Coordination between the ciliary pump and the branchial muscles. Two video endoscopic observations suggest that the lateral cilia and the gill muscles act in a coordinated fashion. First, when the gills of a unionid, *Pyganodon cataracta*, stop pumping, the water tubes constrict, but re-open when pumping resumes (Tankersley, 1996). Second, when the valves of *Mercenaria* are closed, the lateral cilia are immobile, and the gills are tonically contracted, both longitudinally and dorsoventrally (Baker, pers. comm.).

When the clam is actively pumping, we expect that serotonergic stimulation of the muscles is reduced and the muscles are relaxed. Under these circumstances, the ostia, water tubes, and blood vessels would be open, so the flow of water and hemolymph would be maximized. When the clam closes, the dopaminergic innervation would become active, switching the lateral cilia off and stimulating the serotonergic plexus. The longitudinal and dorsoventral muscles and the water-tube muscles would then constrict, closing the ostia and constricting the water tubes and blood vessels.

Comparative aspects of branchial muscle pharmacology

Although there is an extensive literature on the pharmacology of bivalve muscles, it is largely focused on the anterior byssus retractor muscle of *Mytilus* and isolated ventricles of a variety of bivalves including that of *Mercenaria*. In contrast, the pharmacology of branchial muscles has been studied in relatively few species of bivalves, in part because the branchial musculature is not an advantageous model for the study of muscle cells *per se*. Branchial muscles are small and are embedded in a complex organ; thus they cannot be directly attached to a recording apparatus. Furthermore, their neural supply is complex, and the innervation of specific muscles is not readily accessible. However, the pharmacology of these muscles has been studied by those interested in the physiology of bivalve gills; the available data are summarized in Table 4.

The effect of 5HT on the muscle varies with species. The gills of *Mytilus* and *Mercenaria* are contracted by 5HT, whereas those of *Dreissena polymorpha, Anodonta grandis*, and *Ligumia subrostrata* are relaxed. There is no taxonomic order in these data; but 5HT contracted the gills of the two marine species and relaxed those of the three freshwater species. Because the sample size is miniscule, however, the

apparent relationship between 5HT action and habitat may be coincidental.

ACh had a net excitatory effect on five of the six species on which it was tested; one species (*Ligumia subrostrata*) showed no effect, but the gills were not pretreated with eserine. The effect of ACh on the gills of *Mercenaria* was revealed only after pretreating them with eserine; in addition, the inhibitory effect of ACh became evident only when the gills were exposed to a 5HT antagonist and eserine. The relaxing effect of ACh has not been seen in any of the other gills tested, but then the pharmacological analysis reported here was not used in the other studies.

Painter and Greenberg (1982) examined the effects of 5HT and FMRFamide on the ventricles of 50 species of bivalves and remarked that "the responses were strikingly diverse, varying qualitatively with dose as well as species." In their analysis, however, clear taxonomic relationships were discernable. In comparison to ventricles, gills are much more complex and interact directly with the environment. For example, sodium transport in the gills of freshwater mussels appears to be regulated by a serotonergic neural mechanism (data summarized in Dietz *et al.,* 1985).

The odd response of the control gills in experiments with antagonists

When two successive equal doses of any agonist (*i.e.*, 5HT, DA, or ACh) were applied to the control gills, the second contraction was typically larger than the first, and this result was initially inexplicable. Later, however, we discovered that the gills produce nitric oxide (NO) in response to 5HT, and that NO potentiates gill muscle contractions (Gainey *et al.,* 1999b). This mechanism may also explain another experimental observation: that ultrasonic transducers record higher maximal contractions than force transducers, and they produce dose-response curves with lower EC_{50}s. Thus, when force transducers were used, demibranchs could be exposed only to a single dose of agonist, so the individual contractions constituting the dose-response curves were not potentiated by NO. In contrast, when ultrasonic transducers were used, the demibranchs could be exposed to a set of increasing doses of agonist, so NO was produced, the contractions were potentiated, and the resulting dose-response curves were steeper.

Summary

The gills of *Mercenaria* are equipped with an array of muscles and four distinct sets of cilia, and the activity of these effectors—coordinated by a complex neural network—transports water and particles in support of respiration and feeding. This paper and a previous one on the modulation of ciliary activity (Gainey *et al.,* 1999a) lay the groundwork for studies of the integrated control of gill function.

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