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THE INITIATION OF PRE-ECDYSIS AND ECDYSIS BEHAVIORS IN LARVAL *MANDUCA SEXTA*: THE ROLES OF THE BRAIN, TERMINAL GANGLION AND ECLOSION HORMONE

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Summary

Each larval molt of *Manduca sexta* culminates in the sequential performance of pre-ecdysis (cuticle loosening) and ecdysis (cuticle shedding) behaviors. Both behaviors are thought to be triggered by the release of a peptide, eclosion hormone (EH), from brain neurons whose axons extend the length of the nervous system. EH bioactivity appears in the hemolymph at the onset of pre-ecdysis behavior, and EH injection can trigger pre-ecdysis and ecdysis behaviors prematurely. The present study examined the effects of removing or disconnecting portions of the central nervous system prior to the time of EH release on the initiation of pre-ecdysis and ecdysis behaviors at the final larval molt. We found that the initiation of pre-ecdysis abdominal compressions at the appropriate time required the terminal abdominal ganglion (AT) but not the brain; the initiation of pre-ecdysis proleg retractions at the appropriate time required neither the AT nor the brain; the initiation of ecdysis at the appropriate time usually required the brain but did not require the AT; and premature pre-ecdysis (but not ecdysis) could be elicited in isolated abdomens by injection of EH. Finally, pre-ecdysis behavior performed by brainless larvae was not associated with the normal elevation of EH bioactivity in the hemolymph or the normal loss of EH immunoreactivity from peripheral neurohemal release sites.

Key words: moth, molting, insect, *Manduca sexta*, ecdysis, eclosion hormone.

Introduction

The behaviors performed by an animal throughout its lifetime must be initiated at the appropriate time, under the appropriate circumstances and be sequenced in an adaptive way. Although much is known about the neural circuits underlying certain behaviors (e.g. Grillner *et al.* 1991; Katz, 1991; Lukowiak, 1991; Calabrese and De Schutter, 1992; Arshavsky *et al.* 1993; Friesen and Pearce, 1993; Mulloney *et al.* 1993), there remains a great deal to be learned about the mechanisms by which behaviors are selected, initiated and performed in an appropriate sequence (Kien *et al.* 1992; Brodfuehrer *et al.* 1995).

In this study, we investigated neural and hormonal factors involved in the initiation of two behaviors associated with the molting cycle of *Manduca sexta*. *M. sexta* undergoes several molts as a caterpillar (larva) before molting to a pupa and then emerging as an adult moth. At the culmination of each larval molt, two sequential behaviors – pre-ecdysis and ecdysis – are used to shed the old larval cuticle. Larval pre-ecdysis behavior, which appears to loosen the cuticle, includes two major components: (1) rhythmic, dorso-ventral compressions of the abdomen, which occur synchronously on the left and right body sides and in all segments (Fig. 1B), and (2) rhythmic retractions of the abdominal prolegs (Copenhaver and Truman, 1982; Miles and Weeks, 1991; Novicki and Weeks, 1993). The compression pattern is quite stereotyped, while the proleg retraction pattern is more variable: proleg retractions in different segments are not tightly coordinated, and their phasing with respect to the compression pattern varies (Novicki and Weeks, 1993). Ecdysis behavior, which immediately follows pre-ecdysis, consists of (1) rhythmic waves of peristaltic contractions that begin in the terminal abdominal segment and progress anteriorly (Fig. 1C; Weeks and Truman, 1984*a*,*b*), and (2) rhythmic proleg retractions. These movements rupture the old cuticle and propel it posteriorly to be cast off. Although pre-ecdysis and ecdysis behaviors involve similar components, the major difference is in the phasing of the movements: pre-ecdysis compressions are synchronous throughout the abdomen whereas ecdysis contractions are metachronous. At the final larval molt (from the fourth to fifth larval instar), pre-ecdysis behavior lasts for

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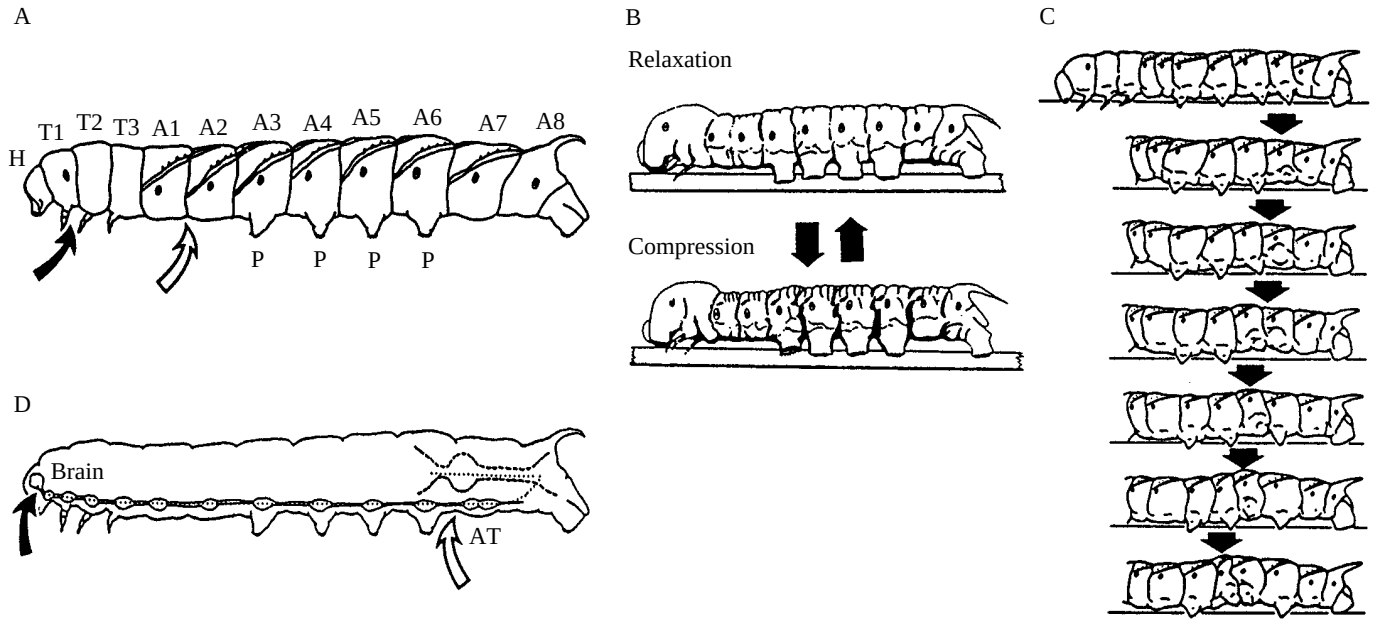

Fig. 1. Anatomy and behaviors of *Manduca sexta* larvae. Anterior is to the left in all drawings. (A) Body segmentation and sites of ligation. The head (H), three thoracic segments (T1–T3) and series of abdominal segments (A1–A8) are labeled. A3–A6 bear prolegs (P). Isolated abdomens were produced by ligating between A1 and A2 (open arrow); 'headless' larvae were produced by ligating between T1 and T2 (filled arrow). (B) Larval pre-ecdysis compression behavior. Drawings show a relaxation–compression cycle (period approximately 5 s); the dorsal–ventral compressions are synchronous throughout the abdomen. Modified from Miles and Weeks (1991, Fig. 1; reprinted with permission from Springer-Verlag). (C) Larval ecdysis behavior. Drawings show an ecdysing larva at approximately 1 s intervals (time runs from top to bottom). Arrows indicate the segmental location of the peristaltic wave, which travels from A6 to A4 in this series of drawings. Modified from Weeks and Truman (1984*a*, Fig. 2A; reprinted with permission from Springer-Verlag). (D) Central nervous system (CNS) anatomy and sites of connective cuts. The CNS includes the brain, subesophageal ganglion, three thoracic ganglia, six abdominal ganglia and a terminal ganglion (AT) located in segment A7. The eclosion hormone (EH)-containing ventromedial (VM) cells are located in the brain, with axons (dotted lines) that extend the length of the nerve cord through the proctodeal nerves to neurohemal release sites on the hindgut (hindgut shown in dashed lines). Arrows show the sites of connective cuts used in this study: 'brain-disconnected' larvae were produced by cutting between the brain and subesophageal ganglion (filled arrow) and 'AT-disconnected' larvae were produced by cutting between A6 and AT (open arrow). Modified from Truman and Copenhaver (1989).

approximately 50 min and ecdysis lasts for approximately 15 min (see below; Weeks and Truman, 1984*a*; Miles and Weeks, 1991; Novicki and Weeks, 1993).

Larval pre-ecdysis and ecdysis behaviors in *M. sexta* are both believed to be triggered by eclosion hormone (EH), a 62 amino acid neuropeptide (Truman *et al.* 1981*a*,*b*; Copenhaver and Truman, 1982; Weeks and Truman, 1984*a*; Marti *et al.* 1987; Miles and Weeks, 1991). EH may control the performance of ecdysis-related behaviors in all insects (Truman *et al.* 1981*b*). In *M. sexta*, EH is found in two pairs of ventromedial brain neurons, the VM cells. The axons of the VM cells travel the length of the nerve cord, exit the terminal ganglion in the terminal nerves and project *via* the proctodeal nerves to neurohemal release sites along the hindgut (Fig. 1D; Truman and Copenhaver, 1989; Hewes and Truman, 1991; Riddiford *et al.* 1994). EH is released at the culmination of each molt to trigger behaviors associated with cuticle shedding (reviewed by Truman, 1992). At the final larval molt, EH bioactivity appears in the hemolymph at approximately the time of onset of pre-ecdysis behavior and persists for approximately 2.5 h (see Fig. 2; Copenhaver and Truman, 1982). EH release occurs both peripherally, into the hemolymph, and centrally, within ganglia; central release of EH appears sufficient to trigger ecdysis behavior (Hewes and Truman, 1991). Injection of EH extract prior to the expected time of endogenous EH release can prematurely initiate the appropriate sequence of pre-ecdysis and ecdysis behaviors (Copenhaver and Truman, 1982). The neural activity patterns underlying pre-ecdysis and ecdysis behaviors have been studied in semi-intact preparations and isolated nerve cords from EH-treated insects (Weeks and Truman, 1984*a*,*b*; Miles and Weeks, 1991; Novicki and Weeks, 1993, 1995), and other studies have investigated the biochemical consequences of EH action on the nervous system (Morton and Truman, 1988; Morton and Giunta, 1992; Ewer *et al.* 1994; Morton, 1995, 1996). Recently, Zitnan *et al.* (1996) have described another peptide, the *M. sexta* ecdysis-triggering hormone (Mas-ETH), which may act between the release of EH and the initiation of pre-ecdysis and ecdysis behaviors (see Discussion).

The objective of the current study was to identify neural loci involved in the initiation of larval pre-ecdysis and ecdysis behaviors in *M. sexta* and to further investigate the role of EH in the initiation of pre-ecdysis behavior. Intact neural connections with the terminal abdominal ganglion (AT) were

found to be required for the initiation of larval pre-ecdysis compression behavior, whereas intact neural connections with the brain were usually required for the initiation of larval ecdysis behavior. The initiation of larval pre-ecdysis proleg retractions required neither the AT nor the brain. These requirements for connections with the AT or the brain for the expression of behaviors were the same whether or not larvae were injected with exogenous EH. An unexpected finding was that pre-ecdysis behavior performed by brainless larvae was not associated with the normal elevation of EH bioactivity in the hemolymph or the normal loss of EH immunoreactivity from the proctodeal nerves. These findings suggest that the regulation of larval pre-ecdysis behavior may be more complex than appreciated previously.

Materials and methods

Insects

Manduca sexta (L.) were reared individually on a 17 h:7 h L:D photoperiod and 27 °C:25 °C temperature cycle, and fed an artificial diet (modified from Bell and Joachim, 1976). Late fourth-instar larvae of both sexes were used. The time of onset of pre-ecdysis behavior was designated as 0 h, and larvae were staged by characteristic morphological markers relative to this time (Copenhaver and Truman, 1982). Fig. 2 illustrates the timing of events for the insects used in this study (staged at 27 °C). Brown coloration of the new mandibles occurred at −7.8±0.1 h (mean ± S.E.M.; N=45). The old head capsule became air-filled at −4.1±0.6 h (N=35) and ecdysis behavior began 0.8±0.2 h (N=35) after the onset of pre-ecdysis behavior.

Injected test substances

EH extract was prepared from pharate adult corpora cardiaca–corpora allata (CC-CA) complexes (Reynolds and Truman, 1980; Weeks and Truman, 1984*a*). Subjects were injected with the equivalent of 0.5 complexes in 25 µl of saline (Ephrussi and Beadle, 1936). Beginning approximately 1 h after the air-filled head capsule stage, injection of this amount of EH extract is sufficient to trigger premature expression of both pre-ecdysis and ecdysis behaviors (see below; Copenhaver and Truman, 1982). Purified EH derived from a recombinant baculovirus line was a gift from Dr David B. Morton at the University of Arizona (Morton and Giunta, 1992). Subjects were injected with approximately 2.5 pmol of recombinant EH in 25 µl of saline, which is equivalent to the EH bioactivity in five CC-CA complexes (Terzi *et al.* 1988). Other subjects were injected with 5 µmol of 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; Sigma Chemical Company, St Louis, MO, USA) in 25 µl of saline. This quantity is sufficient to evoke premature pupal ecdysis behavior (Morton and Truman, 1985), and pilot experiments indicated that it also triggered premature larval pre-ecdysis behavior (data not shown).

Surgical procedures

To isolate portions of the body, larvae were selected as they reached the air-filled head capsule stage, anesthetized with CO_2 gas for approximately 5 min, and ligated with silk thread. For some experiments, larvae were ligated between abdominal segments 1 and 2 (A1 and A2) and the head and thorax were removed (Fig. 1A). Control ‘intact’ larvae were anesthetized but not ligated. After 1 h, the isolated abdomens and controls were injected with test substances and observed as described below. In other experiments, larvae were ligated between thoracic segments 1 and 2 (T1 and T2) (Fig. 1A) and the head was removed. Control larvae were anesthetized but not ligated. The behaviors of ‘headless’ and control larvae were observed, after which the hemolymph of a subset of subjects was analyzed for EH bioactivity or their proctodeal nerves were processed for EH immunoreactivity (see below).

For connective transections, larvae were selected as they reached the brown mandibles stage, anesthetized with CO_2 gas and positioned ventral side up in a saline-filled (Weeks and Truman, 1984*a*), Sylgard-lined (Dow Corning Corp., Midland, MI, USA) dish. After making a small incision at the appropriate location, the connectives were cut either between the brain and the subesophageal ganglion (‘brain-disconnected’) or between ganglia A6 and the AT (‘AT-disconnected’) (Fig. 1D). In sham-operated larvae, the connectives were exposed but not cut. The incision was blotted dry and sealed with melted wax. Individual subjects were then marked with a code such that observers did not know whether the connectives had been cut. After behavioral observations (see below), the larvae were dissected to confirm the status of the connectives.

Behavioral observations

All behavioral observations were made at 27 °C. To score

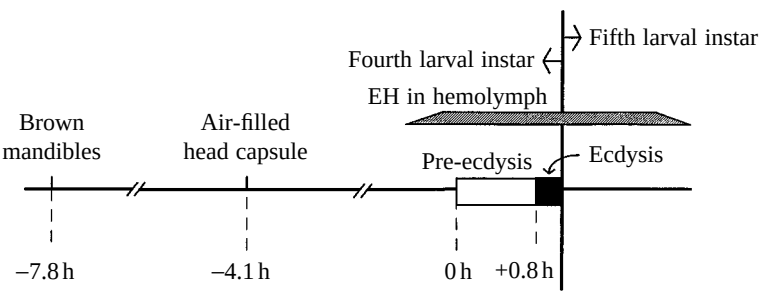

Fig. 2. Timing of events during the final larval molt. The timing of developmental events (at 27 °C) for larvae used in this study is shown relative to the initiation of pre-ecdysis behavior (t=0 h; see Materials and methods). Open and filled horizontal bars indicate the time of pre-ecdysis and ecdysis behaviors, respectively. The solid vertical line indicates the completion of ecdysis and entry into the fifth larval instar. The duration of EH bioactivity in the hemolymph (shaded bar) is taken from Copenhaver and Truman (1982); the duration of ecdysis behavior is taken from Weeks and Truman (1984*a*). Note that the time axis is discontinuous. See text for details.

behavior, groups of larvae were placed on a large Petri dish under a dissecting microscope; the dish was gently rotated to observe each subject for at least 10 s every 10 min. Pre-ecdysis compression behavior was scored when the characteristic rhythmic, dorso-ventral contractions (Fig. 1B) were produced in at least three abdominal segments. In some experiments, pre-ecdysis proleg movements (rhythmic retractions and extensions; Copenhaver and Truman, 1982; Novicki and Weeks, 1993) were also scored. Ecdysis was scored when rhythmic peristaltic contractions of the caudal abdominal segments began (Fig. 1C). Subjects scored as failing to initiate pre-ecdysis and/or ecdysis behavior were observed for at least 2 h (mean 3.0±0.2 h; N=35) after the onset, or expected time of onset, of pre-ecdysis behavior (Fig. 2).

Eclosion hormone bioassay

A prepupal bioassay was used to assess EH bioactivity in the hemolymph of some subjects (Truman *et al.* 1980). Hemolymph was collected from larvae at the appropriate stage (see Results) by clipping the tip of a proleg. The hemolymph was collected in chilled tubes, heat-treated at 80 °C for 5 min, then centrifuged for 15 min. Within experimental groups, the supernatant from multiple subjects was pooled and stored at −70 °C until use. Samples of treated hemolymph (0.2 ml) were injected into staged prepupae (anterior shrink stage; Truman *et al.* 1980), which were observed at room temperature (approximately 22 °C) until pupal ecdysis began. Other prepupae were injected with EH extract (0.5 CC-CA complexes, see above) or saline. The latency from injection to the initiation of pupal ecdysis is inversely related to the amount of EH injected (Truman *et al.* 1980). Hemolymph was collected from larvae, pooled and bioassayed on two separate occasions. Both replicates gave similar results and the data are combined in the Results section.

Immunocytochemistry

The proctodeal nerves, which are the major peripheral release site for EH (Truman and Copenhaver, 1989; Hewes and Truman, 1991), were stained for EH immunoreactivity using a rabbit polyclonal antiserum raised against partially purified EH (Copenhaver and Truman, 1986). The terminal ganglion and proctodeal nerves were dissected from staged larvae under cold saline (Weeks and Truman, 1984*a*) and fixed overnight in 4 % buffered paraformaldehyde at room temperature. The tissue was coded by leaving different lengths of nerve cord attached, and tissue from different groups was combined and processed together. The tissue was rinsed in phosphate-buffered saline with 0.3 % Triton X (PBST), treated in 0.5 mg ml^{-1} collagenase (type IV; Sigma Chemical Co.) in PBST for 30 min at room temperature, rinsed in PBST and incubated in the primary antiserum (1:100) in PBST and 2 % normal goat serum for 36 h at 4 °C. After rinsing in PBST, the tissue was incubated with biotinylated goat anti-rabbit immunoglobulin (1:200; Vector Labs, Burlingame, CA, USA) in PBST and 2 % normal goat serum for 2 h at room temperature, rinsed in PBST, and incubated with Avidin-Cy3 (1:2000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) in PBST and 0.1 % normal goat serum for 1 h at room temperature. The tissue was then rinsed in PBST and in PBS, and mounted on polylysine-coated coverslips and either dehydrated, cleared and mounted in Permount or mounted in 80 % glycerol, 20 % PBS with 2 % propylgallate. Tissues were viewed, scored and photographed on a Zeiss Axioplan microscope under epifluorescent illumination. Tissue collection and processing was performed on two separate occasions. Both replicates gave similar results and are combined in the Results section.

To confirm the identity of the antigen recognized by the anti-EH antiserum, an additional series of tissues was processed using primary antiserum that was preincubated with various concentrations (approximately 0.3, 3 or 30 nmol l^{-1}) of recombinant EH for 1 h at room temperature. All three tested concentrations blocked immunostaining of the proctodeal nerves (see Results).

Photomicrographs were scanned and Fig. 6 was composed in Adobe Photoshop (Adobe Systems, Inc. Mountain View, CA, USA) without manipulating the content of the images.

Statistics

All data are expressed as mean ± S.E.M. (standard error of the mean). Data were compared using Student's two-tailed t-tests, χ^2-tests or analyses of variance (ANOVA) followed by *post hoc* tests, as appropriate (Excel, Microsoft Corporation, Redmond, WA, USA, or BMDP Statistical Software, Inc., Los Angeles, CA, USA). Significance was assumed when P<0.05.

Results

Behavior of isolated abdomens

To investigate the role of the head and thorax in the initiation of pre-ecdysis and ecdysis behaviors, the behaviors of isolated abdomens and intact, age-matched larvae were compared. Larvae at the air-filled head capsule stage (Fig. 2) were briefly anesthetized and either ligated between A1 and A2 or left intact. Abdomen isolation removed the somata of the VM cells as well as their axons and processes in several rostral ganglia (Fig. 1D), which could potentially interfere with EH release. Accordingly, failure of pre-ecdysis or ecdysis behavior to occur in isolated abdomens could result from a failure to release EH, an inability to respond to EH or both mechanisms. To distinguish among these possibilities, half the subjects in each group were injected with EH extract while the remainder were injected with saline. All injections were given 1 h after ligation or sham surgery (i.e. approximately 3 h before the expected time of EH release and initiation of pre-ecdysis behavior; Fig. 2). The times at which pre-ecdysis compressions and ecdysis behavior began were scored for each subject.

All subjects in each group performed pre-ecdysis behavior, with the exception of one intact EH extract-injected larva (Fig. 3A). The reason that this larva failed to respond is unknown. The latency to the onset of pre-ecdysis behavior depended on whether EH extract or saline was injected (Fig. 3B). Both intact larvae and isolated abdomens injected

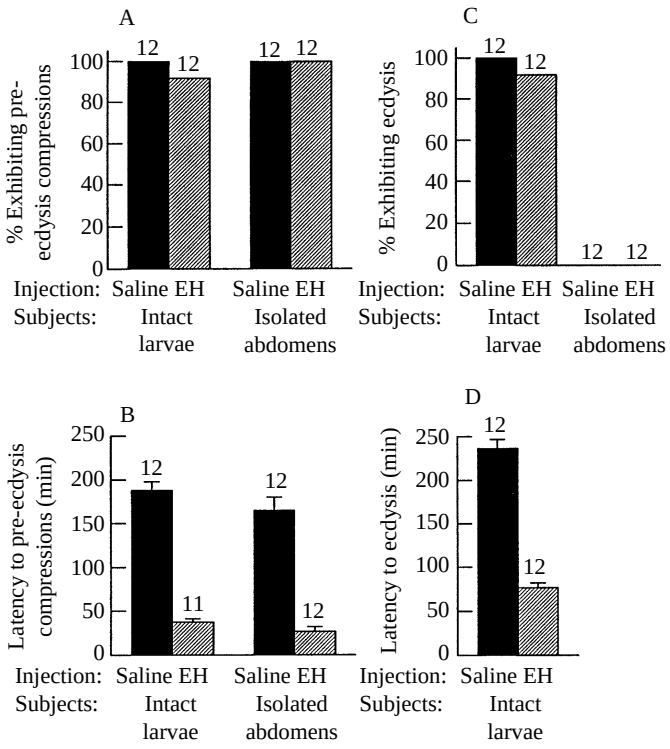

Fig. 3. Behaviors exhibited by isolated abdomens and intact larvae. In each panel, the first two bars show data from intact larvae and the second two bars (when present) show data from isolated abdomens. Solid bars refer to saline-injected subjects and hatched bars to subjects injected with EH extract (0.5 CC-CA equivalents; see text). Data in all panels are from the same subjects; the number of subjects is shown above each bar. (A) Percentage of subjects that exhibited pre-ecdysis compression behavior. (B) Latency from the time of injection of saline or EH extract to the initiation of pre-ecdysis compression behavior. Only data from subjects that performed pre-ecdysis are shown (see A). A two-way ANOVA showed a significant main effect of the substance injected (P<0.0001), with the EH-extract-injected subjects initiating pre-ecdysis compression behavior after a shorter latency. There was no significant main effect of surgery and no significant two-way interaction (P>0.05). (C) Percentage of subjects that exhibited ecdysis behavior. (D) Latency from the time of injection of saline or EH extract to the initiation of ecdysis behavior. Only data from subjects that performed ecdysis are shown (see C). The latency to the initiation of ecdysis differed significantly between the saline-injected and EH extract-injected intact larvae (t-test, P<0.01), with EH-extract-injected larvae initiating ecdysis after a shorter latency. Latency data are means + S.E.M.

with saline initiated pre-ecdysis approximately 3 h after injection, at the expected time of endogenous EH release. In contrast, the latency to the initiation of pre-ecdysis was significantly reduced (to approximately 35 min) in subjects injected with EH extract. The ability of injected EH extract to advance the onset of pre-ecdysis behavior was seen in both intact larvae and isolated abdomens (Fig. 3B). These results suggest (1) that the head and thorax are not necessary for the initiation of pre-ecdysis compression behavior, (2) that removal of the head and thorax does not alter the time of onset of pre-ecdysis compression behavior, and (3) that the head and thorax are not required for injected EH to trigger premature pre-ecdysis compression behavior.

Abdomen isolation had a different effect on ecdysis behavior. All intact larvae produced ecdysis behavior (Fig. 3C), with the exception of one subject that also failed to produce pre-ecdysis behavior (see above). In contrast, none of the isolated abdomens produced ecdysis behavior, even after injection with EH extract (Fig. 3C). Just as for pre-ecdysis behavior, injection of EH extract in intact animals advanced the onset of ecdysis behavior (Fig. 3D). The latency to ecdysis behavior in EH-extract-injected intact larvae was significantly shorter (approximately 1 h) than the latency in saline-injected intact larvae (approximately 4 h). The saline-injected intact larvae initiated ecdysis behavior at the time appropriate for endogenously released EH. These results indicated that the head and thorax are necessary for ecdysis behavior to be initiated at the appropriate time, and the inability of isolated abdomens to initiate ecdysis behavior cannot be overcome by injection with EH extract.

Roles of the brain and terminal ganglion in pre-ecdysis and ecdysis behavior

Abdomen isolation removed the brain, subesophageal ganglion and three thoracic ganglia (Fig. 1D). The preceding results (Fig. 3) suggested that some or all of these structures are required for the initiation of ecdysis behavior. Previously, we found that the AT is required for the maintenance of the compression component of pre-ecdysis behavior (Novicki and Weeks, 1993, 1995), but its role in initiation was not examined. The next experiment was designed to test the roles of the brain and the AT in the initiation of pre-ecdysis and ecdysis behaviors. Larvae were selected at the brown mandibles stage, approximately 8 h before the expected time of EH release (Fig. 2), and four experimental groups were prepared: in one group, the connectives to the brain were cut (‘brain-disconnected’); in another group, the connectives to the AT were cut (‘AT-disconnected’); and the remaining two groups had sham surgeries in which one or the other set of connectives was exposed but not cut. None of the subjects was injected with EH extract, because the previous experiments (Fig. 3) showed that EH injection affected only the latency of the behaviors and not whether they occurred. Subjects were observed for pre-ecdysis compressions, pre-ecdysis proleg retractions and ecdysis behavior.

Essentially all (21 of 22) sham-operated larvae initiated both pre-ecdysis and ecdysis behaviors (Fig. 4); thus, the non-specific effects of surgery were minimal. All brain-disconnected larvae initiated pre-ecdysis compressions, whereas AT-disconnected larvae failed to initiate pre-ecdysis compressions in the abdominal segments *anterior* to the cut connectives (Fig. 4A). However, all AT-disconnected larvae did exhibit compressions at the appropriate time in the segments *posterior* to the cut connectives, which are innervated by the AT (N=11; data not shown). Although disconnecting the AT prevented the initiation of pre-ecdysis

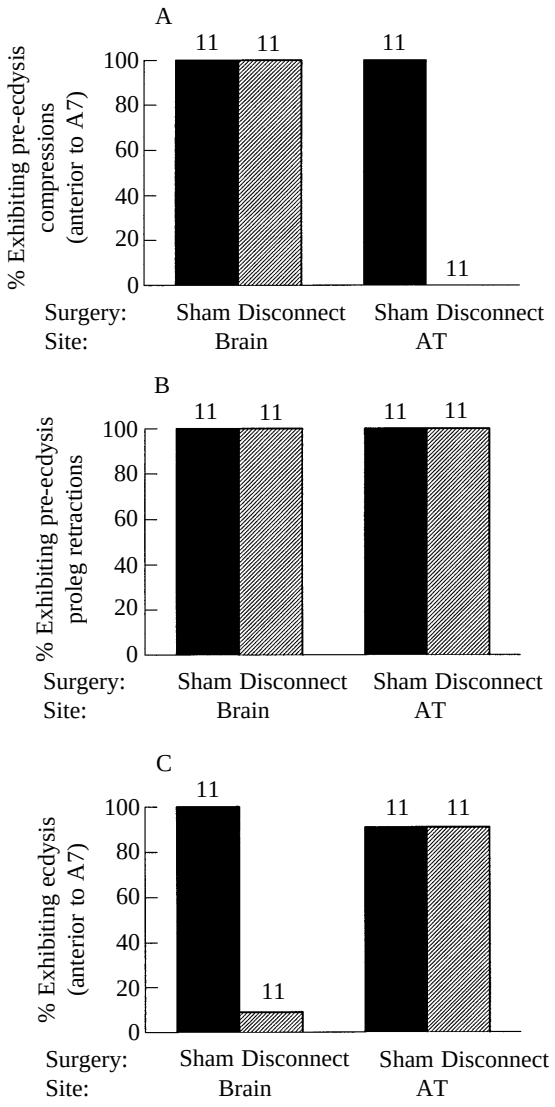

Fig. 4. Behaviors exhibited following connective transections. Filled bars indicate sham-operated subjects while hatched bars indicate brain-disconnected (left) and AT-disconnected (right) subjects. The number of subjects is shown above each bar. In sham surgeries, the relevant connectives were exposed but not cut. Each subject was observed for pre-ecdysis compressions (A), pre-ecdysis proleg retractions (B) and ecdysis (C). (A) Percentage of larvae that exhibited pre-ecdysis compressions in segments anterior to A7; subjects were scored as in Fig. 3A, except that only segments A1–A6 were observed (to assess the effect on anterior segments of disconnecting the AT, see text). (B) Percentage of subjects that performed the proleg retraction component of pre-ecdysis behavior (scored in segments A3–A6). (C) Percentage of subjects that performed ecdysis behavior in segments anterior to A7; subjects were scored as in Fig. 3C, except that only segments A1–A6 were observed.

compressions in anterior abdominal segments, these segments did perform the other major component of pre-ecdysis behavior, proleg retractions (Fig. 4B). This result is consistent with previous findings that pre-ecdysis proleg retractions are controlled in a differently from abdominal compressions (see Discussion; Novicki and Weeks, 1993).

The latency from surgery to the initiation of pre-ecdysis behavior (compressions and/or proleg retractions) did not differ significantly among the four groups of experimental subjects, with pre-ecdysis behavior beginning around the expected time of endogenous EH release (latency from brown mandibles to pre-ecdysis in brain-disconnected larvae, 7.9±0.2 h; in brain-sham-operated larvae, 8.2±0.2 h; in AT-disconnected larvae, 7.4±0.3 h; in AT-sham-operated larvae, 7.8±0.3 h; single-factor ANOVA; N=11 per group; P>0.1). These results suggested that intact connections with the AT, but not the brain, are necessary for the initiation of the compression component of pre-ecdysis behavior in ganglia anterior to the AT, whereas the AT can initiate pre-ecdysis compression behavior independently of the remainder of the central nervous system. Furthermore, initiation of the proleg retraction component of pre-ecdysis behavior requires neither the brain nor the AT.

Connective cuts had different effects on the initiation of ecdysis behavior. Nearly all (10 of 11) AT-disconnected larvae initiated ecdysis behavior (Fig. 4C). In these subjects, the anteriorly directed peristaltic waves were initiated in segment A6 and posterior segments did not participate. In contrast, most brain-disconnected larvae (10 of 11) failed to initiate ecdysis behavior (Fig. 4C). The latency from brown mandibles to the initiation of ecdysis behavior in the AT-disconnected larvae did not differ significantly from the latency in the AT-sham-operated group (8.6±0.3 h and 8.7±0.2 h, respectively; t-test, N=10 subjects per group; P>0.5). The sole brain-disconnected larva that initiated ecdysis did so 8.2 h after surgery. Thus, all larvae that initiated ecdysis did so at the appropriate time, i.e. within 1 h after the expected time of EH release (Fig. 2). These results suggested that initiation of ecdysis behavior at the appropriate time normally requires intact neural connections with the brain, although in one case ecdysis did occur at the appropriate time in a brain-disconnected larva. In contrast, intact connections with the AT are not necessary for the initiation of ecdysis behavior at the appropriate time.

Truman (1972) reported that most larvae which were neck-ligated approximately 12 h after the initiation of the molt from the fourth to fifth instar (more than 24 h earlier than the brown mandible stage; Fig. 2) nevertheless performed larval ecdysis behavior at the appropriate time. This result contrasts with our finding that all isolated abdomens, and nearly all brain-disconnected larvae, failed to ecdyse at the appropriate time (Figs 3C, 4C). In an attempt to replicate the results of Truman (1972), we neck-ligated larvae at approximately the same developmental stage as in the earlier study and visually observed their behavior during the time that age-matched control larvae performed pre-ecdysis and ecdysis behaviors. The ligated animals performed vigorous pre-ecdysis behavior but not ecdysis behavior (N=9). Therefore, we obtained no evidence that neck-ligated larvae produce ecdysis behavior at the appropriate time. Larval pre-ecdysis behavior in *M. sexta* had not been described at the time of Truman's (1972) study, and it is possible that the automated device he used to monitor

the behavior of headless larvae may have recorded pre-ecdysis rather than ecdysis behavior.

Bioassay of hemolymph during pre-ecdysis behavior

The finding that isolated abdomens or brain-disconnected larvae initiated pre-ecdysis behavior at the expected time of EH release (Figs 3, 4) was unexpected, because the brain contains the cell bodies of the VM cells (Fig. 1D). The VM cells are believed to be the only source for EH release at larval molts (Truman and Copenhaver, 1989; Hewes and Truman, 1991; Riddiford *et al.* 1994). To investigate whether EH appeared in the hemolymph in the absence of the brain, EH bioactivity in the hemolymph was assayed in intact larvae and in larvae from which the heads had been removed. Larvae were selected at the air-filled head capsule stage (Fig. 2), briefly anesthetized, and either left intact (N=25) or ligated between T1 and T2 (Fig. 1A), after which the head was cut off (N=29). Hemolymph was removed from a subset of intact (N=11) and headless (N=14) larvae 0.5 h later (i.e. approximately 3.5 h before the expected time of EH release), when EH bioactivity in the hemolymph is normally low (Copenhaver and Truman, 1982). Hemolymph was removed from the remaining intact (N=14) and headless (N=15) larvae 0.5 h after the initiation of pre-ecdysis behavior, when EH bioactivity is normally elevated in the hemolymph of intact larvae (Fig. 2; Copenhaver and Truman, 1982). Hemolymph samples were heat-treated, centrifuged and the pooled supernatants frozen until the time of bioassay (see Materials and methods).

EH bioactivity was measured using a prepupal bioassay (see Materials and methods). Hemolymph samples were injected into staged prepupae and the latency to the initiation of pupal ecdysis was measured; this latency is inversely related to the amount of EH in the injected sample (Truman *et al.* 1980). To validate the bioassay, two groups of staged prepupae were injected with saline or EH extract (Fig. 5A). Injection of saline into prepupae did not advance pupal ecdysis, and these animals initiated ecdysis at the time appropriate for endogenous release of EH. In contrast, injection of EH extract into prepupae significantly advanced the initiation of pupal ecdysis (Fig. 5A), signifying elevated EH bioactivity.

As shown in Fig. 5B, hemolymph taken from intact larvae 3.5 h before the expected time of EH release did not advance pupal ecdysis in the bioassay animals. In contrast, hemolymph taken from intact larvae 0.5 h after the initiation of pre-ecdysis behavior did significantly decrease the latency to pupal ecdysis in the bioassay animals, indicating elevated EH bioactivity (compare with saline and EH controls in Fig. 5A). Hemolymph collected from headless larvae 3.5 h before the expected time of pre-ecdysis behavior failed to advance pupal ecdysis in the bioassay animals, as in the corresponding group of intact larvae. However, hemolymph collected from headless larvae 0.5 h after the initiation of pre-ecdysis behavior also failed to advance pupal ecdysis significantly in the bioassay animals, suggesting that the elevation of hemolymph EH bioactivity normally seen in intact larvae at this time failed to occur in the headless larvae. These results suggested that the head is required for the appearance of

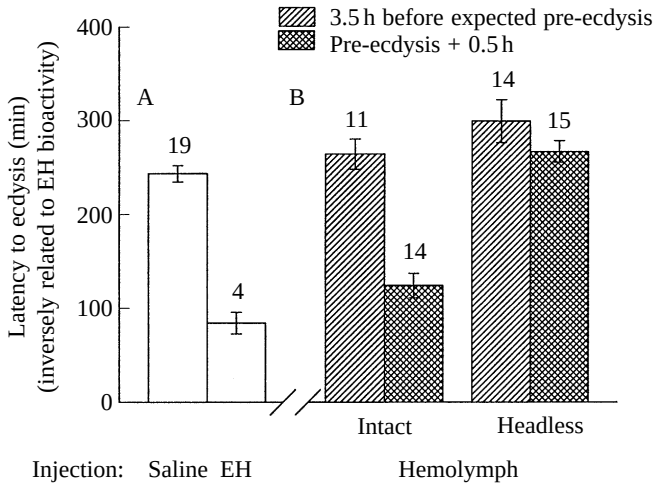

Fig. 5. Bioassay of EH activity in the hemolymph of intact and headless larvae. Staged prepupae were injected with saline, EH extract or hemolymph samples and the latency to pupal ecdysis was measured (see Materials and methods). The latency to ecdysis is inversely related to the amount of EH bioactivity. The number of subjects is shown above each bar. (A) Validation of the bioassay. Staged prepupae were injected with saline or 0.5 corpora cardiaca–corpora allata (CC-CA) equivalents of EH extract. The latency to ecdysis was significantly shorter in prepupae injected with EH extract (t-test, P<0.00001). (B) Hemolymph samples were taken from headless and intact larvae either 3.5 h before the expected onset of pre-ecdysis (hatched bars) or 0.5 h after the onset of pre-ecdysis behavior (cross-hatched bars), and injected into prepupae. A two-way ANOVA showed a significant interaction between the time of hemolymph removal and surgical state (P<0.005). The group of prepupae injected with hemolymph from intact larvae 0.5 h after the onset of pre-ecdysis had a significantly shorter latency to ecdysis than those in the other three groups (*post hoc* Tukey's test, P<0.01). Values are means ± S.E.M.

elevated levels of EH bioactivity in the hemolymph during the performance of larval pre-ecdysis behavior.

The finding that headless larvae produced pre-ecdysis behavior in the absence of detectable EH bioactivity in the hemolymph was unexpected, given the prevailing view that EH is the normal trigger for this behavior (see Introduction). Accordingly, we performed several additional experiments to investigate assumptions about the role of EH in pre-ecdysis behavior. One piece of evidence that EH is the normal trigger for pre-ecdysis is that injected EH extract can prematurely trigger the behavior (Fig. 3B; Copenhaver and Truman, 1982). However, because EH extract is prepared from CC-CA complexes, it is possible that some other factor in the extract is the active component rather than EH itself. To investigate this possibility, headless larvae were prepared at the air-filled head capsule stage and 1 h later were injected with saline, recombinant EH (Morton and Giunta, 1992) or 8-Br-cGMP, a membrane-permeant form of cyclic GMP (see Materials and methods). Cyclic GMP is believed to be the second messenger of EH in the nervous system (Truman *et al.* 1979; Morton and Truman, 1985; Morton and Giunta, 1992). The latency to pre-

ecdysis was significantly reduced in headless larvae injected with either recombinant EH or 8-Br-cGMP compared with saline-injected controls [latency to pre-ecdysis in subjects injected with: recombinant EH, 46.7±6.5 min (N=7); 8-Br-cGMP, 60.6±7.2 min, (N=7); saline, 120.2±22 min (N=6); single-factor ANOVA followed by *post hoc t*-tests; P<0.05]. Larvae in all three groups displayed both pre-ecdysis compressions and proleg movements. These results are consistent with EH being the active factor in CC-CA extract that induces premature pre-ecdysis behavior, and confirm that EH is sufficient to trigger both the compression and proleg components of the behavior. Furthermore, these data are consistent with the proposal that EH acts *via* cyclic GMP to trigger pre-ecdysis behavior.

Another possible explanation for the failure to detect EH bioactivity in the hemolymph of headless larvae during pre-ecdysis behavior was that the prepupal bioassay was not sufficiently sensitive to detect reduced but biologically active levels of EH. We attempted to quantify the sensitivity of the bioassay by injecting serial dilutions of EH extract into staged prepupae and measuring the latency to initiation of pupal ecdysis. In two separate replications, the highest doses of EH extract decreased the latency to ecdysis whereas the lower doses did not; however, there was not a continuous dose–response relationship between the amount of EH injected and latency to ecdysis (N=71 prepupae, each injected with 0–1.0 CC-CA equivalents of EH extract; data not shown). Repetition of this experiment in another group of prepupae, using dilutions of hemolymph from intact larvae during pre-ecdysis behavior, showed that the ability of injected hemolymph to advance ecdysis was lost after the hemolymph was diluted by 50 % or more (N=43 prepupae, each injected with undiluted hemolymph, hemolymph diluted up to 20-fold, or saline; data not shown). Therefore, if EH levels in the hemolymph of headless larvae during pre-ecdysis behavior were reduced by half or more, the prepupal bioassay may have failed to indicate EH bioactivity. Attempts to increase the sensitivity of the bioassay by injecting larger volumes of hemolymph or using methanol extraction to concentrate the EH (Riddiford *et al.* 1994) had deleterious effects on prepupae, rendering the bioassay unusable. Because of these uncertainties regarding the ability of the prepupal bioassay to indicate whether EH was present in the hemolymph of headless larvae, we turned to an independent technique – immunocytochemical staining of EH in the proctodeal nerves – to further evaluate whether headless larvae released EH.

Eclosion hormone immunoreactivity in proctodeal nerves during pre-ecdysis behavior

EH immunoreactivity in the proctodeal nerves, which are the sites of peripheral release of EH (see Fig. 1D; Hewes and Truman, 1991; Truman and Copenhaver, 1989), was examined using a polyclonal antiserum raised against partially purified EH (Copenhaver and Truman, 1986; see Materials and methods). Control experiments indicated that the antiserum was specific for EH (see below). Proctodeal nerves from four groups of subjects were processed for EH immunoreactivity:

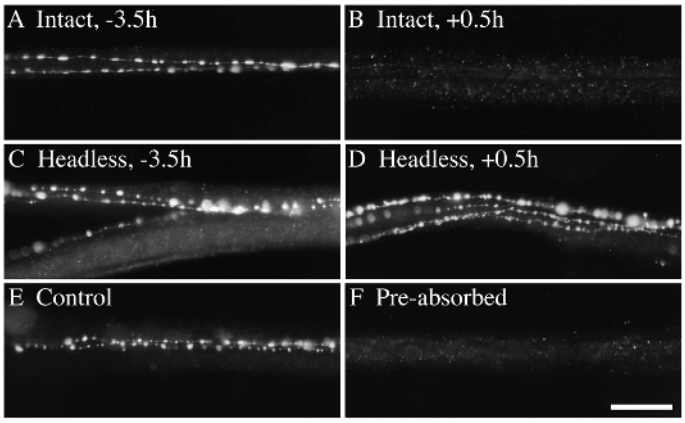

Fig. 6. EH immunoreactivity in the proctodeal nerves of intact and headless larvae. Each panel shows a fluorescence photomicrograph of a proctodeal nerve from a larva in the indicated group. (A) Intact larva 3.5 h before the expected onset of pre-ecdysis. (B) Intact larva 0.5 h after the onset of pre-ecdysis behavior. (C) Headless larva 3.5 h before the expected onset of pre-ecdysis. (D) Headless larva 0.5 h after the onset of pre-ecdysis behavior. Proctodeal nerves in A, C and D showed positive EH immunoreactivity, whereas the nerve in B did not. E and F show proctodeal nerves from intact larvae 3.5 h before the expected onset of pre-ecdysis. E shows a positive control in which the nerves were processed normally. In F, the primary antiserum was pre-absorbed with recombinant EH, which abolished immunoreactivity. Scale bar, 50 μm.

intact and headless larvae at 3.5 h before the expected time of EH release and 0.5 h after the onset of pre-ecdysis. These were the same experimental groups as used for hemolymph bioassays. After processing, proctodeal nerves were scored for EH immunoreactivity by two independent observers who were unaware of the identity of the material; agreement between the observers was 100 %. Positive EH immunoreactivity was indicated by bright, varicose processes of the VM cells running within the proctodeal nerves (Fig. 6A,C,D,E). The release of EH is associated with a loss of EH immunoreactivity (Fig. 6B; Hewes and Truman, 1991) and bioactivity (Truman and Copenhaver, 1989; Truman and Morton, 1990) in the proctodeal nerves.

Table 1 lists the percentage of larvae in each of the four experimental groups that exhibited positive EH immunoreactivity in the proctodeal nerves, and Fig. 6 shows representative examples. Among intact larvae examined 3.5 h before the expected onset of pre-ecdysis, most showed positive EH immunoreactivity (Fig. 6A; Table 1). This finding is consistent with the accumulation of EH in the VM cells. In contrast, a significantly smaller proportion of intact larvae showed positive EH immunoreactivity when examined 0.5 h after the onset of pre-ecdysis (Fig. 6B; Table 1), consistent with the release of EH from VM cells at the expected time (see Fig. 2). All or nearly all headless larvae showed positive EH immunoreactivity in the proctodeal nerves both 3.5 h before the expected onset of pre-ecdysis (Fig. 6C) and 0.5 h after the onset of pre-ecdysis (Fig. 6D; Table 1). The latter observation suggests that EH release from the proctodeal nerves at the

Table 1. *Percentage occurrence of positive eclosion hormone immunoreactivity in proctodeal nerves of intact and headless larvae*

Group	Time nerves removed	Percentage positive	*N*
Intact	3.5 h before expected pre-ecdysis	83	12
	0.5 h after onset of pre-ecdysis	17*	12
Headless	3.5 h before expected pre-ecdysis	100	12
	0.5 h after onset of pre-ecdysis	92	12

The percentage of positive eclosion hormone immunoreactivity in the proctodeal nerves was not equal in the four groups ($P<0.001$, χ^2-test). The percentage of positive immunoreactivity in intact larvae 3.5 h before expected pre-ecdysis, and in both groups of headless larvae, did not differ significantly ($P>0.5$). The percentage of positive immunoreactivity did differ significantly in the group of intact larvae 0.5 h after the onset of pre-ecdysis (*$P<0.001$).

N is the number of larvae examined.

onset of pre-ecdysis behavior in headless larvae was either absent or substantially reduced from the level of release seen in intact larvae (see Discussion). These results, using immunocytochemical methods, paralleled the findings from the hemolymph bioassay experiments: both sets of data suggest that pre-ecdysis behavior performed by headless larvae is not associated with the normal release of EH into the hemolymph.

In a separate experiment, proctodeal nerves were taken from intact larvae 3.5 h before the expected time of pre-ecdysis. For a positive control, some nerves were processed normally (Fig. 6E), while other nerves were processed using EH antiserum that had been pre-incubated with recombinant EH (see Materials and methods). Preabsorption of the antiserum with EH blocked immunostaining in 100 % of the nerves (Fig. 6F; $N=18$ larvae). This result indicates that the primary antiserum was specific for EH.

Discussion

This study investigated neural and hormonal factors involved in the initiation of pre-ecdysis and ecdysis behaviors in *M. sexta* larvae. We found that the requirements for intact neural connections with the brain and/or the AT differed for the initiation of ecdysis and two components of pre-ecdysis behavior, and that the requirements for the brain and/or the AT were the same whether or not exogenous EH was injected. We also obtained evidence suggesting that the initiation of pre-ecdysis behavior in headless larvae occurs without the normal elevation of EH bioactivity in the hemolymph or normal loss of EH immunoreactivity from the proctodeal nerves, prompting a re-examination of the role of EH and the VM cells in this behavior.

The initiation of pre-ecdysis behavior

Compression component

Pre-ecdysis compression behavior began at the appropriate time both in isolated abdomens that lacked the head and thorax, and in brain-disconnected larvae (Figs 3A,B, 4A; see Results). Furthermore, injection of EH extract (Fig. 3B) or recombinant EH into isolated abdomens advanced the onset of pre-ecdysis compressions. Therefore, exposure of neural and endocrine structures in the head and thorax to EH is unnecessary, and exposure of abdominal structures to EH is sufficient, to activate the neural circuit for pre-ecdysis compressions. The AT was essential for the ability to initiate pre-ecdysis compressions, as shown by the finding that disconnection of the AT prevented the initiation of this behavior in ganglia anterior to the severed connectives (Fig. 4A). Previous electrophysiological studies have indicated that the compression motor pattern is generated in the AT and then relayed to anterior ganglia. For example, cutting abdominal connectives during the pre-ecdysis motor pattern eliminates the pattern in ganglia anterior (but not posterior) to the cut, and the AT continues to produce the compression motor pattern after isolation from the rest of the nervous system (Novicki and Weeks, 1993, 1995). Similarly, in the present study, pre-ecdysis compression behavior was initiated at the correct time in the body segments innervated by the AT, even when the AT was disconnected from the rest of the central nervous system. One possibility, which could be tested experimentally, is that the AT is the only portion of the nervous system required for the EH-induced initiation and generation of the pre-ecdysis compression motor pattern.

Proleg retraction component

Initiation of the other component of larval pre-ecdysis behavior, rhythmic proleg retractions, at the correct time required neither the brain nor the AT (Fig. 4B; see Results). Even when disconnection of the AT prevented the initiation of compression behavior, rhythmic proleg retractions began at the appropriate time. Thus, performance of the proleg retraction component does not require the concomitant performance of abdominal compressions. As was also observed for the compression component, exposure of just the abdomen to EH was sufficient to initiate the proleg retraction component of pre-ecdysis behavior. In a previous study, we ligated larvae in segment A5 during pre-ecdysis behavior and found that prolegs both anterior and posterior to the ligation continued to retract rhythmically (unlike the compression movements, which stopped anterior to the ligation; Novicki and Weeks, 1993). Thus, neither the brain nor the AT is required to maintain the proleg retraction pattern after the behavior has been initiated. These findings suggest that, in contrast to the absolute requirement for the AT exhibited by pre-ecdysis compression behavior, the ability to initiate the pre-ecdysis proleg retraction motor pattern in response to EH may be present in individual abdominal ganglia or short chains of ganglia. It is unknown why the neural control of the compression and proleg retraction components of pre-ecdysis behavior differs. The motor neurons involved in the two components participate in many other behaviors (including ecdysis), and differences in

the neural loci involved in their control may provide flexibility for combining their activity during different behaviors.

Role of eclosion hormone

A surprising finding was that abdomen isolation or brain disconnection performed hours before the expected time of EH release did not affect the time at which pre-ecdysis behavior was initiated; in these subjects, pre-ecdysis behavior began at the same time as in age-matched intact controls (Fig. 3B; see Results). If release of EH from the VM cells is the trigger for larval pre-ecdysis behavior, it is difficult to imagine how pre-ecdysis could begin at the correct time after disconnection or partial removal of the VM cells; e.g. in isolated abdomens, the VM cell somata as well as their axons and processes in the brain through the metathoracic ganglion were removed (Fig. 1). That larval pre-ecdysis behavior is normally triggered by the release of EH is supported by two main lines of evidence: EH bioactivity in the hemolymph first becomes detectable at about the time pre-ecdysis begins (Copenhaver and Truman, 1982), and injection of EH extract or recombinant EH can prematurely activate pre-ecdysis behavior (see Results; Copenhaver and Truman, 1982; Miles and Weeks, 1991; Novicki and Weeks, 1993). Several lines of evidence also suggest that the VM cells are the sole source of EH (Truman and Copenhaver, 1989; Hewes and Truman, 1991; Riddiford *et al.* 1994).

To evaluate further the role of EH, we measured EH bioactivity the hemolymph, and EH immunoreactivity in the proctodeal nerves, of intact and headless larvae during pre-ecdysis behavior. As expected, EH bioactivity in the hemolymph of intact larvae was elevated during pre-ecdysis behavior, consistent with release of the peptide, but headless larvae did not exhibit elevated EH bioactivity during their pre-ecdysis behavior (Fig. 5B). Immunostaining of proctodeal nerves for EH during pre-ecdysis behavior gave parallel results: a loss of EH immunoreactivity, consist with release of the peptide from the VM cells, occurred in the nerves of intact larvae but not in the nerves of headless larvae (Table 1; Fig. 6). Thus, two independent techniques suggested that EH was not released at the onset of pre-ecdysis behavior in headless larvae. However, it was also possible that headless larvae released a small amount of EH, which was undetected by our methods; i.e. the bioassay may have failed to detect EH if its levels were half or less than normal (see Results), and the release of a small amount of EH may not have reduced the intensity of immunoreactivity in the proctodeal nerves. These two different interpretations – the complete absence of EH release, or the release of an abnormally small amount of EH – suggest different underlying mechanisms, as discussed below.

The first interpretation, that EH is *not* released during pre-ecdysis behavior of headless larvae, suggests that some other cue triggers the behavior to occur at the correct time. In this scenario, EH exposure (by endogenous release or injection) is sufficient but not necessary to trigger pre-ecdysis behavior and, in the absence of EH, other mechanisms can initiate the behavior at the appropriate time. One possible hormonal candidate is the drop in hemolymph ecdysteroid levels near the end of the molt cycle which, for example, triggers a 'preparatory behavior' in *M. sexta* that precedes ecdysis to the adult moth (Truman, 1984). Another important candidate is the recently discovered neuropeptide, *Manduca sexta* ecdysis-triggering hormone (Mas-ETH; Zitnan *et al.* 1996). Mas-ETH is produced by a segmentally distributed system of gland cells (Inka cells) associated with the trachea. Release of Mas-ETH from the Inka cells coincides with the initiation of larval pre-ecdysis behavior, and injection of Mas-ETH initiates larval pre-ecdysis behavior after a shorter latency than does EH injection (Zitnan *et al.* 1996). The relationship between EH and Mas-ETH remains to be elucidated, but one possibility is that EH causes Mas-ETH release, which then triggers pre-ecdysis and ecdysis behaviors (discussed in Truman, 1996; Zitnan *et al.* 1996; S. Hesterlee and D. B. Morton, 1996). A possible explanation for the initiation of pre-ecdysis behavior in headless larvae is if Mas-ETH were released at the correct time even in the absence of EH release.

The alternative interpretation of our data is that EH is released at the correct time in headless larvae, but at levels too low to be detected by bioassay or immunostaining. How might this release occur? In headless larvae, the somata and rostral processes of the VM cells have been removed. The VM cells have release sites throughout the length of the central nervous system and in the periphery (Fig. 1D), and under normal circumstances the release of EH is believed to occur at both the central and peripheral sites (Hewes and Truman, 1991). Hewes and Truman (1991) showed that removal of the hindgut and proctodeal nerves in otherwise intact animals eliminated the appearance of EH bioactivity in the hemolymph but did not prevent ecdysis behavior, suggesting that central release of EH is sufficient to initiate ecdysis (pre-ecdysis behavior was not examined in this study). One possibility is that the distal, severed axons of the VM cells in headless larvae release a small amount of EH at the correct time, with the amount released centrally being sufficient to activate pre-ecdysis behavior while peripheral release is undetectable. Assuming that EH release involves a transition from quiescence to spiking activity in the VM cells (Hewes and Truman, 1994), this scenario requires that the severed VM cell axons are able to generate impulses after removal of the cell body and initial axon segment (e.g. Thompson and Stent, 1976; Calabrese, 1980), and that the ability to switch from quiescence to activity at the correct time is retained in the severed axon. For instance, if a drop in ecdysteroid levels prior to brain removal commits the VM cells to become active after a certain delay (Hewes and Truman, 1994), this commitment might be retained by the severed axon (by some unknown mechanism). The possibility of EH release distal to a neck ligation has also been suggested in *Drosophila melanogaster* (Kimura and Truman, 1990). The potential involvement of Mas-ETH in this scenario also has to be considered. If EH, in fact, acts by triggering the release of Mas-ETH, which then triggers pre-ecdysis behavior, our findings suggest that a very low level of peripheral EH release

(undetectable by the bioassay or immunostaining methods used in this study) is sufficient to evoke release of Mas-ETH by the Inka cells. This possibility is also consistent with the experiments of Hewes and Truman (1991). Clearly, further elucidation of the relationship between EH and Mas-ETH in regulating pre-ecdysis behavior is essential.

One unequivocal conclusion from the present findings is that the regulation of the initiation of larval pre-ecdysis behavior in *M. sexta* is more complex than appreciated previously.

The initiation of ecdysis behavior

The initiation of larval ecdysis behavior at the correct time showed a clear dependence on the brain. Isolated abdomens failed to initiate ecdysis behavior (Fig. 3C), and all but one brain-disconnected larva failed to do so (Fig. 4C). These manipulations would be expected to potentially interfere with EH release (see above), but injection of EH into isolated abdomens did not restore ecdysis behavior (Fig. 3C). This finding suggests that the absence of ecdysis behavior resulted from an inability to respond to EH (see below). In these experiments, subjects were observed for at least 2 h after the expected onset of pre-ecdysis behavior before being scored as failing to ecdyse. We did not investigate whether ecdysis occurred belatedly in subjects that failed to ecdyse at the appropriate time. In contrast to the effects of removing or disconnecting the brain, disconnection of the AT did not affect the initiation of ecdysis behavior (Fig. 4C). In AT-disconnected larvae, the peristaltic waves were initiated anterior to the cut connectives rather than at the posterior tip of the abdomen. Hewes and Truman (1991) reported similar results for *M. sexta* prepupae clamped with hemostats at various abdominal levels prior to larval–pupal ecdysis. The finding that AT-disconnected larvae failed to produce pre-ecdysis compressions but nevertheless produced ecdysis indicates that performance of the former behavior is not a prerequisite for the latter. Similarly, under certain circumstances, adult ecdysis (eclosion) of cecropia silkmoths can occur without the preceding pre-eclosion behavior (Truman, 1978).

Why does disconnection of the brain prevent the initiation of ecdysis behavior, even when exogenous EH is injected? The Inka cells are unperturbed in brain-disconnected larvae, so the failure of these larvae to ecdyse would not be expected to result from a disruption of Mas-ETH release. One possibility is that neural input from the brain to the abdominal ganglia is necessary to initiate ecdysis. Some insight into this issue has been provided by disconnecting the brain at different times following injection of EH into *M. sexta* larvae (Ewer *et al.* 1994). Normally, EH injection induces the appearance of cyclic GMP immunoreactivity in a population of central neurons, which is temporally correlated with the performance of ecdysis behavior. These investigators showed that the appearance of cyclic GMP immunoreactivity and the performance of ecdysis behavior both require that the brain remain connected to the rest of the central nervous system for approximately 30 min following EH injection (Ewer *et al.* 1994; J. Ewer, personal communication). After this time, the brain is no longer required. These data suggest that the brain is a necessary site of action for EH and/or Mas-ETH in triggering larval ecdysis behavior. Whether the abdominal ganglia require only input from the brain, or both input from the brain and hormonal exposure, to initiate ecdysis remains to be tested. Once larval ecdysis is under way, however, the central motor pattern continues to be generated in isolated abdominal nerve cords (Weeks and Truman, 1984*a*). Thus, the brain appears to be necessary only for the initiation of larval ecdysis behavior and not for its maintenance.

The involvement of the brain in ecdysis may be different at pupation. Taghert *et al.* (1980) reported that, following brain removal during the final larval instar of *M. sexta*, the majority of insects nevertheless performed larval–pupal ecdysis behavior. The insects' development was delayed by surgery but ecdysis occurred at the appropriate time according to cuticular markers. These insects also showed positive EH bioactivity in the hemolymph during ecdysis, despite removal of the cell bodies and rostral processes of the VM cells in the brain.

In conclusion, at the culmination of each larval molt in *M. sexta*, pre-ecdysis and ecdysis behaviors occur at predictable times, for specific durations and in a characteristic sequence. The motor patterns underlying the behaviors are likewise stereotyped (e.g. Weeks and Truman, 1984*a*; Miles and Weeks, 1991; Novicki and Weeks, 1993). Although in these respects larval pre-ecdysis and ecdysis behaviors appear simple and invariant, the present study reveals that different neural loci are involved in controlling these behaviors. The inferred sites of hormone action in the central nervous system, and the neural structures required for initiating and/or maintaining the behaviors, differ for the two components of pre-ecdysis behavior and for ecdysis behavior. Furthermore, the involvement of EH in initiating larval pre-ecdysis behavior is more complex than previously thought. The involvement of multiple neural centers as hormonal targets and loci of behavioral initiation and maintenance presumably permits greater flexibility in the production of adaptive behavior.

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