The control of anterior foregut motility during a larval molt of the moth *Manduca sexta* involves the modulation of presynaptic activity

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Summary

In the moth, *Manduca sexta*, anterior foregut motility is modulated during the larval–larval molts in order to control the timing of molting fluid (MF) ingestion. MF is the enzymatic mixture that destroys the outer cuticle so that it can be shed at the end of the molt. The onset of the larval–larval molt is characterized by a dramatic decline in the amplitude of the anterior foregut contractions so that MF is not prematurely ingested. As the end of the molt approaches, the robust contractions of the anterior foregut return and the MF is ingested, enabling the larva to free itself from its old cuticle. In the present study we examine possible mechanisms involved in modulating anterior foregut motility during a larval–larval molt. Our results reveal that the release of a blood-borne factor plays a role in the decline in anterior foregut peristaltic activity during the molt. This blood-borne factor reduces the efficacy of the presynaptic endings of the motorneurons, resulting in a reduction in the amplitude of the excitatory junctional potential (EJP) recorded from the anterior foregut musculature. We also present evidence that crustacean cardioactive peptide (CCAP) targets the motorneuron terminals and its actions are sufficient to trigger the dramatic increase in EJP amplitude and anterior foregut contractions. Finally, the surgical ablation of the subesophageal ganglion, which has been previously described to be a source of CCAP neurons and the CCAP projections to the anterior foregut region, blocks both the increase in anterior foregut motility and the ingestion of MF that normally occur at the end of a larval–larval molt.

Key words: molting, foregut, *Manduca sexta*, insect, ecdysis, CCAP, modulation.

Introduction

Animals use many different strategies to ensure that series of motor patterns are initiated at the correct time and in the proper order. One common strategy involves the coordinated release of neuromodulators and hormones to activate and/or inhibit motor systems selectively. The molt-related behaviors of the moth *Manduca sexta* serve as a model system in the effort to define the neural and endocrine signals involved in regulating the timing and order of a series of stereotyped motor patterns. From this work it is recognized that the changing titers of the steroid hormones, the ecdysteroids, act as the master regulators of insect molting by controlling the release of several peptide hormones and neuromodulators, including eclosion hormone, the ecdysis triggering hormones and crustacean cardioactive peptide (CCAP) (Kingan et al., 1997; Truman et al., 1998).

Recently, we found that the proper modulation of anterior foregut motility is essential for the successful completion of a larval–larval molt of *M. sexta* (Bestman and Booker, 2003). As described by Miles and Booker (Miles and Booker, 1994) the anterior foregut of *M. sexta* larvae is composed of a muscular section comprising the buccal cavity, the pharynx, esophagus, and the crop. The rhythmic contractions of the anterior foregut musculature are controlled by neurons originating from the frontal ganglion (Miles and Booker, 1994). During the intermolt larval stages, the alternating constrictions and dilations of the muscles of the foregut (exclusive of the crop) power ingestion and together constitute the peristaltic contractions of the anterior foregut that we describe here (Miles and Booker, 1994). Early in the molt between the 4th and 5th larval instars, the old, outer cuticle separates from the newly forming cuticle that lies beneath it. An enzymatic cocktail, referred to as molting fluid (MF), is secreted into the space created as the two cuticle layers separate. The first 16–18 h of the molt after the old and new cuticles begin to separate, the MF digests and weakens the old cuticle to aid in its removal. As the end of the molt approaches MF is ingested before the old cuticle is shed a few hours later. The movement of the MF during a larval–larval molt is correlated with the level of anterior foregut motility. Just prior to the first appearance of MF in the space between the old and newly developing cuticle, the robust contractions of the anterior foregut are suspended, only to return some 16 h later to power the ingestion of MF prior to ecdysis.

During a larval–larval molt, the presynaptic terminals on the anterior foregut musculature are targeted for modulation (Bestman and Booker, 2003). With the beginning of the molt, the decline in anterior foregut activity is accompanied by a sharp decline in the amplitude of the excitatory junctional potentials (EJPs) recorded from the esophageal constrictor muscles of the anterior foregut. Using the styryl dye, FM1-43 it was demonstrated that the decline in EJP amplitude could be accounted for by a reduction in the efficacy of the synaptic endings on the anterior foregut musculature. The return of anterior foregut peristalsis near the end of the molt is correlated with a dramatic rebound in EJP amplitude and synaptic efficacy. Here we examine the potential roles of two signals in modulating anterior foregut motility during a larval–larval molt. We present evidence that an element present in the hemolymph of early-molt stage larvae may act to suppress the contractions of the anterior foregut during the molt. The return of the robust peristaltic contractions of the anterior foregut near the end of molt can be triggered by the release of the nonapeptide, CCAP. In both instances the presynaptic endings on the anterior foregut musculature appear to be the targets of these potential modulators.

Materials and methods
Experimental animals and staging

Manduca sexta L. larvae were reared in individual plastic cups on a wheat germ-based artificial diet modified from that of Bell and Joachim (Bell and Joachim, 1976). A 27°C, 16 h:8 h L:D photoperiod regimen was provided for the *M. sexta* colony. Under these conditions, the durations of the 4th and 5th instar stages were 3–4 days and 4–5 days, respectively. Because head capsule slippage (HCS), when the old head cuticle separates from the developing new cuticle and the resulting space fills with MF, is an easily detected and externally recognizable feature of the molt cycle it was used as the starting reference point for the molt cycle. Under the colony conditions used, the duration of the molt after HCS between the 4th and 5th instars was 25 h. We used a number of developmental markers to track the progress of the molt between the 4th and 5th larval instars. We define the early-molt stage as the first 16 h of the molt after HCS, during which time the newly developing mouthparts lack pigmentation. The late-molt stage, some 16–18 h after HCS, is marked by the new mouthparts becoming yellow-brown as they begin to tan. Approximately 21 h after HCS, air bubbles are first observed in the head capsule, marking the first sign of the ingestion of the MF. The molt typically ends some 4 h later with the shedding of the old cuticle during ecdysis.

Anterior foregut preparation and recording techniques

To gain access to *M. sexta* anterior foregut musculature and nervous system, larvae were dissected along the dorsal midline and the anterior foregut, brain and frontal ganglion were surgically isolated according to methods described (Bestman and Booker, 2003). The 'semi-intact' anterior foregut preparations prepared in this manner were transferred and pinned to a 35 mm plastic tissue culture dish lined with silicone elastomer (Sylgard; Dow Corning, Midland, MI, USA). This preparation preserves synaptic input to the constrictor and dilator muscle groups of the foregut, and is capable of producing both the alternating, posterior-directed muscle contractions associated with the peristaltic swallowing motor pattern of the foregut as well as the 'squeezing' pattern described (Miles and Booker, 1994). Instances of the squeezing motor pattern were very infrequent; this is probably because of the absence of sensory input from an intact and full crop, which has been suggested to provide sensory feedback to the foregut central pattern generator (Miles and Booker, 1994). The preparations were bathed in physiological saline [in mmol l^{-1}: NaCl, 140; KCl, 5; CaCl$_2$, 4; dextrose, 28; Hepes, 5; MgCl$_2$6H$_2$O, 2; trehalose, 5 (modified from Trimmer and Weeks, 1989)]. For Ca^{2+}-free saline, the CaCl$_2$ was replaced by 20 mmol l^{-1} MgCl$_2$, NaCl was lowered to 124 mmol l^{-1}, and 0.5 mmol l^{-1} EGTA was added. For high K$^+$-free saline, the KCl was increased to 90 mmol l^{-1} and NaCl lowered to 55 mmol l^{-1}. The CCAP (Peninsula Labs, San Carlos, CA, USA) was diluted in physiological saline and applied to the anterior foregut preparations by exchanging the regular saline with the CCAP saline solution of appropriate dilution. Extracellular recordings from the muscles were obtained using custom-made glass-pipette-tipped suction electrodes. In some experiments, a movement transducer, constructed from a piezoelectric phonograph cartridge, was attached to the anterior esophageal constrictor muscles in order to monitor and record anterior foregut contractions. The brain and frontal ganglion were removed in the 'isolated anterior foregut' preparation and contractions were elicited by delivering 500 ms trains of 5 ms pulses delivered at 40 Hz to the severed recurrent nerve using a Grass S48 stimulator with a suction electrode. The outputs of the movement transducer and the extracellular suction electrodes were amplified using a differential amplifier (A-M systems, Carlsborg, WA, USA). To elicit EJPs from the anterior foregut musculature, 10 ms pulses were delivered through a suction electrode to the recurrent nerve. To record intracellularly from the anterior foregut constrictor muscles, a piece of Sylgard was inserted into the lumen of the gut in order to stabilize the foregut. Muscle recordings were made with loosely suspended 20–25 MΩ electrodes amplified with a Neuroprobe 1600 (A-M Systems) amplifier. All signals were stored to a VHS cassette tape (A. R. Vetter Instruments, Rebersburg, PA, USA) and real-time playback was conducted using a high-speed chart recorder (Astro-Med MT95000, West Warwick, RI, USA).

Preparation of the active hemolymph fraction

To collect hemolymph, larvae were anesthetized on ice and their hemolymph was extracted through a small incision cut on the proleg with scissors. The hemolymph was collected in microcentrifuge tubes containing phenylthiocarbamide (Sigma Chemicals, St Louis, MO, USA) to prevent oxidation. The hemolymph was then spun in a bench top centrifuge at

20 000 **g** for 5 min. The supernatant was collected, boiled for 10–15 min, cooled and filtered under vacuum through Watmann no. 1 paper. The sample was fractionated by serial liquid/liquid extraction first with ethyl acetate followed by *n*-butanol. The peristalsis of the intact anterior foregut preparation was used as a bioassay; intact anterior foregut preparations were exposed to a sample of each blood fraction and the effects on the ongoing contractions of the anterior foregut were noted.

FM1-43 labeling

The fluorescent dye, FM1-43 (Molecular Probes, Eugene, OR, USA) was used to monitor the effects of potential modulators of the presynaptic endings on the anterior foregut musculature. In all experiments, the anterior foregut preparations were viewed under a 40×, 0.8 water-immersion lens using a Nikon Eclipse 600-FN epifluorescent microscope equipped with a 100 W mercury lamp. The images were captured with a CCD SPOT2 camera (Diagnostic Instruments, Sterling Heights, MI, USA) with identical acquisition settings throughout each experiment. Using PhotoShop 7 (Adobe Systems, San Jose, CA, USA), the amount of FM1-43 taken up by the nerve endings was calculated by measuring the average luminosity of individual FM1-43 fluorescent puncta from which average background levels for each image were subtracted. We estimated the density of FM1-43 labeled puncta by counting the number of fluorescent puncta per 20 μm^2.

To monitor the effects of the inhibitory agent found in the hemolymph of early-molt stage larvae, the anterior foreguts of intermolt 5th instar larvae were first 'loaded' with the FM1-43 dye by incubating anterior foregut preparations in normal saline containing 10 μm FM1-43 for 15 min (Bestman and Booker, 2003). The preparations were quickly rinsed in Ca^{2+}-free saline, and then incubated for 20 min in Ca^{2+}-free saline. The presynaptic endings on the esophageal dilator muscles terminals were then imaged to determine the amount of dye loaded. The dye was then allowed to unload for 20 min by placing the anterior foregut preparations in either normal saline or in saline to which the fraction from the hemolymph of early-molt larvae had been added. The anterior foregut preparations were then rinsed in Ca^{2+}-free saline, and imaged. Terminals were selected individually and the 'percentage of FM1-43 unloading' was calculated by taking the ratio of the average luminosity of each selected terminal after and before the period of unloading (unloaded luminosity/loaded luminosity). Although synapses all over the anterior foregut became loaded with FM1-43, the signal of the constrictor muscles was difficult to resolve because of the autofluorescence of the cuticular lining of the foregut. In addition, synapses tended to lie between constrictor muscles and even beneath the constrictors, making them more difficult to image. By contrast, the esophageal dilator muscles are flat and in many places are just a single fiber thick. In the generation of foregut peristalsis, the activities of the dilator and constrictor groups are temporally and spatially interdependent, and therefore because the esophageal dilator muscles provide a better FM1-43 signal, they were used for these imaging experiments.

To assess the potential role of CCAP in triggering the increase in synaptic activity observed near the end of the molt, the intact anterior foregut preparations of early-molt 4th instar larvae (<16 h after HCS) were loaded in normal saline containing FM1-43 and imaged as above. Next, the preparations were incubated for 20 min in normal saline containing 10^{-8} mol l^{-1} CCAP. The preparations were then returned to normal saline solution containing FM1-43 for 20 min then quickly rinsed in Ca^{2+}-free saline and imaged. After each treatment the density of the FM1-43 labeled endings was determined.

CCAP immunocytochemistry

Cold-anesthetized larvae that were in the process of molting between the second and third instar were cut along the dorsal midline, pinned out and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1–2 h at room temperature. The fixed preparations were then rinsed in PBS for 2 h at room temperature before being switched to PBS containing 0.3% Triton X-100 (PBST) overnight at 4°C. Tissues were then blocked for 4 h in 10% normal horse serum in PBS. A rabbit anti-CCAP antiserum (prepared by Dr H. Agricola, University of Jena, Germany) was diluted 1:5000 in PBST and the tissues were incubated for 24 to 36 h at 4°C. The specificity of this antibody has been previously characterized (Ewer and Truman, 1996). After repeated washings in PBST, the preparations were incubated in Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes) diluted 1:200 in PBST for 2 h at room temperature or overnight at 4°C. After repeated washings, the preparations were dehydrated through an ethanol series, cleared in methyl salicylate, mounted in DPX (Fluka, Sigma-Aldrich Corp., St Louis, MO, USA) and examined using a Bio-Rad 600 confocal microscope. As discussed above, because the dilator muscles have an improved signal to noise ratio, the photomicrographs show CCAP labeling of the anterior foregut dilator muscle group.

Surgical manipulations

Late 4th instar larvae that were committed to entering the molt (mass >1 g) were anesthetized under CO_2 gas. A small incision was made in the cuticle near the target tissue and the CNS lesions made using iris scissors. The procedures for the sham surgeries were similar except that the neural tissue was touched with forceps through the incision. The incisions were sealed with dermatological adhesive (New-Skin, Medtech Laboratories, Jackson, WY, USA) and the larvae were placed back into the colony to recover.

Results

The hemolymph of early-molt stage larvae triggers a reduction of anterior foregut motility

During the molt between the 4th and 5th instars of *M. sexta* larvae, MF is secreted into the space between the old and the newly developing cuticles. At this time there is a sharp decline in anterior foregut motility, preventing the premature ingestion

of the MF (Bestman and Booker, 2003). We decided to test the possibility that a blood-borne factor might play a role in triggering the decrease in anterior foregut motility observed during the molt cycle. Hemolymph was extracted from larvae during the first 2–4 h after larvae showed signs of apolysis during the molt between the 4th and 5th larval instars. As a control we used hemolymph obtained from nonmolting, feeding 5th instar (intermolt) larvae. We then assayed the effect of the hemolymph extracts on the contraction patterns of semi-intact anterior foregut/frontal ganglion preparations isolated from intermolt larvae (see Materials and methods). In saline, anterior foreguts isolated from intermolt larvae show regular alternating rhythmic contractions of the buccal and esophageal regions of the anterior foregut, resulting in robust posteriorly directed peristaltic contractions associated with swallowing (Bestman and Booker, 2003). Upon exposure to hemolymph collected from early-molt stage larvae, we failed to detect peristaltic contractions in 77% (69/90) of the treated semi-intact anterior foreguts preparations. This percentage was significantly greater than when the semi-intact anterior foreguts were exposed to hemolymph collected from intermolt larvae, where only 15% (2/13) of the preparations showed disruption of anterior foregut activity ($P<0.001$, χ^2 test). The hemolymph from early-molt stage larvae was subjected to fractionation (see Materials and methods) resulting in the peristalsis-blocking activity of the hemolymph of early-molt stage larvae remaining in the aqueous fraction. The aqueous extract of early-molt larval hemolymph suppressed peristalsis by 84% (24/29) compared with that of the active semi-intact anterior foregut preparations assayed. In comparison, the ongoing peristalses of significantly fewer, just 15% (3/17) of the anterior foregut preparations assayed, were disrupted after application of aqueous extract of intermolt 5th instar hemolymph ($P<0.001$, χ^2 test).

Our previous results suggested that the presynaptic terminals on the anterior foregut musculature are modulated during a larval–larval molt (Bestman and Booker, 2003). The decline in anterior foregut peristalsis during the early stages of the molt is accompanied by a sharp reduction in synaptic efficacy, resulting in attenuated EJP amplitude recorded from the esophageal constrictor muscles of the anterior foregut (Bestman and Booker, 2003). If the inhibitory agent found in the hemolymph of early-molt stage larvae plays a key role in modulating anterior foregut motility during a larval–larval molt then it should do so by targeting the presynaptic terminals on the anterior foregut musculature. In an effort to identify potential targets of the inhibitory hemolymph factor, we used an isolated anterior foregut preparation (without the brain and frontal ganglion) and elicited foregut contractions through exogenous stimulation of the recurrent nerve (Bestman and Booker, 2003). We quantified the strength of the elicited anterior foregut contractions by attaching a movement transducer to the anterior esophageal constrictors. When isolated anterior foregut preparations obtained from intermolt larvae were bathed in saline, all of the foreguts were responsive to the stimulation and the average amplitude of the elicited
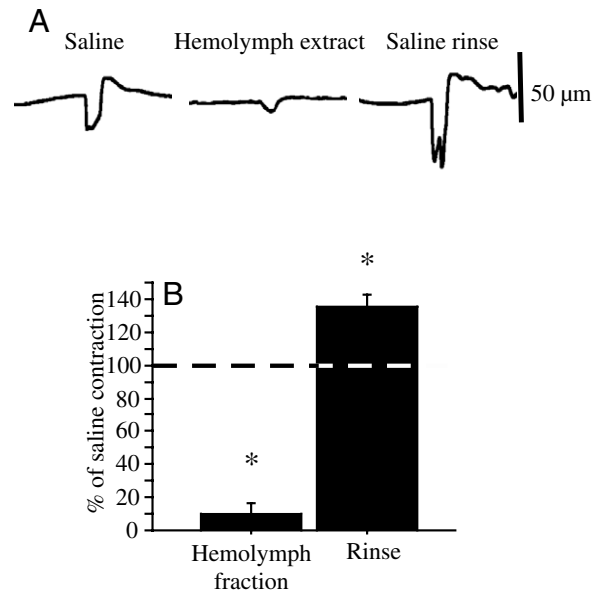

Fig. 1. Exposure to the active factor present in the hemolymph of early-molt stage larvae triggers a dramatic decline in anterior foregut contraction amplitude. (A) In saline, anterior foreguts isolated from nonmolting 5th instar larvae respond to a 10 ms stimulation of the severed recurrent nerve with a large amplitude contraction. Within a few minutes of exposure to the hemolymph fraction from early-molt stage larvae, however, the amplitude of the elicited contractions was markedly decreased. The response rebounded following a saline wash. The anterior foregut contractions were recorded using a movement transducer attached to the anterior esophageal constrictor muscles. (B) Summary of the results ($N=6$; $P<0.001$, paired *t*-test). In saline rinse we found that the average contraction amplitude recovered and surpassed the initial control levels ($P<0.05$, paired *t*-test). *Significant difference from saline value.

contractions was 51.4 ± 5.0 μm (Fig. 1A; $N=6$). However, following the application of the hemolymph fraction from early-molt stage larvae, the average amplitude of the contractions dropped to less than 10% of the control values, and we failed to elicit a measurable contraction in 66% of the anterior foreguts tested ($N=6$; $P<0.001$, paired *t*-test; Fig. 1B). In all preparations, there was a complete recovery of activity once the anterior foregut preparations were washed with fresh saline. Treating the hemolymph isolated from early-molt stage larvae with a number of proteases destroyed all activity, consistent with the active factor being a peptide (J.E.B. and M. del Campo, unpublished observations).

The decline in anterior foregut motility following exposure to the active fractionated early-molt stage hemolymph is also accompanied by a decrease in the amplitude of the EJP recorded from the anterior foregut esophageal constrictor musculature. In saline, the average EJP amplitude elicited by stimulation of the recurrent nerve was 12.5 ± 1.1 mV ($N=10$; Fig. 2A). Following exposure to the aqueous hemolymph fraction from the early-molt larvae, the average EJP amplitude fell to 5.6% of the control value (Fig. 2B; $N=10$; paired *t*-test,
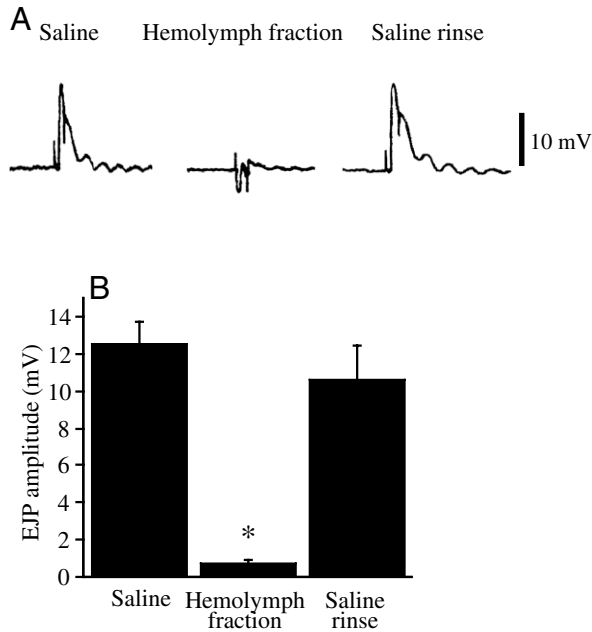
Fig. 2. (A) Example of excitatory junctional potentials (EJPs) recorded from musculature of active anterior foreguts in response to a 10 ms stimulation of the severed recurrent nerve. Following exposure to the active hemolymph factor the amplitude of the EJP dropped significantly but recovered after a saline rinse. (B) We found a significant decrease in the average amplitude of the EJPs recorded from anterior esophageal constrictor muscles of the anterior foreguts (N=10; paired t-test, P<0.001). The average EJP amplitude returned to the initial saline level in the rinse. *Significant difference from both saline and rinse levels.
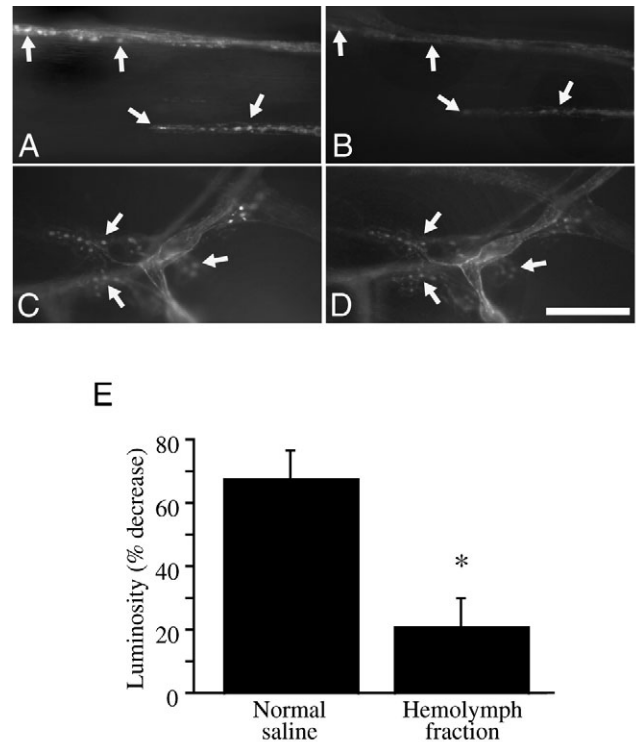
Fig. 3. The activity-dependent dye FM1-43 reveals that the active hemolymph fraction triggers a decline in the efficacy of the synaptic terminals on the anterior foregut musculature. Presynaptic terminals of intermolt 5th instar larvae were first preloaded with FM1-43 (A,C), and then allowed to unload under one of two conditions: (B) after a continued 20 min incubation in normal physiological saline or, (D) after a 20 min incubation in the hemolymph fraction. (E) In the preparations incubated in the hemolymph fraction, the average percentage change in the fluorescence of the terminals was significantly lower than that of the preparations that had been incubated in saline (unpaired t-test, P<0.01) *Significant difference from the normal saline levels. Scale bar, 50 μm.

P<0.001). Rinsing the preparations with fresh saline resulted in the full recovery of the EJP amplitude compared with initial values (N=5; P>0.05, paired t-test). The decline in the contraction amplitude of the anterior foregut musculature following exposure to the hemolymph fraction was not due to a drop in the resting potential of the muscle fibers. The resting membrane potential of the muscles recorded in control saline and following exposure to early-molt stage hemolymph aqueous fraction was –40.5±1.5 mV (N=12) and –36.8±0.8 mV potential (N=12) respectively; they were not significantly different (P>0.05, paired t-test).

Next we used the styryl dye, FM1-43, to determine whether the hemolymph fraction from early-molt stage larvae targeted the presynaptic endings on the esophageal dilator muscle group of the anterior foregut. FM1-43 is taken up exclusively into the presynaptic terminals that have undergone a vesicle exocytosis/endocytosis event while exposed to the dye (Betz and Bewick, 1992; Cochilla et al., 1999) and therefore the amount of dye present in the terminal serves as an indicator of the level of synaptic activity. To examine the effect of the early-molt larval hemolymph fraction on the presynaptic endings we used active semi-intact anterior foregut preparations from intermolt larvae. We first allowed endogenous frontal ganglion-driven activity to preload the FM1-43 dye into synaptic

terminals on the anterior foreguts. The dilator muscles of the foreguts were imaged and the amount of dye loaded was determined by measuring the luminosity of the synaptic terminals. We next tested whether exposure to the active hemolymph fraction influenced the rate that FM1-43 was unloaded from the terminals. For these experiments the pre-loaded anterior foreguts (Fig. 3A,C) were allowed to unload for 20 min in either control saline (Fig. 3B) or saline containing the hemolymph fraction (Fig. 3D). At the end of this period the previously identified terminals were located and their level of luminosity was measured. As expected, when the anterior foreguts with preloaded terminals were bathed in control saline, there was a dramatic drop in the amount of dye remaining in the presynaptic terminals (Fig. 3E). Within 20 min the luminosity of the FM1-43-loaded terminals bathed in control saline declined by 67.6±9.4% (N=14). In comparison, a significantly greater FM1-43 signal remained in the anterior foregut terminals exposed to the active hemolymph, dropping

just 20.9±9.1% (*N*=19; *P*<0.01, unpaired *t*-test). Thus exposure to the blood fraction resulted in a significant decrease in the efficacy of the presynaptic terminals on the foregut dilator musculature.

Crustacean cardioactive peptide triggers the precocious increase in anterior foregut motility

As the end of the molt approaches, there is a sudden rebound in the amplitude of the anterior foregut contractions, followed a short time later by the first signs of the MF ingestion (Bestman and Booker, 2003). We were curious to determine what factor(s) might play a role in the return of anterior foregut peristalsis. For a number of reasons we focused on CCAP as a potential modulator of anterior foregut motility around the time of MF ingestion. CCAP is a nonapeptide and acts as a neuro- and myomodulator in a number of different arthropods (Breidbach et al., 1995; Groome and Lehman, 1995; McNeil et al., 1998; Mulloney et al., 1997). In the central nervous system (CNS) of *M. sexta* there are over 90 neurons that express the CCAP gene (Loi et al., 2001). There is also evidence for a role for CCAP in triggering the ecdysial motor patterns responsible for removing the old cuticle at the end of the molt (Ewer et al., 1997; Truman et al., 1998).

We found it intriguing that one of the four pairs of CCAP-immunoreactive (CCAP-ir) neurons, which have been repeatedly identified in the subesophageal ganglion (SEG), has also been reported to send out processes terminating in the immediate region of the anterior foregut (Davis et al., 2001; Ewer et al., 1994; Klukas et al., 1996; Loi et al., 2001). Using CCAP antiserum, we labeled possible release sites on the muscles of the anterior foregut (shown in Fig. 4, the anterior foregut esophageal dilators). This labeling was reminiscent of CCAP-ir neurohemal structures found on other *M. sexta* tissues and in other

species (Davis et al., 2001; Donini et al., 2002). With these data in mind, we wanted to test whether CCAP also plays a role in triggering the return of the peristaltic contractions of the anterior foregut observed at the end of the molt.

We set out to examine this possibility by using the movement transducer to measure the effect of CCAP on inactive semi-intact anterior foreguts prepared from early-molt stage larvae (between 10 and 14 h after HCS; Fig. 5). When these largely inactive anterior foreguts were bathed in control saline, only low amplitude contractions were recorded (Fig. 5A). Within 3 min of exposure to 10^{-8} mol l^{-1} CCAP (Fig. 5B), the average contraction amplitude increased more than 12-fold (*N*=5; *P*<0.05, paired *t*-test). Within 30 min of the application of the CCAP, the average maximum contraction amplitude increased more than 20-fold compared to the control values (Fig. 5B; *P*<0.05, paired *t*-test). We failed to detect any changes in the period of the anterior foregut contractions following exposure to CCAP. In saline the average period of peristalsis was 3.1±0.6 s, and after application of CCAP it was 2.9±0.7 s (*N*=5, *P*>0.05, paired *t*-test).

Fig. 5. Exposing the inactive anterior foreguts isolated from early-molt stage larvae to crustacean cardioactive peptide (CCAP) triggers a dramatic increase in anterior foregut motility. (A,B) Examples of anterior foregut activity in saline conditions (A) and of the same preparation after 3 min in 10^{-8} mol l^{-1} CCAP (B). The top trace is the endogenous activity recorded by a movement transducer attached to the anterior esophageal constrictor muscle group. The bottom trace is a concurrent extracellular recording of excitatory junctional potentials (EJPs) from the esophageal constrictor muscles. (C) Within 30 min of the CCAP application to the anterior foreguts isolated from larvae early in the molt cycle, the average contraction amplitude increased significantly (*P*<0.05, paired *t*-test) to levels similar to that observed around the time of molting fluid ingestion. *Significant difference from the initial saline contraction levels.
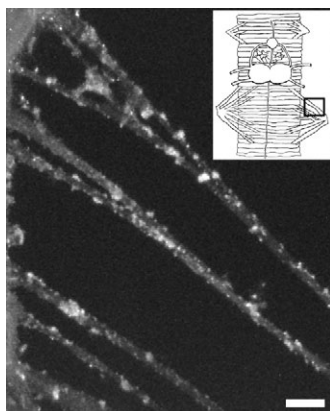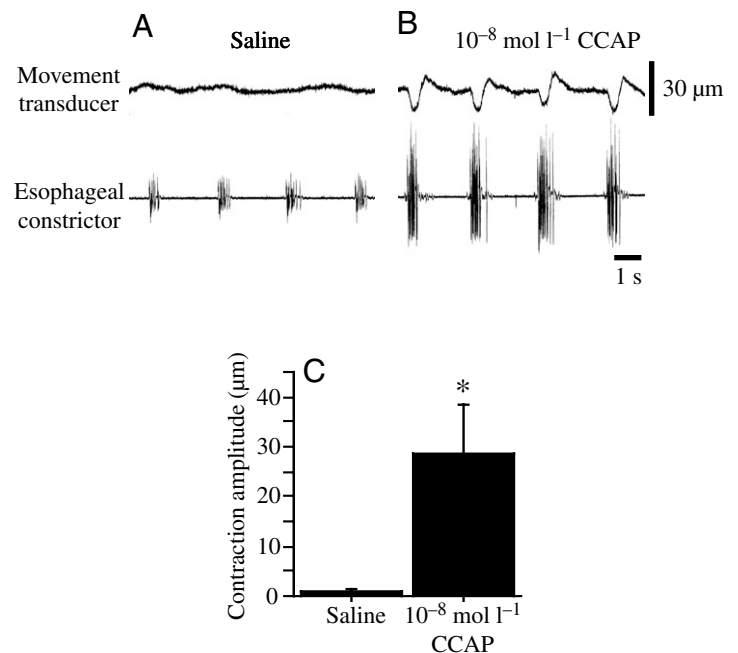
Fig. 4. Crustacean cardioactive peptide (CCAP) immunostaining of the esophageal dilator muscles of an early-molt larva. The diffuse, punctate staining is indicative of neurohemal release sites. Inset: diagram of the anterior foregut musculature, brain and frontal ganglion. The box indicates approximate position of the esophageal dilator muscles shown in the figure. Scale bar, 50 μm.

A dose–response curve was constructed by measuring the amplitude of the anterior foregut contractions in the initial saline condition and then following a single exposure to saline containing CCAP (Fig. 6). For these experiments we used isolated anterior foregut preparations obtained from early-molt stage larvae and elicited anterior foregut contractions through stimulation of the attached recurrent nerve (see Materials and methods). In all instances the magnitude of the response was determined within 3 min of the single application of various CCAP doses diluted in physiological saline, and identical stimulation settings were used before and after CCAP application. Under these conditions the threshold concentration of CCAP to produce a detectable response was 10^{-12} mol l^{-1} ($N=10$; $P<0.05$, paired t-test). At a concentration of 10^{-7} mol l^{-1} CCAP the maximal response recorded was over fivefold that of the saline only values ($578\pm217\%$, $N=8$), with the response reaching a plateau or slightly declining at higher concentrations. The anterior foreguts prepared from intermolt larvae also responded to the application of CCAP with an increase in contraction amplitude. However, although significant, these increases were smaller relative to the results obtained for the early-molt stage anterior foregut preparations. For example, following the application of 10^{-8} mol l^{-1} CCAP to the anterior foreguts isolated from intermolt larvae, the amplitude of the contractions increased less than twofold compared to controls (Fig. 6B; $170\pm42\%$, $P>0.05$, paired t-test).

CCAP also triggered a rapid increase in the EJP amplitude recorded from the esophageal constrictor muscle fibers of an isolated anterior foregut preparation obtained from early-molt stage larvae (Fig. 7). In control saline the average muscle EJP recorded was 8.2 ± 1.3 mV ($N=20$), significantly lower than the 14.6 ± 5.9 mV ($N=6$) potential recorded from the esophageal constrictor muscle of intermolt larvae (Fig. 7B; unpaired t-test, $P<0.01$). Within 10 min of exposure to 10^{-8} mol l^{-1} CCAP, the average EJPs recorded from anterior foreguts isolated from early-molt stage larvae increased to 16.3 ± 2.1 mV ($N=18$; paired t-test, $P<0.001$). By contrast, the application of CCAP had no significant effect on the amplitude of the EJPs recorded from muscles of anterior foreguts isolated from intermolt larvae.
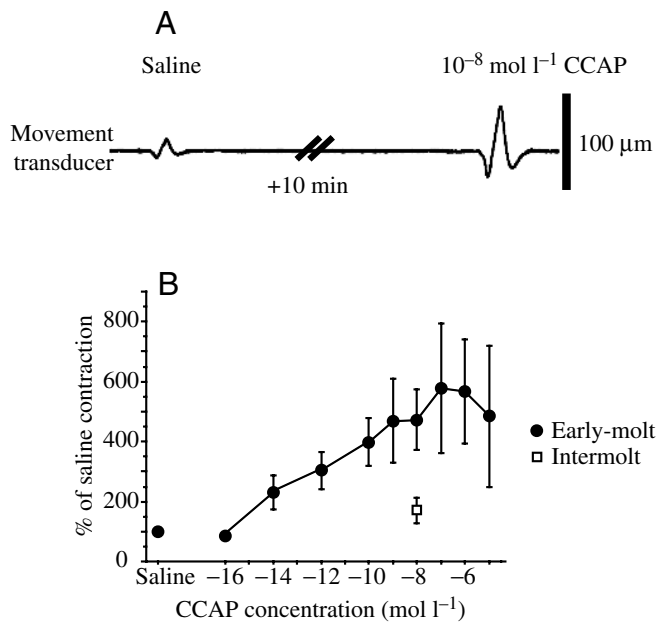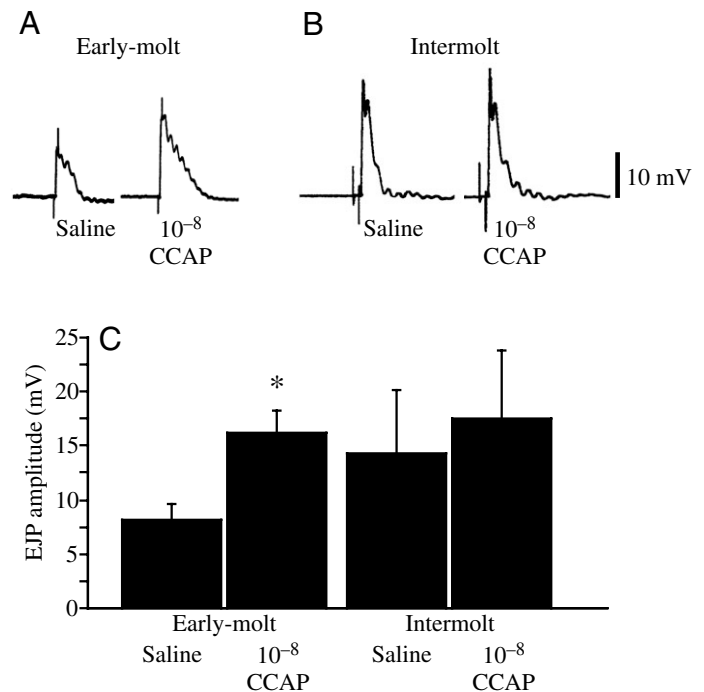

Fig. 6. Crustacean cardioactive peptide (CCAP) dose response. Anterior foregut contractions were elicited by delivering 5 ms pulses at 40 pulses s^{-1} for 500 ms duration to the severed recurrent nerve. (A) An example of a movement transducer record from an early-molt larva initially in saline and then 10 min after exposure to 10^{-8} mol l^{-1} CCAP. (B) There is a strong dose–response relationship between the amount of contraction and increasing concentrations of CCAP. Plotted are the percentage increases in contraction amplitude elicited from stimulation of the recurrent nerve relative to the maximum response elicited in the initial saline conditions. Each point is an average of the responses of between five and 22 animals.

Fig. 7. Excitatory junctional potentials (EJPs) elicited in the presence of 10^{-8} mol l^{-1} crustacean cardioactive peptide (CCAP) produced significantly greater responses than those in saline. EJP responses recorded intracellularly from the anterior foregut muscles of early-molt larvae (A), and intermolt larvae (B), in control saline and after a 10 min exposure to saline plus 10^{-8} mol l^{-1} CCAP. The early-molt larvae produced significantly greater EJP responses after incubation in CCAP (paired t-test, $P<0.0001$), reaching values that were similar to those of the intermolt larvae in saline (unpaired t-test, $P>0.5$). After treatment with 10^{-8} mol l^{-1} CCAP, the average EJP amplitude recorded from the intermolt larvae increased, but was not significantly different from the saline values (paired t-test, $P>0.05$). *Significant increase over initial saline levels (paired t-test, $P<0.0001$) but is not significantly different from the intermolt saline values (unpaired t-test, $P>0.05$).
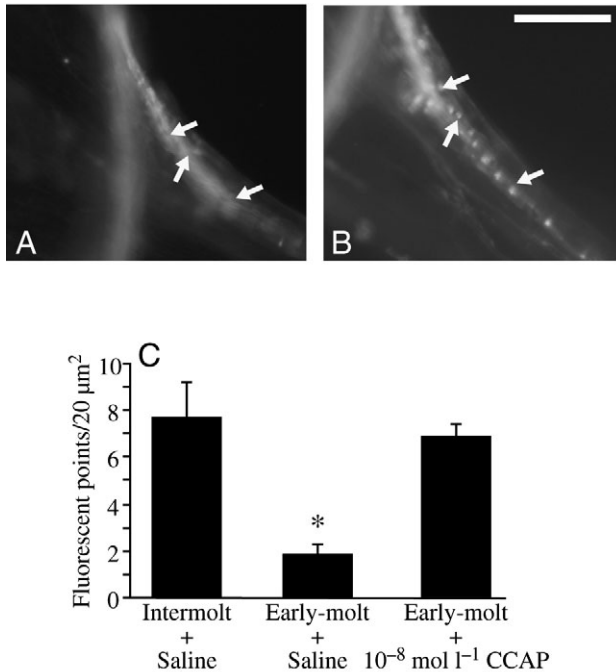
Fig. 8. Application of crustacean cardioactive peptide (CCAP) results in a significant increase in the activity of the synaptic endings on the anterior foreguts of early-molt stage larvae. For these experiments the density of FM1-43 loaded puncta was used to estimate the endogenous activity of the presynaptic terminals found on the anterior foregut musculature. (A) Under control conditions there were relatively few FM1-43-labeled puncta found on the esophageal dilator muscles of the anterior foregut of an early-molt larva. (B) However, following a 15 min incubation in 10^{-8} mol l^{-1} CCAP, there is a dramatic increase in the density of FM1-43-labeled puncta on the muscle. (C) A plot summarizing the FM1-43 results. When loading occurred in control saline the density of FM1-43 puncta on the early-molt stage anterior foreguts was only about a quarter of the value calculated for intermolt larvae. Following exposure to 10^{-8} mol l^{-1} CCAP for 15 min, the density of labeled puncta on the anterior foregut muscles of early-molt stage larvae was similar to that observed in anterior foreguts isolated from intermolt larvae ($P>0.05$, unpaired t-test). *Significant difference from both intermolt saline level ($P<0.005$, unpaired t-test) and from the 10^{-8} mol l^{-1} CCAP level ($P<0.005$, paired t-test). Scale bar, 20 μm.

We also examined the effect of the application of CCAP on the efficacy of the presynaptic terminals on the esophageal dilator muscles of the anterior foregut using the dye FM1-43. In semi-intact anterior foregut preparations isolated from early-molt stage larvae, the reduced synaptic efficacy is reflected in a failure of the synaptic terminals on the dilator muscles to load FM1-43 (Fig. 8A). Following exposure to 10^{-8} mol l^{-1} CCAP the density of labeled endings of early-molt stage larvae increased 3.7-fold (Fig. 8C; $N=8$, paired t-test, $P<0.01$) to levels comparable to those observed for the active anterior foreguts from intermolt larvae ($P>0.05$, unpaired t-test; Fig. 8C). These results are consistent with CCAP playing a role in triggering the rapid increase in the synaptic efficacy of the

terminals located on the anterior foregut musculature observed during the final hours of a larval–larval molt.

The subesophageal ganglia and brain are necessary for molting fluid ingestion

The data presented above reveal that the application of exogenous CCAP was sufficient to trigger a return of the robust peristaltic contractions of the anterior foregut late in the molt cycle. Although the published immunocytochemical studies cited above indicate that the SEG is the source of the CCAP-ir endings found on the anterior foregut musculature, at this point there is no direct evidence supporting a role for these CCAP inputs contributing to the modulation of anterior foregut activity during a larval–larval molt. In an effort to establish that the SEG is necessary for MF ingestion, we carried out a series of surgical manipulations on fourth instar larvae that had already committed to the molt cycle. We then determined whether the surgically manipulated animals ingest their MF and initiated ecdysis within 35 h of the onset of the molt (Table 1). Larvae that had undergone sham surgery served as controls. All the sham-operated control larvae ingested their MF and initiated ecdysis within 35 h of the onset of the molt. By contrast, only 23% (3/13) of the SEG-lesioned larvae ingested their MF and 8% (1/13) initiated ecdysis. We next tested whether the connections between the SEG and the rest of the CNS were necessary for MF ingestion and the initiation of ecdysis. All of the larvae in which the connectives between the SEG and T1 were severed swallowed their MF, however, they all failed to initiate ecdysis. Similarly, 78% of the larvae in which the connectives between the brain and SEG were severed ingested their MF, while only 20% initiated ecdysis. To determine whether the brain also plays a role in regulating MF ingestion, the brain itself was also ablated but the frontal ganglion and its projections to the anterior foregut were left intact. Following the removal of the brain none of the molting larvae swallowed their MF or initiated ecdysis. These data indicate that both the brain and the SEG were necessary for MF ingestion, but that they did not need to be physically connected to the rest of the CNS in order for MF ingestion to occur.

Discussion

A blood-borne factor acts to suppress anterior foregut motility

Anterior foregut peristaltic activity is one of a series of motor patterns that must be properly modulated if *M. sexta* is to complete a larval–larval molt successfully (Bestman and Booker, 2003). Near the beginning of the molt, the robust contractions of the anterior foregut are suspended to prevent the premature swallowing of the MF. About 4–5 h prior to the initiation of ecdysis, as the end of the molt approaches, the anterior foregut is reactivated and the MF is swallowed. Results using the dye FM1-43 reveal that during a larval–larval molt it is the presynaptic terminals on the anterior foregut musculature that are the primary target for modulation (Bestman and Booker, 2003).

The results of this work reveal that there are at least two

Table 1. *Results of surgical manipulation of the CNS on the ability of molting larvae to ingest molting fluid and initiate ecdysis*

Surgical manipulation		% Ingesting MF	% Initiating ecdysis
	Sham	100 (9/9)	100 (9/9)
	No SEG	23 (3/13)	8 (1/13)
	SEG→T1	100 (8/8)	0 (0/6)
	Br→SEG	78 (7/9)	20 (1/5)
	No Br	0 (0/6)	0 (0/6)

Br, brain; SEG, subesophageal ganglion; SEG→T1, connectives severed between the subesophageal ganglion and T1; Br→SEG, connectives severed between the brain and subesophageal ganglion.

factors involved in modulating anterior foregut motility during a larval–larval molt – an inhibitory factor found in the hemolymph of larvae having recently started the molt cycle, and later in the molt cycle, CCAP. The yet unidentified inhibitory factor is present only in the hemolymph of the early-molt stage larvae and is sufficient to suspend anterior foregut peristalsis when applied to the fully active anterior foreguts of intermolt larvae (Fig. 2). The decline in anterior foregut motility triggered by the inhibitory factor is accompanied by a 90% decline in the average amplitude of the EJPs recorded from the esophageal constrictor muscles of the anterior foregut. This inhibitory factor appears to target the presynaptic terminals located on the anterior foregut musculature. Results using FM1-43 reveal a sharp decline in the efficacy of the presynaptic terminals of the esophageal dilators within a few minutes of the application of the active factor. Because the changes in FM1-43 loading were found to coincide with changes in anterior foregut contractions, it also suggests that the inhibitory blood borne factor acts directly on the presynaptic neurons as opposed to acting through a polysynaptic mechanism. Given that the activity of the hemolymph fraction is lost following exposure to several proteolytic enzymes, it is consistent that the active factor is a peptide (J.E.B. and M. del Campo, unpublished observations). There are, however, still many questions that need to be addressed. For example, we have not examined and cannot comment on the many other myomodulatory agents including biogenic amines such as octopamine (Zilberstein et al., 2004) or the numerous peptide hormones (Predel et al., 2001a; Predel et al., 2001b) that have been found to affect insect gut contractions outside of the context of the molt. The isolation and a detailed chemical characterization of the active factor, mechanisms governing its release, as well as the identification of its source would provide valuable clues in the effort to understand the mechanism responsible for coordinating anterior foregut activity relative to the other motor patterns during a molt.

Reactivation of anterior foregut peristalsis by CCAP

As we have described, about two-thirds of the way through the molt cycle there is a sharp increase in anterior foregut motility followed shortly by the first signs of MF ingestion (Bestman and Booker, 2003). We tested whether CCAP application was effective in triggering the increase in anterior foregut peristalsis and found that after CCAP application the foregut began peristaltic contractions similar to those occurring normally as the end of the molt approaches. Within minutes of exposure to CCAP, there was a dramatic increase in the contraction amplitude of all of the early-molt stage anterior foreguts to levels similar to that observed near the time of the onset of MF ingestion (Figs 5, 6). The application of CCAP also triggered a doubling of the amplitude of the EJPs recorded from the esophageal constrictor muscles of the anterior foregut of early-molt stage larvae to a range similar to that observed around the time before the onset of MF ingestion (Fig. 7). Our results using the dye FM1-43 reveal that CCAP targets the presynaptic terminals of the anterior foregut. The larval–larval molt is accompanied by a 75% decrease in the density of active terminals as determined by FM1-43 labeling (Bestman and Booker, 2003). Shortly after the application of CCAP, the density of FM1-43-labeled terminals on the dilator muscles of the anterior foreguts of early-molt larvae increased to levels typically observed for anterior foreguts isolated from late-molt stage and intermolt larvae (Fig. 8).

Additional evidence in support of a role for CCAP in the modulation of anterior foregut activity comes from previous neuron-labeling and immunocytochemical studies. Using cobalt backfills, two cells that project from the SEG to the esophageal dilator muscles through nerves of the corpora cardiaca secretory system were uncovered (Copenhaver and Truman, 1986). Later Ewer and coworkers described how these SEG neurons are homologous to CCAP and cGMP immunopositive neurons in the more posterior ganglia (Ewer et al., 1994; Ewer and Truman, 1996). Klukas et al. confirmed that these SEG neurons were CCAP-IR (Klukas et al., 1996), which was also consistent with neurons identified in the CCAP gene expression study (Loi et al., 2001). Lastly, Davis et al. provided evidence for neurohemal release sites from the SEG of CCAP in the vicinity of the aorta and anterior foregut (Davis et al., 2001). Our finding of CCAP-IR neurohemal-like structures on the muscles of the anterior foregut (Fig. 4) is consistent with these previous studies.

The SEG-CCAP neurons that project to the anterior foregut are two of approximately 90 neurons in the CNS of larval *M. sexta* that express the CCAP transcript (Loi et al., 2001). Many of the CCAP-ir neurons play a role in coordinating the motor patterns that characterize insect molting, and a tight correlation exists between transient increases in cGMP expression in these neurons, the release of CCAP and the triggering of the ecdysis motor pattern (Ewer et al., 1994; Ewer et al., 1997; Ewer and Truman, 1997; Fuse and Truman, 2002; Zitnan and Adams, 2000). In the SEG all but one pair of CCAP-ir neurons show an increase in cGMP expression within the final hour of the start of the ecdysis motor patterns. The lone exception is the pair of SEG CCAP-ir neurons that send projections that terminate on the anterior foregut musculature (Davis et al., 2001; Ewer et al., 1994). This pair of CCAP-ir anterior foregut-projecting neurons exhibits an increase an in cGMP levels at the time of MF ingestion, approximately 6–8 h prior to ecdysis (Ewer and Truman, 1996; Ewer and Truman, 1997; Zitnan and Adams, 2000). The results of our lesion experiments reveal that the SEG is necessary for MF ingestion (Table 1) and together this collection of data suggests a further role of CCAP in the regulation of larval–larval molts of *M. sexta* to include triggering an increase in anterior foregut peristaltic activity to power the ingestion of MF.

There is precedent for CCAP's role as a modulator of anterior foregut motility in a number of crustaceans. In crabs, immunocytochemistry reveals that CCAP is delivered to both central and peripheral targets of the stomatogastric (foregut) motor system (Christie et al., 1995; Dircksen and Keller, 1998; Stangier et al., 1987; Stangier et al., 1988). The effects of CCAP on the stomatogastric system of crabs were examined (Weimann et al., 1997) and it was found that concentrations in the 10^{-10} mol l^{-1} range produced effects on the pyloric rhythm and the concentrations in the 10^{-7} mol l^{-1} range increased nerve-evoked contractions of a subset of pyloric muscles. Although many modulators were tested, only the additions of CCAP to the silent lobster stomatogastric system can routinely produce a recovery in its tri-phasic motor pattern (Marder and Richards, 1999). CCAP also plays a role in modulating the stomatogastric system of crustaceans during the molt cycle. As many crustaceans progress through their molt cycle, they swallow water in order to swell their bodies and rupture their old carapace. In at least two species of decapod crustaceans, transient increases in CCAP titers coincide with an increase in anterior foregut activity and the swallowing of water (Phlippen et al., 2000). The target of CCAP during the molt cycle of these Crustacea is thought to be the stomatogastric motor system, but this has yet to be confirmed. The recent identification of the CCAP receptor from *Drosophila* represents a key step in identifying the potential targets of CCAP (Cazzamali et al., 2003; Park et al., 2002).

A factor from the brain is also necessary for the initiation of MF ingestion (Table 1). Predictably, molting larvae in which the connectives between the brain and the rest of the CNS were transected or the brain itself removed, failed to initiate ecdysis. The neurons that release eclosion hormone, one of the peptide hormones essential for triggering ecdysis, reside in the brain and send projections the length of the nerve cord to release sites in the proctodeal nerve of the terminal ganglion (Copenhaver and Truman, 1986; Truman and Copenhaver, 1989). Animals in which the brain connectives were severed failed to initiate ecdysis, but they were still capable of ingesting MF. However, animals in which the brain was completely removed also failed to ingest their MF. At this point there is no information available on the nature or the target of the brain-derived signal controlling anterior foregut peristaltic activity. The possibilities include direct inputs from the brain to the larval anterior foregut motor system through, for example, the many fine nerves of the retrocerebral complex. Alternatively, the brain itself could be the source of a hormonal signal targeting the SEG or the anterior foregut directly.

In summary, arthropod molts are characterized by the strict coordination of a series of behavioral events including the swallowing of MF and swallowing of air or water in order to inflate the new cuticle (Carlson and O'Gara, 1983; Hughes, 1980; Miles and Booker, 1998; Park et al., 2003). During the larval–larval molts of the moth *M. sexta,* the ongoing activity of the anterior foregut is modulated to control the timing of MF ingestion. The data outlined above suggest that *M. sexta* uses a combination of hormones and neuromodulators to regulate anterior foregut motility during a larval–larval molt. It also appears that it is the presynaptic endings on the anterior foreguts that are the primary target of these modulators. The regulation of the anterior foregut motor system of molting *M. sexta* larvae offers an experimentally accessible system to study the cellular basis of the modulation of complex behaviors.

List of abbreviations

CCAP	crustacean cardioactive peptide
EJP	excitatory junctional potential
HCS	head capsule slippage
MF	molting fluid
SEG	subesophageal ganglion

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