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Modulation of ecdysis in the moth *Manduca sexta*: the roles of the suboesophageal and thoracic ganglia

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Summary

The sequential behaviours shown by insects at ecdysis are due to the sequential release of various hormones, but the transition from one phase to the next can be fine-tuned by inhibitory influences. The ecdysis sequence in the moth *Manduca sexta* was initiated by injecting sensitive animals with the neuropeptide ecdysis-triggering hormone (ETH). Exposure to ETH stimulates the release of eclosion hormone (EH) which, in turn, activates a set of neurons containing crustacean cardioactive peptide (CCAP) by elevating their levels of intracellular cyclic GMP. We characterized a set of non-CCAP containing neurons that also appear to be EH targets because of their response to cyclic GMP at ecdysis. The neurons did not display leucokinin-, diuretic-hormone- or FMRFamide-like immunoreactivity. They are probably the bursicon-containing cells described previously. After release of EH, there is a transient inhibition of the abdominal centers responsible for ecdysis. Transection experiments suggested that this suppression is *via* descending inhibitory units from the suboesophageal and thoracic ganglia. The duration of this inhibition appears to depend on the levels of cyclic GMP and can be extended by pharmacologically suppressing cyclic GMP breakdown. We further found that brief exposure to CO_2 caused premature ecdysis. Since the CO_2 treatment was effective only after EH release, it probably acts by suppressing descending inhibition. Studies on adult eclosion suggest that CO_2, given at the appropriate time, can uncouple the basic larval motor program from modulatory influences provided by the adult pterothoracic ganglion. CO_2 therefore appears to be a novel and non-invasive tool for studies of ecdysis behavior in insects.

Key words: ecdysis-triggering hormone, eclosion hormone, crustacean cardioactive peptide, cyclic GMP, inhibition, eclosion, neuron, carbon dioxide, tobacco hornworm, *Manduca sexta*.

Introduction

Neuromodulators play a key role in timing and coordinating complex behaviours in animals (Marder, 2000). Such control can originate from both neural and endocrine inputs. In insects, growth is punctuated by moults, which provide a new cuticle to accommodate larger or different body forms. The motor patterns and behaviours associated with the shedding of the old cuticle are collectively called the ecdysis sequence and depend on the action of a number of neuromodulators and neurohormones. In the moth *Manduca sexta*, the ecdysis sequence has become a model for studying the neural and endocrine regulation of stereotyped behavioural programs. A neuromodulatory network has been proposed for the ordering and timing of the various behavioural phases that comprise this sequence. While the network itself appears to be activated by a decline in steroid titres (Hewes and Truman, 1991; Kingan et al., 1997), the specific behaviours and motor programs leading to the shedding of the old cuticle are coordinated and maintained by the subsequent release of neuropeptides from the central nervous system and the periphery.

Four key players in the regulation of the ecdysis sequence have been identified. They include pre-ecdysis-triggering hormone (PETH) (Zitnan et al., 1999), ecdysis-triggering hormone (ETH) (Zitnan et al., 1996), eclosion hormone (EH) (Truman and Riddiford, 1970) and crustacean cardioactive peptide (CCAP) (Gammie and Truman, 1997b). These peptides probably play a role in the ecdysis behaviours of many, if not all, insects (Adams and Zitnan, 1997; Ewer and Truman, 1996; Truman et al., 1981), but their roles have been especially well described in the moth *Manduca sexta* (e.g. Ewer et al., 1997; Kingan et al., 1997).

In *M. sexta*, there is a working model for the organization of the pre-ecdysis and ecdysis behaviours. Declining steroid titres and possibly an early release of EH are thought to initiate the release of PETH and ETH from epitracheal glands lining the body wall. PETH triggers pre-ecdysis behaviour I, while ETH triggers pre-ecdysis behaviour II, both of which help the animal to loosen the old digested cuticle (Zitnan et al., 1999). Once ETH has been released, there is a positive feedback cascade between ETH and EH in which ETH stimulates release of EH and EH stimulates further release of ETH (Ewer et al., 1997; Gammie and Truman, 1999; Kingan et al., 1997). This feedback results in the massive release of

both hormones from their respective cells prior to the onset of ecdysis. EH then stimulates the release of CCAP from a group of 50 cells in the ventral nerve cord, called the cell 27/704 group (Davis et al., 1993; Ewer et al., 1994). Two major functions of CCAP are to release the ecdysis motor pattern itself, enabling the animal to shed its old cuticle, and to inhibit the ETH-induced pre-ecdysis behaviours (Ewer and Truman, 1997; Gammie and Truman, 1997b). The action of EH on the 27/704 group of neurons involves an increase in cyclic GMP (cGMP) levels, which increases cell excitability (Ewer et al., 1994; Gammie and Truman, 1997a; Gammie and Truman, 1999).

Measurements of changes in intracellular levels of cGMP have been instrumental in determining the timing of many events associated with EH and ETH activity (e.g. Ewer et al., 1994). The timing of many of these events appears to depend on descending inputs from the head (Baker et al., 1999; Ewer and Truman, 1997; Zitnan and Adams, 2000). In the case of larvae and pupae, there is a delay of 20–30 min between the release of EH and the onset of ecdysis behaviours, whereas this delay is 2–3 h in adults. This paper examines the nature of this inhibition through the use of surgical manipulations, ligatures and pharmacological manipulations. It further characterizes other neurons activated during ecdysis, of which little is currently known.

Materials and methods

Animals

Tobacco hornworms, *Manduca sexta* (L.), were raised under a 17 h:7 h L:D photoperiod at 26 °C on an artificial diet (Bell and Joachim, 1976). Pharate fifth-instar larvae and pharate pupae were staged using external morphological markers (Copenhaver and Truman, 1982; Truman et al., 1980). They were injected with ETH 1–2 h after the fourth-instar head capsule became filled with air (air-filled brown mandible stage; 'AFBM'). Pharate pupae were injected during the anterior shrink stage or no later than the early posterior shrink stage; these stages occur 4–2.5 h before expected ecdysis. Two weeks before adult emergence, pupae were transferred to a 17 h:7 h L:D photoperiod with a 28 °C:25 °C superimposed thermoperiod, based on the method of Mesce and Truman (1988). The thermoperiod enhanced the precision of the timing of adult ecdysis. Animals were isolated in individual vials 1 day before eclosion, based on morphological markers such as dark coloration due to pigmentation of the underlying adult cuticle and the advanced digestion of the old pupal cuticle (Schwartz and Truman, 1983).

ETH was synthesized by the Howard Hughes Macromolecular Synthesis Unit at the University of Washington. The peptide was stored as a 1 mmol l^{-1} stock in phosphate-buffered saline (PBS) and frozen until needed for experiments. Experimental amounts were diluted in modified Weever's saline (Trimmer and Weeks, 1989). Larvae and pupae were injected in the terminal abdominal segment with 10 µl of 2×10^{-5} mol l^{-1} ETH (200 pmol of ETH per animal).

Immunohistochemistry

Animals were anesthetized on ice, and their nerve cords were dissected in cold saline. Tissues were fixed in 4 % buffered paraformaldehyde for 1–2 h at room temperature, then rinsed in PBS containing 1 % Triton-X 100 (PBST; Sigma Chemical Co., St Louis, MO, USA. Tissues being processed for cGMP immunoreactivity (cGMP-IR) were immediately transferred to sheep anti-cGMP antibody (diluted to 1:10 000 in PBST; a generous gift from Dr Jan De Vente). The other tissues were treated with collagenase (Type IV; Sigma) in PBST at 0.5 mg ml^{-1} for 60 min at room temperature, then rinsed in PBST and blocked with 5 % normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 15 min at room temperature. Double-labeled tissues were incubated with a combination of sheep anti-cGMP antibody (1:10 000) and one of various rabbit antibodies diluted to appropriate concentrations with PBST; FMRFamide (1:1000, a gift from D. O. Willows), Leucokinin IV (1:2500, a gift from J. Veenstra) and *Manduca* diuretic hormone (1:2500, a gift from J. Veenstra). All tissues were incubated on a shaker for 36–48 h at 4 °C then rinsed in PBST and processed with secondary antibodies (1:1000) on a shaker for 24 h at 4 °C.

Tissues stained for cGMP-IR were transferred to peroxidase-conjugated donkey anti-sheep IgG (1:1000; Jackson Laboratories) for 24 h. After rinsing, the antibody complex was visualized using the chromagenic diaminobenzidene (DAB) reaction. Tissues were incubated with ammonium chloride (0.4 mg ml^{-1}), beta-D-glucose (2 mg ml^{-1}), DAB (50 µl ml^{-1}) and glucose oxidase (6 µl ml^{-1}) in PBST. Reactions generally took 5–30 min and were stopped by several rinses in PBST. The tissues were then dehydrated through an ethanol series, cleared in xylene and mounted in DPX (Fluka, Buchs, Switzerland) on poly-L-lysine-coated coverslips.

Double-labeled tissues were transferred to fluorescene isothiocyanate (FITC)-conjugated donkey anti-sheep IgG (1:1000; Jackson Laboratories) together with Texas-Red-conjugated donkey anti-rabbit IgG (1:1000; Jackson Laboratories) for 24 h. After rinsing, the tissues were dehydrated and mounted as described above. The samples were viewed on a confocal microscope (BioRad MRC 600; BioRad, Hercules, CA, USA).

Control tissues were treated as described above, with the omission of either the primary or the secondary antibody. No staining was noted in these preparations.

Signal quantification

The intensity of immunoreactivity based on the DAB colour reaction was quantified subjectively using a scoring system from 0 (no stain) to 3 (maximal staining of cell bodies and axons), as described by Ewer and Truman (1997).

Surgical procedures

Animals were anesthetized on ice then placed on a wax mount or immersed in cold saline. Debrained larvae and pupae had their brain removed through a small incision in the head capsule. Sham-operated animals had forceps inserted into the

same opening but without removing the brain. After surgery, the incision was sealed with wax and the animals were left to resume ecdysis. For the ligations, a blood-tight ligature was placed at various segments, using fine surgical thread, and the tissue anterior or posterior to the ligature was then cut off using surgical scissors. Neck ligatures were placed between the head and thorax to eliminate the brain and the suboesophageal ganglion (SOG). Thoracic ligatures were placed between thorax and abdomen to eliminate all thoracic ganglia. Terminal ligatures were placed between the last two abdominal segments to eliminate the terminal abdominal ganglion (TAG). Animals were dissected after the experiments to ensure that the ligatures had eliminated the appropriate ganglia.

Sham-ligated animals had neck ligatures quickly removed after tightening to test for the effects of stress induced by the initial tightening. No incisions or visible injuries were produced.

Animals were treated with CO_2 or N_2 gas, for 1 min, at different times after ETH injection. The animals were placed in loosely sealed containers under a constant stream of gas for the 1 min interval.

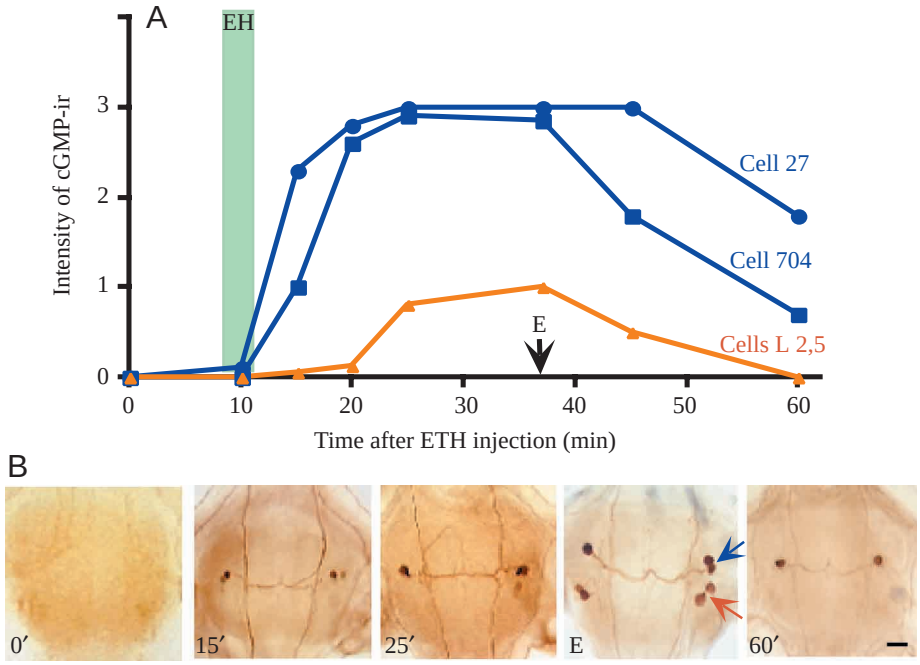


Fig. 1. Time course of cyclic GMP immunoreactivity (cGMP-IR) in abdominal neurons during larval ecdysis after injection of ecdysis-triggering hormone (ETH). Animals were injected with 200 pmol of ETH. (A) The intensity of cGMP-IR was quantified, as described in Materials and methods, for 5–15 samples per time point. Standard error bars were generally small, but have been omitted for clarity. Blue lines represent immunoreactivity in cells 27 and 704. The orange line represents immunoreactivity in paired cells $L_{2,5}$. The green column represents the time of eclosion hormone (EH) release, based on Ewer and Truman (1997). The black arrow represents the mean time of the start of ecdysis (E). (B) Video micrographs show the cGMP response in neurons. Each photograph is a video montage of 2–3 optical sections to show neuron morphology optimally. Cells 27 and 704 are marked with a blue arrow, and the $L_{2,5}$ cells are marked with an orange arrow. Times of dissection (in minutes) are noted in the lower left-hand corners. Scale bar, 150 μm.

Phosphodiesterase inhibitors

The cGMP-specific phosphodiesterase inhibitor Zaprinast (Sigma) was dissolved in 100 % dimethyl sulfoxide (DMSO) and injected into the animals in a similar manner to ETH injections. Animals were injected with 10 μl of Zaprinast 10 min before ETH injection. Control animals were injected with 10 μl of 100 % DMSO. Ganglia from some Zaprinast-injected animals were also fixed and processed for cGMP-IR, as described above.

Adult behaviours and videography

Adult eclosion was monitored and timed by eye and was videotaped using a Sony DCR-TRV11 digital camcorder. Images were captured on a Macintosh iMac using iMovie software.

Results are presented as means ± S.E.M.

Results

cGMP immunoreactivity in cells of the larval ventral nerve cord

Ewer and Truman (1997) quantified changes in cGMP levels in the cell 27/704 group of *Manduca sexta* ventral nerve cords during ecdysis using a cGMP-specific antibody. In animals triggered to ecdyse prematurely with injections of EH or epitracheal gland extracts, the appearance of cGMP in the cell 27/704 group coincided with the release of endogenous EH and preceded ecdysis by 20–25 min. It was suggested that the activity of the epitracheal gland extract was due to the presence of ETH, but that the increase in cGMP levels was due to the ETH-induced release of EH (Gammie and Truman, 1999; Zitnan and Adams, 2000). Fig. 1 shows the time course of cGMP-IR (blue lines in Fig. 1A and blue arrow in Fig. 1B) in the cell 27/704 group after injection of ETH. The time course was similar to that produced by epitracheal gland extracts (Ewer and Truman, 1997) and coincided with time points described after injection of ETH (Zitnan and Adams, 2000).

Fig. 1 also shows the time course of cGMP-IR in the two other pairs of neurons that are not CCAP-containing cells (Zitnan and Adams, 2000). Similar cells have been described during ecdysis of locusts (Truman et al., 1996). As shown in Fig. 1, cGMP was detected in these cells approximately 10–15 min after it appeared in the cell 27/704 group. Peak levels of cGMP-IR were evident at ecdysis, with a rapid decay

thereafter. The intensity of cGMP staining in these cells did not reach the levels seen in the cell 27/704 group. These ventrolateral cells were located posterior to cells 27 and 704, and their axons projected ipsilaterally through the ventral nerve. Hence, these cells would be in the L cell group on the basis of the nomenclature of Davis et al. (1993) (see Discussion). Homologous pairs of cells were noted in each abdominal ganglion, with the greatest intensity of staining occurring in abdominal ganglia 1 and 2 and progressively weaker staining occurring in cells of the more posterior segments.

Double labeling with anti-cGMP and anti-FMRFamide (Fig. 2B), anti-cGMP and anti-leucokinin IV (Fig. 2C) or anti-cGMP and anti-*Manduca* diuretic hormone (Fig. 2D) revealed that these neurons did not contain FMRFamide-, leucokinin- or diuretic-hormone-like peptides. There was no apparent overlap between peptide immunoreactivity and cGMP-IR. The immunohistochemical data suggested that cGMP-positive cells were not the $L_{3,4}$ neurons described in the moth central nervous system (Chen et al., 1994). We will therefore refer to them as the L_2 and L_5 cells.

Effects of surgery and decapitation on the timing of ecdysis

Larvae were decapitated at various times after ETH injection, and the time to ecdysis was determined (Fig. 3). In controls, larval ecdysis began on average 37 min after ETH injection. If animals were decapitated 10 min after ETH injection, after the onset of pre-ecdysis behaviours but prior to detectable levels of cGMP, the animals did not ecdyse. In contrast, larvae decapitated at 15, 20 or 25 min after ETH injection all began ecdysis significantly sooner than controls (P<0.001). The onset of ecdysis occurred approximately 28 min after ETH injection, irrespective of when the animals were decapitated. Similarly, for pupal ecdysis, decapitation at 10 min resulted in no ecdysis whereas the same treatment at 15, 20 or 30 min after ETH injection resulted in animals ecdysing at least 15 min earlier than controls.

As with previous studies (Baker et al., 1999; Ewer and Truman, 1997; Zitnan and Adams, 2000), these results indicated that the head has a dual role in the control of ecdysis. It is necessary to activate the behaviour, presumably through the release of EH (Reynolds et al., 1979), but it also has an inhibitory function. The locus of this inhibition was examined by assessing the effects of removing various ganglia on the timing of ecdysis. The influence of the brain, SOG, thoracic ganglia or terminal abdominal ganglion (TAG) was assessed by direct removal (debraining) or by ligature. Larvae did not ecdyse when the brain was removed 10 min after ETH injection (data not shown). When animals were debrained 20 min after ETH injection (Fig. 4; –Brain), they ecdysed at the same time as the controls, whereas the application of a neck ligature accelerated ecdysis (Fig. 4; –Br/SOG). Interestingly, ligation

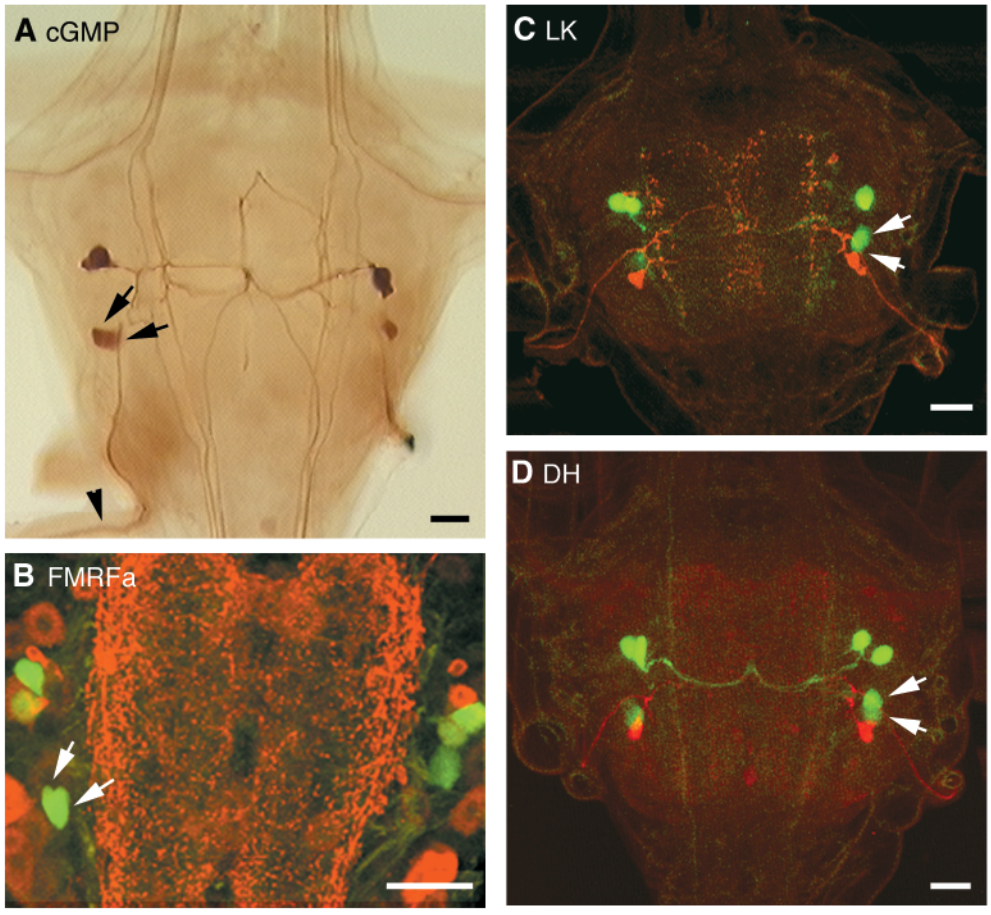


Fig. 2. Photographs of single-labeled (A) and double-labeled (B–D) preparations of ganglion A3 from ecdysis-triggering hormone (ETH)-injected larvae dissected at ecdysis. Arrows in all panels point to the $L_{2,5}$ cells. (A) Video montage of 2–3 optical sections of a cGMP-stained preparation, as visualized with a color reaction. Axons of the $L_{2,5}$ cells can be seen exiting the ventral nerve (black arrowhead). (B–D) Collapsed Z-series confocal images of double-labeled preparations. Red is FMRFamide (FMRFa), leucokinin (LK) or *Manduca sexta* diuretic hormone (DH) staining in panels B–D, respectively. cGMP is indicated by green (B–D) and is marked by white arrows. Co-localization was not noted in cells $L_{2,5}$ in any panel. Scale bars, 100 μm.

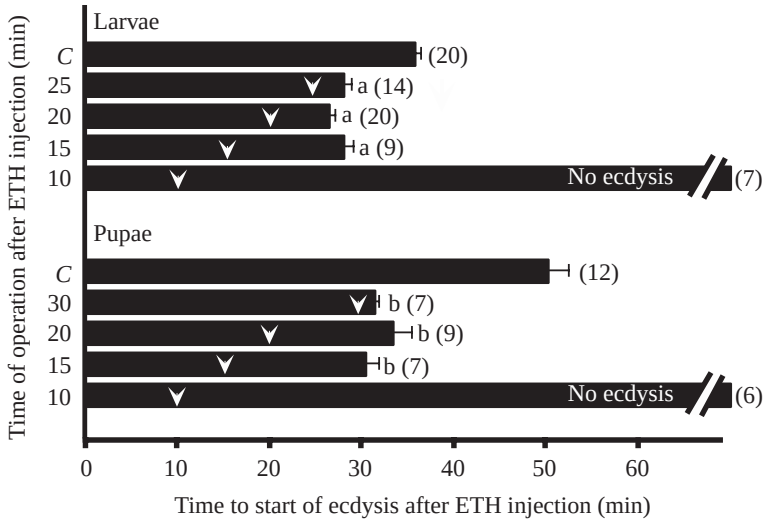


Fig. 3. Timing of the commencement of ecdysis in larvae and pupae decapitated at various times after injection of 200 pmol of ecdysis-triggering hormone (ETH). Animals were injected with ETH (at 0 min) and decapitated at the times listed on the *y*-axis (indicated by white arrowheads). Control larvae (*C*) were not decapitated. Animals decapitated 10 min after ETH injection did not ecdyse ('No ecdysis'), which is shown by a broken bar. Bars are mean times + S.E.M. Sample sizes are indicated in parentheses. Letters represent significant differences compared with controls within groups (P<0.001), as determined by analysis of variance using the Tukey test for differences (α=0.05).

between the third thoracic ganglion (T3) and the first abdominal ganglion (A1) to remove the brain, SOG and thoracic ganglia (Fig. 4; –Br-TG) resulted in a strikingly faster onset of ecdysis compared with the neck ligatures (P<0.001). No other treatments affected the timing of ecdysis, including sham operations (Fig. 4; ShamBr) and stresses such as sham ligatures (Fig. 4; ShamLig), sharp pinching of the cuticle (data not shown) or ligation of the terminal abdominal segments (Fig. 4; –Terminal).

As shown in Fig. 4, a similar relationship is evident for pupal ecdysis. Sham ligations, however, were omitted in pupae because these inevitably ruptured the new pupal cuticle. The high internal pressure in pupae led to excessive haemolymph loss during the course of ecdysis and usually to the eventual cessation of the ecdysis behaviours before the cuticle was shed. If the ligatures were not removed, as was the case for brain/SOG removal, haemolymph loss was minimized and ecdysis occurred prematurely, without noticeable differences in the quality of the visualized behaviours. Because terminal ligatures were followed by a normally timed, robust ecdysis, the stress of ligation did not seem to be sufficient to trigger premature ecdysis.

Thus, it appears that, after the release of EH, the brain can be removed without affecting the subsequent onset of the ecdysis behaviour. The removal of the SOG and thoracic ganglia, in contrast, results in a progressive advance in the onset of ecdysis in both larvae and pupae.

Effects of ligation on the duration of the ecdysis motor program

To determine whether there were differences in the roles of the SOG and thoracic ganglia during ecdysis, the duration of the ecdysis motor program was measured in both neck-ligated and thorax-ligated larvae. Duration was defined as the persistence of peristaltic waves in the abdomen, once ecdysis had started, irrespective of whether the cuticle was shed. This definition was necessary, since ligatures artificially held the cuticle in place during ecdysis in most animals. As can be seen

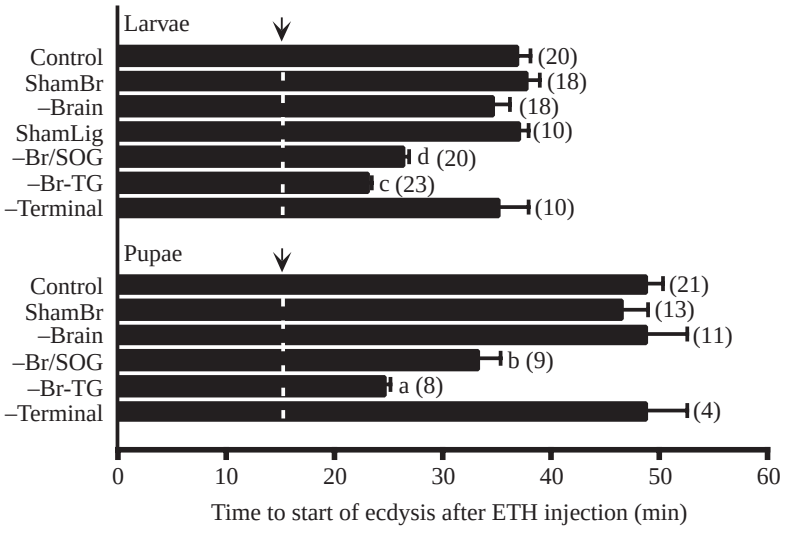


Fig. 4. The effects of surgical manipulations on the initiation of ecdysis in larvae and pupae. Animals were injected with 200 pmol of ecdysis-triggering hormone (ETH) (at 0 min), and surgery was performed 20 min later (black arrow). Controls were not treated surgically. ShamBr had their head capsule punctured and forceps inserted into the head region. ShamLig had a ligature placed and tightened between the suboesophageal ganglion (SOG) and the thoracic ganglia (TG); the ligature was then removed. Major ganglia were eliminated as described in the Materials and methods. Decerebration, –Brain; removal of the brain and SOG, –Br/SOG; removal of the brain, SOG and thoracic ganglia, –Br-TG; removal of the terminal abdominal ganglion, –Terminal. Bars are mean times + S.E.M. Sample sizes are indicated in parentheses. Letters represent significant differences from controls within groups (P<0.001), as determined by analysis of variance using the Tukey test for differences (α=0.05). Different letters denote significantly different values within a group (P<0.001).

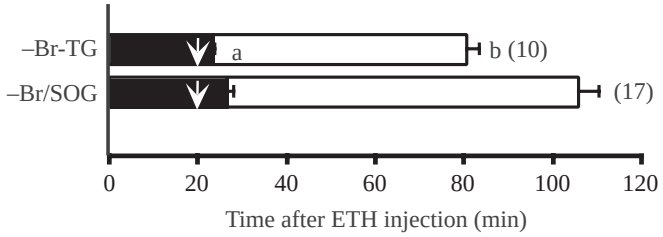


Fig. 5. Commencement and duration of the ecdysis motor program in larvae. Animals were injected with ecdysis-triggering hormone (ETH) (at 0 min) and ligated 20 min later (white arrowheads). Ligatures removed the brain and suboesophageal ganglion (SOG) (–Br/SOG) or the brain, SOG and thoracic ganglia (–Br-TG), as described in Materials and methods. The solid bars represent the mean time of onset of ecdysis, while the open bars reflect the mean duration of the motor program. Values are means + S.E.M. A letter represents a significant difference between the two treatments, as assessed with the unpaired *t*-test (P<0.005).

in Fig. 5, thoracic ligatures significantly decreased the duration of the ecdysis motor program compared with that of animals ligated at the neck. Therefore, the thoracic ganglia may have dual roles, to aid in delaying the onset of ecdysis and to maintain the motor program.

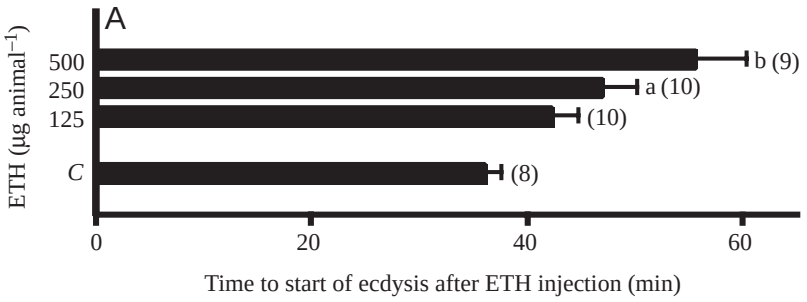
Effects of altered cGMP levels on the timing of ecdysis

The importance of cGMP to the timing of ecdysis was examined by injecting larvae with Zaprinast, a specific cGMP phosphodiesterase inhibitor (Burns et al., 1992). Animals were injected with Zaprinast 10 min before (Fig. 6A) or after (data not shown) injection of ETH, and the time to the onset of ecdysis was determined. When larvae were injected with the solvent DMSO prior to ETH injection, they showed an ecdysis latency of 38 min, which is not significantly different from that of controls injected with ETH without prior DMSO treatment (P>0.5). Treatment with Zaprinast, in contrast, produced a dose-dependent delay in the time of ecdysis onset (Fig. 6A).

Zaprinast has been used in *Drosophila melanogaster* as a specific cGMP phosphodiesterase inhibitor (Chyb et al., 1999). To assess its effectiveness in *Manduca sexta* in maintaining cGMP levels, we used immunocytochemistry to monitor the levels of cGMP in the ventral ganglia 60 min after ETH injection. Using the scoring system illustrated in Fig. 1, the intensity of cGMP-IR in the 704 cells of ganglion A3 of controls and of animals treated with 500 µg of Zaprinast was 1.0±0.4 (N=6) and 2.8±0.2 (N=6), respectively. The controls showed the expected waning of the cGMP response, whereas Zaprinast-treated animals still showed high levels of this second messenger in the cell bodies and processes of these neurons (P<0.001; Fig. 6B), showing that Zaprinast was, in fact, affecting cGMP levels. Preliminary results in naturally ecdysing larvae also showed that injection of Zaprinast after the start of the pre-ecdysis behaviour delays the onset of ecdysis and increases cGMP staining in CCAP-containing neurons (J. Fisher and J. W. Truman, unpublished observations).

Effects of CO_2 on the timing of ecdysis in larvae and pupae

Brief exposure to CO_2 after ETH injection proved to be a non-invasive method of triggering early ecdysis. As seen in Fig. 7A, a 1 min exposure to CO_2 given 20 min after ETH injection resulted in the onset of ecdysis within 4 min after the treatment. This response was significantly faster than that seen in neck-ligated larvae (P<0.001), but not from that of thorax-ligated animals (P>0.5; see Fig. 4). This suggested that CO_2 may relieve the inhibition provided by the SOG and the thoracic ganglia. Application of CO_2 to pharate pupae similarly triggered premature ecdysis (data not shown). The response to CO_2 did not appear to be due to simple anoxia since there was no significant change in the timing of ecdysis in larvae treated with N_2 compared with controls (P>0.5).



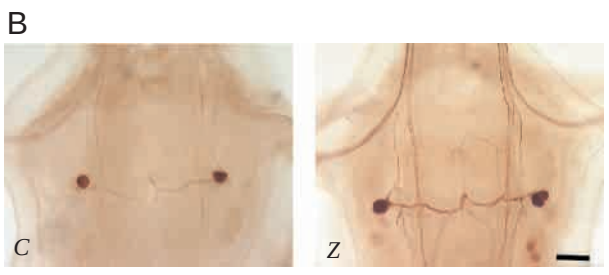


Fig. 6. The timing of the onset of ecdysis-triggering hormone (ETH)-induced ecdysis in larvae injected with the cGMP-specific phosphodiesterase inhibitor Zaprinast. (A) Animals were injected with Zaprinast 10 min prior to injection with 200 pmol of ETH (at 0 min), at the amounts listed on the *y*-axis. Controls (*C*) were injected with the Zaprinast solubilizing agent, 100 % dimethylsulfoxide (DMSO), before injection of ETH. The timing of ecdysis was not significantly different in these controls from that in untreated controls (data not shown). The bars represent mean times + S.E.M. Letters represent significant differences from the control and from each other, as determined by analysis of variance (P<0.001) using the Tukey test for differences (α=0.05). (B) Representative micrographs of cGMP immunoreactivity in abdominal ganglion 3 in DMSO- (*C*) and Zaprinast-treated (*Z*) animals 60 min after injection with ETH. Scale bar, 100 µm.

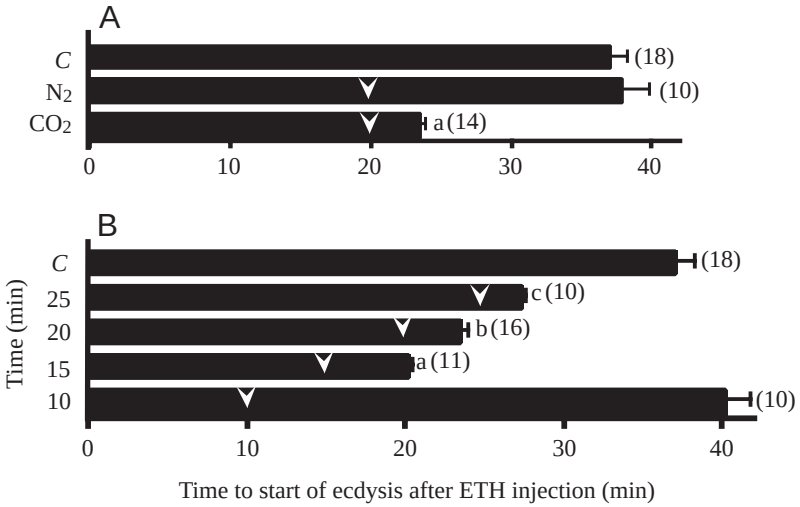


Fig. 7. The commencement of ecdysis-triggering hormone (ETH)-induced ecdysis in larvae after treatment with CO_2 or N_2. (A) Larvae were injected with 200 pmol of ETH (at 0 min) and 20 min later were treated with CO_2 or N_2 for 1 min (white arrowheads). (B) Larvae were treated with CO_2 for 1 min, at various times after ETH injection, as noted on the *y*-axis and by arrowheads. Controls (*C*) were handled at 20 min after ETH injection. The bars represent mean times + S.E.M. Letters represent significant differences from controls and from each other within groups ($P<0.001$), as determined by analysis of variance using the Tukey test for differences ($\alpha=0.05$).

The response of larvae to CO_2 was subsequently tested at various times after ETH injection (Fig. 7B). Treatment always consisted of a 1 min exposure to the gas, and the time of onset of ecdysis was then determined. At 10 min after ETH injection, application of CO_2 did not advance the onset of ecdysis compared with controls ($P>0.5$). In contrast, CO_2 triggered the rapid onset of ecdysis when applied 15, 20 or 25 min after ETH injection, at times when cGMP staining was apparent in the CCAP-containing neurons. At each successive treatment time, the lag between CO_2 application and the onset of ecdysis was reduced, although this was not significantly different.

Effects of CO_2 on the timing of ecdysis in adults

For adult eclosion, the delay between the appearance of cGMP in the cell 27/704 group and the onset of ecdysis behaviours is 2–3 h (Ewer and Truman, 1997). To determine whether CO_2 affected the timing of adult eclosion, animals were first entrained with a light and thermal regime, which resulted in the majority of moths (approximately 90 %) eclosing within a narrow window of time (09:00–13:00 h; see Fig. 9A). Adult ecdysis was generally completed within less than 1 min, with abdominal and thoracic behaviours occurring within seconds of each other. These events are depicted as open and filled black circles, respectively, overlaid because of the rapid succession of the two behaviours (see Fig. 9A). Eclosion began with one or two waves of peristaltic contractions that moved anteriorly along the abdomen. These rapidly evolved into a two-phase movement involving the retraction of the entire abdomen followed by its forceful extension (Fig. 8A). The extension was coupled to a vigorous flexing of the wing bases. The latter served to rupture the sutures over the head and thoracic cuticle, and the abdominal extensions helped to push the animal out of the rigid pupal cuticle. These behaviours have been described in detail by Mesce and Truman (1988). Once the moths had escaped from the pupal cuticle, they searched for a perch, upon which they remained to inflate their wings.

Entrained pharate adults were given a 1 min pulse of CO_2 at various times prior to ecdysis. If CO_2 was applied at 07:30 h, animals did not respond to the treatment. The moths subsequently emerged within their expected period and their

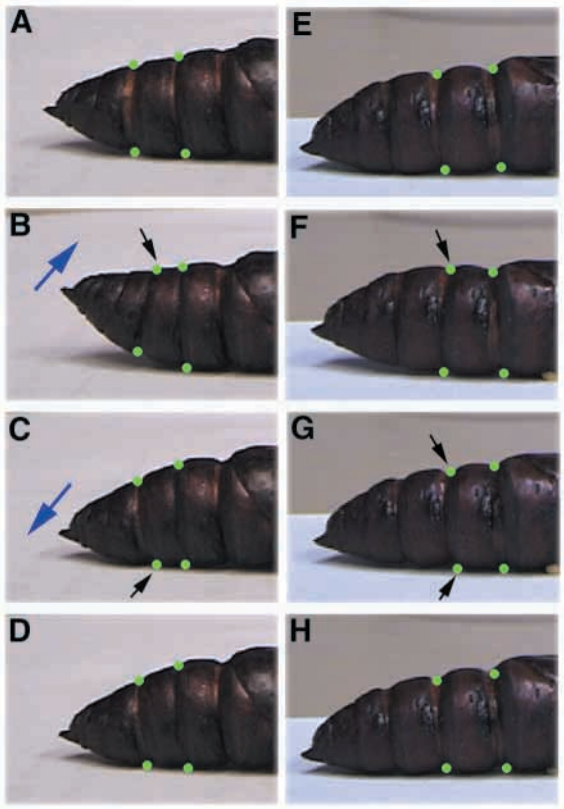


Fig. 8. Photographic sequence of adult abdominal peristalsis during normal eclosion (A–D) and CO_2-induced premature eclosion (E–H). A lateral view of the abdomen showing the retraction (B; upward-pointing blue arrow) and extension (C; downward-pointing blue arrow) of normal eclosion. These movements are lacking in CO_2-treated animals (E–H). Peristaltic contractions of segments are identified by green circles and black arrows.

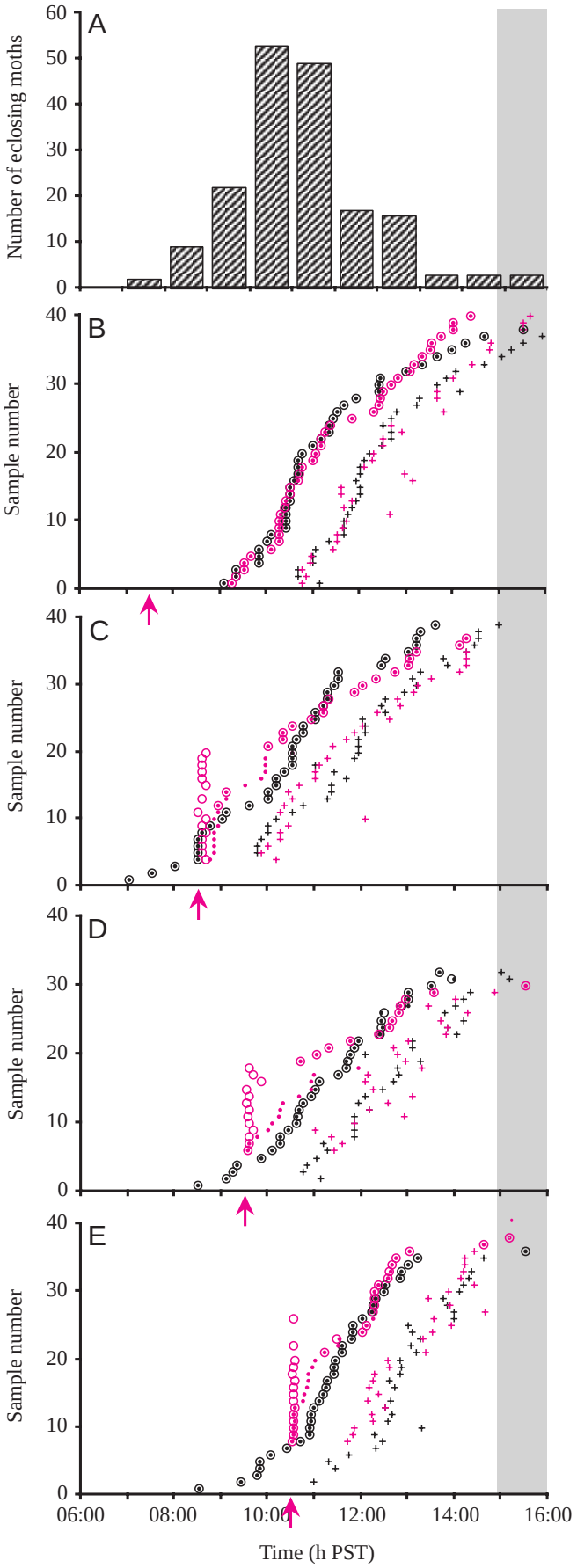


Fig. 9. Distribution of entrained adult eclosion (A) and responses to CO_2 during adult eclosion (B–E). (A) The entrainment period consisted of 2 weeks in a 17 h:7 h light:dark cycle coupled to a thermoperiod of 28 °C:25 °C. The start of the scotophase is depicted by the shaded column at 15:00 h Pacific Standard Time (PST). The frequency of eclosion is depicted for 189 animals. (B–E) The ability of CO_2 to induce premature eclosion in entrained pharate adults. Animals were treated with CO_2 for 1 min at various times (pink arrows) before lights out. Each pair of points (open and filled circle) and a cross represent eclosion behaviours from one animal (sample). Since the onset of the abdominal and thoracic components of ecdysis (pink open and filled circles, respectively) often occurred separately in CO_2-treated animals, they are separated temporally on the graph. When behaviours occurred in immediate succession, the dots were overlaid as a circle with a dotted center. Controls, in which abdominal and thoracic behaviours always occurred in immediate succession (thus open circles containing black dots), are shown in black. The time when the wings are fully expanded and move to the 'moth' position is depicted by a cross for each animal.

behaviour was indistinguishable from that of controls (compare overlaid open and filled pink and black circles; Fig. 9B). With successively later pulses of CO_2, a variety of responses was observed (Fig. 9C–E). A proportion of the animals ignored the CO_2 treatment and emerged at their expected times (overlaid pink circles). A large group of pharate adults responded with the initiation of abdominal peristaltic movements within minutes of the CO_2 treatment.

In some cases, the initial abdominal peristalsis quickly progressed to the two-phase abdominal movements coordinated with the strong thoracic flexing of the wing bases (early overlaid pink circles). These movements usually brought about the rapid shedding of the entire cuticle. In many of the treated animals, however, the abdominal peristalsis occurred in isolation with little or no thoracic activity (isolated open pink circles). These movements occasionally ruptured the abdominal cuticle. The peristaltic movements were transient and the animals then became quiescent, although every 5 min or so, 2–3 waves of abdominal peristalsis were noted which then rapidly disappeared. The quiescence lasted for 20–90 min, at which time the remaining abdominal cuticle was removed by initiation of the retraction/extension movements, and thoracic shrugging resulted in the shedding of the thoracic and head portions of the cuticle (isolated filled pink circles). The moths that showed a split ecdysis behaviour (separate open and filled pink circles) appeared to finish ecdysis approximately 30–60 min earlier than the controls.

Once the entire cuticle had been shed, all the moths perched and inflated their wings in a manner similar to controls (pink and black crosses). For example, the time from complete shedding of the cuticle until the completion of wing expansion and rotation of wings to the 'moth' position (Truman and Endo, 1974) was 94±2 min (N=31) for controls and 93±1 min (N=24) for animals treated with CO_2 at 9:30 h. For the subgroup of CO_2-treated animals that showed premature peristalsis behaviour, the time was 97±4 min (N=12) between the final

completion of ecdysis and the termination of wing spreading. These values were not significantly different (P>0.5).

Discussion

Ventrolateral neurons and the ecdysis response

The ability of an insect to shed its cuticle successfully at the end of a moult relies on close coordination between behaviour and physiology. From studies on *Manduca sexta* and *Drosophila melanogaster*, it appears that this coordination is maintained by the interplay between a number of hormones and neurohormones. Most attention has been given to the peripheral Inka cells, the EH neurons and a group of 50 CCAP-positive neurons that are apparently activated at the time of ecdysis. The peptides from these cells often have physiological as well as behavioural actions. For instance, EH has been shown to trigger tracheal air-filling during ecdysis in *Drosophila melanogaster* (Baker et al., 1999), and CCAP has actions on the heart as well as on ecdysis motoneurons (Gammie and Truman, 1999; Tublitz and Truman, 1985). Other peptides are likely to be involved in coordinating this complex behavioural and physiological program. For example, Zitnan and Adams (2000) described increases in cGMP-IR in ventrolateral neurons of the abdominal ganglia of *Manduca sexta* during ecdysis. We have quantified these increases to show that they are coincident with the onset of ecdysis (Fig. 1). These cells may have a conserved function during ecdysis since their cGMP-positive homologues also appear during ecdysis in grasshoppers (Truman et al., 1996).

Double-labeling experiments (Fig. 2B,C) show that these lateral neurons are not the diuretic hormone/leucokinin-containing $L_{3,4}$ cells (Chen et al., 1994), as was suggested recently (Zitnan and Adams, 2000). This is not surprising, because the paired lateral leucokinin-containing cells are not detected in the first or second abdominal ganglia (Chen et al., 1994), and it is in these ganglia that cGMP-IR is strongest in these ventrolateral neurons. This and the fact that these neurons project ipsilaterally through the ventral nerves (Fig. 2A, arrowhead) suggest that they are lateral neurons L_2 and L_5 instead, based on the nomenclature of Davis et al. (1993). In *Manduca sexta* larvae, cells in this lateral cluster contain cardioacceleratory peptides (other than CCAP) (Tublitz and Sylwester, 1990), but during metamorphosis they change their peptide content to bursicon (Taghert and Truman, 1982). While such cardioacceleratory peptides and bursicon have been attributed to this cluster (Tublitz and Sylwester, 1990; Taghert and Truman, 1982), immunological confirmation of the peptide content of the L_2 and L_5 cells is still required. The time of appearance of cGMP in these cells, at the time of ecdysis, suggests that they may play a role in the postecdysial phase of the behavioural sequence.

A role for FMRFamide-related peptides during ecdysis has been suggested on the basis of immunohistochemical patterns of expression (Miao et al., 1998). However, on the basis of cGMP-IR, cells that contain FMRFamide-related peptides appear not to be direct targets of EH. These peptides are not found in the cell 27/704 group nor did we find them in the $L_{2,5}$ cells (Fig. 2B). A set of thoracic neurons show changes in expression levels of FMRFamide-related peptides before and after ecdysis (Miao et al., 1998), but these are not among the cells that show cGMP-IR during ecdysis (data not shown). Hence, activation of FMRFamide-positive neurons may be caused by ETH or CCAP, but most probably not directly by EH.

The role of descending inhibition in the timing of ecdysis onset

A model for the interactions between the hormones regulating the ecdysis sequence in insects suggests that peptides from peripheral glands and from the central nervous system interact to trigger the pre-ecdysis and ecdysis behaviours (e.g. Gammie and Truman, 1999). Declining ecdysteroid titres initiate the release of various peptides from the epitracheal glands. These peptides activate pre-ecdysis behaviours. One of these peptides, ETH, also promotes the release of EH from the brain (Ewer et al., 1997; Gammie and Truman, 1999). EH stimulates the CCAP-containing neurons, cells 27 and 704, in the ventral nerve cord *via* an increase in cGMP levels (Ewer et al., 1994; Gammie and Truman, 1997a). It may also stimulate the L_2 and L_5 neurons *via* cGMP. As discussed above, these cells may contain cardioacceleratory peptides or bursicon (Tublitz and Sylwester, 1990), but their role in the ecdysis sequence is unclear. CCAP from cells 27 and 704 then releases the ecdysis motor program (Gammie and Truman, 1999). The onset of the ecdysis phase, however, involves an interplay between excitatory and inhibitory influences (Baker et al., 1999; Ewer and Truman, 1997; Gammie and Truman, 1997b; Zitnan and Adams, 2000). It is clear that the brain is required for the onset of the ecdysis behaviour itself (Novicki and Weeks, 1996), but this requirement ends with the release of EH (Ewer et al., 1997; Novicki and Weeks, 1996). Inhibition, however, does not require the brain, but rather the lower ganglia (Fig. 4). The sites of inhibition include the SOG and one or more of the thoracic ganglia. This is the case for both larvae and pupae (Fig. 4) and is probably also the case for adults (Ewer and Truman, 1997; Mesce and Truman, 1988). It will be interesting to determine whether this is a common feature of ecdysis in insects in general since inhibitory factors from the head that affect ecdysis behaviours have also been noted in Diptera (Baker et al., 1999) and Orthoptera (Carlson, 1977).

There are two options for the regulation of inhibition during ecdysis: (i) EH may co-activate the CCAP cells and the descending inhibitory cells or (ii) suppression may be tonic, as is seen for behaviours such as walking (Roeder, 1967), and thereby be independent of control by EH. While this issue is not easily resolved, we favour the first option. In *Drosophila melanogaster*, mutants lacking EH-positive neurons (EH cell knockouts) show no signs of this descending inhibition, while the controls show the same pattern of descending inhibition seen in *Manduca sexta* (Baker et al., 1999). Inclusion of the descending inhibitory neurons among the EH target cells is

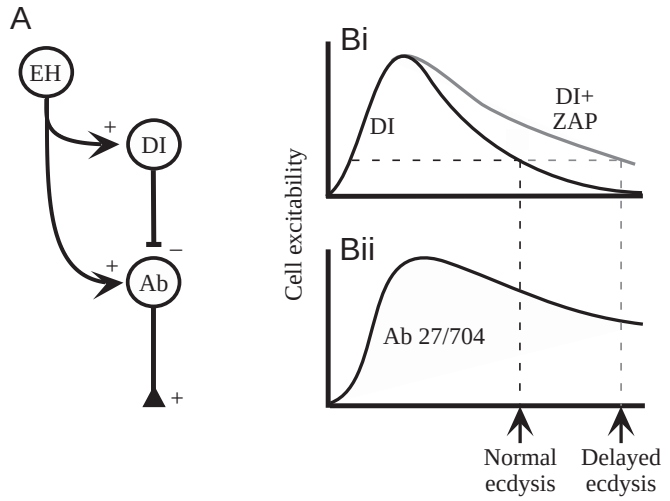


Fig. 10. Schematic diagram illustrating the interactions between inhibitors and activators of ecdysis behaviours and the role of cGMP levels in neurons regulating these behaviours. (A) Diagram of eclosion hormone (EH) neurons activating (+) both descending inhibitors (DI) from the suboesophageal ganglion or thoracic ganglia and crustacean cardioactive peptide (CCAP)-containing abdominal neurons (Ab). (B) Representational plots of cell excitability of the DI (Bi) and abdominal 27/704 neurons (Ab27/704) (Bii). The excitability of DI neurons declines below threshold (black dashed line) sooner than that of the abdominal 27/704 neurons. Thus, inhibition is lifted and CCAP is released from the abdominal 27/704 neurons. If a phosphodiesterase inhibitor (ZAP) is present (gray curve), cGMP levels are elevated and cell excitability stays high for longer (gray dashed line). Release of CCAP, and subsequently the onset of the ecdysis motor pattern, is therefore delayed.

also consistent with the anatomy of some of these neurons and with the dynamics of cGMP seen in these cells. A prominent feature of the cell 27/704 system is the paired 704 cells in the labial neuromere of the SOG and in each of the thoracic ganglia. These neurons have axon collaterals that descend into the abdomen and make apparent contact with the abdominal 27/704 neurons. Interestingly, these neurons show an increase in cGMP levels coincident with that of the abdominal 27/704 cells, but their cGMP levels decline more rapidly in the descending processes compared with the abdominal neurons (Ewer et al., 1994). The rise in intracellular cGMP levels causes an increase in the excitability of EH target cells (Gammie and Truman, 1997a). Thus, the rapid rise in cGMP levels in the abdominal 27/704 cells, and in their putative inhibitors, would cause an activation of the network, but CCAP release from the abdominal neurons would be held in check by the activity of the descending inhibitors. The early decline in cGMP levels in the inhibitors would then result in the waning of the suppression, with the eventual 'escape' of the abdominal neurons, leading to the release of CCAP and, hence, ecdysis (Fig. 10). This hypothesis is consistent with the results of experiments blocking cGMP breakdown using a specific cGMP phosphodiesterase inhibitor, Zaprinast (Fig. 6). Addition of Zaprinast caused a prolonged latency to the onset of ecdysis, presumably by prolonging the time that the descending neurons continued to be active because of their elevated cGMP levels.

The increase in cell excitability elicited by cGMP in descending inhibitory neurons, as well as in the abdominal CCAP neurons, is a simplistic model that assumes that increases in firing rates of each neuron alone provide the inhibition noted in *Manduca sexta* ecdysis. However, it does generate predictions that can be tested experimentally in future studies.

One or more of the thoracic ganglia form a second source of inhibition modulating the onset of ecdysis. The thoracic ganglia also appear to be necessary for maintaining the ecdysis motor program once it has commenced (Fig. 5). They have already been shown to play a large role in modulating eclosion in adults (Mesce and Truman, 1988) and they are the source of neuronal inputs, which modulate the conserved larval abdominal motor program to produce the adult motor pattern used for shedding the rigid pupal cuticle and for digging through the soil. Whether they are also the source of inhibition modulating the timing of ecdysis in adults is not yet known.

CO_2 and release of the ecdysis behaviour

CO_2 provides a powerful tool for studying ecdysis (Fig. 7). Treatment of animals with a short pulse of CO_2 shortly before the onset of ecdysis results in its premature onset. This response is conserved in larvae (Fig. 7), pupae (data not shown) and in adults (Fig. 9). The response does not appear to be a result of anoxia since treatment with nitrogen gas (Fig. 7A) or cold (data not shown) has no effect on larvae.

CO_2 does not affect the timing of ecdysis if it is applied prior to EH release, when cGMP is not yet detectable in the CCAP neurons, suggesting that CO_2 does not act directly on (i) the ecdysis motoneurons, (ii) the muscles involved in ecdysis or (iii) the as yet inactive CCAP neurons. CO_2 treatment becomes effective only after EH release. In the context of the EH target cells, CO_2 may act directly to further excite EH-activated CCAP cells or it may act to block the descending inhibition onto these cells. We cannot yet distinguish experimentally between these two possibilities. However, once the CO_2 has acted, the system cannot revert to its 'waiting' mode.

Unlike simple anoxia, CO_2 can act as an anesthetic in insects by blocking synapses (Clark and Eaton, 1983; Perron et al., 1972; Sillans and Biston, 1979). Since insects go through a transient hyperexcitable phase before they become anesthetized, it may be that inhibitory circuits are more sensitive to CO_2 than are excitatory ones. CO_2 has been shown to inhibit cercal grooming in cockroaches (Eaton and Farley, 1969), but also specifically reduces the efficacy of γ-aminobutyric acid (GABA) activity in inhibitory neurons of honeybees (Kashin, 1973). We favour the hypothesis that the mechanism for CO_2 action on the central nervous system of larval and pupal *Manduca sexta* during ecdysis is probably through releasing the inhibition provided by the SOG and thoracic ganglia.

The eclosion behaviour of the adult is strikingly different from that of larvae and pupae because adults need to shed a rigid sheath of pupal cuticle and to dig out from their underground pupation cell. This is accomplished through two-phase, retraction/extension movements of the abdomen in which the extension phase is coupled with 'shrugging' movements at the wing base (Kammer and Kinnamon, 1977; Mesce and Truman, 1988). Although the adult behaviour is markedly different from the larval and pupal behaviours, it is, nevertheless, based on the larval ecdysis central pattern generator (Mesce and Truman, 1988). This was shown by nerve cord transection or by using a cold block to uncouple the abdomen from descending units from the thorax. In the absence of the thoracic input, the abdominal extension phase of the cycle immediately disappeared and the retraction phase transformed into the characteristic larval peristaltic wave. In the animals that showed only abdominal movements after the CO_2 treatment (Fig. 8), the movements were similar to those seen when the descending thoracic units are blocked. That is, they were primarily peristaltic in character. These movements did not, however, persist with any regularity in CO_2-treated animals.

In pharate adults that have released EH, decapitation results in the rapid onset of a normal adult ecdysis behaviour that includes both the thoracic and abdominal components (Ewer and Truman, 1997). CO_2 treatment also triggers premature ecdysis and, as in larvae and pupae, it appears to do so only in those that have already released EH (Fig. 9B–D). The CO_2 effects differ from decapitation, however, in that often only the abdominal component is displayed immediately after treatment. After a lag, CO_2-treated animals eventually activate thoracic components that allow them to shed their entire pupal cuticle, and this also appears to be early relative to controls (Fig. 9C–E). These differences suggest that the larval- and adult-specific components of ecdysis are under inhibitory control, but that each is under a distinct inhibitory pathway. The abdominal component of adult ecdysis shows the same type of CO_2 sensitivity as seen in larvae and pupae. Thus, as in the earlier stages, CO_2 appears to relieve descending inhibition that controls the activation of the persisting larval ecdysis pattern generator. A separate set of descending units may regulate the adult-specific portions of the behaviour that include the thoracic flexions and coupled abdominal extensions, but these seem not to be readily activated by CO_2 exposure. The thoracic portion of the behaviour eventually appears with time, and the entire adult behaviour is typically expressed somewhat earlier than in controls. Thus, the adult form of the behaviour appears to include not only the larval ecdysis pattern generator but also some of the neurons that control the timing of its expression. At metamorphosis, the animal appears to add additional motor components and also additional regulatory circuitry to produce the final adult behaviour.

Although CO_2 treatment has a marked effect on the timing of ecdysis, it does not appear to affect other aspects of the ecdysis sequence, such as the release of EH itself. Also, the timing of the post-eclosion event, wing expansion, remains undisturbed relative to the time of ecdysis. CO_2 treatment may, therefore, turn out to be a non-invasive and far more useful tool than decapitation for studying the inhibitory influences on ecdysis. Moreover, multiple components of eclosion may be uncoupled in adults by this means.

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