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Neural control of the velum in larvae of the gastropod, *Ilyanassa obsoleta*

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Summary

Larval molluscs commonly use ciliated vela to swim and feed. In this study we used immunohistochemistry to demonstrate innervation of velar cilia and muscles by monoaminergic and peptidergic fibres in the caenogastropod, *Ilyanassa obsoleta*. Photoelectric recordings from pre-oral cilia on isolated pieces of velum revealed that serotonin increased, whereas catecholamines (dopamine and norepinephrine) decreased beat frequency at concentrations of 10^{-6} to 10^{-9} mol l^{-1}. Catecholamines also increased the frequency of momentary, isolated arrests of pre-oral cilia, but failed to suppress beating of the post-oral cilia at these concentrations. The neuropeptides, FMRFamide and Leu-enkephalin, did not affect the frequency of ciliary beating or of isolated ciliary arrests, but did induce numerous muscular contractions, which were accompanied by sustained ciliary arrests. In terms of whole animal behaviour, serotonin caused larvae to concentrate toward the top of a water column and to increase feeding, whereas catecholamines caused larvae to concentrate toward the bottom of a water column and decrease feeding. Monoamine analogues which facilitated or opposed the effects of synthetic transmitters on larval behaviour, further suggested that these transmitters are released endogenously to control velar function. Finally, applications of peptides to whole larvae caused increased frequency of locomotory arrests. Together these findings demonstrate several potential roles for the nervous system in controlling larval behaviour in gastropods.

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Key words: serotonin, dopamine, norepinephrine, monoamines, FMRFamide, Leu-enkephalin, neuropeptides, cilia, veliger, muscle, mollusc.

Introduction

Many animals have complex life cycles accompanied by changes in anatomy, physiology and behaviour, appropriate to the different ecological niches inhabited by each stage. Detailed knowledge of one stage may, therefore, provide inadequate background for understanding fundamental processes operating in another stage. For example, molluscs have been the subjects of extensive research as models for understanding neural underpinnings of behaviour (Chase, 2002; Kandel, 1976), but such work has focused almost exclusively upon adults with little attention paid to the larvae. And yet, examination of larval behaviour and its neural control would provide insight into how these organisms survive this critical life stage and further our understanding of the development and evolution of the molluscan nervous system.

Molluscan larvae typically use a velum, which is rimmed by one or two bands of cilia, to swim and feed (Fretter, 1967; Mackie et al., 1976). The longer, pre-oral cilia are located on the outer edge of this organ and generate the propulsive forces necessary for swimming. Shorter, post-oral cilia, when present, are located on a nearby parallel ridge and collect food particles downstream from the pre-oral band. Separating the two bands is a food groove, in which the combined currents of ciliary activity entrap and transport food particles to the mouth (Chia and Buckland-Nicks, 1984; Strathmann and Leise, 1979).

The basic anatomy and function of the velum have been well established, but little is known about neural control over this organ, although velar innervation has long been known to exist. For example, Carter (Carter, 1928) first provided histological evidence for neural innervation of the velum in larval nudibranch molluscs. More recently, Mackie et al. (Mackie et al., 1976) and Marois and Carew (Marois and Carew, 1997b) used electron microscopy to confirm innervation of pre-oral cells in *Mangelia nebula* and *Aplysia californica*, and in the latter species, demonstrated that serotonergic fibres contributed to such innervation. In fact, immunocytochemical studies have now demonstrated serotonergic innervation of the vela of various gastropods (Dickinson et al., 2000; Dickinson et al.,

1999; Kempf et al., 1997; Page and Parries, 2000) and bivalves (Croll et al., 1997; Plummer, 2002), although the cell types of the targets are generally unknown. Finally, physiological evidence also exists suggesting that momentary arrests of pre-oral cilia on the velum might be elicited by neural input (Arkett, 1987; Leise and Hadfield, 2000).

Serotonergic control of ciliary beating in developing molluscs has been best studied in the fresh water gastropod *Helisoma trivolvis*, which, like other pulmonates, develops *in ovo* and has a greatly reduced velum and larval nervous system (Croll, 2000; Croll and Dickinson, 2004). *H. trivolvis* uses a ciliated foot to rotate within the egg capsule to enhance aeration of the capsular fluid (Diefenbach et al., 1991; Marois and Croll, 1991). A putative sensorimotor neuron detects intracapsular hypoxia and stimulates ciliary beating *via* local release of serotonin (Kuang et al., 2002). Although these findings may be generally applicable to closely related species (Uhler et al., 2000), questions arise about whether direct-developing pulmonate gastropods are representative models for understanding the control of ciliary activity on vela of free-living molluscan larvae. In particular, ciliated cells on the foot of *H. trivolvis* are only known to possess serotonergic innervation whereas serotonergic, catecholaminergic and peptidergic axons extend into the vela of larval caenogastropods (Dickinson and Croll, 2003; Dickinson et al., 1999; Page and Parries, 2000), heterobranch gastropods (Dickinson et al., 2000; Kempf et al., 1997) and bivalves (Croll et al., 1997; Plummer, 2002).

Questions also arise regarding the neural control of the larval retractor muscle, which extends bilaterally into the velum. Large contractions of this muscle occur either spontaneously or following collisions with the surface or other objects and result in complete retraction of the velum into the shell (Fretter, 1967). Subtle changes in the tone of this muscle could also reposition the velum and affect the direction of swimming. Neural innervation of the velar musculature has been described in developing caenogastropods (Dickinson and Croll, 2003; Page and Parries, 2000) and heterobranchs (Kempf et al., 1997), but it remains unclear how this innervation contributes to maintenance and control of muscle activity.

In the present study we characterized the neural control of the velum and ultimately the behaviour of the developing caenogastropod, *Ilyanassa obsoleta*. We first used immunocytochemistry to examine neurotransmitters previously identified in larval *I. obsoleta* (Dickinson and Croll, 2003). We then tested the hypotheses that these putative neurotransmitters affect the activity of cilia and muscles in the isolated velum and the behaviour of intact larvae. Finally, we also tested the hypothesis that monoamines are released endogenously to regulate normal swimming behaviour.

Materials and methods

Larvae of *Ilyanassa obsoleta* Say 1822 were collected as described previously (Dickinson and Croll, 2003). Briefly, egg capsules were gathered from aquaria containing adults collected from the wild and were kept in 1 l containers of aerated, filtered (0.22 μm pore size) seawater (FSW; from the Dalhousie University Aquatron System). Upon hatching, larvae were transferred to culture systems similar to those described previously (Miller and Hadfield, 1986) and fed daily with *Isochrysis galabana* (Clone T.ISO; Provasolin Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME, USA) at a final concentration of ~15 000–20 000 cells ml^{-1}. All larvae used for experiments described below were 7–10 days post-hatching.

Morphology

Immunocytochemistry

Immunolabelling followed procedures outlined previously (Dickinson and Croll, 2003). Larvae were anaesthetized using a 7.0% solution of $MgCl_2$ for several minutes until fully extended from their shells. Specimens labelled with polyclonal anti-FMRFamide, anti-serotonin (ImmunoStar, Inc., Hudson, WI, USA), or anti-Leu-enkephalin (Chemicon International, Inc., Temecula, CA, USA) antibodies or a monoclonal anti-alpha-tubulin antibody (DM1A clone; Sigma Chemical Co., Mississauga, ON, Canada) were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 50 mmol l^{-1} Na_2HPO_4 and 140 mmol l^{-1} NaCl, adjusted to pH 7.2) for 10–30 min at room temperature (22–24°C). For immunolabelling with a monoclonal tyrosine hydroxylase (TH) antibody (ImmunoStar, Inc., Hudson, WI, USA), larvae were fixed in 100% methanol for 10–30 min at –20°C.

After fixation, shells were decalcified for 12–24 h with a solution of 80% 0.23 mol l^{-1} ethylenediaminetetraacetic acid (EDTA) and 20% 0.1 mol l^{-1} sodium acetate and specimens were then permeabilized with 4% Triton X-100 in PBS overnight at 4°C. Tissues were next incubated for 3–7 days (4°C) in one of the primary antibodies listed above, diluted 1:500–1:1000 in PBS with 1.0% goat or sheep serum and 1.0% Triton X-100. (No significant differences were noted in staining over these dilutions and incubation times.) This was followed by 1–2 days incubation in secondary antibodies: goat anti-rabbit (for polyclonal primary antibodies) or sheep anti-mouse (for monoclonal primary antibodies) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA), FITC or rhodamine (Bio/Can Scientific, Mississauga, ON, Canada) and diluted 1:50 in PBS.

Muscle labelling

Some larvae fixed in 4% PFA and labelled with anti-5-HT, Leu-enkephalin or FMRFamide were double labelled with phalloidin (which marks F-actin) as described (Degnan et al., 1997). Briefly, after incubation in secondary antibodies conjugated with FITC or Alexa Fluor 488 and subsequent washing, specimens were incubated in a 1:100 dilution of phalloidin (labelled with tetramethlyrhodamine B isothiocyanate; TRITC; Sigma Chemical Co.) for 1–4 h. The alcoholic fixation needed for localization of TH-like immunoreactivity was incompatible with subsequent F-actin staining.

Table 1. *Chemical compounds used for in vivo studies on isolated vela and in vitro studies on intact larvae*

Chemical	Common name	Distributor	Action in vertebrates	Effectiveness in molluscs	Concentration tested (mol l^{-1})	
					On isolated velum	In intact larvae
5-Hydroxy-tryptamine hydrochloride	Serotonin	Sigma			10^{-6}–10^{-9}	10^{-4}–10^{-6}
Arterenol bitartrate salt	Nor-epinephrine	Sigma			10^{-6}–10^{-9}	10^{-4}–10^{-6}
Dopamine hydrochloride	Dopamine	Sigma			10^{-6}–10^{-9}	10^{-4}–10^{-6}
Mianserin hydrochloride	Mianserin	Sigma	5-HT_2 receptor antagonist (Willins et al., 1999)	(Diefenbach et al., 1991; Uhler et al., 2000)	NA	10^{-5}
Fluoxetine hydrochloride	Fluoxetine	Eli Lilly[a]	Serotonin re-uptake inhibitor (Fuller, 1996)	(Couper and Leise, 1996; Fong et al., 1998; Uhler et al., 2000)	NA	10^{-5}
Spiperone hydrochloride	Spiperone	Sigma	D_2 antagonist (Amenta et al., 1999); alpha-adrenergic antagonist (Testa et al., 1993); 5-HT2/5-HT1 (Geerts et al., 1999)	(Green et al., 1996; Pechenik et al., 2002)	NA	10^{-5}
Haloperidol	Haloperidol	Sigma	$D_{2\text{-}4}$ receptor antagonist with possible action on 5-HT receptors (Seeman, 2002)	(Heiss et al., 1976; Voronezhskaya et al., 1993)	NA	10^{-5}
Alprenolol	Alprenolol	Sigma	β-adrenergic antagonist (Surman and Doggrell, 1993)	(Marsden and Hassessian, 1986) (polychaete)	NA	10^{-5}
FMRFamide	FMRFamide	Sigma			10^{-6}–10^{-9}	10^{-6}
Leu-enkephalin	Leu-enkephalin	Sigma			10^{-6}–10^{-9}	10^{-6}

Additional references to transmitters are given in the text or involve common knowledge. NA, not applicable.
[a]Eli Lilly, Toronto, ON, Canada.

Mounting and viewing

Specimens were mounted in glycerol (3:1) in 0.1 mol l^{-1} Tris buffer (pH 8.0) with 2% *n*-propyl gallate added to prevent fading (Longin et al., 1993) and then viewed with a Zeiss LSM 510 confocal microscope. Images were created by superimposing stacks of 10–80 images obtained through stepped sequences of focal planes at 0.10–0.80 μm intervals. Projections were created with Zeiss LSM 510 software. Images were assembled into plates and labelled using Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA, USA).

Controls

As negative controls, larvae were processed without incubation in primary antibodies; such specimens exhibited no detectable fluorescence. Pre-absorption controls were also performed for anti-FMRFamide and anti-Leu-enkephalin. Synthetic FMRFamide and Leu-enkephalin (Sigma Chemical Co.) were added at a concentration of 200 μg ml^{-1} to the 1:500 dilutions of their respective antibodies. The antibodies were pre-absorbed for 24 h at 4°C and then spun for 10 min at 5000 r.p.m. in a bench-top centrifuge. Larvae were incubated in the supernatant and processed as described above. These specimens did not exhibit immunoreactivity. Positive controls included parallel processing of embryonic and larval *Lymnaea stagnalis* and *Aplysia californica* with known labelling patterns (Croll and Voronezhskaya, 1995; Marois and Carew, 1997a; Marois and Croll, 1992).

Photodiode recordings of ciliary beating in isolated velar lobes

Preparation of the isolated velum

Larvae were anaesthetized with 7% $MgCl_2$ and fully extended vela were transected lateral to the eyespots, hence peripheral to the apical organ and developing cerebral ganglia. This produced single isolated velar lobes on which the cilia continued to beat. Pieces of velum were next pipetted through three washes of fresh FSW and then suspended in 1 ml FSW within a silicone compartment adhered to a glass slide. A glass pipette, pulled to a fine tip and then broken and polished to yield a lumen of 20–30 μm, was mounted on polyethylene tubing embedded in the wall of the chamber and connected to a syringe. Vela were positioned near the tip of the pipette, and application of light suction immobilized the specimens. Such vela were left to acclimatize for 2 min during which FSW was continuously replaced (2 ml min^{-1}).

Drug administration

Drug perfusions of 10 ml were used because preliminary experiments indicated that this volume completely washed a dye solution from the chamber.

Drugs were introduced in ascending order of concentration from 10^{-9} mol l^{-1} to 10^{-8} mol l^{-1} at intervals of at least 2 min after cilia resumed their normal beat patterns following washouts of approximately 30 ml FSW. For higher drug concentrations different specimens were used for each trial. Six specimens were tested at each of four concentrations (10^{-6}–10^{-9} mol l^{-1}) for each drug (see Table 1), all at room temperature (22–24°C).

Photoelectric recordings

Immobilized vela were observed with a 40× Zeiss long-working-distance, fluid-immersion objective. Beating cilia could be viewed through the oculars while images were also projected onto a white screen *via* the camera port of a Leitz Aristoplan microscope in a darkened room. Such images were positioned over a small hole (diameter 1 mm) in the screen. A photodiode mounted behind the hole recorded changes in light intensity caused by the shadows of beating cilia. The roughly sinusoidal output was displayed on an oscilloscope (Tektronix, Beaverton, OR, USA) and further downloaded to a computer through Digidata 1320A software in conjunction with Axoscope 8 (both from Axon Instruments, Inc., Union City, CA, USA). Shadows caused upward deflections on the traces, in which height corresponded to the intensity, and width to the duration, of the shadowing. Data acquisition was started approximately 2 min after completion of drug perfusions and continued for 3 min per trial. Calculations of ciliary beat frequency (CBF) were based upon time intervals required for eight maximal downward deflections during a regular beating pattern (i.e. without ciliary arrests; see below).

Comparisons between experimental groups were performed through standard one-way analyses of variance (ANOVA) and Dunnett's tests for multiple pairwise comparisons (P<0.001). All statistics were performed with SPSS (Chicago, IL, USA) statistical software. Oscilloscope traces selected for presentation were filtered (lowpass filter: 20–30 Hz) with Clampfit 8.0, and assembled using Photoshop 7.0 (Adobe, San Jose, CA, USA). Schematic diagrams were constructed using Corel Draw 11 (Corel Corp. Ltd., Ottawa, ON, Canada).

Photoelectric measurement of post-oral ciliary beating frequency

Activity of post-oral cilia was assessed with similar recording procedures. However, these cilia could not be monitored when the pre-oral cilia were beating, since the larger cilia obscured their smaller neighbours. We therefore measured activity of the post-oral cilia only during drug-induced arrest of the pre-oral cilia (see below).

Measurements of muscular contractions and ciliary arrests

Temporary cessations of regular ciliary beating were occasionally noted in the photoelectric recordings. Simultaneous viewing of the velum through microscope oculars permitted further description and counting of these ciliary arrests. A computerized event marker was used to record the occurrence of such events before and during applications of different compounds. Observations were made of four specimens at each drug concentration for 1–2 min during measurement of ciliary beating. Differences in frequency were analyzed through one-way analyses of variance (ANOVA) and Dunnett's tests for multiple pairwise comparisons (P<0.05).

Behavioural tests on whole larvae

Vertical distribution test

Approximately 50 larvae were placed into vertical polystyrene pipettes (30 cm× 1 cm outer diameter), containing 10 ml of seawater and one neuroactive chemical (Table 1). After 30 min, each column was drained into two fractions: the bottom *versus* the top of the column. (Preliminary experiments indicated that monoamines were effective at 10^{-4}–10^{-6} mol l^{-1} concentrations in whole larvae.) The larvae in each fraction were then counted. A total of eight trials were performed for each chemical at each concentration. Neuropeptides were not tested in this experiment since preliminary studies indicated that they did not retain their effectiveness for the 30 min duration of the experiment.

Feeding rates

A matched pair design was used to calculate relative feeding rates of larvae (equation 1) when exposed to the various chemicals (Table 1). Tests were performed in 50 ml plastic tissue culture flasks (VWR, Canlab, Mississauga, ON, Canada) containing *Isochrysis galbana* (Clone T.ISO) in seawater at concentrations generally ranging between 100 000 and 200 000 cells ml^{-1} at which larvae ingest at relatively constant rates (Dickinson, 2002). Each trial (N=8 per drug), consisted of a pair of flasks with one containing algae and the chemical tested but no larvae (A in equation) and the other containing equal concentrations of the algae and chemical plus 25 larvae (B in equation). After each trial, flasks were sampled (1 ml) and the algae killed by adding 20 μl of 4% PFA. The algal concentrations were determined by standard counting methods using a Brightline haemocytometer (VWR, Canlab). Additional controls, serving to normalize the data, consisted of averaged algal counts for flasks containing algae plus larvae without any chemical (D in equation), or alternatively algae alone (C in equation). Neuropeptides were not tested in this experiment since their effectiveness did not last the length of the experiment.

$$\text{A–B/C–D} \times 10\ \text{ml}\ 4\ \text{h}^{-1}\ 25\ \text{animals}^{-1} = \text{relative feeding rate}, \quad (1)$$

where A is the number of cells ml^{-1} (algae + chemical), B is the number of cells ml^{-1} (algae + chemical + larvae), C is the average number of cells ml^{-1} (algae) and D is the average number of cells ml^{-1} (algae + larvae).

Frequency of locomotory arrests: ciliary arrest and velar contractions

The frequency of locomotory arrests and effects of drugs (Table 1) on this behaviour were counted over 5 min periods with intact larvae swimming freely in small depression slides

(2 cm diameter). This arrangement permitted continuous monitoring of the animals at low magnification (15–30×), but did not permit discrimination between isolated ciliary arrests and subtle contractile arrests. We therefore pooled all visible arrests into a single count. A total of 10 larvae were tested for each chemical.

Statistics

Data were subjected to tests for normality and a one-way analysis of variance (ANOVA). Alternatively, for those trials that failed to show normal distribution a Kruskal–Wallis one-way ANOVA on ranks was conducted. Dunnett's tests for multiple pairwise comparisons were used to identify drug trials that differed significantly from the controls (P<0.05).

Results

Morphology

The velum of *I. obsoleta* was rimmed with long (50–70 μm) cilia, which exhibited intense tubulin-like immunoreactivity. These pre-oral cilia arose from slender columnar epithelial cells that were located on the outer edge of the velum (Fig. 1A,B). The more cuboidal post-oral ciliated cells were arranged as a band along the posterior edge of the food groove parallel with the pre-oral ciliated cells. Each post-oral cell gave rise to several bundles of short (15–25 μm) cilia. Along the basal edges of the pre-oral ciliated cells, tubulin-like immunoreactivity revealed what appeared to be axons (Fig. 1B).

Axons radiated into each velum from the apical and/or cerebral ganglia (Dickinson and Croll, 2003). Fibres containing serotonin (Fig. 1C), FMRFamide and Leu-enkephalin (Fig. 1D) possessed numerous varicosities along the basal edges of the pre- and post-oral ciliated cells (also see Fig. 2) and along the velar musculature (serotonin, Fig. 2A; FMRFamide, Fig. 2B,C; Leu-enkephalin, Fig. 2D), which originated in the main retractor muscles and formed attachments near the basal edges of the pre-oral cells. Additional bands of muscle fibres ran parallel to the food groove along the basal edges of pre-oral cells. TH-immunoreactive cells were located along the posterior edge of the food groove close to the post-oral ciliated cells (Fig. 2E). Processes from TH-immunoreactive cells ran along the basal edges of the post-oral ciliated cells and also extended across the food groove to the basal edges of the pre-oral cells (Fig. 2F).

Ciliary beat frequency and arrests in isolated velar lobes

Photoelectric recordings from beating cilia

When viewed at 400×, pre-oral ciliary beating was clearly observed as metachronal waves which travelled along the rim of the isolated pieces of velum. Photoelectric recordings of ciliary activity were influenced by the location of the photodiode with respect to the projected images of the beating cilia (see Fig. S1 in supplementary material), but placement of the photodiode at mid-length of the cilia consistently yielded three-phase waveforms (Fig. 3A). All recordings used for analysis of ciliary beat frequency (CBF) were from mid-length positions. From all pooled recordings (N=30), we estimated that pre-oral cilia beat at a mean CBF of 9.19 Hz (±1.05 Hz) under normal control conditions.

Serotonin applied to the isolated velum significantly increased CBF in a dose-dependent manner (Fig. 4) for all concentrations tested (P<0.001). Notably, when administered at 10^{-6} mol l^{-1}, serotonin led to an approximately threefold increase in ciliary beating (Fig. 3B). Analyses of photodiode recordings with increased temporal resolution (Fig. 5) demonstrated that the effects of serotonin on CBF were accompanied by graded changes in the beat pattern. Thus, with increasing concentrations, phase 1 remained clearly

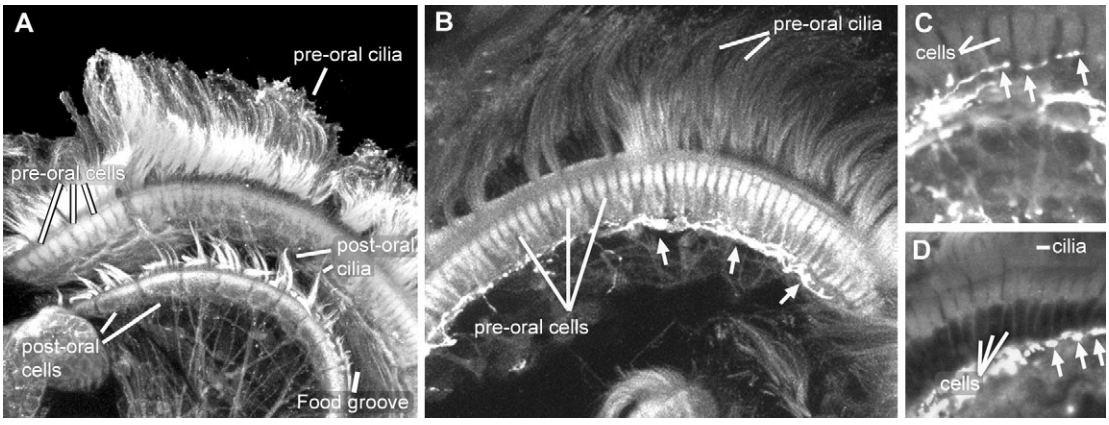


Fig. 1. Anatomy and innervation of ciliated cells on the velum. (A) Lateral view of a velar lobe showing tubulin-like immunoreactivity in cell bodies and cilia along the pre- and post-oral bands. Scale bar, 20 μm. (B) Lateral view of the velum at a slightly deeper focal plane showing details of the pre-oral ciliated cells and a bundle of tubulin-like immunoreactive axons (arrows) at their bases. Scale bar, 20 μm. (C) At higher magnifications, serotonin-immunoreactive fibres and varicosities (arrows) can be seen near the bases of the pre-oral ciliated cells. Scale bar, 10 μm. (D) Lines of varicosities (arrows) exhibit Leu-enkephalin immunoreactivity. Pre-oral cells possess slender nuclei at their bases. Scale bar, 10 μm.

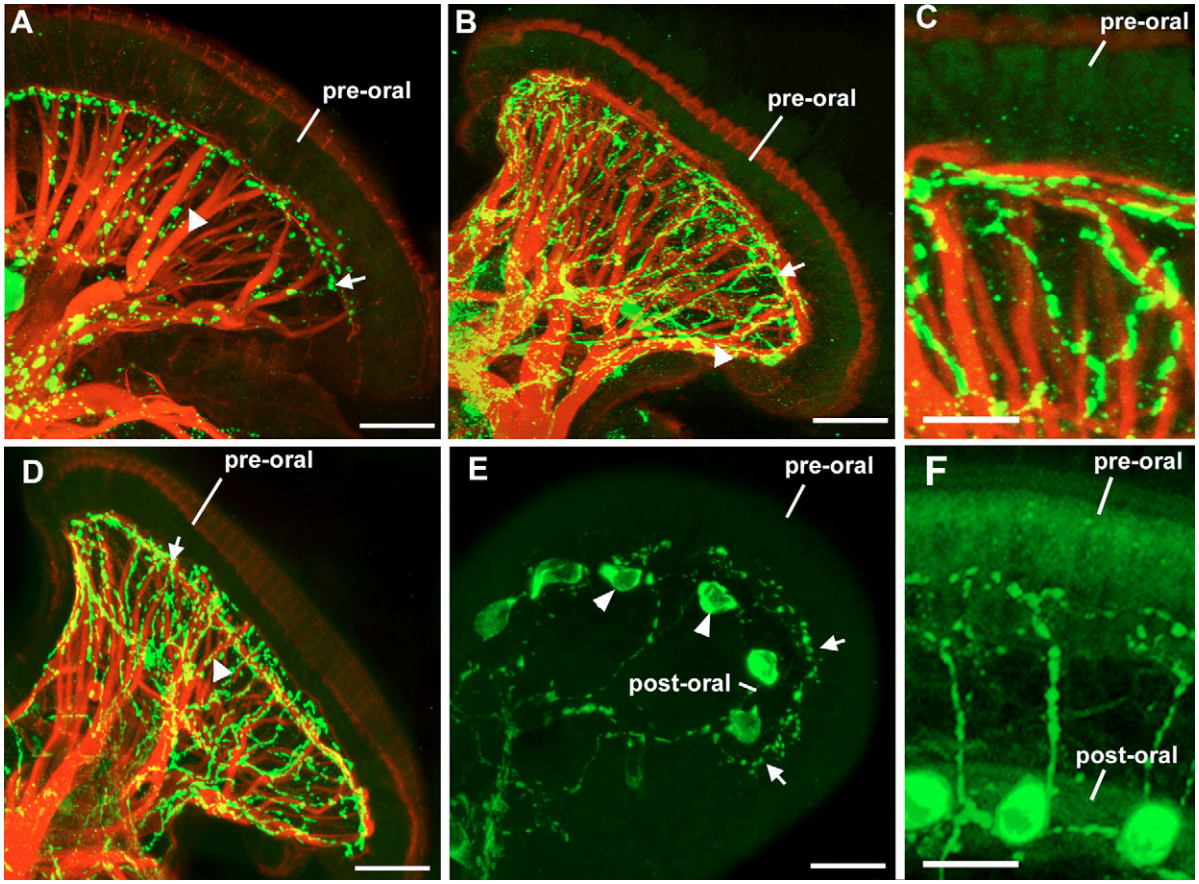
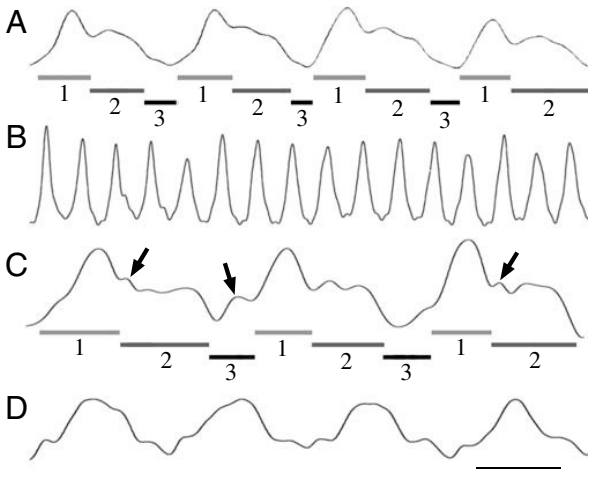


Fig. 2. Immunocytochemical localization of neural elements (green) and F-actin labelling of muscle (red) in the velum. (A) Lateral view of a velar lobe showing 5-HT-immunoreactive axons (green) with varicosities along the muscles and concentrated distally near the pre-oral cells. Scale bar, 30 μm. (B) Lateral view of a velar lobe showing FMRFamide-immunoreactive axons (green) and muscle fibres (red). Scale bar, 30 μm. (C) High magnification of the edge of a velar lobe showing FMRFamide immunoreactive axons (green) and muscle fibbers (red). Scale bar, 25 μm. (D) Lateral view of a velar lobe showing Leu-enkephalin-immunoreactive axons (green) and muscle fibres (red). Scale bar, 30 μm. (E) Lateral view of a velar lobe showing TH-like immunoreactive cells and axons. Scale bar, 10 μm. (F) High magnification of the edge of a velar lobe showing TH-like immunoreactive cells and axons and cells containing pre-oral and post-oral cilia. Scale bar, 10 μm.

recognizable, while phases 2 and 3 became less distinct, thereby suggesting possible changes in the stiffness of the cilia, their stroke angles or their positions.



By contrast, catecholamines caused significant dose-dependent decreases in CBF (Fig. 4). Application of dopamine at 10^{-6} mol l^{-1} caused complete arrest of the pre-oral cilia in five of six specimens (see below). Perfusions of dopamine at lower concentrations (10^{-7}–10^{-9} mol l^{-1}) did not halt ciliary activity but led to significant dose-dependent decreases of CBF in all groups (P<0.001). Photodiode recordings obtained during dopamine perfusions suggest that the three-phase cycling of

Fig. 3. Effects of transmitters on pre-oral ciliary beating. (A) Under control conditions, cilia beat at approximately 8–9 Hz and all three phases are evident. (B) Perfusions of 10^{-6} mol l^{-1} serotonin caused a threefold increase in ciliary beat frequency (CBF). Different phases are no longer evident in this recording (but see Fig. 5). (C) Perfusion of 10^{-7} mol l^{-1} dopamine slowed the ciliary beat frequency. All three phases of activity are apparent, but additional, irregular peaks (arrows) were also observed. (D) Perfusion of 10^{-7} mol l^{-1} Leu-enkephalin did not alter ciliary beat frequency but change the waveform of the beat cycle, with the normal three-phase form no longer evident. Scale bar, 150 ms.

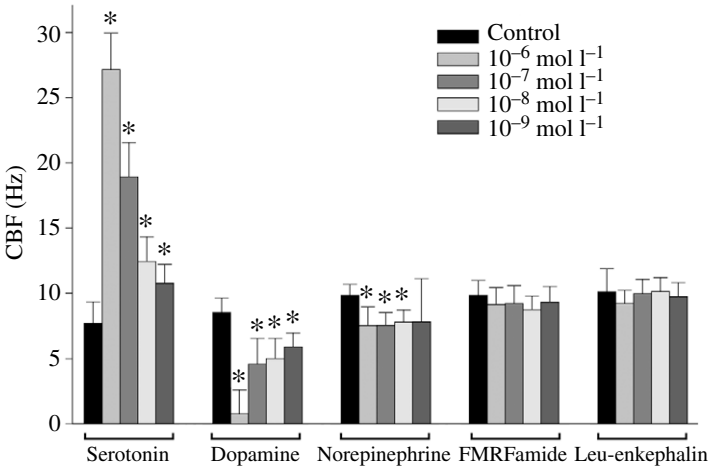


Fig. 4. Summarized effects of putative transmitters (serotonin, dopamine, norepinephrine, FMRFamide and Leu-enkephalin) at different concentrations (10^{-6} mol l^{-1} to 10^{-9} mol l^{-1}) on ciliary beat frequency (CBF) of the pre-oral cilia. Asterisks indicate significant difference when compared to the respective control groups (P<0.001).

ciliary activity was largely preserved (Fig. 3C), although there were several irregular peaks on the traces. Norepinephrine also caused slowing of CBF (Fig. 4), although it was less effective than dopamine in reducing ciliary beating, and did not cause complete, sustained ciliary arrests at the highest concentration administered.

Neuropeptides did not significantly alter the CBF of pre-oral cilia at any of the concentrations tested (Fig. 4). However, application of both FMRFamide (Fig. 3D) and Leu-enkephalin (data not shown) markedly changed the three-phase waveform

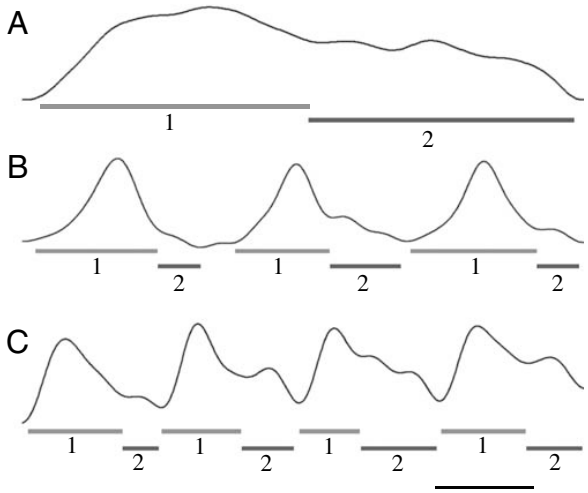


Fig. 5. Serotonin affects both the frequency and the waveform of the ciliary beat as seen at higher temporal resolution. (A) Control trace of cilia in seawater without serotonin. Both the phases 1 and 2 are evident. The subsequent phase 3 is not shown. Perfusion with 10^{-7} mol l^{-1} (B) and 10^{-6} mol l^{-1} (C) serotonin increases the speed of ciliary beating. Phase 2 is less regular and phase 3 is no longer apparent. Scale bar, 50 ms for all traces.

Fig. 6. Photodiode recordings from post-oral ciliary activity during perfusion with 10^{-6} mol l^{-1} dopamine. Different phases of the beat waveform are not as distinct as those recorded in large pre-oral cilia. Scale bar, 150 ms.

normally recorded in control conditions; recordings obtained from these experimental groups generally had single, broad, upward deflections that were flanked by irregular smaller peaks.

Post-oral ciliary beat frequency

When pre-oral cilia were beating, they obscured the activity of the smaller post-oral cilia, thereby preventing the measurement of their CBF. During dopamine-induced arrests of the pre-oral cilia (10^{-6} mol l^{-1} concentration), however, we observed that the post-oral cilia continued to beat (Fig. 6) at an average CBF of 13.34 Hz (±2.11 Hz; N=4), which was significantly faster than the pooled normal CBF of the pre-oral cilia (P<0.001). Recordings of post-oral ciliary activity did not yield the clear patterns of multiphasic cycles of the pre-oral cilia. Rather, each cycle was generally characterized by a single large upward and a variable (one to two) number of smaller deflections.

Ciliary arrests and the velar musculature contractions

All measurements of pre-oral CBF described above were obtained during periods of sustained and regular beating. However, recordings were often interrupted by ciliary arrests of varying durations and frequencies. Visual inspection revealed two types of ciliary arrests. In the first type, the pre-oral cilia stopped beating without visible contractions of the velum and without interruption of post-oral ciliary activity. Such isolated ciliary arrests generally lasted only briefly (<1 s), before the pre-oral cilia resumed beating (Fig. 7A). In the second type, large contractions of the velum accompanied arrests of both pre- and post-oral cilia. The post-oral cilia

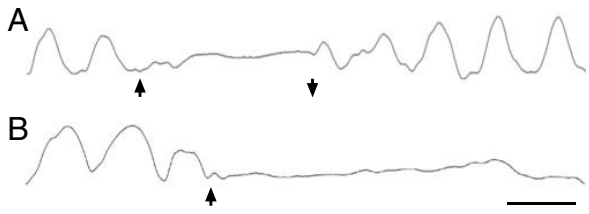


Fig. 7. Photodiode recordings of ciliary arrests along the pre-oral band. (A) Brief, isolated ciliary arrest (onset and offset indicated by arrows). (B) Longer duration contractile arrest, which occurred during muscular contraction (onset indicted by arrow) and often repositioned the entire velum and prevented further recordings. Scale bar, 150 ms.

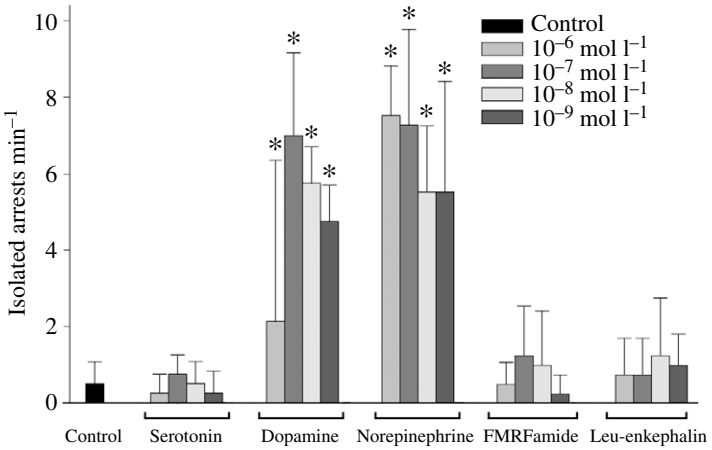


Fig. 8. Summarized effects of putative transmitters (serotonin, dopamine, norepinephrine, FMRFamide and Leu-enkephalin) at different concentrations (10^{-6} mol l^{-1} to 10^{-9} mol l^{-1}) on the frequency of isolated ciliary arrests. Asterisks indicate significant difference when compared with the control group (P<0.05).

always resumed beating shortly after initiation of the contraction, whereas the pre-oral cilia remained quiescent for as long as the contraction persisted. These contractile arrests often changed the shape of the velum or moved it with respect to the photodiode with no subsequent recording of activity (Fig. 7B).

Isolated ciliary arrests were seldom observed in control conditions, occurring on average 0.50 (±0.57) times m^{-1} (Fig. 8). The frequency of these arrests did not change significantly following administration of serotonin at any concentration (Fig. 8; P>0.05). By contrast, perfusions of dopamine at 10^{-6} mol l^{-1} concentration had profound effects on ciliary arrests. Specifically, the pre-oral cilia completely stopped beating with no visible contractions of the velum in 83% (5/6) of the tested specimens during the course of dopamine exposure. When administered at lower concentrations, dopamine did not cause sustained arrests of ciliary beating, but increased the frequency of isolated ciliary arrests in a dose-dependent manner (Fig. 8). Similarly, norepinephrine also increased the frequency of isolated ciliary arrests (Fig. 8), however, unlike dopamine, this compound did not lead to complete cessations of ciliary beating at the highest administered dose. Neither neuropeptide was observed to significantly affect the occurrence of isolated ciliary arrests (Fig. 8).

In control specimens, contractile arrests also occurred at a low mean frequency of 1.50±1.29 contractions m^{-1} (Fig. 9). Serotonin, dopamine and norepinephrine had no significant effects on the frequency of muscular contractions compared to the control level. A striking effect, however, was observed after application of FMRFamide, which caused much more frequent contractile arrests at all concentrations tested (Fig. 9). At the highest FMRFamide dose administered, the contractions accompanying the arrests were more vigorous and occurred in immediate succession to one another, thus causing the velum to twitch constantly for sustained periods. During such periods, the pre-oral cilia remained inactive and were curled onto the velum. Leu-enkephalin had a similar effect to FMRFamide in enhancing the number of contractile arrests (Fig. 9).

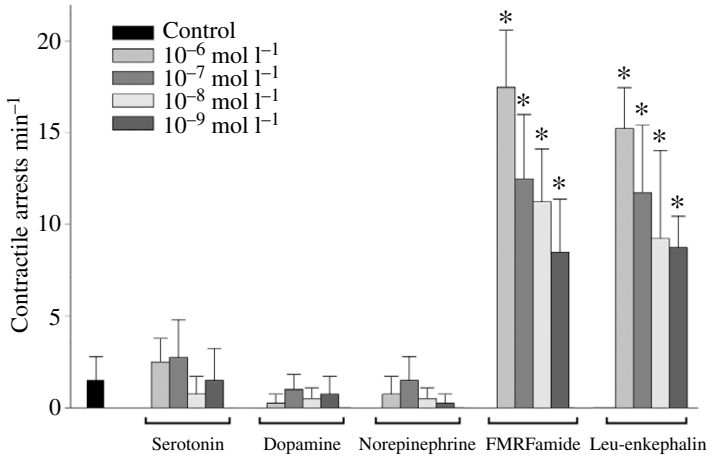


Fig. 9. Summarized effects of putative transmitters (serotonin, dopamine, norepinephrine, FMRFamide and Leu-enkephalin) at different concentrations (10^{-6} mol l^{-1} to 10^{-9} mol l^{-1}) on the frequency of contractile ciliary arrests. Asterisks indicate significant difference when compared with the control group (P<0.05).

Behavioural tests in whole larvae

Vertical test

Bath applications of neuroactive compounds resulted in an overall significant difference in the average vertical distribution of larvae, as expressed by the percentage of animals in the top half of the column (Fig. 10; P<0.001). Under control conditions, an average of 46.70% (±6.63) of the larvae were within the top half of the water column. Serotonin generally increased the percentage of larvae in the top half of the water column in a dose-dependent manner (Fig. 10). Larvae bathed in 10^{-4} mol l^{-1} serotonin, however, were less concentrated toward the top of the column. Closer examination revealed that many of these larvae were contracted into their shells and lying immobile at the bottom. The percentage of larvae in the top half of the column was significantly increased by 10^{-7} mol l^{-1} fluoxetine (the only concentration tested based on preliminary experiments; Fig. 10), whereas mianserin, at a concentration of 10^{-5} mol l^{-1} (the only concentration tested) resulted in a decreased percentage of larvae in the top of the vertical column (Fig. 10).

In contrast to serotonin, both dopamine and norepinephrine significantly decreased the percentage of larvae in the top half of the water column in dose-dependent manners (Fig. 10). Spiperone, conversely, resulted in a significant increase in the percentage of larvae in the top half of the column. However, alprenolol induced a significant decrease in the percentage of larvae

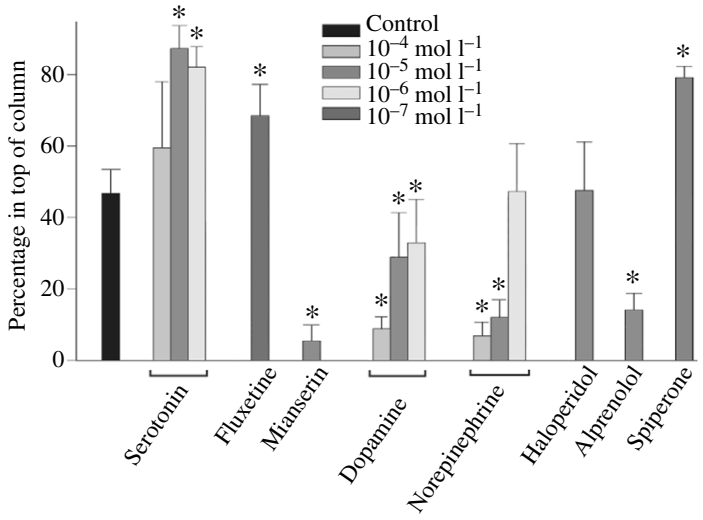


Fig. 10. Summarized effects of putative transmitters and respective analogues on swimming behaviour, expressed as the average percentage of larvae in the top of the water column. Tested compounds included serotonin (10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}), fluoxetine and mianserin (both at 10^{-5} mol l^{-1}), dopamine and norepinephrine (both from 10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}), haloperidol, alprenolol and spiperone (all at 10^{-5} mol l^{-1}). Asterisks indicate significant difference when compared with the control group (P<0.05).

in the top half of the water column whereas haloperidol had no significant effect.

Feeding rate

An overall significant difference in feeding rates was observed following exposure of larvae to transmitters (Fig. 11; P<0.001). When larvae were exposed to 10^{-6} mol l^{-1} and 10^{-5} mol l^{-1} serotonin, the feeding rates increased significantly. Exposure to 10^{-4} mol l^{-1} serotonin resulted in no significant differences from control levels but again a number of larvae were observed to be lying on the bottom of the test chamber at this concentration. Applications of both dopamine and norepinephrine decreased feeding rates in dose-dependent manners from 10^{-6} mol l^{-1} to 10^{-4} mol l^{-1} concentrations.

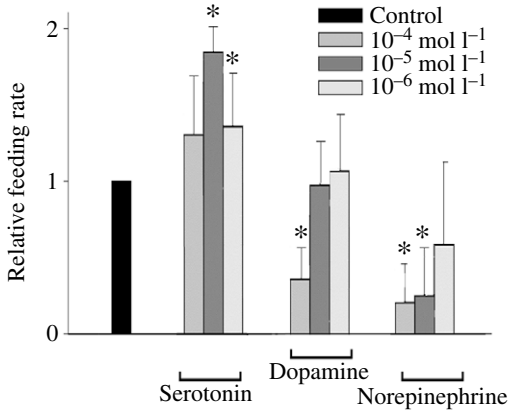


Fig. 11. Summarized effects of putative transmitters on algal intake of *I. obsoleta* larvae expressed as percentage of the control (100%). Tested compounds included serotonin (from 10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}), dopamine and norepinephrine (from 10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}). Asterisks indicate significant difference when compared with the control group (P<0.05).

Locomotor arrests

Applications of neurotransmitters resulted in an overall significant difference in the average number of locomotor arrests (Fig. 12; P<0.001). The control larvae produced approximately 0.87 (±0.99) arrests m^{-1}. Both serotonin and dopamine significantly increased the average frequency of locomotor arrests at concentrations of only 10^{-5} mol l^{-1}. At 10^{-4} mol l^{-1}, both serotonin and dopamine immobilized many larvae, however, in serotonin they were contracted into their shells whereas in dopamine they appeared to be flaccid and extended. Norepinephrine at a concentration of 10^{-4} mol l^{-1} also significantly increased the average frequency of locomotor arrests. Both FMRFamide and Leu-enkephalin caused significant dose-dependent increases in the frequency of arrests with 40- to 50-fold increases over control values at the highest concentrations.

Discussion

The velum of *I. obsoleta* is typical of planktotrophic gastropod larvae with its double rows of cilia, which mediate swimming and feeding behaviours. The velum also contains a network of musculature that can position the velar lobes to

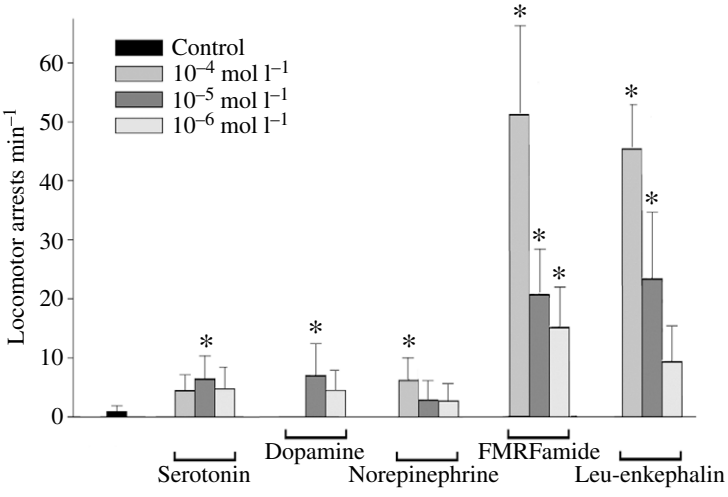


Fig. 12. Summarized effects of putative transmitters on the number of locomotor arrests per minute during exposure of free swimming larvae to the following compounds: serotonin (from 10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}), dopamine and norepineprhine (from 10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}), FMRFamide and Leu-enkephalin (from 10^{-4} mol l^{-1} to 10^{-6} mol l^{-1}). Asterisks indicate significant difference when compared with the control group (P<0.05).

adjust swimming direction and also contract the entire velum to cause cessation of swimming. Together the cilia and musculature allow the larva to position itself in the water column, feed and evade predators. Here we present evidence for neural elements in the velum that modulate the frequencies of ciliary beating, ciliary arrests and velar muscle contractions.

Serotonin and catecholamine regulation of pre-oral cilia

Serotonergic axons were localized throughout the velum and particularly along the bases of the pre-oral cells, and serotonin caused dose-dependent increases in CBF on the isolated velum. Serotonin also increased swimming and feeding in whole larvae, thus reflecting the heightened ciliary activity seen *in vitro*. These results are largely consistent with previous research, which has shown that serotonin stimulated ciliary beating in adult (Aiello, 1990; Audesirk et al., 1979; Cadet, 2004; Catapane, 1983) and developing molluscs (Kuang et al., 2002; Uhler et al., 2000) and in animals from other phyla (Wada et al., 1997).

We also provide evidence that catecholamines decrease ciliary activity and thus have opposite effects to serotonin. Catecholamine-containing neurones are located near the post-oral cells, but fibres arising from these cells appear to contact the pre-oral cells. Dopamine and norepinephrine had dose-dependent actions leading to a gradual decline and ultimately arrest of pre-oral ciliary beating on the isolated velum. Similarly, application of these transmitters depressed swimming and feeding in intact larvae (see below).

There is ample anatomical evidence, to suggest that catecholaminergic innervation of ciliated cells is widespread in adult (Cadet, 2004; Cain and Woodward, 2002) and developing (Croll et al., 1997; Dickinson et al., 1999; Plummer, 2002; Voronezhskaya et al., 1999) molluscs. The identity of endogenous catecholamine(s), however, generally remains unclear. Here we used immunocytochemistry to detect tyrosine hydroxylase, an enzyme involved in the synthesis of both dopamine and norepinephrine. Chemical analyses of adult and larval molluscs suggested that dopamine is the most abundant catecholamine (Cann-Moisan et al., 2002; McCaman, 1984; Pani and Croll, 1995; Pires et al., 2000a), in accordance with descriptions of various actions for dopamine in molluscs (Ascher, 1972; Swann et al., 1982a; Swann et al., 1982b; Swann et al., 1982c). However, many studies (see above) have also detected significant concentrations of norepinephrine (see also Pires et al., 2000b), while yet other evidence suggests possible roles for norepinephrine in modulating larval behaviours (Coon et al., 1985; Pechenik et al., 2002). Clearly, further work must determine the specific roles for dopamine and norepinephrine in the larval nervous systems of molluscs.

Possible mechanisms for aminergic effects

The cellular actions of serotonin and catecholamines are not always clearly understood in other populations of ciliated cells, although serotonergic enhancement of ciliary beating generally appears to be mediated by an increase of intracellular calcium and activation of downstream messengers (e.g. nitric oxide) in molluscs (Cole et al., 2002; Doran et al., 2003) and mammals (Gertsberg et al., 2004). In fact, nitric oxide synthase has been identified in *I. obsoleta* veligers and might therefore play a role in modulation of larval behaviours (Thavaradhara and Leise, 2001). The cellular mechanisms that underlie the actions of catecholamines on ciliated cells are also relatively unknown, but appear to be more complicated than those underlying serotonergic effects. Both excitation and inhibition of ciliated cells have been suggested (Beiras and Widdows, 1995; Cain and Woodward, 2002; Malanga, 1975). Furthermore, both central (Cadet, 2004) and peripheral (Paparo and Murphy, 1975) mechanisms have been postulated to account for the effects of catecholamines. For example, centrally acting dopamine depresses beating of the lateral cilia in *M. edulis*, possibly through inhibition of peripheral serotonin release (Cadet, 2004). However, it was shown in this same species that local catecholamines could also inhibit ciliary beating, possibly by increasing intracellular calcium within the ciliated cells (Paparo and Murphy, 1975), and may resemble calcium dependent ciliary arrests in other molluscs (Arkett et al., 1987; Stommel and Stephens, 1985). Thus, paradoxically, both serotonergic excitation and catecholaminegic inhibition may initially arise from increases in intracellular calcium but modulatory pathways diverge thereafter.

Regulation of pre-oral ciliary beat waveform

Although serotonin and catecholamines clearly affect CBF, changes in waveforms in the photodiode recordings suggest other possible actions as well. One limitation of photoelectric recordings is that they most clearly represent the movement of cilia along a single plane. However, cilia normally move in three dimensions during a single beat cycle (Blake and Sleigh, 1974). Moreover, dopamine has previously been shown to affect ciliary beat plane in sea urchin larvae (Wada et al., 1997). The changes in waveform that we recorded following administration of neurotransmitters may thus represent changes in beat plane or stiffness of the cilia. Such changes in waveform were particularly evident following administration of FMRFamide and Leu-enkephalin, which had no effect on CBF. Thus, while various neuropeptides have previously been shown to affect CBF in adult *T. diomedea* (Willows et al., 1997) and also in the lateral gill cilia of the clam *Mercenaria mercenaria* (Gainey et al., 1999), our results suggest that such substances may also change the plane of ciliary beating. Clearly, high speed video microscopy will be a necessary component of future studies aimed at understanding the full spectrum of effects that neurotransmitters may induce upon ciliary beating.

Neural control over post-oral cilia

Our data suggest that the pre-oral and post-oral cilia may be affected differently by neural input. For example, whereas both pre-oral and post-oral cilia stop during muscular contractions, only the pre-oral cilia stop during spontaneous, isolated arrests or in isolated arrests following applications of catecholamines. Also, pre-oral cilia were completely immobilized by dopamine at high concentrations, but post-oral ciliary beat at speeds that

exceeded normal pre-oral ciliary beating. This suggests that pre-oral ciliary cells are inhibited by catecholamines, but post-oral ciliated cells may be unresponsive to dopamine or may in fact respond to catecholamines with an increase in ciliary beating, although this hypothesis must be tested by observations of post-oral CBF under normal conditions. Similar variations in the responsiveness of different populations of ciliated cells have been reported for serotonin in *H. trivolvis* (Doran et al., 2004) and neuropeptides and dopamine in the clam *Mercenaria mercenaria* (Gainey et al., 1999), suggesting that a different catecholamine receptor or even a lack thereof on the post-oral ciliated cells is plausible.

Neural control over the velar musculature

The cilia on the velum play essential roles in locomotion and feeding. The velar musculature also serves important functions in the generation of such behaviours (Fretter, 1967). Our anatomical data indicated that serotonergic and peptidergic fibres are abundant along muscles, which both radiate into the velum and form a circumferential band near its outer rim. Catecholamine-containing axons, in contrast, are sparse along the radial muscle fibres although they are present near circumferential muscle.

Consistent with numerous previous reports of peptidergic effects on molluscan muscles (Brezden et al., 1999; Hernadi et al., 1998), we found that both FMRFamide and Leu-enkaphalin induced vigorous muscular contractions at all concentrations administered. Such contractions were also often correlated with ciliary arrests suggesting that these two actions are coupled, possibly *via* the peptidergic neural elements. Finally, in addition to eliciting repeated short contractions in the velar muscles, tonic contractions of muscles may also be commanded by the larval nervous system to modulate the angle of ciliary beating, which ultimately may account for both changes in the waveforms of ciliary activity (see above) and for changes in direction of swimming of the whole larvae (see below).

The lack of effects of serotonin on musculature in the present study may be explained in view of known effects of this amine on other molluscan muscles. For example, in buccal muscles of adult *Aplysia californica*, application of serotonin does not cause contractions but rather enhances subsequent contractions elicited by other inputs (Fox and Lloyd, 2002; Hurwitz et al., 2000; Kupfermann and Weiss, 1982). It will therefore be interesting to observe interactions between synaptic inputs to the velar muscles in future research.

Neurotransmitters and analogues affect whole larval behaviour

Administration of monoamines and neuropeptides to whole larvae elicited changes in behaviour which were largely consistent with those expected from observed effects on cilia and muscles in isolated vela; however, such effects were only seen at higher concentrations, presumably because of restricted access to internal receptors. The pre-oral cilia provide the propulsive force used by the larvae to swim upward against the pull of gravity, and thus, one would expect that increased ciliary beating caused by serotonin would result in net upward movement in a water column as observed in the vertical test. Conversely, decreased ciliary beating and heightened levels of ciliary arrests caused by both dopamine and norepinephrine would be predicted to result in net downward movement of larvae within the column.

Ciliary beating also underlies feeding, and our behavioural observations are again consistent with predictions derived from findings in the isolated velum. Thus as expected, serotonin increased feeding rates whereas catecholamines decreased algal intake. The highest concentrations of serotonin and dopamine, however, also appeared to have non-specific effects, thus confounding the results.

In addition to supporting our observations of the effects of neurotransmitters on cilia on isolated vela, these behavioural studies also permitted tests of the hypothesis that monoamines are released endogenously in whole larvae to tonically regulate ciliary beating. For example, fluoxetine, a selective serotonin re-uptake inhibitor and mianserin, a serotonin antagonist, both known to be effective in molluscs (see Table 1), would only be expected to affect swimming in the specific inverse manners observed if serotonin was being released endogenously. By contrast, the catecholamine antagonist spiperone, suggested to be a nonspecific antagonist of catcholaminergic pathways in molluscs (see Table 1), caused a net upward movement of larvae in the water column, as would be the predicted outcome from inhibiting tonically released catecholamines. However, alprenolol (see Table 1) caused net downward movement in the column, which is opposite to the hypothesized effect, whereas haloperidol (see Table 1) had no significant effect at all. Clearly, future studies would be aided by a more extensive pharmacological characterization of the catecholaminergic receptors involved in controlling ciliary beating in the larvae.

In a final set of behavioural experiments, we demonstrated that locomotor arrests in freely swimming larvae are affected by neuropeptides. As expected from findings in the isolated velum, applications of both FMRFamide and Leu-enkephalin led to large, dose-dependent increases in the number of locomotor arrests displayed by intact larvae. Smaller increases in the frequency of arrests elicited by catecholamines were also expected from our observations of isolated vela, but brief arrests of only the pre-oral cilia may have been overlooked in our low magnification observations of swimming larvae. Flaccid paralysis of larvae exposed to the highest concentration of dopamine also confounded these results. Thus, only the slight increase in the frequency of arrests elicited by serotonin was unexpected from our findings of the effects of transmitters on isolated vela. We suggest that this increased frequency of arrests may have been the result of increased numbers of collisions by more rapidly swimming larvae with the water surface and small suspended objects, and therefore not an accurate indicator of the rate of spontaneous arrests. More detailed observations of either free-swimming larvae in larger containers or tethered larvae to prevent such collisions, should be a goal of future studies.

Conclusions

Data from the present study offer new insight into neural control mechanisms over the cilia and muscles of the velum, which together mediate major larval behaviours of free-living veligers, such as *I. obsoleta*. The findings suggest that neural control over the velum is more complex than might have previously been expected for such a simple organism, and in some regards it is reminiscent of autonomic control in vertebrates, with effectors often innervated by dual, antagonistic controls and where blocking receptors of one division reveals its significant, ongoing tonus (Nilsson and Holmgren, 1994).

We specifically investigated regulation of the CBF and ciliary arrest rates of the pre-oral cilia and provided data concerning the CBF of the post-oral cilia. We also provided evidence of neural regulation of an intricate network of velar muscles. Together these effectors are responsible for generating larval swimming and feeding, which we showed to be influenced by neurotransmitter analogues in whole larvae, thereby confirming the endogenous roles of the identified neural substrates.

Clearly, much more work is required to understand neural control of larval behaviour in gastropods. Electrophysiological studies are particularly needed since only a very small number of studies have been published to date using this approach (Arkett et al., 1989; Arkett et al., 1987; Leise and Hadfield, 2000). Furthermore, the larval nervous system of *I. obsoleta* probably contains other transmitters, [as suggested by Dickinson and Croll (Dickinson and Croll, 2003)], which potentially mediate additional controls over velar effectors than those examined in this initial survey. Nonetheless, the present paper, together with previous detailed studies of larval neuroanatomy (Dickinson and Croll, 2003; Lin and Leise, 1996) demonstrate that *I. obsoleta* may prove to be a valuable system for basic understanding of how neurons regulate behaviours in such planktotropic larvae. [But see for preliminary results (Pires and Penniman, 2003) using another model species, *Crepidula fornicata*].

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