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RESEARCH ARTICLE

Eclosion gates progression of the adult ecdysis sequence of *Drosophila*

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SUMMARY

Animal behavior is often organized into stereotyped sequences that promote the goals of reproduction, development and survival. However, for most behaviors, the neural mechanisms that govern the order of execution of the motor programs within a sequence are poorly understood. An important model in understanding the hormonal determinants of behavioral sequencing is the ecdysis sequence, which is performed by insects at each developmental transition, or molt. The adult ecdysis sequence in *Drosophila* includes the emergence of the insect from the pupal case followed by expansion and hardening of the wings. Wing expansion is governed by the hormone bursicon, and stimulation of the bursicon-expressing neurons in newly eclosed flies induces rapid wing expansion. Here we show that that such stimulation delivered prior to eclosion has no immediate effect, but does cause rapid wing expansion after eclosion if the stimulus is delivered within 40 min of that event. We observe a similar delayed effect upon stimulation of a single pair of bursicon-expressing neurons previously identified as command neurons for wing expansion. We conclude that command neuron stimulation enables the motor output pathway for wing expansion, but that this pathway is blocked prior to eclosion. By manipulating the time of eclosion, we demonstrate that some physiological process tightly coupled to adult ecdysis releases the block on wing expansion. Eclosion thus serves as a behavioral checkpoint and complements hormonal mechanisms to ensure that wing expansion strictly follows eclosion in the ecdysis sequence.

Key words: hormones, behavior, innate, circuit.

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INTRODUCTION

One of the most salient features of behavior is its temporal organization. Over both short and long time scales, the motor output of the nervous system is assembled into relatively identifiable sequences. The mechanisms used by the nervous system to generate such sequences range from the central pattern generating networks used to produce rhythmic motor sequences underlying such things as locomotion and feeding (Marder and Bucher, 2001), to the broadly distributed neuroendocrine networks that organize complex behavioral sequences, such as those that characterize developmental or reproductive cycles in many animals (Ewer and Reynolds, 2002; Pfaff et al., 2006). Sequences of the latter type typically depend on the patterned release of hormones to either direct motor output or alter behavioral priorities, and the timing of release of successive hormones is typically dependent upon both intrinsic neural and endocrine signaling as well as on environmental cues (Wingfield, 2006). Developing a detailed understanding of how neuroendocrine networks function to ensure the type, timing and order of hormonal release – and consequently the correct progression of a behavioral sequence – is a major challenge of behavioral neurobiology.

A well-studied model for understanding the mechanisms that govern hormonally regulated behavioral programs is the ecdysis sequence, which is used by insects to periodically shed their exoskeletons (Truman, 2005; Žitňan et al., 2007). This sequence can be divided into three phases, typically called pre-ecdysis, which involves breaking the attachments to the cuticle of the previous developmental phase; ecdysis, which involves shedding the old cuticle; and post-ecdysis, which typically consists of expanding and hardening a new cuticle into which the insect can grow. Evidence indicates that progression through these three phases is orchestrated by the patterned release of hormones and neuropeptides, with sensory cues able to catalyze particular steps. The hormonal mechanisms responsible for the initiation of the ecdysis sequence and for the transition from pre-ecdysis to ecdysis have been largely elucidated for the hawkmoth *Manduca sexta* (Žitňan and Adams, 2012). The transition from ecdysis to post-ecdysis, however, is less well understood in any insect, though there is evidence that the onset of post-ecdysial behaviors in both crickets and grasshoppers is triggered when sensory signals report the removal of the old exoskeleton (Carlson, 1977; Hughes, 1980).

The behavioral transition from ecdysis to post-ecdysis is particularly well defined for the final, adult molt in holometamorphic insects, such as *Drosophila melanogaster*, where the insect first completes ecdysis by emerging from the pupal case and then proceeds to expand and harden its newly formed wings along with the adult cuticle (Baker and Truman, 2002; Peabody et al., 2009). Both the somatic and behavioral aspects of wing expansion in flies are known to require the hormone bursicon (Dewey et al., 2004; Honegger et al., 2008; Lahr et al., 2012). Here we probe the mechanisms that regulate bursicon release and the transition from ecdysis to post-ecdysial behavior by using the cold-activated TRPM8 (transient receptor potential cation channel, subfamily M member 8) channel to perform targeted activation of the bursicon-expressing neurons at different times relative to natural eclosion. We find that both bursicon release into the hemolymph and the wing expansion motor output pathway are inhibited prior to eclosion, and

that release from inhibition is dependent, at least in part, upon eclosion. Eclosion therefore acts as a checkpoint to guarantee that wing expansion is executed strictly after eclosion.

MATERIALS AND METHODS

Fly culture and crosses

All flies (*Drosophila melanogaster* Meigen 1830) were grown on corn meal–molasses medium and maintained at 25°C in a constant 12 h:12 h light:dark cycle. The $w^{1118};+;+$ line was from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA). The following lines used in this study have been described previously: *w;Burs-Gal4;+* (Peabody et al., 2008); $w;ET^{VP16AD}$*-99;* $Burs^{Gal4DBD}$*–U6A1* and $w;ET^{VP16AD}$*-N9A88A;* $Burs^{Gal4DBD}$*-U6A1* [hemidriver combinations that specifically target the bursicon-expressing neurons in the subesophageal (B_{SEG}) and abdominal ganglia (B_{AG}), respectively] (Luan et al., 2012); *w;CCAP-Gal4;+* (Luan et al., 2006a); and $w;+;UAS\text{-}TRPM8^{C4A}$ (Peabody et al., 2009).

Behavioral observations and analysis

The inhibitory 'minichamber' environment has been described before (Peabody et al., 2009) and consists of a 0.3×0.7 cm (diameter × length) glass cylinder. For video recordings, late-stage pharate adults were first collected from food vials using a wet brush and their puparia were placed on a glass slide and scored for developmental stage according to criteria developed by the Truman laboratory (Baker et al., 1999; Kimura and Truman, 1990). The puparia of animals at the grainy to extended ptilinum stages (i.e. within several hours to minutes of eclosion) were selected for studies of naturally eclosing flies. These puparia were placed into minichambers before being temperature shifted from 24 to 18°C for 15 min by transfer to an incubator. For experiments in which pharate adults were forced to eclose early, we selected animals at the extended ptilinum stage (i.e. within 15–45 min of eclosion) and removed their opercula with forceps. This manipulation alone often led to eclosion within 2 min, but when it did not, vigorous stroking of the bristles and antennae usually succeeded in getting the animals to emerge. To delay eclosion, we chose animals at the extended ptilinum stage, placed the puparia in minichambers and occluded the operculum with a cotton plug for 90 min (making them on average ~60 min late for eclosion). After a 15 min temperature shift (note: negative control animals were maintained at 24°C), the cotton plug and operculum were carefully removed, allowing the animals to eclose into a minichamber. Finally, for experiments involving adults that had eclosed normally and were then subjected to a temperature shift, standard food vials were cleared every 5 min and recently eclosed flies were placed individually into minichambers and subjected to temperature shifts as described above. Box plots were generated using the PTS Charts (www.peltiertech.com) add-on for Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Videorecording was carried out at 24°C using either a Sony HDR-FX7 or HDR-XR550 digital video camera (Sony, Tokyo, Japan), and records were analyzed using Sony PMB software (version 5.6). Wing 'expansion time' was measured as the time of maximum abdominal flexion, which normally coincides with wing expansion. We chose this measure because it can be used both with flies that successfully expand their wings, and also with flies that exhibit wing expansion behavior but fail to expand their wings, a situation that sometimes occurs after considerable delays in the minichamber. Wing expansion failure can also be seen in positive controls and is likely due to progressive stiffening of the cuticle because of 'secondary' tanning processes (Cottrell, 1962).

To assay for air swallowing, animals were dissected as described previously (Peabody et al., 2009) to expose the intestinal tract and crop after pre-eclosion temperature shift. Air bubbles, if present, were typically small and were recorded as either present or absent without being measured.

Immunohistochemistry and immunoblotting

In most cases, flies were killed for hemolymph collection either directly after the end of a temperature shift (t=0 min) or 15 min later. In the case of 'negative control' animals, the exact genotype and procedure varied. Negative controls typically were not subjected to a temperature shift (i.e. remained at 24°C throughout), but had identical genotypes (and were otherwise treated in parallel) to the experimental animals. Hemolymph from these animals was collected at the same time as it was from the experimental animals. For the experiment in which N_{Burs} stimulation occurred pre-eclosion and animals were killed 5 min after eclosion, negative control animals lacked a driver (genotype: w;+;UAS-TRPM8) and were treated identically to experimental flies.

Hemolymph collection from newly eclosed adults was carried out essentially as described previously (Peabody et al., 2009). A somewhat different protocol was used for pharate adults in that hemolymph was collected directly into microcapillary tubes from the impaled ptilinum after opening of the operculum. Collected hemolymph was then injected into buffer. Hemolymph for positive control (+) samples was collected from either wild-type Canton S or Burs-Gal4>UAS-TRPM8 flies that were killed within an hour of eclosion and had expanded their wings. An exception was the Burs-Gal4>UAS-TRPM8 flies that had eclosed normally but were forced to expand their wings by subjecting them to a 15 min temperature shift from 24 to 18°C, followed by incubation at 24°C for an additional 15 min before collection. In general, samples were pooled, with 14–17 flies needed to collect 1 µl of hemolymph. Western blotting was carried out as described previously (Peabody et al., 2009) using anti-bursicon alpha-subunit antibody at 1:2000.

RESULTS

Pre-eclosion activation of bursicon neurons does not acutely induce wing expansion

Wing expansion results from the combined execution of two principal motor patterns, namely, air swallowing and persistent abdominal contraction, coupled with the hormonally mediated plasticization of the wing pads (see Baker and Truman, 2002; Peabody et al., 2009 and references therein). The hormone responsible for both wing plasticization and for wing expansion behaviors is bursicon, which is expressed in a small set of neuroendocrine cells in the abdominal ganglion (B_{AG}) and a single pair of cells in the subesophageal ganglion (B_{SEG}) (Peabody et al., 2008). The B_{SEG} secrete bursicon into the central nervous system and principally mediate the behavioral effects of the hormone, while the B_{AG} secrete bursicon into the hemolymph to mediate its somatic effects (Luan et al., 2012). Under normal circumstances, bursicon secretion is initiated shortly after eclosion and results in wing expansion within ~20 min of that event. However, if a fly is placed in a tightly confined space (a so-called 'minichamber') only several times larger than its body length, it will delay expansion for up to several hours (Peabody et al., 2009). We have previously demonstrated that this inhibitory effect of confinement can be overcome by acutely activating either the full set of bursicon-expressing neurons (N_{Burs}) or, remarkably, by activation of the B_{SEG} alone (Luan et al., 2012). Confinement may mimic the pre-eclosion constraints of the puparium, and we asked whether acute activation

of N_{Burs} might similarly force wing expansion in flies that had not yet eclosed.

To examine the effects of pre-eclosion stimulation of N_{Burs} on wing expansion, we selected pharate adults within 0–2 h of eclosion and activated N_{Burs} using the cold-sensitive UAS-TRPM8 channel. In contrast to post-eclosion stimulation in Burs-Gal4>UAS-TRPM8 flies, which results in rapid wing expansion following a 15 min temperature shift from 24 to 18°C, we found that pre-eclosion stimulation in such flies never caused wing expansion within the puparium, nor did it induce precocious wing expansion behavior. No abdominal movements similar to the characteristic elongation and flexion seen during normal wing expansion were observed. Dissection of some animals (N=11) revealed no air in the gut, indicating that none had initiated air swallowing in response to N_{Burs} stimulation. Indeed, stimulated flies exhibited only the abdominal muscle movements characteristic of normal pre-eclosion behavior. All also extended their ptilina and eclosed properly without any obvious aberrations in wing morphology.

All of the above experiments were conducted on pharate adults placed in glass minichambers so that behavior both before and after eclosion could be monitored. Interestingly, we found that after eclosion a subset of N_{Burs}-stimulated flies lacked the environmental sensitivity typical of normal flies and expanded their wings within ~50 min (Fig. 1), despite the confines of the minichamber. By systematically correlating the time between wing expansion and eclosion with the time between N_{Burs} stimulation and eclosion, we determined that flies that eclosed within 40 min of stimulation exhibited rapid expansion (35±20 min, N=29), whereas flies that remained in the puparium for longer periods exhibited delayed wing expansion times, similar to those of genetically identical control animals that were not subjected to the temperature shift (i.e. N_{Burs} was not stimulated). These control animals took a mean of 246±67 min (N=56) to expand their wings after eclosion. Additional control animals that lacked the UAS-TRPM8 transgene were subjected to the 15 min temperature shift and likewise exhibited

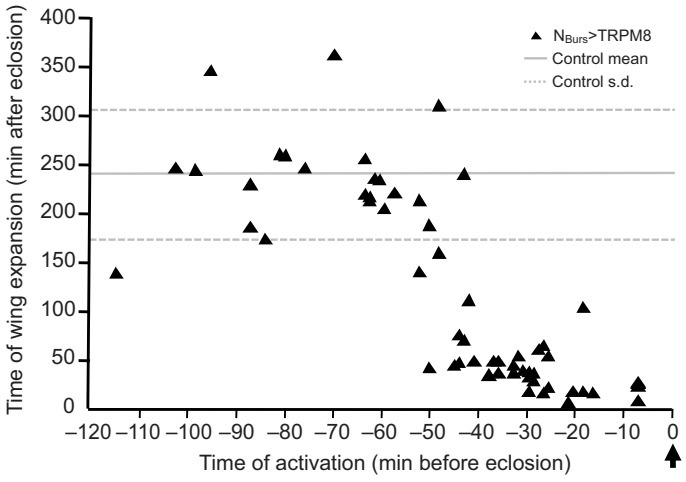

Fig. 1. Pre-eclosion stimulation of bursicon-expressing neurons accelerates wing expansion after eclosion if delivered within 40 min of emergence. N_{Burs}>TRPM8 pharate adult *Drosophila melanogaster* (N=62, ▲) were stimulated and then scored for both the time to eclose and the time to expand their wings after eclosion. The mean time of wing expansion for control flies (solid line, N=52), in which bursicon-expressing neurons (N_{Burs}) were not activated, is shown together with the standard deviations from the mean (dashed lines). All experiments were conducted in the confined environment of minichambers (see Materials and methods). Arrow, time of eclosion.

delayed wing expansion (mean=219±82 min, N=31), indicating that rapid wing expansion in N_{Burs}-stimulated animals was not an artifact of the cooling temperature shift. Our observations of flies in which N_{Burs} neurons were stimulated prior to eclosion leads to three interesting conclusions: (1) execution of the behavioral program for wing expansion is blocked prior to eclosion; (2) this program is, however, available for execution immediately after eclosion provided N_{Burs} stimulation is carried out within ~40 min of that event; and (3) N_{Burs} stimulation is ineffective in causing rapid wing expansion if it is delivered any time prior to ~50 min before eclosion. This 50 min period corresponds to the so-called 'extended ptilinum' phase defined by the staging criteria of Kimura and Truman (Kimura and Truman, 1990).

Pre-eclosion activation of bursicon neurons induces bursicon release only after eclosion

In addition to specific behaviors, wing expansion requires somatic changes – likely at the level of the epidermis – which are mediated by bursicon release into the hemolymph (Honegger et al., 2008). These changes include the 'plasticization' of the wings (rendering them capable of expansion) followed by 'tanning' of the expanded wing cuticle. Tanning, which involves both hardening and pigmenting of the cuticle, occurs not just at the level of the wing, but body-wide to form the new exoskeleton of the adult fly. We have previously shown that newly eclosed flies show robust release of bursicon into the hemolymph upon stimulation of bursicon-expressing neurons (Luan et al., 2012), but our examination of the flies in which stimulation occurs prior to eclosion showed no evidence of either premature plasticization or tanning. It remained possible, however, that bursicon was released into the hemolymph upon stimulation in these flies, but that the epidermis was not yet competent to respond to it. To directly determine whether bursicon release occurs, we performed western blot analysis on hemolymph collected from N_{Burs}>TRPM8 pharate adults immediately after a 15 min temperature shift to 18°C (from 24°C), or 15 min later, but still prior to eclosion. Contrary to what we observed in recently eclosed adults (Peabody et al., 2009), this manipulation did not result in detectable amounts of bursicon either immediately after TRPM8 activation (Fig. 2A, lanes 3 and 4) or 15 min later (Fig. 2A, lanes 5 and 6).

Because bursicon is required for wing expansion (Dewey et al., 2004), and flies in which N_{Burs} is stimulated within 40 min of eclosion expand their wings shortly after eclosion, we reasoned that bursicon release into the hemolymph in these flies must, like the behavioral response, be delayed until after eclosion. To test this, we extracted hemolymph from flies that eclosed within 5 min of a pre-eclosion temperature shift and examined it by western blot for bursicon. As shown in Fig. 2B (lane 3), we found detectable levels of bursicon in these hemolymph samples, indicating that the hormone is indeed released in a delayed fashion that correlates with the behavioral response. Control flies that lacked the Burs-Gal4 driver and underwent the same experimental procedures showed no detectable bursicon in the hemolymph (Fig. 2B, lane 2). These results indicate that not only the motor, but also the neuroendocrine, outputs of the wing expansion pathway are suppressed prior to eclosion.

The effects of TRPM8 activation are strictly correlated with eclosion

Our results have shown that pre-eclosion N_{Burs} stimulation results in some persistent change in the nervous system – at the level, or downstream, of the bursicon-expressing neurons themselves – that causes rapid wing expansion, but only after the fly has eclosed. A

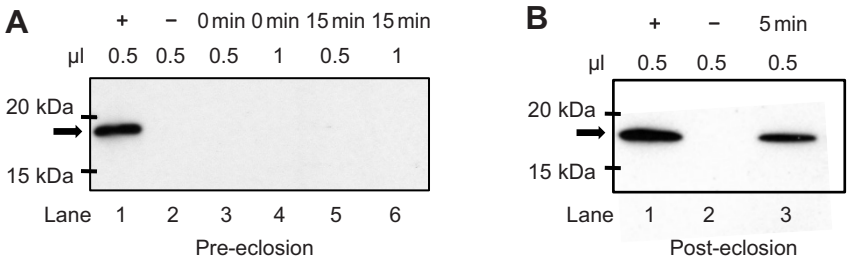

Fig. 2. Pre-eclosion activation of N_{Burs} does not cause release of bursicon until eclosion has been completed. (A) Western blot of hemolymph extracted from pharate adult *Drosophila melanogaster* subjected to pre-eclosion N_{Burs} stimulation, and killed either immediately (0 min) or 15 min later, as indicated. (B) Western blot of hemolymph extracted from adults subjected to N_{Burs} stimulation just prior to eclosion, and killed 5 min after eclosion. For each blot, hemolymph from positive (+) and negative (–) control flies (containing and not containing bursicon, respectively; see Materials and methods) are included for reference. Negative control animals in B (lane 2) were not subjected to N_{Burs} stimulation, but were otherwise treated identically to the experimental animals (lane 3). Presence of bursicon was assayed using an anti-bursicon alpha-subunit antibody (arrow). Hemolymph volume (µl) is shown; molecular weight markers (kDa) are as indicated.

crucial question is whether eclosion itself acts as a gate to allow the effects of the pre-eclosion stimulus to be realized, or whether eclosion is merely coincident with some other physiological change that serves as such a gate. It is possible, for example, that some hormone (e.g. eclosion hormone) coordinately regulates the timing of eclosion and the physiological change that permits wing expansion in response to N_{Burs} stimulation. If this were true, we reasoned that it should be possible to dissociate eclosion from the fly's ability to respond to N_{Burs} stimulation (by expanding the wings). To test this, we manipulated the timing of eclosion by mechanical means, either forcing flies to eclose prematurely or delaying their eclosion for up to an hour, and then examined the effects of these manipulations on the ability of flies to expand their wings in response to N_{Burs} stimulation.

In the course of obtaining hemolymph from pharate adults at the extended ptilinum stage, we made the observation that many animals quickly eclosed upon removal of the operculum. Those that did not immediately eclose could often be encouraged to eclose by stimulating the exposed head with a brush. We took advantage of this observation to force N_{Burs}>TRPM8 flies to eclose prematurely, after which we immediately subjected them to a 15 min temperature decrement to 18°C (from 24°C) and examined them for either bursicon release into the hemolymph (Fig. 3A) or behavior (Fig. 3B). Western blot analysis demonstrated that these flies released bursicon into the hemolymph immediately after the temperature shift (Fig. 3A, lane 1), just like naturally eclosing adults (Fig. 3A, lane 3). Flies forced to eclose prematurely took on average 26±3 min (N=10) to expand their wings in minichambers, after the start of the temperature shift, compared with 244±56 min (N=13) for control flies that lacked a Gal4 driver but were treated identically (Fig. 3B). The observed acceleration of wing expansion in flies forced to eclose prematurely was similar to that seen in flies that had eclosed normally in minichambers: these N_{Burs}>TRPM8 flies expanded their wings within 27±4 min (N=13) of the temperature shift, whereas control flies that lacked a Gal4 driver showed no response to the temperature shift and took 160±57 min (N=20) to expand their wings (Fig. 3B). We conclude that flies forced to eclose during the extended ptilinum phase have the capacity to respond to N_{Burs} stimulation by releasing bursicon into the hemolymph and executing wing expansion. The fact that they do not do so while they are still in the puparium suggests that it is this confinement that inhibits the expansion processes.

In a similar set of experiments, we examined the effects of delaying eclosion by placing a cotton plug over the operculum and removing it approximately an hour after the anticipated time of eclosion (based on ptilinum extension). Removal of the plug together with the operculum typically led to rapid eclosion of animals trapped in the puparium in this manner. To test the response of such animals to N_{Burs} stimulation, we subjected N_{Burs}>TRPM8 flies to a 15 min temperature decrement directly prior to releasing them. Despite their artificially delayed eclosion time, these animals exhibited a similar response to stimulation as N_{Burs} flies stimulated just prior to natural eclosion: they showed no overt behavioral response to the pre-eclosion temperature shift, but rapidly expanded their wings once allowed to eclose into the environment of the minichamber. Their mean time to wing expansion (16±2 min, N=13) was over 10-fold faster than that of controls (189±36 min, N=10) not subjected to TRPM8 activation by a temperature shift (Fig. 4A). Western blot analysis in animals prevented from eclosion further showed that bursicon was absent in the hemolymph of N_{Burs}>TRPM8 flies directly after stimulation, but appeared in the hemolymph within 5 min of eclosion (Fig. 4B).

Taken together, our results show that release of bursicon into the hemolymph and the execution of wing expansion behaviors in response to N_{Burs} stimulation correlates strictly with eclosion even when the timing of that event is altered. This strongly suggests a causal relationship between eclosion and the ability to respond to N_{Burs}>TRPM8 activation and implies that a change in some physiological variable(s) closely coupled to eclosion permits rapid bursicon release and wing expansion under environmentally inhibitory conditions.

Pre-eclosion stimulation of the B_{SEG} alone results in post-eclosion activation of the wing expansion pathway

The failure of pre-eclosion N_{Burs} stimulation to directly promote detectable bursicon release into the hemolymph was striking given that a subset of bursicon-expressing neurons in the abdominal ganglion (i.e. the B_{AG}) is directly responsible for this release. Indeed, we have previously shown that selective activation of the B_{AG} after eclosion elicits bursicon release into the hemolymph (Luan et al., 2012). Our results therefore suggest that the B_{AG} are electrically suppressed prior to eclosion. If this is the case, selective pre-eclosion stimulation of these neurons should be without either immediate or delayed effect, whereas stimulation of the bursicon-expressing neurons located in the subesophageal ganglion (i.e. the B_{SEG}) should replicate the effect of stimulating all of N_{Burs}, as occurs in newly eclosed animals. To test these predictions, we used the Split Gal4 system (Luan et al., 2006b) and previously described hemidrivers

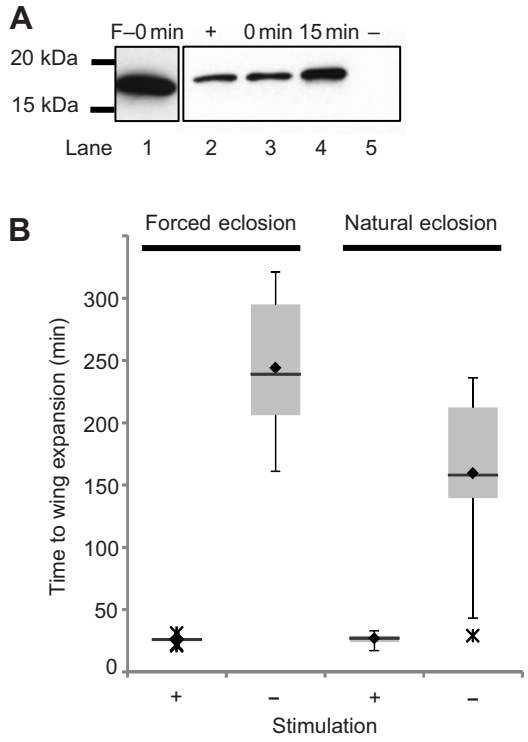

Fig. 3. *Drosophila melanogaster* forced to eclose ~30 min prior to natural eclosion release bursicon and expand their wings rapidly in response to N_{Burs} activation. (A) Western blots of hemolymph collected immediately after stimulation (0 min) from flies forced to eclose early (F, lane 1), and from naturally eclosing flies, extracted either 0 or 15 min after N_{Burs} stimulation (lanes 3 and 4, respectively). Hemolymph from positive (+) and negative (–) control flies is shown for comparison. Lane 1 contained 1.0 µl of hemolymph, and lanes 2–5 contained 0.5 µl of hemolymph each. (B) Time to wing expansion of flies forced to eclose early and then subjected (+) or not subjected (–) to N_{Burs} stimulation, or flies allowed to eclose naturally and treated similarly. The inner quartiles of each sample are represented by light gray boxes, separated at the median by a thin line. The height of the gray boxes together makes up the interquartile (IQ) range. The range of data falling within 1.5 IQ ranges of the median is represented by whiskers. Any outliers that fall between 1.5 and 3 IQ ranges from the mean are represented by asterisks. The mean of each sample is represented by a diamond overlying the box and whisker for that sample.

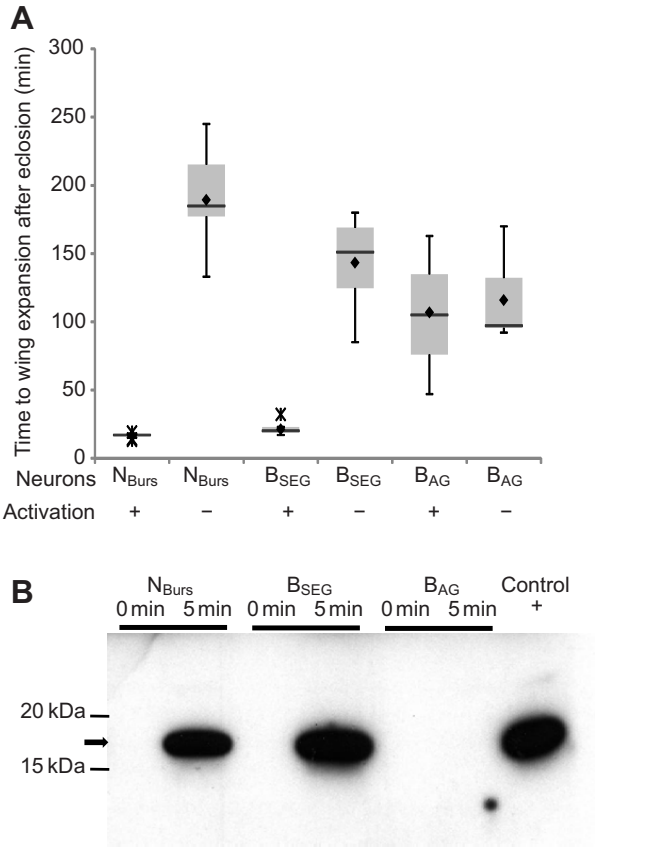

Fig. 4. *Drosophila melanogaster* prevented from eclosion respond to activation of either N_{Burs} or the B_{SEG} alone, with accelerated wing expansion and bursicon release after eclosion. (A) Time to wing expansion after eclosion. Activation of TRPM8 in the indicated cell group was performed by temperature shift of animals from 24 to 18°C (+). Unshifted control animals (–) remained at 24°C throughout. Pairwise comparisons of the distributions for each neuronal group (+ and – activation) showed the differences to be very significant (P<<0.01) except for the B_{AG} (P=0.3, Student's t-test). (B) Western blot analysis of hemolymph taken from flies in which the indicated cell group was activated as in A. Flies were killed immediately after the temperature shift and induced eclosion (0 min), or after an additional 5 min incubation in minichambers at 24°C (5 min). Control, control hemolymph containing bursicon for reference. B_{SEG} and B_{AG}, subsets of bursicon-expressing neurons in the subesophageal and abdominal ganglia, respectively. Note that the strength of bursicon immunoreactivity (arrow) in some lanes (e.g. 5 min lanes) bleeds into the adjacent (i.e. 0 min) lanes on the blot.

(Luan et al., 2012) to selectively target each of the two subsets of N_{Burs} for TRPM8 stimulation. Hemolymph collection from multiple animals is easier when their eclosion times are similarly staged, so we tested the effects of pre-eclosion stimulation on animals in which eclosion was artificially delayed for 1 h. As before, the 15 min temperature shift to 18°C was performed just prior to releasing the animals and allowing them to eclose.

As in our previous experiments, we noted no immediate behavioral response to the temperature shift in either B_{AG}>TRPM8 or B_{SEG}>TRPM8 flies, and in neither case was bursicon found in the hemolymph immediately after eclosion (Fig. 4B, 0 min lanes). B_{AG}>TRPM8 flies temperature-shifted prior to eclosion also did not exhibit rapid wing expansion after eclosion, taking on average 107±40 min (N=9) to expand their wings, as compared with 116±32 min (N=6) for non-temperature-shifted control flies (Fig. 4A). Importantly, western blot analysis revealed that B_{AG}>TRPM8 flies also lacked detectable bursicon release into the hemolymph both immediately after, and within 5 min of, eclosion (Fig. 4B, B_{AG} lanes). This was in contrast to the results obtained with B_{SEG}>TRPM8 flies, which, when subjected to temperature shift prior to eclosion, showed robust levels of bursicon in the hemolymph 5 min after eclosion (Fig. 4B, 5 min B_{SEG} lane), and also rapidly expanded their wings (21±5 min, N=9) despite the confined environment of the minichamber. B_{SEG}>TRPM8 flies not subjected to the pre-eclosion temperature shift took 143±36 min (N=7) to complete wing expansion (Fig. 4A). Because the effects of pre-eclosion stimulation of the B_{SEG} alone mimic those of stimulating all bursicon-expressing neurons and stimulation of the B_{AG} is without apparent effect, we conclude that the B_{SEG} are the relevant site of action in pre-eclosion N_{Burs} stimulation.

DISCUSSION

To successfully expand its wings, the newly metamorphosed fly must delay wing expansion until after eclosion. Although wing

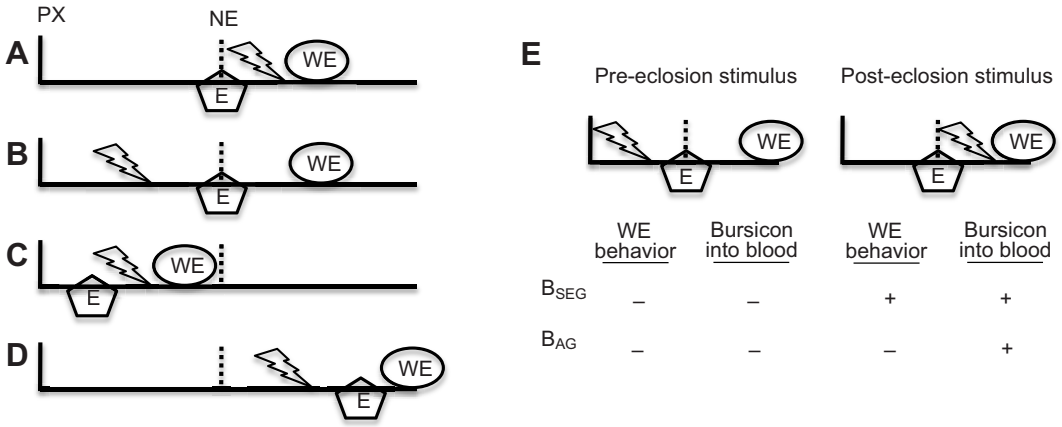

Fig. 5. Summary schematics of the effects of N_{Burs} stimulation in *Drosophila melanogaster*. (A–D) Wing expansion is elicited by stimulation of the N_{Burs} only after eclosion, even when the timing of eclosion is artificially shifted. Natural eclosion (NE, dotted vertical line) normally occurs ~40 min after the onset of ptilinum extension (PX, solid vertical line). (A) If N_{Burs} stimulation (lightning bolt) is delivered to flies that eclose (E, pentagon) naturally under confined conditions, which normally delays wing expansion for hours, wing expansion (WE, oval) is rapidly induced. (B) If N_{Burs} stimulation is delivered between ptilinum extension and eclosion, accelerated wing expansion is induced, but only after eclosion. (C) If N_{Burs} stimulation is delivered to flies forced to eclose prior to natural eclosion, wing expansion is elicited. (D) If N_{Burs} stimulation is delivered well after natural eclosion to flies forced to remain in the puparium, WE is induced, but only after the fly is permitted to eclose. (E) Stimulating the two subsets of N_{Burs} before eclosion (left side) has no immediate effect (–) on either wing expansion behavior or bursicon release into the hemolymph. This is in contrast to post-eclosion stimulation (right side) (from Luan et al., 2012), where rapid induction (+) of both behavior and bursicon release follows B_{SEG} stimulation, and bursicon release alone follows B_{AG} stimulation. The differential results of pre- and post-eclosion stimulation indicate different mechanisms of inhibition of these neurons before and after eclosion.

expansion is known to require secretion of the hormone bursicon into both the hemolymph and the nervous system, the mechanisms that govern bursicon release remain obscure. In the present study, we have shown that these mechanisms are tightly regulated prior to adult emergence. Specifically, we demonstrate that the activation of bursicon-expressing neurons, a manipulation previously shown to rapidly elicit bursicon release and wing expansion in an eclosed fly, fails to do so in a fly prior to eclosion. Interestingly, pre-eclosion neuronal activation exerts a delayed effect, and induces rapid bursicon release into the hemolymph and wing expansion shortly after eclosion. These results, which are summarized in Fig. 5, permit several interesting conclusions. First, both bursicon release into the hemolymph and execution of the motor patterns that support wing expansion are suppressed prior to eclosion; second, this suppression is relieved by some physiological change that is tightly coupled to emergence from the pupal case; and third, stimulation of the bursicon-expressing neurons – or even the single pair of bursicon-expressing neurons that act as command neurons for wing expansion – induces a relatively persistent change in the nervous system that causes rapid wing expansion after eclosion under environmental conditions that would normally inhibit it.

The first of these conclusions is broadly consistent with the finding that inhibitory pathways are thought to play a role in gating adult ecdysis in insects (Fuse and Truman, 2002; Žitňan and Adams, 2000). Eclosion in the fly typically follows the release of eclosion hormone (EH) by ~45 min, but animals in which the heads are ligated after EH release (i.e. during the extended ptilinum stage) will eclose within approximately 1 min (Baker et al., 1999). This suggests that the motor program for eclosion is available for execution after EH release, but is inhibited by signals descending from the head for approximately 45 min, presumably to allow certain physiological changes to occur. It remains to be determined whether wing expansion is inhibited by the same circuits that inhibit eclosion or by some other mechanism, but preliminary experiments suggest differential mechanisms of inhibition in that animals head-ligated after a pre-eclosion TRPM8 stimulation showed no wing expansion behavior if forced to remain in the puparium (N.C.P. and B.H.W., unpublished data). The data presented here make clear, however, that the downstream effectors of wing expansion are blocked prior to emergence in that neither bursicon secretion into the hemolymph nor the motor patterns for wing expansion – namely, air swallowing and sustained abdominal contraction – are induced by stimulation of the bursicon-expressing neurons prior to eclosion. The fact that bursicon release into the hemolymph is inhibited indicates that the block of the B_{AG} is direct, as these neurons are responsible for bursicon secretion into the hemolymph. The mechanism of block appears to differ from the mechanism that operates after eclosion under conditions of confinement, when animals execute an environmental search program using eclosion-related behaviors (Peabody et al., 2009). Under the latter conditions, direct stimulation of the B_{AG} elicits bursicon release into the hemolymph, whereas stimulation prior to eclosion, as shown here, fails to do so. It is worth noting, however, that the levels of bursicon release observed in response to post-eclosion stimulation are reduced relative to those seen upon either B_{SEG} stimulation or natural wing expansion. This suggests that the B_{AG} remain partially inhibited even after eclosion. This inhibition may reflect the persistent effects of pre-eclosion inhibition, or more likely the effects of post-eclosion suppression, for example by the environmental search program. Similar to the B_{AG}, the B_{SEG} are responsive to post-eclosion stimulation, but show no overt signs of response to pre-eclosion stimulation. Whether these neurons, which mediate the behavioral effects of bursicon, are likewise directly blocked is less clear, but as is discussed in greater detail below, we believe it likely that inhibition occurs downstream of them.

Regardless of its mechanisms, our results demonstrate that pre-eclosion inhibition of wing expansion is relieved by eclosion itself. Flies in which N_{Burs} are stimulated within 40 min of natural eclosion will quickly expand their wings only after emerging from the pupal case even though it is clear that they are competent to expand in response to stimulation. Likewise, flies in which eclosion is artificially delayed for an hour respond to N_{Burs} stimulation only

after eclosing, which also indicates that expansion is dependent on this event rather than being under independent control. Overall, our results suggest that the neural circuitry governing wing expansion is released (or partially released) from inhibition by some process tightly coupled to eclosion. One candidate process is the release from contact with all or part of the pupal case. Indeed, in locusts it has been demonstrated that retraction of the head from the old exoskeleton is a prerequisite for post-ecdysial expansion (Hughes, 1980). Similarly, in crickets, the freeing of the abdominal sensory appendages (i.e. the cerci) from the old exoskeleton is coupled to expansion (Carlson, 1977). As noted in the present work, removal of the operculum is often sufficient to induce eclosion after EH release and it is possible that opening of the operculum also releases the machinery of wing expansion from pre-eclosion inhibition. This mechanism is consistent with the observation that pre-eclosion stimulation has an effect only if delivered within 40–50 min of eclosion, the approximate time window of EH release (Baker et al., 1999). EH release may thus 'arm' both the eclosion and wing expansion motor programs, which, however, stay suppressed until relieved of suppression by sensory, and potentially behaviorally generated, signals at the time of eclosion. Further work, however, will be required to test this hypothesis.

Under normal circumstances, wing expansion proceeds immediately after eclosion only in the absence of perturbing environmental conditions, such as confinement (Peabody et al., 2009). In the present work, eclosing animals were always confined, and only those in which N_{Burs} or the B_{SEG} had been stimulated prior to eclosion expanded rapidly. The fact that they did so even when the stimulus was delivered tens of minutes previously demonstrates that the stimulus must have imparted some lasting change, presumably to the nervous system. The nature of this change remains to be determined, but one obvious possibility is a persistent change in B_{SEG} excitability or cellular biochemistry that translates into activation of the wing expansion network upon eclosion. Alternatively, the change may occur downstream of the B_{SEG}. One possible downstream target is suggested by our recent finding that the B_{SEG} negatively regulate the pathway that mediates post-eclosion environmental inhibition (Luan et al., 2012). It may be that pre-eclosion stimulation of the B_{SEG} pre-emptively disarms this inhibitory pathway so that flies eclosing into confined conditions cannot respond to confinement by delay and instead expand quickly. These two possibilities will have to be distinguished by future experiments.

Although the mechanisms of both pre-eclosion inhibition of wing expansion and its release upon emergence are unknown, our results emphasize the importance of emergence as a behavioral checkpoint that prevents wing expansion within the pupal case – an event that would certainly result in disfigured wings. The fact that this safeguard is needed suggests that the circuitry underlying wing expansion is either activated or at risk of being activated prior to eclosion. With respect to the first possibility, and as noted above, EH may play a role in activating this circuit prior to eclosion. This would be consistent with the action of EH on bursicon-expressing neurons in *Manduca* at larval ecdysis (Ewer et al., 1994), and also with the fact that flies in which the EH-expressing neurons have been ablated largely fail to expand their wings (McNabb et al., 1997). With respect to the second possibility, it may be that the wing expansion circuit is armed or activated strictly after eclosion, but that the danger of its accidental activation prior to eclosion exists and must be avoided. Elucidating which of these two possibilities exists should help further clarify how the ecdysis sequence is organized at the level of neural architecture and, in general, further our understanding of how behavioral structure emerges from neural structure.

LIST OF ABBREVIATIONS

B_{AG}	subset of N_{Burs}, 14 neurons in the abdominal ganglion
B_{SEG}	subset of N_{Burs}, two neurons in the subesophageal ganglion
Burs	bursicon alpha subunit
EH	eclosion hormone
N_{Burs}	bursicon-expressing neurons of the adult *Drosophila* nervous system
TRPM8	transient receptor potential cation channel, subfamily M member 8
UAS	upstream activating sequence bound by Gal4

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AUTHOR CONTRIBUTIONS

N.C.P. and B.H.W. conceived, designed and interpreted the experiments described here and wrote the manuscript. N.C.P. conducted all experiments.

COMPETING INTERESTS

No competing interests declared.

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