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Mutations in the *Drosophila* glycoprotein hormone receptor, *rickets*, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program

James D. Baker* and James W. Truman

Department of Zoology, University of Washington, Box 351800 Seattle, WA 91895, USA

*Author for correspondence at present address: Department of Neurobiology and Behavior, SUNY at Stony Brook, Stony Brook, NY 11794, USA (e-mail: jabaker@ms.cc.sunysb.edu)

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Summary

Adult insects achieve their final form shortly after adult eclosion by the combined effects of specialized behaviors that generate increased blood pressure, which causes cuticular expansion, and hormones, which plasticize and then tan the cuticle. We examined the molecular mechanisms contributing to these processes in *Drosophila* by analyzing mutants for the *rickets* gene. These flies fail to initiate the behavioral and tanning processes that normally follow ecdysis. Sequencing of *rickets* mutants and STS mapping of deficiencies confirmed that *rickets* encodes the glycoprotein hormone receptor DLGR2. Although *rickets* mutants produce and release the insect-tanning hormone bursicon, they do not melanize when injected with extracts containing bursicon. In contrast, mutants do melanize in response to injection of an analog of cyclic AMP, the second messenger for bursicon. Hence, *rickets* appears to encode a component of the bursicon response pathway, probably the bursicon receptor itself. Mutants also have a behavioral deficit in that they fail to initiate the behavioral program for wing expansion. A set of decapitation experiments utilizing *rickets* mutants and flies that lack cells containing the neuropeptide eclosion hormone, reveals a multicomponent control to the activation of this behavioral program.

Key words: Receptor, bursicon, neuroethology, behavior, hormone, tanning, eclosion, ecdysis triggering hormone

Introduction

During the last hours of metamorphic development, a cascade of peptide hormones acts to coordinate the emergence of the adult insect from the puparium or pupal case. The behaviors used during emergence can be divided into three distinct phases: the pre-ecdysis, ecdysis (Gammie and Truman, 1997; Truman, 1978; Zitnan et al., 1996) and expansional phases (Carlson, 1977; Hughes, 1980; Truman and Endo, 1974; Truman, 1973). Although the initial stages of adult eclosion have been described in detail for *Drosophila* (Baker et al., 1999; McNabb et al., 1997), a behavioral description of the post-ecdysial, expansional phase of the behavior is lacking. The expansional phase involves a stereotypical set of behaviors that increases the hemolymph pressure needed for expansion of the new cuticle. Changes in the new cuticle occur in concert with this behavioral program. The cuticle first shows a transient plasticization, followed by tanning, a period of sclerotization and melanization. The basis of cuticular tanning has been investigated most extensively in blowflies, where it is triggered by the peptide hormone bursicon (Cottrell, 1962a,b; Fraenkel and Hsiao, 1962; Fraenkel and Hsiao, 1965).

Drosophila and blowflies are higher flies in the suborder of Cyclorapha. These insects show a delay between the emergence of the adult from the puparium and the onset of wing expansion with its accompanying cuticular tanning and melanization. Flies that were decapitated by neck ligation immediately after emergence failed to show normal post-ecdysis cuticular tanning, but tanning could be induced with injections of blood collected from intact flies a few minutes after their emergence (Cottrell, 1962a; Fraenkel and Hsiao, 1962). The blood borne tanning factor, bursicon, is a 33 kDa peptide hormone (Kaltenhauser et al., 1995; Kostron et al., 1995) made by neurosecretory cells in the nervous system (Garcia-Scheible and Honegger, 1989; Honegger et al., 1992; Kostron et al., 1996; Taghert and Truman, 1982). It appears in the blood at or after ecdysis (Reynolds, 1980; Truman, 1981) and induces a transient elevation of cAMP in target tissues. Although the cuticular actions of bursicon have been examined, its possible behavioral actions are poorly understood because most assays for bursicon involve decapitation of the insect. Furthermore, the scope of comparative studies on bursicon action is constrained by the fact that, in many insects, the ecdysis and expansional phases of emergence behavior overlap considerably.

We chose a molecular genetic approach to understand the changes that characterize the final phase of adult eclosion, studying the behavior in wild-type *Drosophila* and in *rickets* (*rk*) mutants that do not complete the post-ecdysial expansion behaviors. The *rk* gene encodes a member of the glycoprotein hormone receptor family of G-protein-coupled receptors (Ashburner et al., 1999; Eriksen et al., 2000), which appears to be an integral component of the bursicon signaling pathway. Experiments using the *rk* mutants give a novel insight into the complex regulatory pathway that controls wing expansion programs in insects and suggest a physiological role for a glycoprotein hormone receptor in insects.

Materials and methods

Sequence tagged site analysis

Using the Prime utility in GCG we designed primers that would amplify 300 bp fragments at approximately 5 kb intervals across the *rk* region. The sequence of the three sets of primers was: STS 1, sense 5′-GCCAACTCGCTGAAA-TATAC-3′, antisense 5′-AAACCACAAAACTTCCCC-3′; STS2, sense 5′-AAAAACCACCCACACTCC-3′, antisense 5′-TTCTAACTCCCCAGATAATCTC-3′; and STS3, sense 5′-CGTTACCGATTTTTGCCC-3′, antisense 5′-CGTATCTT-CGCTGTTTGCC-3′. Using the protocol of Engles, (Engles et al., 1990) we optimized conditions for each primer set so that they could reliably amplify a band of the appropriate size from one eighth of the extract prepared from a single wild-type embryo. Single embryos were ground in 10 mmol l^{-1} Tris-Cl, pH 8.2, 1 mmol l^{-1} EDTA, 25 mmol l^{-1} NaCl and 200 μg ml^{-1} freshly diluted Proteinase K. The extracts were then incubated at 37 °C for 30 min, heated to boiling for 15 min and frozen until use. We used 3 μl of extract for each PCR and each embryo was tested for the presence or absence of all three PCR products.

Stocks

The two strong *rk* alleles we used, rk^4 and rk^1, were maintained as homozygous stocks. The two deficiencies that have been genetically defined to break in the *rk* gene are *Df(2)b-L* and *Df(2)A376* (Ashburner et al., 1982). Both deficiencies are lethal when homozygous so each was maintained as a balanced stock over *CyO*. The progeny of a cross between these two deficiency stocks were viable and fertile but displayed a severe rickets phenotype. We used Canton S as our wild-type stock of *Drosophila melanogaster*.

Extracts

A hormone extract that contained bursicon was made from the abdominal ganglia of *Sarcophaga bullata* (Pkr.). The fused ventral ganglia of pharate adults were dissected and homogenized in saline (Ephrussi and Beadle, 1936) (127.7 mmol l^{-1} NaCl, 4.7 mmol l^{-1} KCl) at a concentration of 1 CNS/5 μl. The extract was centrifuged for 5 min, and the supernatant was divided into portions and frozen until use. For cAMP experiments a 1 mmol l^{-1} solution of 8-Br-cAMP (Sigma) was prepared in saline (Ephrussi and Beadle, 1936). Injections were performed as described below.

Ligations and injections

Newly eclosed adults were collected from bottles onto an ice-chilled Petri plate at 3 min intervals. When enough animals were collected for an experiment, the flies were neck ligated using a very fine nylon monofilament line. They were maintained at room temperature in a humid chamber for 1 h and ligated flies that tanned or spread their wings during this interval were discarded. The remaining flies were injected with the hormone extract, saline, or cAMP, using a picospritzer II (General valve, corp.) and glass micropipettes. Flies were injected with between 180 and 300 nl of solution. The volume was estimated by measuring the volume of a drop injected into oil. Flies that bled excessively following injection were discarded. The cuticular darkening induced by bursicon was evident within 2 h of injection.

For injections of hemolymph, blood samples were collected by removing a wing and compressing the fly between two glass microscope slides. A drop of blood was produced at the site of the injury and was collected in a micropipette injection needle. Blood collected in this way was relatively free of cellular debris and could be injected without clogging the needle. The contents of a needle were injected into a single fly. Blood from wild type was collected after wing expansion had begun. Blood from mutants was collected 15 min after adult eclosion because *rk* mutants do not expand their wings.

Cuticle preparations

Abdomens of flies were opened along the ventral midline, pinned flat in a sylgard (Dow Corning) dish and fixed in 4 % formaldehyde in phosphate buffered saline (PBS). The abdomens were dehydrated through an ethanol series, cleared in Xylene, and mounted in DPX (Fluka Biochimika). Pictures were collected on a Macintosh PowerPC 7600/120 from a Sony CCD camera connected to a Nikon Optiphot microscope. An average of 15–20 focal planes of each portion of the cuticle were collected and assembled into a montage using layers in Adobe PhotoShop 3.0.

Immunocytochemistry

Nervous systems of rk^4 homozygotes were dissected in cold saline (Ephrussi and Beadle, 1936) from either developing adults at the smooth to smooth-grainy stage (approximately 5–9 h before adult eclosion) or from newly eclosed adults. They were fixed in 4 % formaldehyde overnight at 4 °C. After fixation the nervous systems were washed three times in PBS with 0.3 % Triton X-100 (PBS-TX) for 1–3 h. A rabbit anti-EH (eclosion hormone) antiserum was diluted 1:100 in PBS-TX with 1 % normal donkey serum (NDS) (Jackson labs) and 0.005 % sodium azide. The tissue was incubated in the primary antibody overnight at 4 °C. After washing in 5 ml of PBS-TX, 3×, for 1–3 h, the tissues were incubated in a donkey anti-rabbit IgG antibody conjugated to Texas Red (Jackson Labs). Secondary antibodies were used at a dilution of 1:1000 in PBS-

TX with 1 % NDS and 0.005 % sodium azide for either 8 h at room temperature or overnight at 4 °C. The nervous systems were then washed 3 times in PBS-TX for 1–3 h, mounted on poly-lysine coated coverslips and dehydrated, cleared and mounted as described above. Images were collected using a BioRAD MRC-600 confocal microscope.

Behavioral observations

Pharate pupae were collected in small Petri dishes ringed with Whatman paper and observed periodically for the initiation of ecdysis. When ecdysis was completed, a timer was started and observations were made either continuously or at 1 min intervals.

Results

Correspondence of DLGR2 *to* rk

Flies that are mutant for the *rk* locus are typically viable and ecdyse but have abnormal post-ecdysial behavior. These flies display a spectrum of phenotypes related to the failure of the expansional program: kinked femurs, crossed post-scutellar bristles, folded wings, as well as weak post-ecdysial tanning and melanization. Therefore, this gene appears to be intimately involved in the last behavioral phase of the ecdysis sequence. Genetic and molecular mapping experiments have placed *rk* in a molecular interval that contains the gene for a *Drosophila* member of the glycoprotein hormone receptor family, *DLGR2* (Ashburner et al., 1999; Eriksen et al., 2000). To further assess the relationship of *DLGR2* to the *rk* mutation, we mapped two deficiencies known to disrupt the *rk* gene, one from the centromeric side (*Df(2L)A376*) and the other from the telomeric side (*Df(2L)b-L*). Flies trans-heterozygous for these deficiencies are viable and fertile adults that show a strong *rk* phenotype. We used a sequence tagged site (STS) mapping approach to define the extent of these two deficiencies in the region surrounding the *DLGR2* gene. The embryos of a stock of *Df/CyO* flies were collected and incubated at 25 °C. Of these embryos, the *Df/CyO* progeny were viable and hatched within 24 h. Those embryos unhatched after 36 h were an equal ratio of homozygous *CyO* and homozygous deficiency embryos. Single unhatched embryos were used to amplify three different STSs spanning the *DLGR2* transcription unit (Fig. 1A). For both deficiency stocks we saw only two patterns: either all bands were amplified (presumed *CyO/CyO*) or one (Fig. 1B, for the *Df(2L)A376* line) or two (Fig. 1C, for the *Df(2L)b-L* line) bands were missing. For both stocks, embryos of the two patterns were recovered in essentially a 1:1 ratio as expected by Mendelian segregation. These results were repeatable and invariant, leading us to conclude that these two patterns of amplification did represent the genotypes *CyO/CyO* and deficiency/deficiency respectively. We were unable to amplify any product using primers to the first coding exon of *DLGR2* (the STS1 primer set), on homozygous *Df(2L)A376* embryos (Fig. 1B). Consequently, *Df(2L)A376* lacks the 5′ upstream region and at least the first two exons of *DLGR2* but retains the 3′ end of the gene that is represented by the STS2 and STS3 sets. By contrast, in embryos homozygous for *Df(2L)b-L* we were able to amplify a product with the most 5′ primer pair, STS1, but not with STS2 and STS3 primer pairs, which are located in the transmembrane/intracellular exon and in the 3′ UTR of *DLGR2*, respectively (Fig. 1C). Therefore, this deletion removes at least the entire transmembrane and intracellular domains of DLGR2. In the heterozygous deficiency flies (*Df(2L)A376/Df(2L)b-L*), one chromosome

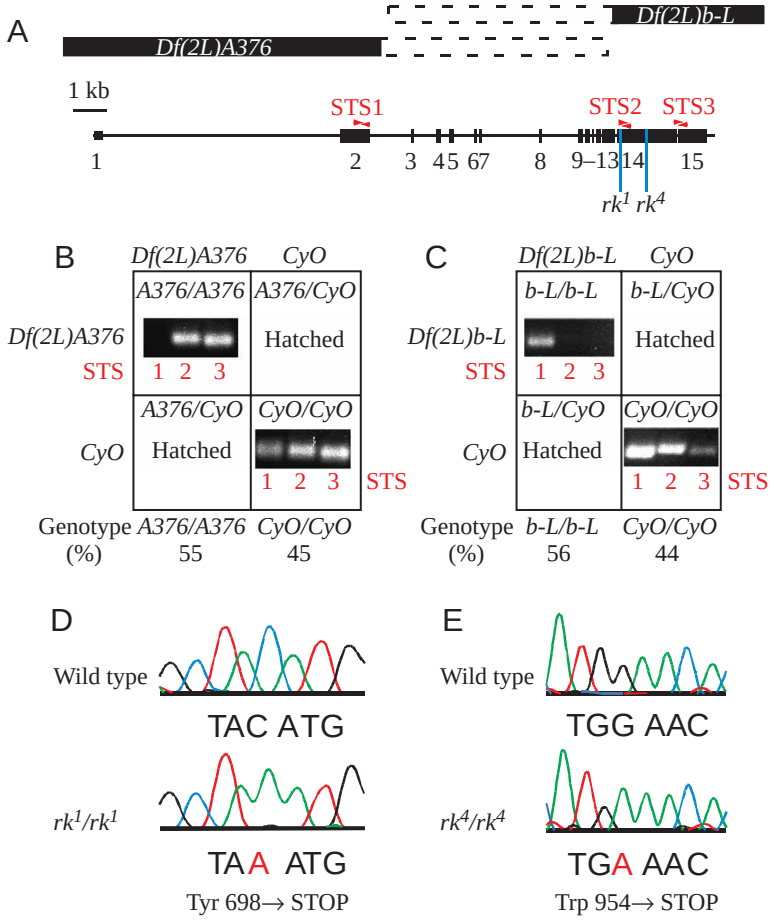

Fig. 1. The *DLGR2* coding region is disrupted in noncomplementing deficiencies that end within the *rk* gene and in two strong *rk* alleles. (A) Non-complementing deficiencies, *Df(2L)b-L* and *Df(2L)A376*, were mapped by testing for the presence of sequence tagged sites, STS1, STS2 and STS3, in a mixed population of homozygous balancer and homozygous deficiency flies. (B) *Df(2L)A376* breaks in the 5′ end of DLGR2 removing STS1, the initiator ATG and most of the first exon. (C) *Df(2L)b-L* removes STS2 and STS3 and breaks in 3′ end of the gene removing at least the entire transmembrane domain. 20 embryos from each cross were tested. (D) Electropherogram from a rk^1 homozygote. The wild-type sequence of codon 698, TAC, is converted to TAA, changing a tyrosine residue to a stop codon. (E) In rk^4 the wild-type sequence of codon 954, TGG, is changed to TGA, altering a tryptophan to a termination codon.

lacks the promoter and first two exons, while the other lacks the entire transmembrane coding region. Flies of this genotype could produce no complete transcript and we have concluded that they show a null or loss of function phenotype.

Eriksen et al. (2000) reported that a p-element insertion into the first exon of the *DLGR2* gene produced an embryonic lethal phenotype. They concluded that DLGR2 performed some vital function during embryogenesis. The embryonic lethality of this allele is at odds with the phenotype of the other 21 described alleles of *rk* and with the phenotype of the overlapping deficiencies described above. Consequently, we wondered whether the lethality was associated with the disruption of the *rk* gene or with a second, unmapped lethal mutation. Therefore, we complementation tested rk^{w11p} against the two mapped deficiencies, *Df(2L)A376* and *Df(2L)b-l*. We found a *rk* phenotype in 29 % (67/229) of the eclosing flies from the *Df(2L)A376*/*CyO* x rk^{w11p}/*CyO* cross and in 26 % (31/119) of the *Df(2L)b-L*/*CyO* x rk^{w11p}/*CyO* cross. These results are close to the expected value of one third of the flies being mutant, indicating that there is a very low level of lethality. Complementation testing against two strong alleles, rk^{1} and rk^{4}, gave similar results, indicating that the embryonic lethality in rk^{w11p} is not associated with the *rk* region. To test whether the rk^{w11p} p-element is separable from the lethality by recombination, w^{w1118}/w^{w1118}; rk^{w11p}/rk^{4} flies were maintained as a red-eyed *rk* stock for several generations. 10 red-eyed males were isolated from this stock and individually crossed to w^{w1118}/w^{w1118};*Sp/CyO* females. w^{w1118}/w^{w1118}; rk^{w11p}/CyO flies were backcrossed and tested for the *rk* phenotype and for retention of the p-element by eye color. One line had red eyes and a strong *rk* phenotype. In a test cross, 31 % (91/203) of progeny showed the *rk* phenotype, consistent with the loss of the embryonic lethality from this chromosome. Flies homozygous for this recombinant chromosome are viable and fertile enough to be maintained as a stock.

The phenotype of *Df(2L)b-L,Df(2L)A376* trans-heterozygotes is essentially identical to the two available strong alleles of *rk*, rk^{1} and rk^{4}. Consequently, we hypothesized that rk^{1} and rk^{4} might be null alleles. We sequenced the *rk* coding region from flies homozygous for rk^{1} and for rk^{4}. The gene consists of 15 exons, 14 of which code for protein (Eriksen et al., 2000). The first 13 exons comprise the leucine-rich extracellular domain while the 14th exon encodes the seven transmembrane domains and the intracellular portion of the protein. In both mutants we found a mutation resulting in a premature termination codon in the transmembrane domain. In rk^{1} the mutation is C to A, converting tyrosine 698 to a stop codon that would terminate translation prior to the first transmembrane domain (Fig. 1D). In rk^{4} the mutation is a G to A change that converts tryptophan 954 to a stop codon, which would truncate the protein between transmembrane domains 5 and 6 (Fig. 1E). Both mutations should prevent the production of a functional membrane receptor.

rk *and the endocrinology of the ecdysis sequence*

In Lepidoptera and Diptera, one steroid (20-Hydroxyecdysone) and four peptide [EH (eclosion hormone), PETH (pre-ecdysis triggering hormone), ETH (ecdysis triggering hormone) and CCAP (crustacean cardioactive peptide)] hormones regulate the ecdysis sequence (Gammie and Truman, 1997; Truman, 1981; Zitnan et al., 1996; Baker et al., 1999; Park et al., 1999). Collectively, these hormones coordinate the pre-ecdysis and ecdysis behaviors. By contrast, a single peptide hormone, bursicon, is known to play a role in the post-ecdysial phase of development (Cottrell, 1962a; Frankel and Hsiao, 1962). Bursicon has been shown to hasten the tanning reaction that normally begins after ecdysis, serving to harden the newly expanded cuticle. The common link between strong and weak alleles of *rk* is a failure to fully expand their wings and thoracic cuticle, giving rise to adults with crossed post-scutellar bristles and the kinked femurs that are the basis of the gene name (Edmondsen, 1948). In addition to these deficits, we observed that strong alleles of *rk* lacked the rapid tanning response characteristic of wild type. By 3 h after eclosion the cuticle of wild-type flies is tanned and the abdominal tergites are fully darkened (Fig. 2A,B). By comparison, flies homozygous for rk^{4} have not begun to darken at this time (Fig. 2C,D). This difference is no longer apparent by 6–9 h after eclosion.

Pre-eclosion and eclosion in wild-type and rk *flies*

We studied rk^{4} homozygotes to see if the events prior to adult eclosion were normal. Mutations in the *rk* gene had no effect on the time course of adult development. For example, wild-type and rk^{4} homozygote flies were collected at the white puparium stage and maintained together in the same humid chamber through metamorphosis (*N*=10 each). Under these conditions all flies of both genotypes eclosed within 1 h of each other. In addition, during the staging of pharate adult rk^{4} homozygotes, we observed all of the stages that characterize the last 12 h of adult development of wild-type flies (Kimura and Truman, 1990), suggesting that the events that immediately precede ecdysis are normal in *rk* mutants. Significantly, the only defect that is evident prior to eclosion is the flattening of the tarsi in severe alleles and deficiency

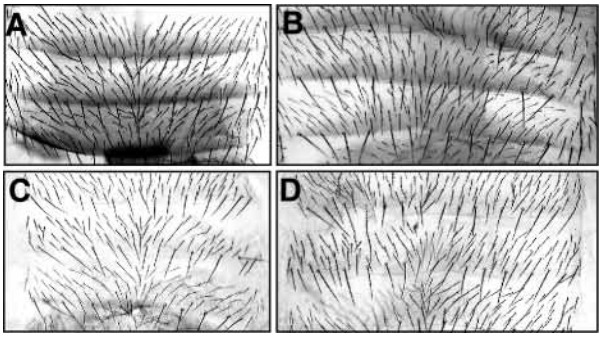

Fig. 2. Homozygous rk^{4} flies show delayed post-ecdysial tanning. Photomicrographs show the dorsal abdominal cuticle dissected from (A) wild-type males or (B) females and (C) rk^{4}homozygous males or (D) females at 3 h after eclosion. Cuticles are mounted to show the extent of melanization.

combinations (Ashburner et al., 1982). This defect arises immediately following head eversion, which is the equivalent of pupal ecdysis in other insects. Hence, this tarsal phenotype is likely also a post-ecdysial defect, albeit of pupal ecdysis rather than adult ecdysis. Based on this phenotype, a recent review suggested that *rk* might be involved in pupal limb development (Brody and Cravchik, 2000). Such an involvement, however, is probably indirect and due to a failure of the pupal cuticle to expand properly. The penetrance of the tarsal phenotype is relatively low in rk^4 homozygotes (17 %, N=60) and rk^4/rk^{w11p} heterozygotes (44 %, N=55), whereas the penetrance of the failure to expand the wings is 100 %.

Flies that have their eclosion hormone (EH) neurons genetically ablated undergo adult emergence but often show a folded wings phenotype similar to *rk* (McNabb et al., 1997). This similarity suggests that EH production or release and *rk* may be involved in the same pathway. We used immunocytochemistry to study the accumulation and release of EH in *rk* mutants. Abundant EH-immunoreactivity was present in the EH neurons of pharate adult rk^4 homozygotes. As in wild type, the EH staining was then depleted during adult ecdysis (Fig. 3A,B). Interestingly, rk^4 homozygotes still showed some residual EH-immunoreactivity in the axons after ecdysis, a result that was seen only occasionally in Canton S flies (Baker et al., 1999). Closer inspection of the axons before and after ecdysis (insets, Fig. 3) shows the loss of the brightly staining varicosities that are characteristically seen in the EH axons prior to ecdysis. It is not clear whether the small amount of residual EH is due to a subtle effect of the disruption of *rk* or due to the genetic background of the rk^4 homozygotes.

In wild-type *Drosophila*, injection of ecdysis-triggering hormone (EH) from *Manduca sexta* (MasETH) into sensitive pharate adults leads to the release of endogenous EH stores and to precocious ecdysis (Baker et al., 1999; Park et al., 1999). Injection of comparably staged rk^4 homozygotes with MasETH also induced premature ecdysis with a latency of about 50 min (Fig. 3C), the same latency as wild-type flies (Baker et al., 1999).

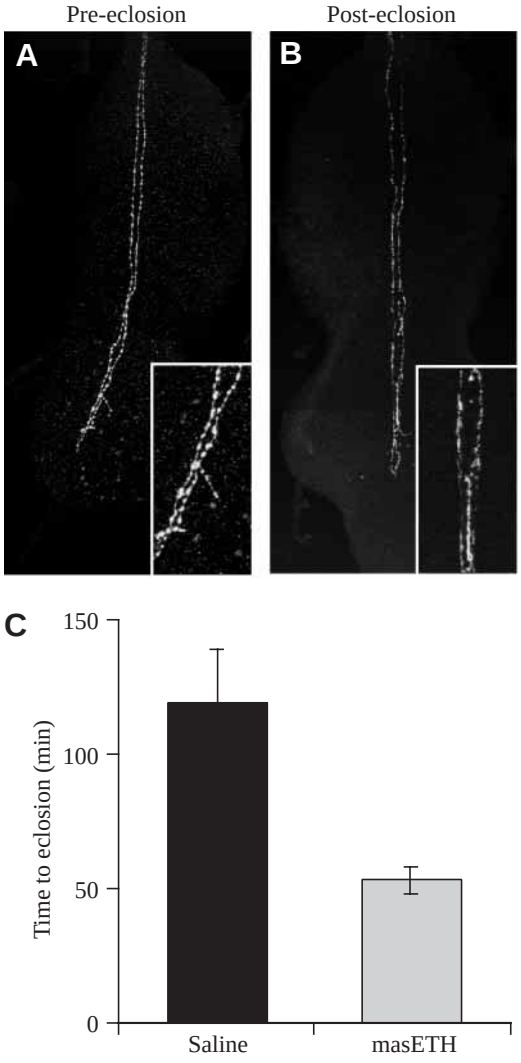

Fig. 3. Homozygous rk^4 flies show normal release of eclosion hormone at ecdysis and are responsive to injections of ecdysis triggering hormone (MasETH). (A,B) The eclosion hormone immunoreactivity in the ventral nervous system of rk^4 flies before (A) and after (B) adult eclosion. The insets show the loss of large immunoreactive swellings after eclosion. (C) The latency to adult ecdysis when pharate adult rk^4 homozygotes were injected with MasETH. The advance in the time to ecdysis is identical to that seen in normal flies. 5 nervous systems from each stage were assayed.

The post-ecdysial phase in wild-type and rk *flies*

An obvious link between *rk* and post-ecdysial processes is evident from the failure of mutant flies to expand their wings or to show the normal time-course of tanning and melanization. If *rk* were associated with the bursicon pathway, it could influence the pathway either upstream or downstream of bursicon release. If the defect in *rk* mutants is upstream of the

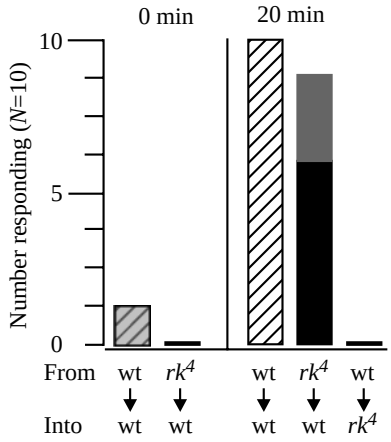

Fig. 4. Bioassays of the blood from wild-type (wt) and rk^4 flies showing that bursicon is released by the mutants. Blood was collected donors at either 0 min or 20 min after ecdysis. It was assayed for bursicon activity by injecting into either wild-type or rk^4 hosts that had been neck ligatured immediately after eclosion. Hatched bars, injection of blood from wild-type into wild-type; black bars, injection of rk^4 blood into wild-type; gray bars, partial melanization responses.

bursicon receptor, then *rk* flies may lack bursicon or fail to release it. To examine this possibility we used newly eclosed, neck-ligatured *Drosophila* (see Materials and methods) to test the blood for bursicon activity (the ability to induce cuticular tanning and melanization). As seen in Fig. 4, blood taken from flies immediately after eclosion showed no tanning activity, but blood taken from flies 20 min later, when they were actively expanding their wings, had strong tanning activity. Similarly, the blood from the rk^4 flies lacked tanning activity immediately after ecdysis but showed substantial activity 20 min later. Interestingly, these same flies did not show wing expansion behavior despite the appearance of bursicon activity in their blood (see below). In summary, the *rk* flies appear to release bursicon but show none of the physiological or behavioral changes that are normally associated with that release.

Bursicon has yet to be sequenced and synthesized, and so we could not examine the response of flies to a synthetic peptide. We did, however, examine the ability of *rk* flies to respond to bursicon-containing extracts. We neck-ligatured flies within 3 min of eclosion and then injected them with extracts prepared from the ventral nervous system of the flesh fly *Sarcophaga bullata*. These extracts caused a normal melanization response in wild-type flies. By contrast, the same extract elicited no response from neck-ligatured rk^4 flies (Fig. 5, Table 1). Ligated rk^4 flies also failed to respond to blood from wild-type *Drosophila* that were actively expanding their wings (Fig. 4). The inability of *rk* mutants to melanize in response to bursicon could occur by disrupting any one of the many components of the receptor-signaling pathway. Studies on blowflies have implicated an increase in cAMP in the action of bursicon (Seligman and Doy, 1972, 1973; Von Knorre et al., 1972; reviewed in Reynolds, 1980). As seen in Fig. 5E and Table 1, injection of rk^4 flies with 8Br-cAMP resulted in strong melanization of the abdominal tergites showing that at least this part of the response machinery is functional in the mutants. Hence, the epidermis is capable of melanization although not in response to bursicon. It should be noted that cAMP injections do not evoke components of the wing expansion program in either *rk* or wild-type flies (data not shown).

Table 1. *Response of ligatured flies to bursicon or cyclic AMP*

	% Tanning	
Solution	Wild type	rk^4/rk^4
Saline	0 (10)	0 (10)
Bursicon	100 (10)	0 (16)
cAMP	100 (6)	92 (13)

Saline solutions containing either bursicon or 8-Br-cAMP (100–200 pmoles) were injected into neck-ligatured wild-type or mutant (rk^4/rk^4) flies.

% Tanning, the percentage of animals that responded to the injection by melanizing their cuticle; the number of animals injected is given in parentheses.

Bursicon was extracted from *Sarcophaga bullata* nervous system and approximately 0.13 nervous system equivalents were injected into each fly.

Fig. 6 shows the timing of behaviors that occur immediately following eclosion in normal and mutant flies. The post-ecdysial behavior can be separated into two phases. The first is a perch selection phase, which is quite variable in duration, depending on environmental conditions, and in the types of behaviors performed. The second is the expansional phase, a stereotyped motor program that is relatively insensitive to environmental stimuli. Perch selection was complete in all flies by 2 min (N=6). Having settled on a perch, the flies then use their legs to clean their head, antennae, legs, wings and abdomen. The expansional phase begins with the ingestion of air, when the flies extend their proboscis and the cibarium begins pumping. Pumping is followed by a tonic contraction of the lateral tergosternal muscles of the abdomen. The expansional behaviors last for 7.6±0.3 min (N=11), and force

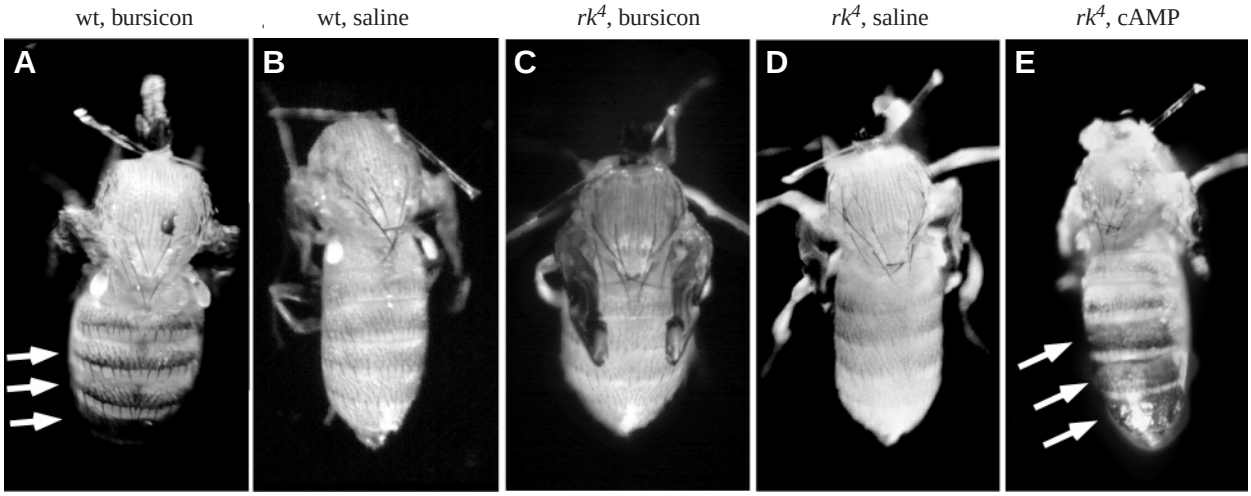

Fig. 5. The effects of the *rk* mutation on the ability of flies to respond to bursicon or its putative second messenger, cyclic AMP. Neck-ligatured wild-type (wt) flies were injected with (A) an extract containing bursicon or (B) saline. Neck-ligatured rk^4 flies were injected with (C) a bursicon extract, (D) saline or (E) 8-Br-cAMP. The arrows indicate the abdominal tergites where darkening has occurred.

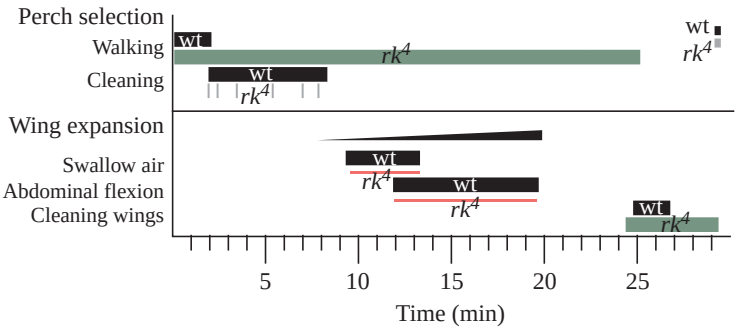

Fig. 6. A summary of the post-ecdysial behavior in wild-type and *rk* mutants. Data were collected by repeated observations during the early post-ecdysial period. Where given, behavioral durations are derived from continuous observation. Bars represent the activation of behavior. Triangles are used to represent the increasing likelihood of behavior during a period. In contrast to wild-type, rk^4 mutants do not become quiescent after eclosion and subsequently fail to initiate the expansional behaviors. A label on a red line marks behaviors missing in mutants.

blood into the thorax and wings, stretching the newly plasticized cuticle in preparation for tanning. If unmolested, the average time to the onset of wing expansion for wild-type flies is 16.6±0.6 min (*N*=16). Once expansion is completed, wild-type flies remain in place for more than 1 h, cleaning and testing the state of their wings. In contrast to wild-type flies, rk^4 flies are much more active over the first 30 min after emergence, repeatedly alternating between cleaning and walking (Fig. 6). They do not show air swallowing behavior (*N*=30), nor do they exhibit the sustained abdominal contraction characteristic of wing expansion (*N*=17).

The effects of decapitation on wing expansion behavior and tanning

In a number of higher flies [*Calliphora* (Cottrell, 1962a); *Sarcophaga* (Fraenkel and Hsaio, 1962); *Drosophila* (Kimura and Truman, 1990)], and in the moth, *Manduca sexta* (Truman and Endo, 1974) decapitation experiments have been used to define the timing of the signal that initiates wing expansion. In *Drosophila*, flies decapitated by neck ligation immediately after adult emergence do not subsequently tan or expand their wings. If the decapitation is delayed until about 10 min post-ecdysis, though, the flies can then initiate and complete wing expansion despite the removal of their head (Kimura and Truman, 1990). Thus, there appears to be a descending signal that occurs about 10 min after ecdysis that is needed to induce both bursicon release and wing expansion. A similar requirement for the head is seen in pharate adult flies that are in the extended ptilinum stage, a stage that begins about 40 min before ecdysis. These flies have just undergone their major release of EH and, when decapitated, they show rapid eclosion but never show wing expansion (Baker et al., 1999).

The requirement for the head in initiating tanning and wing expansion is different, however, in the period prior to EH release. There is a sensitive period between 3 h before ecdysis and the time of extension of the ptilinum (40 min before eclosion), when decapitation is still followed by ecdysis but only after a long and variable latency. Many of these decapitated flies also showed tanning and wing expansion with the percentage increasing as flies got closer to the extended ptilinum stage. For example, for flies decapitated 3 h before ecdysis, 25 % of the decapitated flies that ecdysed also tanned (*N*=20). For flies decapitated 55–48 min prior to ecdysis, 64 % of the ecdysing flies tanned and 36 % spread their wings after ligature (*N*=33) (Fig. 7A). No decapitated flies spread their wings without also tanning. Importantly, those flies that were decapitated early began tanning and wing expansion during or immediately following ecdysis and, thus, did not show the normal 10–15 min delay between eclosion and wing expansion. Thus, relative to ecdysis and wing expansion, the decapitation experiments identify two windows: an early window during which decapitation results in some flies showing a delayed ecdysis along with tanning and wing expansion and a late window during which decapitation is followed by immediate ecdysis but no tanning or expansion.

The involvement of EH in these windows was examined by repeating the ligature experiments on EH cell knockouts, flies that had their EH cells genetically ablated (McNabb et al., 1997). These flies were extremely reluctant to ecdyse after neck ligation, regardless of when the ligation was performed (Baker et al., 1999). Of the few that ecdysed, none tanned or spread their wings after ligation, regardless of when the ligature was performed (*N*= 14) (Fig. 7A). The involvement of *rk* in this phenomenon was examined by ligaturing rk^4 homozygotes at various times before their expected ecdysis. Flies mutant for *rk* ligated during the late window never showed either tanning or wing expansion. Surprisingly though, 54 % of ecdysing *rk* homozygotes decapitated during the first window (*N*=11) showed wing expansion behavior, although none of them tanned (Fig. 7A). The wing spreading in the decapitated *rk* flies was accomplished by sustained contraction of the lateral tergosternal muscles as in wild-type flies (Fig. 7B), but, because the cuticle did not tan, the wings refolded when the abdominal contraction terminated.

Discussion

Relationship of rickets, DLGR2 *and bursicon*

In mammals, mutations in glycoprotein hormone receptors cause developmental defects due to endocrine misregulation (Cocco et al., 1996; Gromoll et al., 1998; Muller et al., 1998). *Drosophila* has three identified members of this family of G-protein-coupled receptors (Hewes and Taghert, 2001). The gene of one of these, *DLGR2*, has been ascribed to the *rickets* mutation (Ashburner et al., 1999; Eriksen et al., 2000). Our data support the conclusion that *rk* mutations arise from changes in the *DLGR2* gene. Our STS analysis showed that

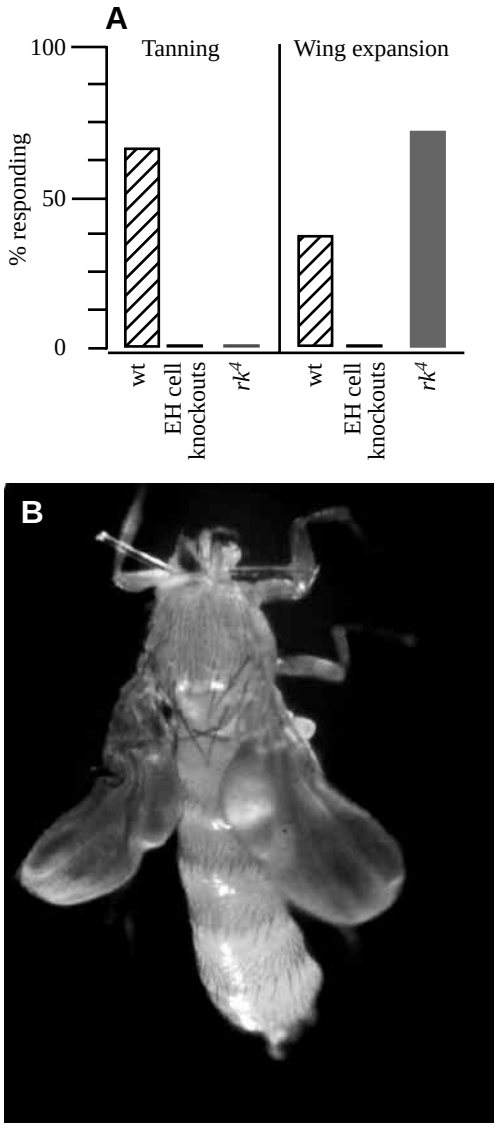

Fig. 7. Ligature experiments link eclosion hormone with the bursicon pathway and separate wing expansion from tanning. (A) Pharate adults were prepared by dissecting away the puparial case and pupal cuticle surrounding the neck region. These flies were staged using morphological markers (Kimura and Truman, 1990) and neck-ligatured. Following ligature, treated flies were maintained in a humid chamber and observed at intervals for up to 5 h. Ligature of wild-type (wt) flies prior to eclosion induces premature tanning and wing expansion behavior upon eclosion. In EH cell knockouts, tanning and wing expansion are eliminated. Flies lacking *rk* function (rk^4) did not melanize but did expand their wings. Ligature-induced wing expansion is accompanied by tonic contraction of the lateral abdominal muscles in both wild-type and (B) rk^4 mutants.

deletions that had been genetically defined as breaking within the *rk* gene (Ashburner et al., 1982) result in a partial removal of the *DLGR2* transcription unit. Also, sequencing of two strong mutant alleles of *rk* showed that these strains had mutations that resulted in premature stop codons in the critical transmembrane domain of the receptor. Hence, we are in full agreement with previous studies that suggest that *rk* mutations arise from disruption of the *DLGR2* gene.

A point of controversy, however, comes from an analysis of the phenotype that arises from the removal of the *rk* product. Eriksen et al. (2000) described the sequence and expression profile of *DLGR2* and showed that flies carrying a p-element inserted into the 5′-untranslated region of *DLGR2* had an embryonic lethal phenotype. Their paper did not, however, address the synonymy of *DLGR2* and *rk* or the fact that out of 22 reported *rk* alleles, rk^{w11p} is the only one that is lethal. The data we present here indicate that complete removal of the *rk* function is not lethal. Flies that carry deficiencies that truncate *DLGR2* from telomeric and centromeric directions (Fig. 1) are unlikely to make functional protein, but are viable and fertile with only a failure of tanning and post-ecdysial expansion. Two strong alleles, rk^1 and rk^4, are shown to contain nonsense mutations in the transmembrane containing exon and these also show the classic *rk* phenotype. Moreover, the phenotype is the same when these mutations are over deficiencies for the region. The embryonic lethality observed in the rk^{w11p} stock seems more likely to be due to the presence of an unidentified lethal mutation elsewhere on the second chromosome, or to an effect of misexpressing *DLGR2* in embryonic tissues in a way that is lethal. Our observation that the lethality and the p-element insert are separable by recombination supports the second-site lethal interpretation for the embryonic lethality. Also, crosses of rk^{w11p} with either *Df(2L)b-L* or *Df(2L)A376* give a *rk* phenotype but no embryonic lethality. From the loss of function phenotypes, we have concluded that normal *DLGR2* function is associated only with signaling processes that occur around the time of ecdysis.

The *rk* mutations specifically interfere with the last phase (the expansional phase) of the ecdysis sequence. These flies fail to expand their wings and thorax and they delay the onset of tanning and melanization. This phenotype is also observed in flies that have had their EH cells genetically killed (McNabb et al., 1997), implicating EH in the regulation of post-ecdysial expansional behavior as well as of ecdysis itself. Our analysis of the EH system in *rk* flies, however, shows that they respond normally by initiating ecdysis when challenged with MasETH, a response that requires the activity of the EH neurons (McNabb et al., 1997), and that they show EH depletion from the nervous system following eclosion. These results indicate that *rk* is probably downstream of EH release and action.

In blowflies and *Drosophila* decapitation immediately after ecdysis results in a delay in tanning that phenocopies *rk* mutations. As with the *rk* flies, such decapitated flies gradually darken over a period of several hours after decapitation (Cottrell, 1962a; J.D.B., unpublished). The lack of rapid pigmentation has been ascribed to the lack of the tanning hormone bursicon. The assay of blood from *rk* flies shortly after emergence shows that these flies contain bursicon activity and that they release it on schedule. The quantitative difference in bursicon activity between *rk* and wild type is probably due to differences in the timing; we have no behavioral assay for the time of hormone release in the mutants and, therefore, had

to rely on time after eclosion. However, *rk* flies do not respond to injection of bursicon-containing material; neither the blood from normal *Drosophila* that are expanding their wings nor CNS extracts from the fly *Sarcophaga* provoked a response. Although these flies do not melanize in response to bursicon extracts, they show prominent melanization in response to injection of cAMP, the suggested second messenger mediating bursicon action (Seligman and Doy, 1972). This result shows that the cuticle of *rk* flies has all of the machinery needed for melanization, but that it simply cannot activate this machinery in response to bursicon. It also argues that the lesion caused by *rk* probably occurs in the bursicon signaling pathway between the reception of bursicon and the production of cAMP. From the nature of the *DLGR2* product, we think that the most likely possibility is that it is the receptor for bursicon. The large extracellular domain of this family of receptors is used for binding large, glycoprotein hormones (Dufau, 1998). Intriguingly, bursicon is one of the largest of the insect hormones, with an estimated molecular mass of 33 kDa (Kostron et al., 1995). Because of its size, however, this hormone has yet to be isolated and sequenced. Therefore, we cannot yet test directly whether DLGR2 can bind bursicon.

The regulation of wing expansion behavior

Hormonal and behavioral control over wing expansion in *Drosophila* is time dependant and appears to involve both EH and *rk* (and, by inference, bursicon). In wild-type individuals, decapitation within 10 min of ecdysis prevents both the release of bursicon (shown by their lack of rapid tanning) and wing expansion (Kimura and Truman, 1990). During a transition period starting at around 10 min post-ecdysis, decapitated flies would occasionally tan without wing expansion, but not *vice versa*. Shortly thereafter, both tanning and wing expansion consistently followed decapitation. This timing suggests that bursicon release and wing expansion are activated at about 10 min post-ecdysis in a stereotypical sequence, with bursicon release being followed by activation of the wing expansion program. This pattern is consistent with a model in which bursicon release triggers the wing expansion motor programs. We found that in *rk* mutants, the release of bursicon occurs on schedule (Fig. 4) but the expansional behaviors fail to occur (Fig. 6). If *rk* does indeed encode the bursicon receptor, then this is the first direct evidence that bursicon activates the wing expansion program.

In *Drosophila*, the effects of post-ecdysial ligatures mirror those seen in the blowflies *Sarcophaga* and *Calliphora* (Cottrell, 1962a; Fraenkel and Hsaio, 1962; Kimura and Truman, 1990). Surprisingly, we have identified a pre-ecdysial period during which ligation activates the post-ecdysial behavior and melanization rather than inhibiting it. There is an early response window that begins when the animals reach the ‘grainy stage’ approximately 3 h before ecdysis. Pharate adults never eclose if decapitated prior to this time but routinely eclose if decapitated after they start the grainy stage. The latencies from decapitation to ecdysis ranged from 20 min to 5 h, with some flies emerging earlier than expected, based on their developmental stage, and other flies emerging later. None of the late emerging flies were seen to then tan or spread their wings, but, by contrast, most of the prematurely ecdysing individuals showed normal tanning and wing expansion despite lacking their head. Interestingly, these flies began wing expansion during or immediately after ecdysis rather than waiting the 10–15 min that is typical for intact animals. The significance of this difference in timing will be discussed below. During this early window, between 3 h and 50 min before ecdysis (the start of the ‘extended ptilinum stage’; Kimura and Truman, 1990), the fraction of flies showing precocious ecdysis and wing expansion gradually increased with time. A different set of responses were observed starting at about 50 min before ecdysis. During this second window, the latency period before ecdysis, after decapitation, was abruptly reduced down to about a minute or less (Baker et al., 1999), but tanning and wing expansion never occurred. Therefore, in terms of wing expansion, flies decapitated during this second window behaved in the same way as flies decapitated immediately after eclosion.

Two pieces of evidence link these response windows to EH. First, the transitions in the response of developing flies to decapitation correlate with immunocytochemical changes in the EH-expressing cells (Baker et al., 1999). At 4–7 h before ecdysis there is a shift in EH immunostaining that represents either an initial release of EH or a redistribution of EH to the axon terminals. At around 50 min before ecdysis the major depletion of the EH neurons occurs. Second, flies that lack EH neurons do not show premature ecdysis in response to decapitation (Baker et al., 1999), and they do not subsequently tan or expand their wings. In contrast, experiments with *rk* flies suggest that bursicon is not involved in the early window. When decapitated during the initial window, early ecdysing mutant flies also expand their wings, although the wings subsequently collapse because tanning does not occur. Hence, the elements driving the wing expansion program are present in the mutant but cannot be activated in response to bursicon after eclosion.

These data, coupled with our findings that flies decapitated prior to adult ecdysis often spread their wings, suggest that an inhibitory component descending from the brain/SEG suppresses these ecdysial and post-ecdysial motor programs. At about 3 h before ecdysis developmental changes make the ecdysis and post-ecdysis motor programs competent to be expressed. The data from the EH cell knockout flies suggest that the EH neurons are involved with this competence, and it may be a low-level release of EH that initially activates the motor programs. Although expansion occurs along with a response to bursicon in this case, it does not depend on it as shown by the ability of *rk* flies to expand their wings if decapitated at this early time. The major release of EH at 50 min before ecdysis causes a strong activation of the ecdysis program but suppresses both bursicon secretion and the wing expansion program. Decapitation after this EH release is rapidly followed by ecdysis but wing expansion is never displayed (Baker et al., 1999). Subsequent descending

commands from the head that occur after ecdysis bring about bursicon release and this, in turn, is required to activate the expansional program.

This seemingly complex control may have arisen from elaboration of a simpler control system evident in most other insects. In many insects (Carlson, 1977; Hughes, 1980; Mills, 1967, 1966; Reynolds, 1980; Srivavista and Hopkins, 1975) ecdysis and tanning are closely linked and expansion begins as the insect is escaping from the old cuticle. By contrast, insects that pupate underground or in confined sites delay the expansion of delicate wings until the insect has dug its way to freedom. In these insects, the fixed relationship between the ecdysial and expansional phases has been replaced by a period of behavioral flexibility that allows escape from a buried pupation chamber before the initiation of cuticular expansion and hardening. This separation is found in the Cycloraphous Diptera and also in some moths such as the tobacco hornworm, *Manduca sexta* (Truman and Endo, 1974).

Intriguingly, during larval ecdysis of *Manduca* and *Drosophila*, expansion is already underway at the start of ecdysis. Hence, in these early stages both programs may be under the direct activation of EH. This link between ecdysis and expansion must be reconfigured during adult development to give the delay between ecdysis and expansion that is characteristic of adult behavior. It appears that in *Drosophila*, the larval relationships may remain intact but are masked by a strong inhibition that is imposed at the time of the major release of EH, about 50 min before ecdysis. Decapitation prior to this time may reveal the persisting larval circuit that links the ecdysial and post-ecdysial phases under common control of EH.

In most insects, the activation of ecdysis and tanning are linked to limit the risk of desiccation and predation to the soft, flexible post-ecdysis cuticle. In *Drosophila* and *Manduca*, evolution has replaced this program with a period of behavioral flexibility that permits the adult insect to take advantage of sensory information to escape its pupal confinement and to control the time and place in which the expansional behaviors are initiated.

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