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Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency

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SUMMARY

At the end of each developmental stage, insects perform a stereotypic behavioral sequence leading to ecdysis of the old cuticle. While ecdysis-triggering hormone (ETH) is sufficient to trigger this sequence, it has remained unclear whether it is required. We show that deletion of *eth*, the gene encoding ETH in *Drosophila*, leads to lethal behavioral and physiological deficits. Null mutants (*eth*$^{-}$) fail to inflate the new respiratory system on schedule, do not perform the ecdysis behavioral sequence, and exhibit the phenotype *buttoned-up*, which is characterized by incomplete ecdysis and 98% mortality at the transition from first to second larval instar. Precisely timed injection of synthetic DmETH1 restores all deficits and allows normal ecdysis to occur. These findings establish obligatory roles for *eth* and its gene products in initiation and regulation of the ecdysis sequence. The ETH signaling system provides an opportunity for genetic analysis of a chemically coded physiological and behavioral sequence.

Key words: Ecdysis, *Drosophila*, Behavioral sequence, Transgene, Ecdysis-triggering hormone

INTRODUCTION

Insect development proceeds through a series of stages from egg to reproductive adult, each punctuated by ecdysis or shedding of the old cuticle. Initiation of the molt, which culminates in ecdysis, coincides with one or more bouts of ecdysteroid-induced gene expression appropriate to the next stage. The end of the molt is signaled by declining steroid levels, leading to ecdysis of the old exoskeleton surrounding the body and lining the respiratory system and gut.

Ecdysis is controlled by a genetic program specifying a precisely timed developmental sequence. Among the genes involved are those encoding peptide hormones that activate central pattern generators for pre-ecdysis and ecdysis behaviors (Truman, 1992; Zitnan et al., 1999). A foundation for studies of ecdysis behavior was provided by Truman and colleagues, who discovered that ecdysis in Saturniid moths is triggered by a peptide factor from the brain (Truman and Riddiford, 1970), later identified as eclosion hormone (EH) (Truman, 1992). Recently, we showed that EH causes secretion of ecdysis-triggering hormones (ETHs) from endocrine Inka cells (Zitnan et al., 1996; Kingan et al., 1997), which act on the CNS to trigger centrally patterned pre-ecdysis and ecdysis behaviors.

As direct gene products, many peptide signaling molecules provide a link between gene expression and behavior. Orchestration of the ecdysis behavioral sequence depends on coordinated induction and suppression of genes essential to its properly timed initiation. This includes induction of ETH gene expression in Inka cells and CNS sensitivity to ETH 1-2 days before ecdysis (Zitnan et al., 1999; Zitnan and Adams, 2000). The timing of ecdysis initiation is accomplished by ecdysteroid suppression of secretory competence in Inka cells until the animal is ready to perform the behavioral sequence and escape the old cuticle (Kingan and Adams, 2000). Once ecdysteroids drop to low levels during the hours preceding ecdysis, peptide hormones, including ETHs, eclosion hormone and CCAP, are released to initiate the behavioral sequence (Zitnan et al., 1996; Ewer et al., 1997; Gammie and Truman, 1997; Zitnan et al., 1999). Orchestration of ecdysis behavior therefore depends on coordinated expression of genes that are involved in peptide signaling.

Each of these peptides is capable of initiating ecdysis, but it has remained unclear to what extent they play obligatory roles. To test the hypothesis that ETH is required for ecdysis, we sought to use genetic tools in *Drosophila* to delete its gene, *eth* (Park et al., 1999). We describe genetic null *Drosophila* mutants carrying micro-deletions in the *eth* locus. The consequences of this selective hormonal deficit are severe, and include failure both to inflate the new respiratory system and to perform the ecdysis behavioral sequence. Virtually all animals fail to survive the first ecdysis. These findings establish an obligatory role for *eth* and its gene products, and demonstrate how lack of a specific chemical signal results in a lethal behavioral deficit.

MATERIALS AND METHODS

Drosophila stocks and *eth* mutants

To generate *eth* deletion mutants, we used the *EP(2)1065* line carrying a P-element located at the 5′ end of *reg-5*, 'rhythmically expressed gene' (McNeil et al., 1998), 1427 bp down stream of the end of *eth* polyadenylation site (see Fig. 4A). Imprecise excision lines induced by transposase activity in the Δ*2-3* line (*EP(2)1065/CyO; SbΔ2-3/+*) were screened by polymerase chain reaction (PCR). Primer pairs for the PCR screen were ETHupF1 (5′- CAATCTCCCCACATCACAGC) located within *orc4* (~4 kb upstream of *eth*) and ETHpR3 (5′-CAGGCGTCTATTTATGCCAAG) occurring ~1 kb downstream of the P-element insertion. Genomic DNA was isolated from a single fly according to Sullivan et al. (Sullivan et al., 2000) with slight modifications. PCR was performed using genomic DNA from either single individuals or two flies pooled. Reactions, 20 μl, contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM each primers, 2.5 units Taq polymerase (Gibco), and 20 mM Tris-HCl, pH 8.4. The reaction mixture was denatured initially for 5 minutes at 94°C, then subjected to 40 cycles of 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes, with a final 72°C extension for 15 minutes.

Approximately 400 excision lines were screened, yielding three relevant deletions as eth^{27}, eth^{25b} and eth^{196}. Deletions were verified by sequencing the PCR product or a TA clone of the product (Invitrogen) using an Applied Biosystems model 377 sequencer. A GFP balancer for the second chromosome (*CyO, P{w*$^{+mC}$*=ActGFP}JMR1*) was used to distinguish homozygous from heterozygous mutants. CantonS was used as a control line in all experiments.

The transgene *eth3-egfp* was constructed in a *pCaSpeR4* vector kindly provided by Dr Carl Thummel (Thummel and Pirrotta, 1991). The *eth3-egfp* includes 382 bp of sequence immediately upstream of the *eth* open reading frame. This sequence containing a putative ecdysteroid response element (see Fig. 4C) extends to the 13 bp after the stop codon of 5′ upstream gene *orc4* (GenBank Accession Number AF139063) (Chesnokov et al., 1999). The construct was designed for a fusion protein; *egfp* sequence starts right after the third putative amidation site –GRR in the *eth* (see Fig. 4C). Standard procedures for the egg injection and crossings were used for the transformation (Ashburner, 1989). A transformed line expressing *egfp* was obtained. Inverse PCR determined an insertion of the transgene in the 2nd chromosome 58F7, intergenic space between CG11605 and CG3550.

Observation of ecdysis behavior and injection of ETH peptides

Eggs were collected overnight on apple-juice agar plates supplemented with a few granules of yeast. Observations were made under a stereomicroscope after transfer of larvae to a fresh apple-juice agar plate or a yeast-agar plate. Physiology and behaviors prior to ecdysis were recorded on videotape using a Sony CCD camera. The videotape was analyzed with the software program Observer (Noldus) when necessary. In some experiments, living larvae were positioned in a drop of tap water between a slide glass and a cover glass and observed with a compound microscope.

Injections of DmETH1 into staged animals immobilized on sticky tape were made using a microelectrode needle (~2 μm tip opening) attached to a Picospritzer. The needle was inserted laterally through the region of abdominal segment 6 and 7 from back. Injection volumes were determined following calibration of drops under paraffin oil, and adjusted to ~10 pl. Injected larvae were transferred immediately to an agar plate with a wet brush.

Microscopy and immunohistochemistry

We raised rabbit antisera against the C terminus of DrmETH1, which contains a –PRXamide motif. Peptides were conjugated to KLH using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (Hermanson, 1995). An antiserum raised against *Manduca* PETH (MasPETH) was previously described (Zitnan et al., 1999).

For whole-mount CNS or trachea staining, the tissues were dissected in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4) and fixed in 4% paraformaldehyde overnight at 4°C or ~2 hours at room temperature. After two 15 minute washes with PBST (1% Triton X-100 in phosphate-buffered saline (PBS)) and 1 hour blocking with 5% normal goat serum in PBST, the tissues were incubated with primary antisera (1:500 dilution for anti-DrmETH1 and anti-DrmETH2 or 1:2,000 for anti-MasPETH) for 2 days. After a wash with PBST (4 times for 15 minutes), tissue was incubated for 24 hours in Cy-3-labeled goat anti-rabbit secondary antibody (1:500, Jackson). The tissue was mounted on a slide glass in 90% glycerol after four 15 minute washes with PBST. DAPI staining was at 5 ng/ml in PBST for 5 minutes. Colocalization of ETH and EGFP in the *2eth3egfp* transgenic line (see Fig. 3A) was determined by confocal microscopy (Zeiss). We used an antiserum against ETH (described as above), a mouse monoclonal anti-GFP against EGFP (Clontech, Palo Alto, California), and a goat anti-mouse IgG (H+L) conjugate (Alexa Fluor 488) that was highly cross-absorbed (Molecular Probes, Eugene, Oregon).

The amount of EGFP emission is considered to be proportional to the amount of ETHs in the transgene with the fusion protein *eth3-egfp*. Quantification of EGFP emission was performed with individual endocrine cells. Trachea were removed from each staged animal in PBS and mounted in 20% glycerol in PBS. Image capture was performed immediately after dissection with a 40× objective in Zeiss microscope using a cooled CCD camera (TILL Photonics) using a 1 second exposure, 480×640 pixel number, leveling 200 to 1500. This condition was chosen for non-saturation of individual pixels at maximum emission of the EGFP in preliminary experiments (Piston, 1999). A minimum of five cells from each individual, five to nine individuals at each stage, was used for the data collection (Fig. 5).

RESULTS

A stereotypic behavioral sequence precedes larval ecdysis in *Drosophila*

Larval ecdysis in *Drosophila* culminates a sequence of precisely timed morphological, physiological and behavioral events. These include appearance of new mouthparts, tracheal inflation, and pre-ecdysis and ecdysis behaviors. We provide a detailed time line of these events, which occur during the 1 hour interval preceding ecdysis to the 2nd larval instar.

Visible morphological changes become apparent ~1 hr prior to ecdysis, including the appearance of new mouth hooks and vertical plates (Fig. 1A) (Jürgens and Hartenstein, 1993). The first event is sclerotization of new mouth hooks adjacent to the old structures, referred to 'double mouth hooks'. About 30 minutes later, new vertical plates appear, leading to 'double vertical plates' (dVP). We chose dVP as a reference point (time zero) to which all other events are related.

Before the appearance of dVP, the old tracheal lining or 'intima' in the main dorsal tracheal trunk separates from the new intima. About 10 minutes after the dVP stage (Table 1) the old intima collapse, becoming coiled in appearance (Fig. 1A). Tracheal collapse is followed immediately by inflation of the new trachea with air, which takes ~2-5 minutes (Fig. 1A). Before this time, the space between the old and new intima is filled with liquid, presumably molting fluid.

Pre-ecdysis behaviors commence upon completion of tracheal air filling (Fig. 1A). The first stage of pre-ecdysis consists of repetitive 'anteroposterior' contractions (A-P) beginning 15 minutes after dVP. This behavior is characterized by alternating telescopic contractions of 2-5 seconds duration

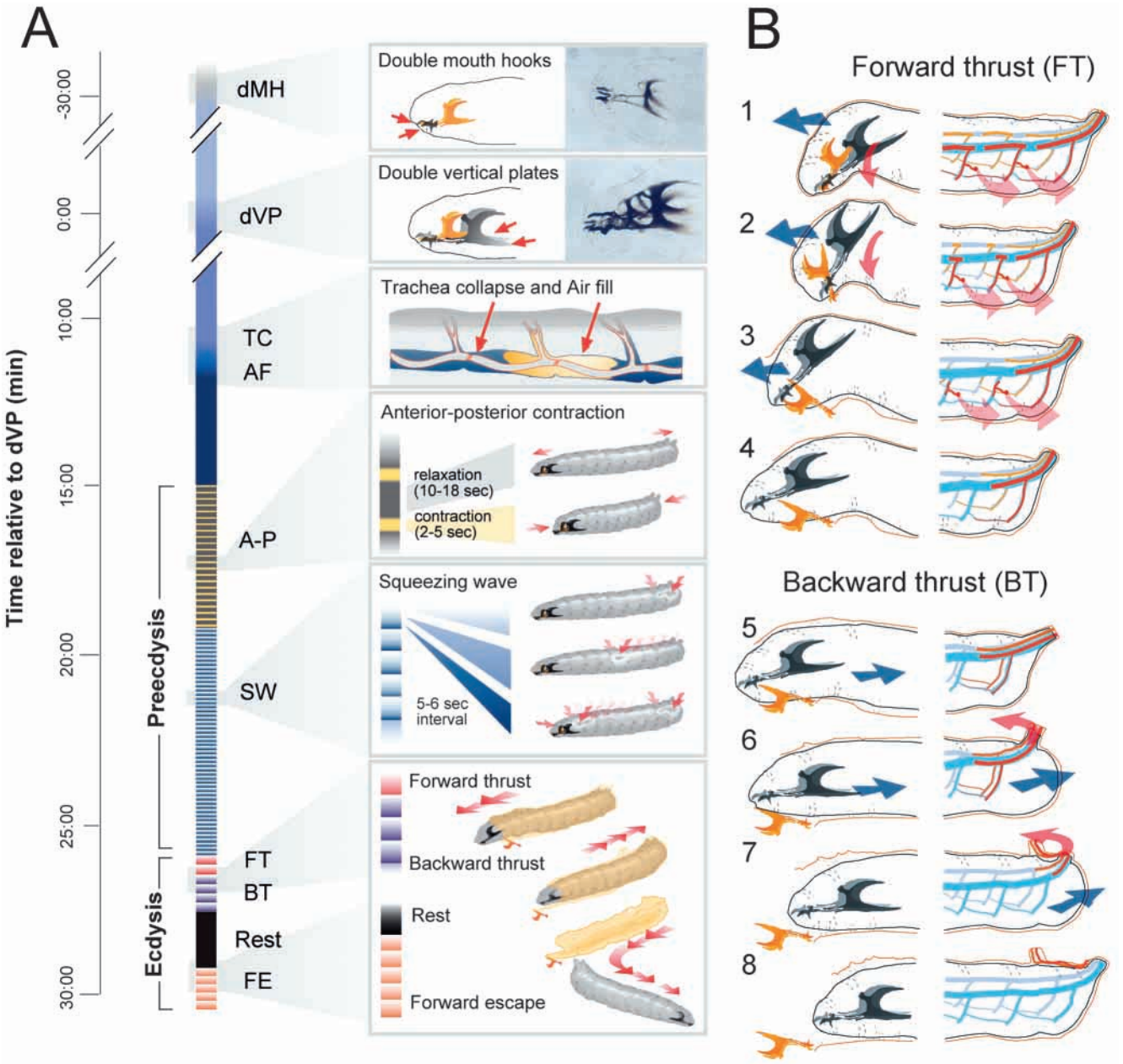

Fig. 1. Timeline depicting the morphological, physiological and behavioral changes preceding ecdysis to the second larval instar in *Drosophila*. (A) All time points are relative to the double vertical plates (dVP) stage as shown. Broken bars (e.g. A-P) indicate periodically repeated contractions in each stage. Bars depicting a gradient of color indicate gradual changes in morphology. (B) Shedding of the mouthparts and old tracheal tubes (red) during ecdysis. Left: the forward thrust (FT) phase of ecdysis results in deposition of the old mouth hooks and vertical plates onto the substrate (forward blue arrow) and removal of the old tracheal tubes, which are pulled out through spiracular pits, functional only during ecdysis. Right: during the backward thrust (BT) phase of ecdysis, the old spiracles are detached and slide forward (curved red arrow) a maneuver that also extracts the old tracheal trunks (red).

and relaxations lasting 10-18 seconds. The second stage of pre-ecdysis beginning at 19 minutes involves rolling contractions called ‘squeezing waves’. Squeezing waves are visible from the dorsal aspect and travel from posterior to anterior at 5-6 second intervals, ending with head retractions (Fig. 1A). During the A-P and SW behaviors, vigorous muscle contractions pull the mouthparts alternately in the posterior and anterior directions. We believe that these movements may be crucial for later detachment of old vertical plates and mouthhooks from the new apparatus during subsequent ecdysis behavior.

Ecdysis behavior begins 25 minutes after dVP, with one or two forward head thrust movements, which detaches old mouthparts and plants them in the substrate (Fig. 1B). The forward movement also coincides with shedding of old tracheal linings through segmental spiracular pits (Fig. 1B) (Keilin, 1944). Upon planting the old mouthparts onto the substrate, the forward thrust is followed by three to five vigorous backward thrusts to detach the old posterior spiracles (Fig. 1A,B). The behavior is interrupted by a 2-5 minute rest period, and is completed by a forward, lateral turning escape movement,

freeing the animal from the old cuticle. Some variation in this pattern was observed, in which subunits of the ecdysis behavior were repeated or even entire recapitulations of the behavioral sequence were observed.

The patterned behavior just described is also accompanied by some irregular behaviors prior to anterior-posterior contractions (A-P) with large variations between individuals and time of onset. These are (1) swinging head, (2) dorso-ventral contractions and (3) alternating anterior and posterior peristaltic squeezing.

Ecdysis from second to third instar follows a similar pattern. Even the time of behavioral onset is similar, with the exception that double mouth hooks appear at –104 minutes relative to dVP, (Table 1, n=7), instead of –30 minutes as observed in the 1st to 2nd instar ecdysis (n=8, P<0.01; Student's t-test).

ETH-IR is expressed in Inka cells and disappears at ecdysis

Immunohistochemical staining using antisera raised against DrmETH1 revealed segmentally repeated cells in both larval and adult stages (Fig. 2A-C,E). These cells appear to be homologous to 'Inka cells' previously identified in *M. sexta* (Zitnan et al., 1996), and henceforth are referred to by the same name. We observed an identical staining pattern using an antiserum raised against the C-terminus of the *M. sexta* peptide, MasPETH (Zitnan et al., 1999) (Fig. 2I,J). In larvae, cells exhibiting ETH-like immunoreactivity (ETH-IR) occur along each of the two dorsal tracheal trunks at the main branch points of transverse connectives (Fig. 2) (Manning and Krasnow, 1993). A total of seven Inka cell pairs occur consistently in each tracheal metamere Tr1 and Tr4 through Tr9 (Manning and Krasnow, 1993) in the larval stage. In adults, cells showing ETH-IR also occur at homologous positions (Fig. 5B,E,F,J), but vary in shape and location. Depletion of ETH-IR is observed at each larval ecdysis.

Antisera to DrmETH1 and MasETH also stained ~20 neurons and axons in the CNS (data not shown). Staining in the CNS presumably results from cross-reactivity with neuropeptides containing the conserved C-terminal sequence motif –PRXamide, which is shared by ETHs (DrmETH1, DDSSPGFFLKITKNVPRLa; DrmETH2, GENFAIKNLK-TIPRIa; MasPETH, SFIKPNNVPRVa; MasETH, SNEA-ISPFDQGMMGYVIKTNKNIPRMa), the cardioactive peptide CAP2b, pheromonotropic and diapause hormones in moths (Gäde et al., 1997), and the *Drosophila* neuropeptides CG15520 and CG6371 (Adams et al., 2000).

Specific expression of the chimeric transgene *2eth3-egfp* in Inka cells

To examine the cellular expression pattern of *eth* (Park et al., 1999), we constructed a fly line carrying the chimeric transgene *2eth3-egfp*. This transgene occurs on the 2nd chromosome and contains the sequence of *eth* up to the 3rd amidation site with chimeric *egfp* encoding the enhanced green fluorescent protein (see Fig. 4D). EGFP fluorescence in *2eth3-egfp* flies is observed in both larval and adult stages, but is confined to the constellation of Inka cells showing ETH-IR (Fig. 2, Fig. 3). No EGFP fluorescence occurs in any other cell or tissue in larvae or adults. These data are consistent with cell-specific expression of *eth*. Observations under laser confocal microscopy revealed an identical distribution of EGFP fluorescence and ETH-IR in Inka cells of wandering 3rd instar (Fig. 3A), suggesting that EGFP and processed ETHs are located in the same subcellular compartments.

In 1st instar larvae, peak EGFP fluorescence occurs at dVP, and declines sharply to 16±3% of peak emission just before tracheal inflation (Fig. 3B). A further drop of EGFP emission to 11±3% occurs by the squeezing wave stage. Loss of EGFP fluorescence suggests that ETH is released naturally *in vivo* during the time interval between dVP and tracheal collapse.

Injection of ETHs induces premature tracheal inflation and ecdysis-related behaviors

The peptides DrmETH1 and DrmETH2, which trigger adult ecdysis (Park et al., 1999), were injected into late first instar larvae to assess their ability to induce ecdysis at this stage of development. Injection of DrmETH1 (0.01 to 10 fmol) at the double mouth hooks stage induced the premature appearance of physiological and behavioral events outlined in Fig. 1. The first response to peptide injection was premature tracheal

Table 1. Morphological changes and patterned behaviors at ecdysis of *Drosophila*

Abbreviation	Description	Time relative to dVP (minutes) (mean±s.d.)* 1st to 2nd	2nd to 3rd
Morphological changes			
dMH	Double mouth hooks indicating appearance of new mouth hooks on the top of old mouth hooks	–30±7	–81±37
dVP	Double vertical plates indicating appearance of entire new mouthparts	0	0
Trachea dynamics			
TC	Trachea collapse in old intima	10±5	15±6
AF	Air filling the space between old and new intima	12±2	17±2
Pre-ecdysis behavior			
A-P	Anterior-posterior contractions	15±2	19±2
SW	Squeezing wave of dorsoventral contraction from back segments to forward	19±3	23±2
Ecdysis behavior			
FT	Forward thrust shading old mouth hook	26±3	30±3
BT	Backward thrust shading old posterior spiracles	27	31
FE	Forward escape completing the ecdysis	29±2	35±2

*n=9 and n=7 for 1st to 2nd and 2nd to 3rd, respectively.

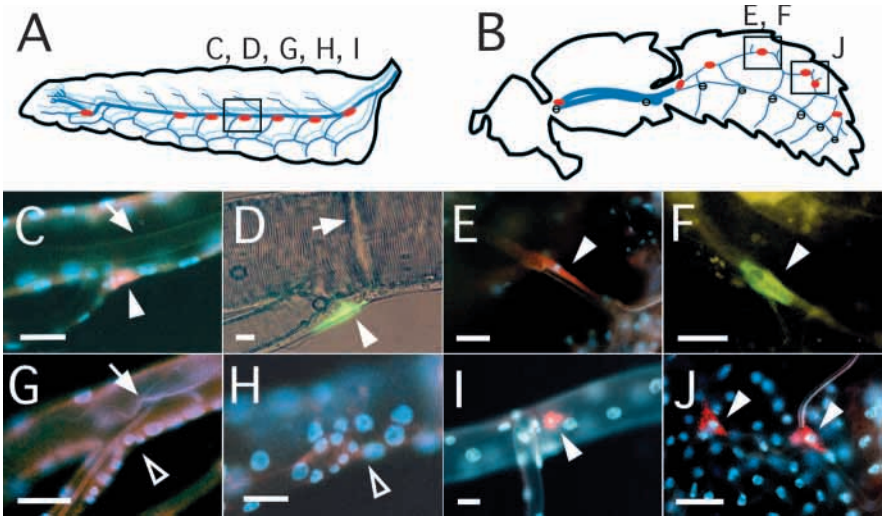

Fig. 2. Location of Inka cells shown by ETH immunohistochemistry and EGFP expression in the *2eth3egfp* transgenic fly line. Red, blue and green colors are for Cy3, DAPI and EGFP, respectively. Scale bars: (C-J) 10 μm. (A) The larval tracheal system and positions of Inka cells (red). (B) Adult tracheal system and positions of the Inka cells (red). Letters correspond to boxed areas in the diagram shown in C-J. (C) Cy3 staining in the late 1st instar using the DrmETH1 antibody. Old intima (arrow) is already separated from new intima. The Inka cell (arrowhead) is located along the main dorsal tracheal trunk at each branchpoint of the transverse connectives. (D) Expression of *eth3-egfp* transgene in late 3rd instar. The cell expressing EGFP (arrowhead) at a node (arrow, see Fig. 1B) is shown with low intensity transmitted light. (E) Cy3 staining (arrowhead) in the adult stage using the DrmETH1 antibody. (F) Expression of the *eth3-egfp* transgene (arrowhead) in the adult stage. (G) The *eth* deletion mutant eth^{25b} Cy3 (late 1st instar) shows no immunohistochemical staining of the Inka cell. The old intima (arrow) is separated from new intima. Open arrowhead indicates location of the epitracheal gland in wild-type flies. (H) Depletion of ETH immunoreactivity in the Inka cell (arrowhead) of wild-type flies (open arrowhead) immediately after ecdysis to early 2nd instar. (I) Immunoreactive Inka cell in the late 3rd instar using an antibody against MasPETH. (J) Inka cell immunoreactivity (arrowheads) in the adult stage using the MasPETH antiserum.

collapse and inflation, with a latency of 3 minutes and 4 minutes, respectively, followed by pre-ecdysis and ecdysis behaviors. Weak anterior-posterior contractions were observed in 33% of injected animal (six out of 18). Strong squeezing waves occurred in all injected animals, with an average latency of 14±2 minutes. Ecdysis behavior consisting of forward thrust, backward thrust and turning escape movements appeared with a latency of 24±3 minutes (Fig. 5).

The timing of DrmETH1 injection was critical for successful ecdysis. All animals (*n*=10) injected at the dVP stage underwent successful ecdysis. However injections performed earlier, for example at double mouth hooks, induced tracheal collapse and inflation, anterior-posterior contractions, squeezing waves and repeated bouts of ecdysis behavior that were unsuccessful in shedding the mouthparts, leading ultimately to death (Fig. 5). These observations indicate that precise timing of events in the ecdysis sequence is critical for successful ecdysis.

DrmETH2 injections were less effective in eliciting tracheal dynamics and behaviors. At relatively high doses (≥10 fmol), DrmETH2 induced tracheal collapse and inflation with a latency of 3 minutes and 4 minutes, respectively, and ecdysis behaviors with a latency of 34±5 minutes (*n*=12, Fig. 5). Strikingly, DrmETH2 (10 fmol) elicits neither anterior-posterior contractions nor squeezing waves. Lower doses of DrmETH2 (1 fmol) induced tracheal collapse and inflation with latencies of 4 minutes and 5 minutes, but had no behavioral effects (*n*=12).

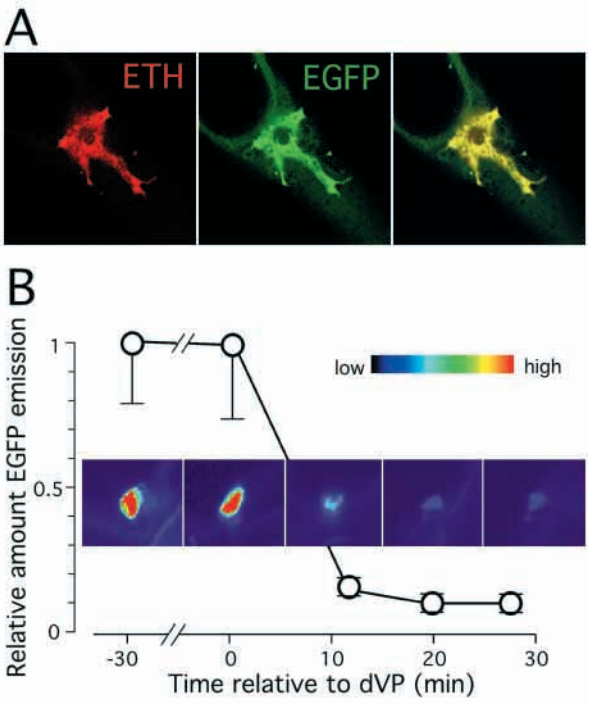

Fig. 3. EGFP co-localized in the Inka cell is depleted during ecdysis to the 2nd instar. (A) Colocalization of ETH and EGFP in an Inka cell in a wandering 3rd instar larva viewed under confocal optics. (B) Depletion of EGFP in a *2eth3egfp* transgenic *Drosophila* Inka cell during ecdysis to the 2nd instar. Sample images of the Inka cell are shown at each time point. Error bars indicate s.e.m. of five to nine individuals for each stage, and more than five cells for each individual.

Excision of *eth* leads to respiratory and behavioral deficits and lethality

To test whether ETH is required for ecdysis, gene deletions were generated by imprecise excisions of EP(2)1065 (Rorth, 1996), a P-element located 1427 bp downstream of the *eth* polyA site (Fig. 4A). These efforts led to creation of three deletion lines, eth^{27}, eth^{25b} and eth^{196}, all of which possess small excisions near or including *eth* (Fig. 4B). The eth^{27} line has a deletion from the P-element site in the 5′ untranslated region of *reg-5* (rhythmically expressed gene 5) (McNeil et al., 1998) up to the 3′ untranslated region of *eth*, 22 bp downstream

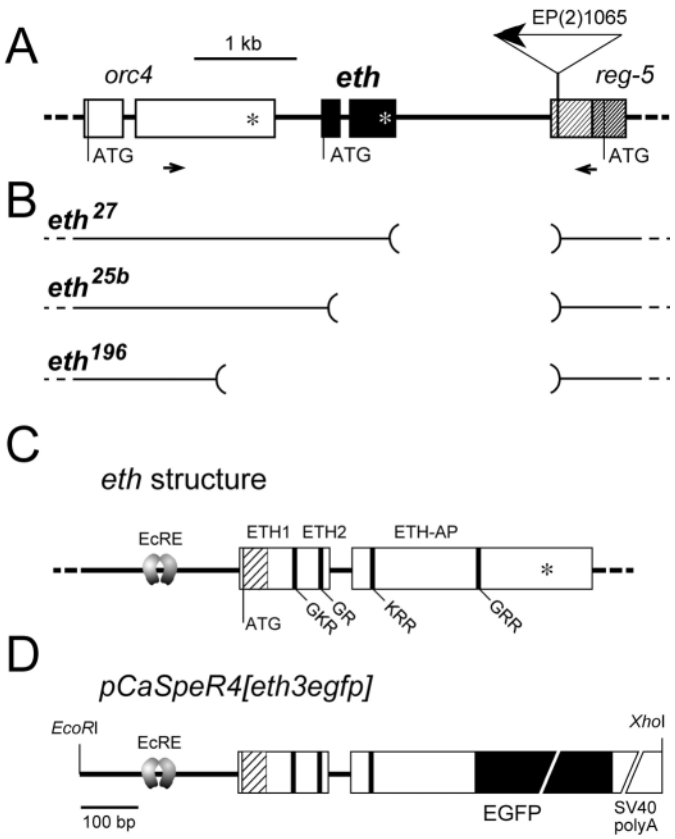

Fig. 4. Molecular map depicting the *eth* gene, deletions following P-element excision, and structure of the *eth-egfp* transgene. (A) Molecular map for the 2nd chromosome right arm (60E) region covering *eth* and adjacent P-element location and direction in the EP(2)1065 line. Boxes are exons for each of the genes, including *orc4*, *eth* and *reg-5*. The primer set used for deletion mutant screening is shown by small arrows. ATGs depict the putative translation initiation sites and directions of the genes. Asterisks indicate the stop codons in each gene. As the transcription initiation site for *reg-5* is ambiguous, a thin hatched bar is used to depict the sequence of Van Gelder (Van Gelder and Krasnow, 1996) (GenBank Accession Number, U65105) and a thick hatched bar is used for the EST clones HL04722.5′, LP09845.5′ and SD04185.5′ sequences (GenBank Accession Numbers, AA698461, AI296050 and AI532618, respectively). (B) *eth* deletion lines generated by imprecise P-element excision from EP(2)1065 line. Three relevant deletion mutant lines are shown. (C) *eth* gene structure. Boxes show exons, EcRE indicates a putative ecdysteroid responsive element (imperfect repeat of aggtca) (Park et al., 1999), ATG shows the putative translation initiation site for *eth*, and the star indicates location of the stop codon. ETH1, ETH2 and ETH-AP are depicted with canonical processing sites GKR, GR, KRR and GRR (Park et al., 1999). (D) The transgene pCaSpeR4[*eth3egfp*]. The ETH chimera with EGFP is constructed after the last canonical processing site GRR. Restriction enzyme sites used for cloning into pCaSpeR4 vector (Thummel and Pirrotta, 1991) are shown as *Eco*RI and *Xho*I.

of the *eth* stop codon. This deletion did not disturb the coding sequence of *eth*, thus serving as a useful negative control for other deletions. The eth^{25b} deletion removes virtually the entire *eth* sequence, extending from the original P-element site up to the *eth*-coding region, leaving only part of the signal sequence (MRIITVLSV) (Park et al., 1999). The eth^{196} deletion occurs from the P-element site through *eth* to the middle of the adjacent gene *orc4*, (origin recognition complex 4) (Chesnokov et al., 1999).

Loss of *eth* in both eth^{25b} and eth^{196} lines causes recessive lethality, while eth^{27} has no obvious phenotype (Table 2). The fact that genotype eth^{25b}/eth^{196} also showed the same ecdysis deficiency phenotype suggests that the *eth* deletions cause this phenotype, rather than other unknown aberrations (Table 2). Lethality is associated with ecdysis deficiencies, whereby double mouthhooks and dVP indicate failure to shed the old mouthparts. These animals show a shrunken body appearance, thick trachea and partial ecdysis of old cuticle both exteriorly and within the tracheal system. We refer to the phenotype resulting from *eth*-deletion as '*buttoned-up*', which describes an inability to extricate old mouthhooks and vertical plates from the new sclerotized structures.

Further analysis revealed disrupted respiratory dynamics and behavioral deficits in *eth* null mutants. Tracheal collapse and inflation of new trachea are delayed for ~1.5 hours, and pre-ecdysis behaviors are completely absent (Fig. 5, *n*=6). In the absence of these events, 'ecdysis-like' behavior occurs early, around the dVP stage with a large variation among individuals (4±23 minutes). Ecdysis-like behavior differed from wild-type ecdysis behavior in several respects. First, normal forward thrust movements to plant the old mouthparts in the substrate were absent. Instead, animals engaged in swinging head movements, and repeated extensions and retractions of the mouth. Second, strong backward thrust movements, which normally result in separation of the spiracles and ecdysis of trachei, were also absent. Although some backward movements were observed, animals were unsuccessful in detaching the old spiracles and tracheal linings. Some turning behavior resembling forward escape was observed, but animals are unsuccessful in this maneuver, owing to the fact that neither mouthparts nor spiracles have been detached. These ecdysis-like behaviors are repeated on an irregular basis for 1 to 3 hours. Some time after the occurrence of delayed tracheal collapse and inflation, ecdysis-like behaviors become more like normal ecdysis. Indeed, the majority of eth^{-} mutants (78%, *n*=23) are able to move through an anterior dorsal opening in the old cuticle that appears after repeated ecdysis movements. This occurs on the average at 2 hours 17 minutes±40 minutes after the dVP stage. This type of exit from the old cuticle contrasts with that of wild-type flies, which ecdyse by moving through the anterior opening created by removal of the old mouthparts. Even though many mutant larvae are able to escape the old cuticle, their mouthparts remain 'buttoned-up'. The *buttoned-up* phenotype remains quiescent, does not feed and dies within 1 to 2 days. A small fraction of eth^{-} larvae undergo successful ecdysis and development through the second instar (~2%, Table 2), but all succumb following ecdysis failure at the 2nd to 3rd instar transition.

Null mutants are rescued by ETH Injection

We found that properly timed injection of DrmETHs rescue ecdysis deficiencies in mutant flies and promotes successful ecdysis. Injection of DrmETH1 (~ 1 fmol) into either eth^{25b} or eth^{196} larvae at the dVP stage restores all missing steps in the ecdysis sequence (Table 2). Specifically, DrmETH1 injections induced tracheal collapse and inflation of trachea (3 and 4 minutes after the injection, respectively, *n*=10). Thereafter, pre-

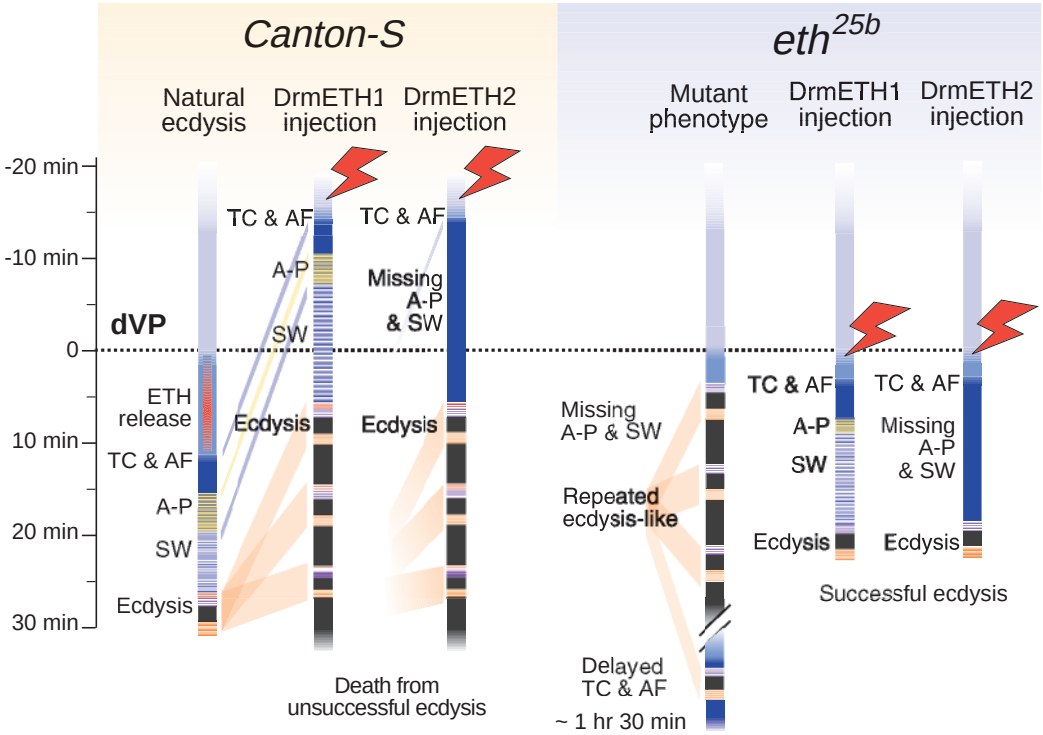

Fig. 5. Comparisons of the time lines for ecdysis-related behaviors in wild-type CantonS and in the *eth* deletion mutant *eth*^{25b}. See abbreviations in Table 1. (Left) Early DrmETH1 or DrmETH2 injections in CantonS induced premature ecdysis-related behaviors, but ecdysis fails. (Right) The null mutant *eth*^{25b} lacks pre-ecdysis behaviors anterior-posterior contractions (A-P) and squeezing waves (SW), and exhibits repeated ecdysis-like behavior, and delayed tracheal collapse (TC) and air inflation (AF). Injection of *eth*^{25b} flies with DrmETH1 or DrmETH2 at the dVP stage rescued the ecdysis deficiency (see text for more details).

ecdysis behaviors appeared, including weak anterior-posterior movements (7±2 minutes) followed by strong squeezing waves (9±2 minutes). A set of typical ecdysis behaviors, including forward and backward thrusts and forward escape, occurred at 18±2 minutes after injection. Rescued flies that succeeded in passing to the 2nd instar succumbed at the transition to the 3rd instar, owing to unsuccessful ecdysis.

Though mutants injected with DrmETH1 showed a normal ecdysis behavioral sequence, some individuals were unsuccessful in completing ecdysis. Approximately 25% of *eth*^{25b} and 41% of *eth*^{196} flies failed to successfully shed the old cuticle (Table 2). Rescued *eth*^{25b} mutants show no further mortality during the 2nd instar, but rescued *eth*^{196} mutants show significant mortality during the early 2nd instar; accumulated mortality rises from 41% to 73% (lethal phase *i* in the 2nd instar, see Table 2). The elevated mortality observed for *eth*^{196} mutants during the 2nd instar may result from partial deletion of the upstream gene *orc4* (Fig. 4B). Maternally deposited *orc4* mRNA (Chesnokov et al., 1999) could promote survival enough the early 1st instar, but be insufficient for development through the early 2nd instar. Further examination of this question requires genetic rescue of the *eth*^{196} line.

Lethality could be reversed also by injection of DrmETH2 at relatively high doses (≥ 10 fmol). These treatments partially rescued behavioral deficits in *eth*^{25b} flies, including induction of tracheal collapse, inflation of trachea and ecdysis. However, DrmETH2 injections failed to induce anteroposterior contractions and squeezing waves (Fig. 5). Lower doses of DrmETH2 (~1 fmol) induced tracheal collapse and inflation of new trachea, but were not effective in eliciting either pre-ecdysis or ecdysis behaviors.

Table 2. Lethal phase of *eth* mutants and wild-type *Drosophila* and rescue of *eth* mutants by injection of ETH1 peptide

		Accumulated lethality			
		1st instar		2nd instar	
Allele	*n*	Death *i**	Death *ii*†	Death *i**	Death *ii*†
CantonS	226	0.9%	1.3%	1.3%	1.3%
eth^{27}	78	2.6%	2.6%	2.6%	2.6%
eth^{25b}	455	5.7%	98.2%	98.2%	100%
eth^{196}	142	8.5%	100%	100%	100%
eth^{25b}/*eth*^{196}	147	5.4%	100%	100%	100%
Rescue by DrmETH1 injections‡ at the 1st to 2nd instar ecdysis					
eth^{25b}	20		25%	25%	100%
eth^{196}	22		41%	73%	100%
Rescue by DrmETH2 injections§ at the 1st to 2nd instar ecdysis					
eth^{25b}	10		30%	30%	100%

*Death with single vertical plate, implying death before ecdysis.
†Death with double vertical plate, implying death at ecdysis.
‡0.1 fmol DrmETH1.
§10 fmol DrmETH2.

DISCUSSION

Ecdysis is a recurring, critical event in insect development. The sequence of physiological and behavioral events preceding ecdysis is stereotypic, and under the control of steroid and peptide hormones. It is well established that ETHs trigger ecdysis upon injection into moths and flies (Zitnan et al., 1996; Ewer et al., 1997; Baker et al., 1999; Park et al., 1999; Zitnan et al., 1999; Zitnan and Adams, 2000). These studies demonstrate the sufficiency of ETHs in triggering ecdysis, but cannot prove their necessary involvement in the natural process. It also should be recognized that the behavioral

functions ascribed to ETHs in this work might be only the most obvious manifestations of their actions. The full range of their biological actions could be much greater.

In this paper, we used *Drosophila* genetics to test the hypothesis that ETH is necessary in the regulation of ecdysis. Excision of *eth*, the gene encoding DrmETH, resulted in profound deficits in the developmental steps culminating in ecdysis, and 98% mortality at the first larval ecdysis. To properly characterize the effects of genetic deletion, we performed a thorough analysis of normal ecdysis, characterized the epitracheal endocrine system in *Drosophila*, and showed it to be the likely source of ETH.

The epitracheal endocrine system and ecdysis in *Drosophila*

During the hour preceding ecdysis of 1st instar *Drosophila* larvae, three main events occur: sclerotization of new mouthparts, tracheal dynamics and a defined behavioral sequence. Appearance of the new mouthparts, including mouthhooks and vertical plates, precedes ETH release, and no disruption of these events occurs in *eth*$^{-}$ flies. However subsequent events are triggered by ETH release from a system of Inka cells homologous to those previously described in moths (Zitnan et al., 1996; Adams and Zitnan, 1997; Zitnan et al., 1999).

Inka cells of *Drosophila* express the gene *eth* and contain the peptide ETH. Expression of *eth* is evident in transformed *Drosophila* carrying the chimeric transgene *eth3-egfp*, where EGFP fluorescence and ETH-like immunoreactivity are colocalized in Inka cells. No other cells exhibit EGFP fluorescence, although some CNS neurons also show ETH-IR. The most intense EGFP fluorescence is observed as larvae approach the first ecdysis, whereas neither EGFP fluorescence nor ETH-IR is detected during embryonic development or early 1st instar. This, together with the observation that no hatching deficiency occurs in *eth*$^{-}$ mutants (data not shown), indicates that ETHs are probably not required for early development, including the patterned behavior associated with egg hatching (Hartenstein et al., 1997). EGFP fluorescence and ETH-IR are observed throughout the adult stage, suggesting possible mating or reproductive functions.

ETH triggers tracheal collapse and inflation. This conclusion is indicated by two observations. First, EGFP fluorescence in Inka cells sharply declines just minutes before these events. As the subcellular distribution of EGFP fluorescence and ETH-IR in Inka cells is identical, we conclude that EGFP and processed ETHs are sorted into secretory granules of Inka cells (Klein et al., 1999), and that these peptides are co-released. Second, injection of either DrmETH1 or DrmETH2 into wild-type larvae or *eth*$^{-}$ mutants induces tracheal collapse and air inflation within minutes. These observations strongly implicate ETH in the control of tracheal dynamics before ecdysis.

It is notable that Inka cells and associated components of the epitracheal endocrine system are situated directly on tracheal tubes (Zitnan et al., 1996). While it has been recognized for some time that ETHs act directly on the CNS to elicit centrally patterned behaviors, this report documents for the first time functions for these peptides in respiratory physiology. What is the functional significance of Inka cell placement directly on tracheal tubes? We speculate that their location in some way senses the readiness of the respiratory system to switch over from old to new trachea in preparation for pre-ecdysis and ecdysis behaviors. Whether the Inka cells indeed have such a sensory function remains to be demonstrated.

The mechanism of tracheal inflation is not known, but we hypothesize that dissolved gas is liberated as a consequence of fluid movement out of the tracheal lumen. The hydrophobic surface of the cuticle lining the tracheal tube may then facilitate a reverse capillary force to complete air filling (Wigglesworth, 1979). This is the first indication that these processes are under hormonal control. Further work is needed to define the signaling steps involved in this process.

Upon completion of tracheal inflation, a behavioral sequence ensues consisting of pre-ecdysis and ecdysis behaviors. These behaviors and successful ecdysis are triggered upon injection of DrmETH1, consistent with our earlier study showing that ETH injection induces premature eclosion behavior in the pharate adult (Park et al., 1999). It is striking that the same chemical signal initiates two quite different behaviors separated by two metamorphic molts.

A previous immunohistochemical study detected myomodulin-like-IR in tracheal cells of *Drosophila* and members of other insect groups (O'Brien and Taghert, 1998). Immunoreactivity in these 'peritracheal cells' also disappears at ecdysis, whose pattern and distribution suggest they could be the Inka cells described in this study. However, subsequent biochemical analyses of Inka cells in *M. sexta* (Zitnan et al., 1999) and *Bombyx mori* (D. Zitnan and M. E. A., unpublished) provide no evidence for the presence of myomodulin or related peptides in Inka cells, so the antigen(s) and functional significance of peritracheal cells and myomodulin-like IR in ecdysis remains unclear.

ETH is both necessary and sufficient for ecdysis

Our results show that DrmETH1 is sufficient to evoke the ecdysis sequence in larval *Drosophila*, as was previously observed for adult *Drosophila* and for MasETH in *M. sexta* and BomETH in *B. mori* (Zitnan et al., 1996; Adams and Zitnan, 1997; Park et al., 1999; Zitnan et al., 1999). The lethal phenotype observed in *eth*$^{-}$ deletion mutants provides confirmation that, in *Drosophila*, ETH is necessary for ecdysis. The same physiological and behavioral deficiencies occur in both *eth*^{25b} and *eth*^{196} lines, where tracheal collapse and inflation are delayed for hours, and pre-ecdysis behaviors are absent. Ecdysis behavior is abnormal and occurs prematurely, soon after the dVP stage. These physiological and behavioral deficits contribute to the *buttoned-up* phenotype.

It is remarkable that tracheal dynamics and the behavioral sequence absent in *eth*$^{-}$ mutants can be completely restored by injection of DrmETH1. Animals rescued by injection of DrmETH1 shed the cuticle normally and develop through the second instar, although a higher mortality is observed in *eth*^{196} mutants. As this line also has a significant deletion of the adjacent *orc4* gene, it may suffer additional defects may account for this. The deletion phenotype and its facile rescue by injection of DrmETH1 provide the best evidence thus far that this blood-borne peptide is a necessary signal in the orchestration of key developmental events culminating in ecdysis. Interestingly, DrmETH2 injection also rescues the buttoned up phenotype, but without triggering pre-ecdysis behaviors. It therefore appears that the pre-ecdysis behaviors

we have described here in *Drosophila* larvae are not crucial for completion of ecdysis.

An obvious physiological consequence of *eth* deletion is failure of the respiratory system to inflate on schedule. This is consistent with our finding that ETH injection regulates collapse of old trachea and inflation of new trachea, and confirms that this peptide plays a vital signaling function for tracheal dynamics associated with ecdysis. Significantly, tracheal collapse and inflation are markedly delayed, but not eliminated in *eth*$^{-}$ larvae. Therefore ETH may act indirectly through downstream regulatory processes, which eventually succeed in mediating tracheal inflation. One candidate signal for tracheal inflation is eclosion hormone. Adult *Drosophila* that lack functional eclosion hormone neurons fail to inflate the tracheal system properly upon adult eclosion and are reported to be insensitive to ETH (Baker et al., 1999).

The actual causes of lethality observed in *eth*$^{-}$ larvae could be a combination of factors, including respiratory and behavioral deficits. Lack of tracheal inflation and incomplete shedding of the old tracheal lining would obviously compromise respiratory functions. Failure to shed old mouthparts at the appropriate time is likely the main factor in production of the *buttoned-up* phenotype. This condition appears in null mutants, as well as in wild-type flies upon premature injection of DrmETH1. In either case, the *buttoned-up* phenotype prevents complete ecdysis and further ingestion of food. All of these factors could be jointly involved in the lethality observed.

Of special significance is the fact that *eth* deletion mutants engage in a premature ecdysis-like behavior. Although quite variable and different from authentic ecdysis (see Results section for more details), these sporadic bouts gradually became more similar to authentic ecdysis more than 2 hours after dVP. Animals were able to shed the old cuticle partially, albeit with *buttoned-up* mouthhooks. The early appearance of ecdysis-like behavior provides further evidence that ETH not only triggers ecdysis, but delays its onset until the appropriate time. Decapitation or removal of cephalic or thoracic ganglia accelerates ecdysis, supporting the hypothesis that ETH activates both excitatory and inhibitory centers in the CNS (Baker et al., 1999; Zitnan and Adams, 2000), with inhibition functioning to delay ecdysis until the appropriate time. In the absence of ETH, these inhibitory centers may not be triggered, leading to premature ecdysis or ecdysis-like behavior.

Functional diversity of ETHs in *Drosophila*

The *eth* gene encodes three predicted amidated peptides: DrmETH1, DrmETH2 and DrmETH-AP. Two of these peptides, DrmETH1 and DrmETH2 have biological activity in both larval and adult stages (Park et al., 1999) (this work). In wild-type larvae, injection of DrmETH1 elicits the entire sequence of ecdysis-related events, although for reasons not yet clear the A-P phase of pre-ecdysis is rather weak. Similarly, DrmETH1 alone rescues the entire sequence in *eth*- mutants, including tracheal dynamics, A-P (weak), SW and ecdysis (Fig. 5). DrmETH2 also elicits tracheal dynamics and ecdysis behavior, but only at higher doses (≥ 10 fmol). This peptide fails to trigger pre-ecdysis (A-P and SW). Lower doses of DrmETH2 (~1 fmol) induce only tracheal dynamics, without pre-ecdysis or ecdysis. With regard to relative potency, these findings are consistent with our earlier work showing that DrmETH1 is more active than DrmETH2 in triggering adult eclosion (Park et al., 1999). However, no functional differences were apparent in that study.

Given that DrmETH1 is sufficient to trigger the entire sequence, the functional role of DrmETH2 is unclear. It might have been expected that DrmETH2 is involved in pre-ecdysis behavior, given that DrmETH1 injection elicits only weak A-P contractions. However, DrmETH2 fails to elicit pre-ecdysis at all, yet elicits tracheal collapse and air filling at lower doses than are needed to generate ecdysis behavior. The higher potency of this peptide in eliciting tracheal collapse and air filling suggests that it may regulate tracheal dynamics in a way that has thus far escaped our attention. It is also possible that, as *eth* is expressed in the adult stage, DrmETH2 plays some role in mating or reproduction. Further work is needed to resolve these issues.

In *M. sexta*, the ETH gene also encodes three peptides, PETH, ETH and ETH-AP (Zitnan et al., 1999). PETH induces only pre-ecdysis I, whereas ETH triggers pre-ecdysis II and ecdysis. It is also known that injection of ETH into naive animals elicits all three behavioral steps, including pre-ecdysis I, pre-ecdysis II and ecdysis (Zitnan et al., 1996). We would therefore predict that, if the ETH gene were deleted in *M. sexta*, ETH alone might be sufficient to rescue the missing behavioral sequence, leaving the role (necessity) of PETH similarly unclear. It might be possible to approach this issue using RNAi followed by injection of each peptide.

The phenotype of *eth*$^{-}$ null mutants resulted in 98-100% lethality at the first larval ecdysis. This is striking in comparison with the phenotype of EH-cell knockout flies that showed only ~50% lethality in larval stages, uncoordinated behaviors during adult eclosion and accumulated lethality of ~70% (McNabb et al., 1997; Baker et al., 1999). Similarly, ablation of CCAP-cells leads to no obvious defects during larval stages but major lethality at pupation (Ewer et al., 2001). McNabb et al. (McNabb et al., 1997) and Baker et al. (Baker et al., 1999) proposed that ETH acts through central release of EH, because although wild-type flies showed premature eclosion behavior upon injection of ETH, EH-cell knockout flies are insensitive to ETH. Furthermore, CCAP is seen as a downstream signal whose release is caused by EH (Gammie and Truman, 1997).

If EH is an obligatory signal downstream of ETH as suggested by Truman and colleagues (Ewer et al., 1997; McNabb et al., 1997; Baker et al., 1999) why is the larval mortality of *eth*$^{-}$ animals shown in this study so much higher than that of EH knockouts? Analysis of behavioral phenotypes in larval ecdysis of CCAP-cell knockout and EH-cell knockout flies may provide invaluable information to aid in the understanding of the roles of each peptide and signaling cascade for the ecdysis.

The cascade of peptides involved in regulation of insect ecdysis is growing. It is clear that ETH participates in a complex cascade that includes EH (Truman, 1992), and crustacean cardioactive peptide (CCAP) (Gammie and Truman, 1997). These molecules appear to be broadly conserved among Lepidoptera, *Drosophila* and other insects, and may be expandable to other arthropods such as crustaceans (Phlippen et al., 2000). A conceptual framework for the peptide signaling cascade regulating ecdysis has been provided by studies of *Manduca sexta* (Ewer et al., 1997; Gammie and Truman, 1999;

Zitnan et al., 1999; Zitnan and Adams, 2000) and *Drosophila* (McNabb et al., 1997). It is thought that a positive feedback pathway between peripheral secretion of EH from the CNS and ETH from Inka cells elevates circulating levels of both peptides (Ewer et al., 1997). Elevated levels of ETH acting on downstream targets within the CNS recruit sequential pre-ecdysis and ecdysis behaviors. Among these targets are CCAP-containing neurons, which activate the central pattern generator for ecdysis behavior (Gammie and Truman, 1997).

Ecdysis provides an excellent model system for analysis of a chemically coded behavioral sequence (Bicker and Menzel, 1989). The behavioral deficits associated with *eth* deletion provide a particularly clear illustration of behavioral failure in the absence of the requisite signaling molecule. It is remarkable that complete rescue of the behavior occurs on simple injection of ETH. These findings indicate that the performance of innate, stereotypic behavioral sequences may depend on achieving proper levels of peptides and other endogenous signals in the nervous system so as to activate and bias central pattern generators appropriate to developmental and sensory context (Bicker and Menzel, 1989; Harris-Warrick and Marder, 1991; Marder and Calabrese, 1996). Other specific examples of how peptides may function as behavioral orchestrators *in vivo* include egg-laying, feeding and maternal behaviors (Stanley and Leibowitz, 1985; De Bono and Bargmann, 1998; Lucas et al., 1998; Waggoner et al., 2000).

Genes required for *Drosophila* ecdysis

Several developmental mutants of *Drosophila* display phenotypes defined by molting deficiencies. Many of these display ‘double mouth hooks’, suggesting a defect in an aspect of the ecdysis program. Many if not most of these phenotypes result from defective events upstream of ETH signaling, and it seems likely that may result in ETH deficiency and the *buttoned-up* phenotype described here.

Mutations that lead to the double mouthhooks phenotype fall into three general categories: ecdysone synthesis and secretion, downstream transcriptional signaling and peptide processing enzymes. Ecdysone synthesis mutants include *ecd* (ecdysoneless) (Belinski-Deutsch et al., 1983), *dare* (defective in avoidance of repellents) (Freeman et al., 1999) and *itpr* (IP-3 receptor) (Venkatesh and Hasan, 1997). Transcription factors regulated by ecdysone signaling include *EcR-B (*ecdysteroid receptor B) (Schubiger et al., 1998; Li and Bender, 2000), *USP* (ultraspiracle) (Perrimon et al., 1985; Li and Bender, 2000), *βFTZ-F1* (*ftz* transcription factor 1) (Yamada et al., 2000) and *crc* (cryptocephal) (Hewes et al., 2000). Finally, mutants displaying the *buttoned-up* phenotype carrying defective peptide processing enzymes are *amontillado*, which encodes the enzyme prohormone convertase 2, are deficient in larval molting (Gooding et al., 2000). Some also are deficient in hatching behavior. Mutations of PHM (peptidylglycine α-hydroxylating monooxygenase), which is required for α-amidation at the C-terminal end, also generate a *buttoned-up* phenotype (Jiang et al., 2000). It will be interesting to determine whether many if not most of the above mutations lead to ETH deficiency.The *eth* deletion mutant and the *eth* reporter fly line *2eth-egfp* also provide opportunities to investigate roles for ecdysone in regulation of expression, processing and secretion of ETH, and its downstream pathways up to ecdysis behavior.

Conclusions

Ecdysis in *Drosophila* is an excellent model for understanding a genetically programmed, hormonally driven sequence of physiological and behavioral events. Physiological studies using large lepidopteran insects, such as *Manduca sexta*, have advanced our understanding of ecdysis-related behaviors. Our studies of the *Drosophila* system complement these findings with the use of genetic tools. Deletion of *eth* has demonstrated the obligatory role of ETH in the performance of a behavioral sequence vital to the survival of the animal. Further studies are under way to define genes for upstream regulators of the expression and secretion of the ETH, as well as those regulating the ETH receptor and downstream cellular and molecular targets within the central nervous system.

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