Characterization of the Decision Network for Wing Expansion in *Drosophila* Using Targeted Expression of the TRPM8 Channel

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After emergence, adult flies and other insects select a suitable perch and expand their wings. Wing expansion is governed by the hormone bursicon and can be delayed under adverse environmental conditions. How environmental factors delay bursicon release and alter perch selection and expansion behaviors has not been investigated in detail. Here we provide evidence that in *Drosophila* the motor programs underlying perch selection and wing expansion have different environmental dependencies. Using physical manipulations, we demonstrate that the decision to perch is based primarily on environmental valuations and is incrementally delayed under conditions of increasing perturbation and confinement. In contrast, the all-or-none motor patterns underlying wing expansion are relatively invariant in length regardless of environmental conditions. Using a novel technique for targeted activation of neurons, we show that the highly stereotyped wing expansion motor patterns can be initiated by stimulation of N_{CCAP} , a small network of central neurons that regulates the release of bursicon. Activation of this network using the cold-sensitive rat TRPM8 channel is sufficient to trigger all essential behavioral and somatic processes required for wing expansion. The delay of wing expansion under adverse circumstances thus couples an environmentally sensitive decision network to a command-like network that initiates a fixed action pattern. Because N_{CCAP} mediates environmentally insensitive ecdysis-related behaviors in *Drosophila* development before adult emergence, the study of wing expansion promises insights not only into how networks mediate behavioral choices, but also into how decision networks develop.

Introduction

A primary function of the nervous system is to execute behavioral choices, that is, to alter an animal's behavior in response to changes in environment or internal demands. The neural mechanisms that underlie such choices have principally been investigated in reduced, invertebrate preparations, where work has focused largely on behaviors that are elicited by simple environmental stimuli and have motor outputs that are easily monitored in the absence of actual behavior (Kristan and Gillette, 2007). The introduction of genetically encoded effectors, which permit the targeted manipulation of specific neurons in intact animals (Miesenböck and Kevrekidis, 2005; Luo et al., 2008), is increasingly allowing the investigation of behavioral decisions that are based on more complex environmental stimuli and on neuromodulatory changes in internal state (Wu et al., 2003; Ada-

mantidis et al., 2007; Dickson, 2008; Yang et al., 2008). Because the new methods are easily deployed in genetic model organisms, such as *Drosophila* (Holmes et al., 2007), which are also amenable to developmental and comparative studies, they promise progress in addressing the broader issues regarding the development, evolution, and adaptive value of the processes and circuits underlying behavioral choice first articulated by Tinbergen (2005).

An extensively analyzed neuromodulatory circuit, for which physiological, developmental, and comparative data are available, is the network underlying ecdysis in insect molting (Ewer, 2005; Truman, 2005; Zitnan et al., 2007). This circuit orchestrates the motor patterns (the so-called "ecdysis sequence") needed to shed the old exoskeleton and expand and harden the new one. This sequence must be executed flawlessly, even in the absence of previous experience, and is therefore largely governed by endogenous factors, but often some components of the sequence are sensitive to external conditions to allow synchronization of hormonal release with environmental cues.

Such coordination is frequently required for the adapted ecdysis sequence used by adult insects to expand their wings, a process which in newly emerged flies requires a suitable perch (Cottrell, 1962; Fraenkel and Hsiao, 1965). If prevented from perching by perturbation or confinement, flies will choose to delay wing expansion for hours. The mechanisms underlying this choice are unknown, as are the neural determinants of the environmentally sensitive perch selection program. However, in *Dro-*

Received Sept. 5, 2008; revised Jan. 9, 2009; accepted Feb. 3, 2009.

This work was supported by the Intramural Research Program of the National Institute of Mental Health. We thank David Julius for providing the TRPM8 cDNA and John Ewer, Paul Taghert, and the Bloomington stock center for fly stocks. We appreciate early help with behavioral assays from Hailyn Nielsen and William Lemon. Special thanks to Grace Gray for editorial assistance and to Howard Nash for insightful comments and persistent insistence on clarity of thought.

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DOI:10.1523/JNEUROSCI.4241-08.2009

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sophila the network underlying wing expansion has been shown to include a set of neurons implicated in both larval and pupal ecdysis in *Drosophila* and in other insects (Gammie and Truman, 1997; Park et al., 2003; Clark et al., 2004; Luan et al., 2006). These neurons (i.e., N_{CCAP}) express crustacean cardioactive peptide, and a subset expresses the hormone bursicon (Dewey et al., 2004; Luo et al., 2005), which governs two motor patterns underlying wing expansion (Baker and Truman, 2002).

Here we combine environmental perturbations with targeted manipulations of N_{CCAP} activity to show that environmental factors prolong the perch selection program and delay activation of the expansion program. We also introduce a method for acutely activating neurons by temperature decrements to demonstrate that N_{CCAP} activation is sufficient to abrogate environmental inhibition and elicit the expansion program.

Materials and Methods

Fly culture/crosses

All flies were grown on corn meal-molasses medium and maintained at 25°C in a constant 12 h light/dark cycle. Wild-type flies (Canton-S strain) were from the Bloomington Stock Center (Indiana University, Bloomington, IN). The Gal4 driver lines used in this study were generous gifts of the following: *yw; CCAP-Gal4; +* and *w; +; CCAP-Gal4* (Park et al., 2003, John Ewer); *w; c929-Gal4; +* (O'Brien and Taghert, 1998, Paul Taghert). The *elav^{C155}-Gal4; +; +*, *yw; 2×UAS-EGFP*; *2×UAS-EGFP*, and *1×*, *2×*, and *3×* EKO lines, which carry one, two, and three copies of the EKO transgene have been described previously (White et al., 2001).

Generation of UAS-TRPM8 constructs and fly lines

The coding sequence of the TRPM8 cDNA (McKemy et al., 2002) was amplified by PCR using primers that introduced an *EcoRI* restriction site and an optimized translation initiation motif [with sequence CAAA, Cavener (1987)] immediately before the ATG start codon and a *KpnI* restriction site just after the stop codon. The amplified fragment was then subcloned into the pUAST plasmid for P-element transformation, using the unique *EcoRI* and *KpnI* restriction sites in the multiple cloning site. P-element injections and isolation of transformants was performed by Genetic Services. Flies with inserts of UAS-TRPM8 on the second (*yw; C4-D; +*) and third (*yw; +; C4-A* and *yw; +; C1-A2*) chromosomes were used in this study, separately or in combination.

Behavioral observations and analysis

Behavior of newly eclosed flies was observed by one of three methods as described in the Results. In all cases, flies were observed for at least 90 min after eclosion and wing expansion phenotypes were assessed at least 24 h after eclosion.

For observations in the low-perturbation condition, the vials used were either cultured at low density and cleared of any adults before observation of the first fly to eclose, or third instar larvae in the wandering stage were transferred individually to new culture vials to pupate and were observed after eclosion. Most observations were made by eye, although in a few cases flies were videotaped and behavior was scored from the video. The onset and offset of walking and abdominal flexion were scored to determine the three principal phases defined below.

For observations in the medium-perturbation condition, individual flies were transferred within 1–3 min of eclosion to a cylindrical chamber (1.4 cm diameter, 0.4 cm thick) formed by placing a plastic ring between two glass cover slides, which were held together by magnets glued to each slide. In some cases, an acetate sheet was attached to one of the glass slides to provide a better substrate for perching. Flies were videorecorded in this chamber using a Sony DCR-PC115 Digital Video Recorder, mounted on an Olympus SZX-12 stereomicroscope, with the video signal streamed to an external hard drive using a Firestore FS-1 (Focus Enhancements) for storage (see supplemental Fig. S1, available at www.jneurosci.org as supplemental material, for a full description of the setup). Videorecords for 40 animals were analyzed for sustained “state” behaviors (e.g., walking, grooming, abdominal contraction, proboscis extension, etc.) using Observer Video-Pro Version 5.0 software (Noldus

Information Technology). Detailed ethograms were constructed for 10 flies, scoring individual movements of the legs, wings, abdomen, and proboscis. Cibarial pumping, which appeared as pulses of light reflected from the base of the proboscis could not be resolved in all flies depending on their orientation relative to the camera.

For the high-perturbation condition, flies were transferred after eclosion into glass tubes (0.3 cm diameter) plugged on each end to form a small chamber 0.7 cm in length. Flies confined within the tubes were videorecorded using a Sony HDR-FX7 digital videocamera, and records were scored for walking, grooming, abdominal flexion, abdominal contraction, and wing expansion.

The behavioral data in the text and in Figures 1*B* and 5*A,B* are presented as mean values $\pm$ SEM. For box-and-whisker plots, the boxes represent the interquartile range of the data separated by the median value, and the “whiskers” extend to the minimum and maximum values of the data, with the exception of outliers. Outliers are defined as values that lie more than three times the interquartile range above the upper quartile value (i.e., top of the upper box). Data were analyzed using nonparametric statistics, due to significant heteroskedasticity based on the Levene test. Comparisons of more than two groups were performed using the Kruskal–Wallis test, followed by repeated *post hoc* Mann–Whitney *U* tests with Bonferroni corrections for multiple comparisons. Pairs of groups were compared with Mann–Whitney *U* tests. All statistical analysis was performed with SPSS version 13.0.

Definitions of behavioral phases

Posteclosion behavior was divided into three phases, similar to those described previously in blowflies (Cottrell, 1962; Zdzarek et al., 1984), defined by the following beginning and end points.

Phase I. From eclosion (or placement in the videorecording chamber) to first perch. A perch is defined as the cessation of walking for more than 5 min. Flies sometimes commenced walking and re-perched, but such episodes were typically brief compared with the initial walking bout.

Phase II. From first perch to the start of sustained abdominal flexion, a condition defined by the elongation and downward flexion of the abdomen. Although short pulses of abdominal flexion could be observed in phase II, sustained abdominal flexion typically lasted 10–15 min.

Phase III. From the onset to the cessation of abdominal flexion.

Quantification of air swallowing

Air swallowing was quantified by measuring the volume of air in the gut of fly. To do so, flies were briefly immersed in 100% ethanol to free the cuticle and bristles of any air, then pinned down through the head and anus in a Sylgard-treated culture dish filled with glycerol. The legs and wings were removed under a SZX-12 Olympus dissection microscope outfitted with a Nikon camera, and the gut was exposed by an incision extending up the midline of the fly. The air inside the gut was then released by gently tearing the gut membrane with forceps and, once liberated, formed a spherical bubble that rose slowly in the glycerol. This bubble was then photographed and its volume calculated from the value of the diameter measured using ImageJ software (W. S. Rasband, National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>) calibrated with a 2 mm micrometer. Typically, air volume was measured at the end of phase III or, in cases where this phase was absent, 90 min after the eclosion time.

Testing UAS-TRPM8 and electrophysiology

The efficacy of the TRPM8 channel was tested using a pan-neural driver (*elav^{C155}-Gal4; +; +*) to express one, two, or three copies of UAS-TRPM8. Up to 10 flies were placed in a large chamber (either a 35 mm culture dish or for the menthol experiments an empty food vial) and then either subjected to a temperature shift or exposed to saturating menthol vapor. The number of flies no longer standing (i.e., “flies down”) was scored every 2.5 min, typically from videorecords or in some cases by eye. Electrophysiological recordings from adult flight muscle were made as follows: Flies were anesthetized with CO₂ just long enough to remove the wings and place them onto a temperature-controlled stage, where they were held in place with vacuum. To record UAS-TRPM8 activated motoneuron activity, electrolytically sharpened tungsten recording electrodes were placed in dorsal longitudinal muscles (DLM) and reference

electrodes in the abdomen. Signals were amplified with Dagan IX2–700 (Dagan Corporation) or A-M Systems 1700 (A-M Systems) differential amplifiers. The temperature-controlled stage consisted of a hollow brass disc through which water of the appropriate temperature was circulated. Temperature changes of $>10^{\circ}\text{C}/\text{min}$ could be achieved by switching between two water baths maintained at target temperatures. Temperature was monitored with a thermocouple probe (T-type, Physitemp Instruments) placed adjacent to the fly, with the signal transduced by a temperature controller (CNI-3252-DC, Omega Engineering). Electrophysiological and temperature data were recorded using pClamp v. 8.2 (Molecular Devices).

Menthol application

Animals were exposed to saturating menthol vapors in plastic vials (94 mm long $\times$ 27 mm in diameter) as follows: flies were introduced into a vial capped by a foam plug in which menthol crystals were embedded. To test the effects of menthol on animals expressing UAS-TRPM8 panneuronally, 1-d-old adults were transiently immobilized by CO_2 and permitted 1 h recovery in an empty vial before menthol exposure. Animals were observed and scored every two and a half minutes for 25 min. To test the effects of menthol on wing expansion time, animals were collected within 5 min of eclosion and immediately transferred into a menthol-saturated vial for 15 min. After 15 min, the plug was gently replaced with one lacking menthol crystals. Individual flies were scored for wing expansion phenotypes at 1 min intervals.

Hemolymph collection and immunoblotting

For experiments on wild-type flies in the low-perturbation condition, hemolymph was extracted (as described below) from flies transferred into individual culture vials directly after eclosion and continuously monitored until they had spent 5 min in phases I, II, or III, or were at least 15 min past completion of phase III. For experiments in which flies expressing UAS-TRPM8 in N_{CCAP} had been subjected to a temperature shift in the high-perturbation condition, hemolymph was taken 1 h after the onset of the temperature shift (i.e., 45 min after return of the flies to 25°C). Hemolymph was extracted in both cases by briefly anesthetizing flies with CO_2 , piercing the thorax with a needle and centrifuging as described previously (Luan et al., 2006). Collected samples were mixed with HE buffer (100 mM KCL, 20 mM HEPES-pH 7.5, 5% glycerol, 0.5M EDTA, 0.1% Triton X-100) containing $2\times$ Halt protease inhibitor (Pierce) and then frozen on dry ice. They were then thawed in equal portions of Laemmli sample loading buffer containing 5% β -mercaptoethanol, and boiled for 5 min before electrophoresis on a 12% Tris-HCl gel (Bio-Rad). Gels were transferred to 0.2 μm nitrocellulose membranes using a Tris-Glycine-20%MeOH buffer, and immunoblotted. The primary rabbit anti-bursicon α -subunit antibody (Luan et al., 2006) was used at a dilution of 1:5000 and a goat anti-rabbit secondary antibody (10 $\mu\text{g}/\text{ml}$, Pierce) at 1:2000. Blots were incubated with West Femto chemiluminescent substrate (Pierce) for 5 min before development on BioMax film (Eastman Kodak) for 10 min.

Immunohistochemistry and confocal analysis

For analysis of whole-mount nervous system preparations, freshly emerged adults (i.e., within 15 min of eclosion) or stage P15i pharate adults (Bainbridge and Bownes, 1981) were dissected in PBS, and the excised nervous systems were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for ~ 20 min, followed by postfixation in 4% paraformaldehyde/PBS plus 0.5% Triton X-100 (Sigma) for 15 min. Procedures for immunostaining were as previously described (Luan et al., 2006). Rabbit anti-TRPM8 antibodies (Novus Biologicals) were used at 1:200 dilution. Secondary antibodies (AlexaFluor 594 goat anti-rabbit from Invitrogen) were used at 1:500 dilution. Confocal imaging was performed using a Nikon C-1 confocal microscope. Z-series through either the brain or ventral nerve cord of each sample were acquired in 1 μm increments using a $20\times$ objective unless otherwise noted, using 488 and 543 nm laser emission lines for EGFP and fluorophore excitation, respectively. Unless otherwise noted, the images shown are maximal projections of the volume rendered Z-stacks of confocal sections taken through the entire nervous system.

The consensus pattern of CCAP-Gal4>UAS-TRPM8 expression was

determined by analyzing the intensity and frequency of labeling of identified CCAP-expressing neurons in multiple wholemount preparations. CCAP-expressing neurons in each preparation were identified in confocal sections by expression of UAS-EGFP and the intensity of TRPM8-associated fluorescence was scored for each neuronal soma on a scale of 0–3. The consensus intensity value (I) for a given neuron was calculated by averaging all nonzero values for this neuron across preparations. The frequency (ν) with which a given neuron was labeled was calculated by dividing the number of preparations in which that neuron had a nonzero labeling intensity by the total number of preparations.

Results

Environmental perturbation delays wing expansion

The behavioral pattern required for wing expansion in *Drosophila* has been described previously as consisting of two principal phases: perch selection, in which the fly walks to identify a site for wing expansion, and expansion itself, during which the stationary fly swallows air and simultaneously contracts its abdomen to drive hemolymph (i.e., blood) into the wings (Baker and Truman, 2002). These behavioral phases are executed immediately after emergence from the pupal case (a process called eclosion) and also have been extensively characterized in blowflies (Zdarek et al., 1984). Genetic data demonstrate that the expansion phase is absent in *Drosophila* bearing null mutations in the *ricketts* gene, which encodes the receptor for the hormone bursicon (Baker and Truman, 2002; Luo et al., 2005). However, apart from the observation of Kimura and Truman (1990) that fruit flies will delay wing expansion if forced to eclose into an empty puparium from which they cannot escape, there has been little investigation of how the behavioral programs underlying perch selection and wing expansion respond to environmental stimuli.

To analyze the behavioral consequences of different environmental conditions, we therefore investigated perch selection and wing expansion in wild-type flies in three different paradigms. In the first, which involved minimal perturbation, the flies were kept in the culture vial in which they had pupated (volume, ~ 30 ml) and were observed by eye after eclosion. This condition required intermittently approaching the vial with a magnifying lens or gently manipulating the vial to optimize viewing as a fly moved. In the second paradigm, flies were gently transferred into a thin cylindrical chamber (volume, 0.616 ml) immediately after eclosion. This configuration (supplemental Fig. S1, available at www.jneurosci.org as supplemental material) was optimized for videomicroscopy to obtain comprehensive behavioral records, as described in Materials and Methods. Flies in the videochamber were exposed to a constant visual environment. In the third paradigm, flies were tapped into an even smaller cylindrical minichamber with cotton endplugs (volume, 4.9×10^{-2} ml) and their behavior was videorecorded. In this paradigm, multiple chambers were monitored simultaneously and flies in adjacent chambers were exposed to each others' movements. The three paradigms thus differed in the degree of handling experienced by the fly after eclosion, the size of—and type of environment within—the chamber, and the visual environment to which the fly was exposed. We did not examine the contributions of each of these variables individually to the behavior of the fly, but each is likely to exert some effect on posteclosion behavior (J. Pohl, N. Peabody, and B. White, unpublished observations). In the following, we refer to the different paradigms as the “low-perturbation,” “medium-perturbation,” and “high-perturbation” conditions to reflect the increasing degrees of stimulation and confinement experienced by the flies after emergence.

We found that the time required for flies to complete wing

expansion varied considerably in the three different conditions, increasing progressively with the level of perturbation. In the low-perturbation condition, flies typically expanded their wings in approximately half an hour (32.8 ± 12.8 min, $n = 20$), whereas in the medium- and high-perturbation conditions they took nearly three and four times this long (83.8 ± 30.7 min, $n = 40$ and 119.3 ± 42.7 min, $n = 44$, respectively). Our preliminary analysis indicated that this difference was largely due to an extension of the perch selection phase, as flies in the smaller chambers persisted in walking for extended periods before finally engaging in wing expansion. This difference was most pronounced for flies in the high-perturbation condition, which sometimes walked for several hours. In addition, these flies frequently executed a distinct “digging” program (Fraenkel, 1935; Reid et al., 1987) when they encountered the cotton plugs at the ends of the chambers. They would attempt to push through the plug by expanding their ptilinum into the gap between the chamber wall and the plug while trying to propel themselves forward by abdominal, and sometimes leg, movements. Animals eclosing in the culture vials never displayed this behavior, and it was observed in the video-chamber only on rare occasions when a small gap was accidentally left between the hard plastic ring and the glass plates that formed the chamber.

Definition of posteclosion behavioral phases

To quantify the differences between the behavioral patterns observed in the three paradigms, we required a baseline description of the post-emergence behavioral program and behavioral endpoints that could be used to define the phases. We therefore analyzed in detail the records of 10 flies observed in the video-chamber where movements could be observed at the highest resolution. The pattern of behavior for all the flies was quite stereotyped, as previously observed by Baker and Truman (2002), and followed the progression shown in the ethogram of Figure 1*A*. Emerging flies, which have compact, folded wings (Fig. 1*Ai*) first walked extensively and then perched. Sustained walking was rarely resumed after perching, although flies occasionally pivoted or walked short distances. While perched (Fig. 1*Aii*), the flies groomed intermittently and occasionally extended their abdomens or probosces. Eventually, the extension of both the proboscis and the abdomen became sustained, with the extended abdomen contracted radially and flexed downward (Fig. 1*Aiii*). During this interval, the wings expanded, initially adopting a downwardly cupped shape, but gradually flattening while angled slightly away from the body axis. As the flattened wings were brought together over the body, the abdomen ceased to be flexed, although the proboscis often remained extended for some time. Grooming continued throughout the period of wing expansion, and persisted for some time afterward.

The overall behavioral pattern divided naturally into three principal phases, which we defined with respect to easily observable behavioral endpoints. As shown in Figure 1*B*, each phase is typically distinguished by a primary category of behavior. The perch selection phase, which we refer to as phase I, is dominated largely by walking and ends when the animal stops and then remains sedentary for at least 5 min. The expansion phase, which we refer to as phase III, is defined by the onset and offset of tonic abdominal contraction. The proboscis also is extended during this phase and in favorably oriented flies ($n = 7/10$), the cibarial pump, which drives air swallowing, was seen to be active (Fig. 1*B*). In the low-perturbation condition, flies dissected immediately after this phase had large bubbles in the abdomen (Fig. 1*Aiiib*; Table 1), confirming the ingestion of air. Although expan-

sion of the wings typically occurs during phase III, this event is thought to be mediated solely by movement of the blood into the wings rather than by exertions of wing-attached muscles. As described below, wing expansion can under some circumstances fail even when phase III and its associated motor patterns (i.e., abdominal contraction and air swallowing) appear normal. Interposed between phases I and III is a period dominated by grooming (Fig. 1*A,B*). We call this period, which also has been noted in blowflies (Cottrell, 1962), phase II. Twenty animals from the low-perturbation condition dissected during this behavioral phase were devoid of air in the gut, indicating that air ingestion is largely or completely restricted to phase III.

We also examined bursicon release as a function of behavioral phase for flies eclosing in their culture vials (i.e., the low-perturbation condition), where we could most readily collect sufficient numbers of similarly staged animals. Hemolymph samples collected from flies during phases I, II, and III, and ~15 min after the end of phase III were analyzed by Western blot (Fig. 1*C*). Bursicon was not detectable in the hemolymph of animals during phase I. It first appeared in phase II, and rose to its highest level during the expansion phase (phase III). Thus, in the low-perturbation condition, bursicon release appears to be initiated within 5 min of perch selection and to peak during wing expansion.

Environmental perturbation extends the perch selection phase

Having defined the behavioral phases, we compared their durations under the three environmental conditions (Fig. 2). This analysis confirmed that the differences in wing expansion time resulted primarily from a progressive increase in the duration of the perch selection phase. Mean phase I durations differed significantly between the three conditions ($p < 0.001$ by the Kruskal–Wallis test), rising 20-fold between the low- and high-perturbation conditions. In contrast, the mean duration of the expansion phase (III) was ~15 min in all cases and did not differ significantly between perturbation conditions ($p > 0.7$ by Kruskal–Wallis test). The stereotyped duration of phase III suggests that the motor patterns underlying expansion, once triggered, run for a fixed time. Bursicon is the likely trigger, and, as noted above, its release begins during phase II. Although phase II durations in the three different conditions were significantly different ($p \leq 0.007$ in pairwise comparisons using the Mann–Whitney test), they showed no obvious trend with respect to the level of perturbation. The observed variability in phase II durations, both within any given condition and across conditions, suggests that the timing or rate of bursicon release may differ considerably among individual animals.

The perch selection phase is insensitive to N_{CCAP} suppression

The above data demonstrate that environmental perturbations delay wing expansion primarily by extending the perch selection phase. The perch selection program is thus modulated by the sensory pathways that mediate environmental perturbation, but it is possible that this modulation is indirect and requires the circuitry underlying the expansion program. For example, the expansion program may be the primary target of environmental modulation, and the perch selection program could simply be active until inhibited by initiation of the expansion program. If the expansion program never starts up, perch selection should continue indefinitely. To directly test the possible involvement of the expansion program circuitry, we examined the effects of in-

Figure 1. Posteclosion behavior in *Drosophila* consists of three phases with distinct patterns of behavior. **A**, Ethogram of a wild-type fly videotaped in the medium-perturbation condition. Bouts of walking, grooming, abdominal (Abd.) elongation and flexion, proboscis extension (ext.), and cibarial pumping can be resolved and scored in the detailed video records as indicated, as can individual movements of the three pairs of legs. The three principal phases of behavior are labeled and shaded, and an image of a representative fly in each phase is placed above the label. Phase I (magenta, **i**) is distinguished principally by walking. At this time, the wings (black arrows) remain unexpanded. In phase II (green, **ii**), the fly is perched and grooms. Wings (white arrow) are still unexpanded and the abdomen (yellow arrowhead) is unflexed. In phase III (blue), the proboscis is extended (gray arrowhead, **iiia**) and the abdomen is elongated and flexed downward (yellow arrowhead) as the wings (white arrow) expand. During this phase, the fly swallows air, filling its gut with a large bubble (white arrowhead, **iiib**). **B**, Bar graph indicating the average frequency of major state behaviors during each phase of flies in the medium-perturbation condition ($n = 10$). Shading is as indicated in the legend. Error bars represent SEM. **C**, Immunoblot of hemolymph samples collected 5 min into each phase (or 15 min after the termination of phase III for the last sample) from multiple flies. The blot was probed with an antibody against the bursicon α -subunit. The position of the bursicon α -subunit (Burs α) is indicated by the arrow. Positions of molecular weight markers (in kDa) are indicated.

Table 1. Air-swallowing deficits correlate with the absence of abdominal contraction

Genotype	Swallowed air (nl)			
	End of phase III		After 90 min	
	Males	Females	Males	Females
Canton S (control)	82 $\pm$ 5 (10)	138 $\pm$ 12 (10)	24 $\pm$ 4 (10)	75 $\pm$ 7 (10)
CCAP-Gal4 > w^{1118} (control)	ND	ND	38 $\pm$ 6 (10)	106 $\pm$ 6 (10)
CCAP-Gal4 > 1 $\times$ UAS-EKO	131 $\pm$ 10 (10)	175 $\pm$ 14 (10)	98 $\pm$ 16 (10)	172 $\pm$ 22 (10)
CCAP-Gal4 > 1 $\times$ UAS-EKO (medium perturbation)	131 $\pm$ 16 (8)	196 $\pm$ 36 (7)	ND	ND
CCAP-Gal4 > 2 $\times$ UAS-EKO (no phase III)	ND	ND	0 $\pm$ 0 (20)	0 $\pm$ 0 (21)
CCAP-Gal4 > 2 $\times$ UAS-EKO (+ phase III)	111 $\pm$ 5 (4)	179 $\pm$ 25 (4)	105 $\pm$ 30 (8)	222 $\pm$ 55 (2)
CCAP-Gal4 > 3 $\times$ UAS-EKO	ND	ND	0 $\pm$ 0 (9)	0 $\pm$ 0 (10)
<i>rickets</i> ⁴	ND	ND	0 $\pm$ 0 (10)	0 $\pm$ 0 (10)

The amount of air (in nanoliters) swallowed by male and female flies of the indicated genotypes is shown together with the SEM. The volume of air found in the gut of dissected animals was determined as described in Materials and Methods. The number of animals assayed for each measurement is shown in parentheses. ND, Not determined.

hibiting activity in N_{CCAP} in the relative absence of environmental stimuli, i.e., in the low-perturbation condition.

We previously showed (Luan et al., 2006) that graded levels of N_{CCAP} suppression can be achieved by using the CCAP-Gal4 driver to express increasing copy numbers of the UAS-EKO suppressor transgene. Expression of three copies of the UAS-EKO transgene (i.e., 3 $\times$ UAS-EKO) in N_{CCAP} completely blocks wing expansion and inhibits bursicon release into the hemolymph (Luan et al., 2006). As expected, we found that in the low-perturbation condition incremental

Figure 2. Environmental perturbation selectively extends the duration of the perch selection phase. Box-and-whisker plots show the distribution of phase durations for flies observed under the low (L), medium (M), and high (H)-perturbation conditions described in the text. Each graph shows the durations of one of the three principal behavioral phases. The duration of the perch selection phase (phase I) increases incrementally with perturbation level, while the expansion phase (phase III) is unaffected by environmental conditions. The grooming phase (phase II) does not vary systematically with perturbation. For each plot, the lower and upper boxes span the second and third quartiles of the data, with the division between them defining the median. The whiskers extend to the minimum and maximum values of the data except when outliers are present (see Materials and Methods), in which case the outliers are represented by individual plus signs. Diamonds represent mean values. Double asterisks indicate very significant differences ($p \leq 0.01$) as determined by the Mann–Whitney test. For the low-, medium-, and high-perturbation conditions, $n = 20, 40$, and 44 , respectively.

N_{CCAP} suppression causes wing expansion deficits of increasing severity and frequency (Fig. 3A, top panel). Consistent with the known requirement for bursicon in expansion behaviors (Baker and Truman, 2002), suppression of N_{CCAP} by $3 \times$ UAS-EKO completely eliminated both the tonic abdominal contraction that defines phase III (Fig. 3A, bottom panel) and air swallowing (Table 1; see also supplemental Movie S2, available at www.jneurosci.org as supplemental material). Despite the absence of the expansion phase, however, the duration of phase I in these animals was indistinguishable from that of the control flies (Fig. 3B). Indeed, there was no significant difference in the length of phase I for any of the experimental groups ($p > 0.2$ by Kruskal–Wallis test), and almost all the flies completed perch selection in <10 min regardless of the level of N_{CCAP} inhibition. Our results demonstrate that N_{CCAP} neurons are not involved in the termination of perch selection, and therefore in the transition to phase II, but that they are essential for the subsequent activation of the expansion program.

N_{CCAP} activity regulates initiation of the expansion program

The behavioral effects of N_{CCAP} suppression have implications not only for the regulation of the perch selection program, but also for the mechanisms governing the wing expansion-related motor patterns. In this regard, the results for animals expressing $2 \times$ UAS-EKO are of particular interest. These flies can be divided into two distinct categories: Approximately half resembled flies expressing $1 \times$ UAS-EKO, which exhibited a robust expansion phase that included tonic abdominal contraction (Fig. 3A, bottom panel) and air swallowing (Table 1), while the rest resembled $3 \times$ UAS-EKO animals, which completely lack phase III behaviors (Fig. 3A, bottom panel; Table 1). This observation implies that there is a threshold level of N_{CCAP} activity which must be exceeded if the expansion phase is to be initiated. Because the two behaviors required for wing expansion either occur or fail in

Figure 3. Graded suppression of N_{CCAP} incrementally inhibits wing expansion and slows or blocks entry into the expansion phase. **A**, Inhibition of N_{CCAP} in the low-perturbation condition by expression of one to three copies of the suppressor transgene, UAS-EKO, incrementally increases wing expansion deficits (top graph) and decreases the percentage of animals that execute phase III (bottom graph). EW, PEW, and UEW represent expanded, partially expanded, and unexpanded wing phenotypes, respectively. All data were taken from animals tested in the low-perturbation condition. **B**, Box-and-whisker plot showing the distribution of phase durations for animals expressing $1-3 \times$ UAS-EKO in the low-perturbation condition. Definitions are as described in the legend of Figure 2. Animals expressing $3 \times$ UAS-EKO failed to enter phase III, and phase II was therefore of indeterminate length. Both phases are rendered as “not defined” (ND) for these animals. Phase II and phase III plots for animals expressing $2 \times$ UAS-EKO include data only from the 12 animals (of 25 total) that executed phase III. Also, the phase I data set for these animals included three outliers, only one of which is represented, because two of the animals walked for the entire observation period and therefore did not end phase I. Only a median value (and no mean) is therefore given for this plot. For $1 \times$ and $3 \times$ UAS-EKO conditions, $n = 10$ and 6 , respectively. Plus signs and double asterisks are as defined in the legend of Figure 2. A single asterisk indicates a significant difference ($p < 0.05$) across conditions as determined by the Kruskal–Wallis test.

tandem, our results also imply that the two motor patterns share a common mechanism of initiation.

The common mechanism of initiation of the phase III motor patterns most likely reflects their shared requirement for bursicon (Baker and Truman, 2002), and their coincident failure at higher levels of N_{CCAP} suppression presumably results from impaired hormone release. Impaired bursicon release is also likely to underlie the observation that animals expressing $1 \times$ UAS-EKO, as well as approximately one-half of animals expressing $2 \times$ UAS-EKO, initiate phase III only after a significant delay, as reflected in the extension of phase II (Fig. 3B). Further work will be required to determine whether the extension of phase II, which appears to result from longer quiescent episodes between grooming bouts, is

caused by the slowing of bursicon release or by its delay (or both), but the former possibility would conveniently explain the observed threshold in N_{CCAP} activity for wing expansion. If EKO-mediated suppression attenuates bursicon release, the threshold for N_{CCAP} activity would simply reflect a threshold requirement for bursicon, with expansion triggered only when bursicon reaches a critical level.

A final implication of the results shown in Figure 3 is that N_{CCAP} modulates not just the expansion program, but also the nonbehavioral processes necessary for wing expansion. This is again most obvious for flies expressing $2 \times$ UAS-EKO. Approximately half of these flies executed phase III (Fig. 3A, bottom panel), but none of these expanded their wings fully, and 70% did not expand them at all (Fig. 3A, top panel). In addition, most flies expressing $1 \times$ UAS-EKO in N_{CCAP} only partially expanded their wings, despite executing normal phase III behavior. While we cannot rule out subtle behavioral deficits in the animals that failed to expand their wings, our results suggest that N_{CCAP} regulates nonbehavioral processes involved in wing expansion, such as plasticizing the wing cuticle to render it pliable. These nonbehavioral processes may well be mediated by bursicon released into the hemolymph, as the blood-borne hormone is known to be required for somatic changes at the level of the wing cuticle (Reynolds, 1976, 1977; Dai et al., 2008). In contrast to motor-pattern deficits resulting from N_{CCAP} inhibition, which are all-or-none, wing expansion defects are often partial (Fig. 3A, top panel) and change in a graded manner. The nonbehavioral deficits associated with N_{CCAP} suppression thus appear to lack a threshold.

Targeted expression of rat TRPM8 can be used to acutely activate neurons

The suppression experiments described above indicate that perch selection normally terminates without input from N_{CCAP} and that the bursicon released from N_{CCAP} during phase II initiates the expansion program. Initiation of expansion thus requires activation (or disinhibition) of N_{CCAP} , and these neurons are probable targets of modulation by circuits that mediate the perch selection program or environmental inputs. To determine whether N_{CCAP} neurons are the sole targets of such modulation, or whether downstream neurons that mediate the execution of the expansion program are also subject to regulation, we sought to artificially activate N_{CCAP} during perch selection. We reasoned that if neurons downstream of N_{CCAP} are normally inhibited during perch selection, either by the circuitry underlying this program or by negative environmental signals, activation of these neurons would fail to initiate the expansion motor patterns. If, on the other hand, artificial activation of N_{CCAP} readily induced expansion, this would provide strong evidence that neural substrates of the expansion program downstream of N_{CCAP} were unlikely regulatory targets of the circuits underlying perch selection and/or environmental inputs. To determine which of these two possibilities might be the case, and more generally to determine whether N_{CCAP} activity is sufficient to initiate expansion behavior, we sought to acutely stimulate these neurons in post-eclosion flies.

Efforts to use the light-sensitive Channelrhodopsin-2 protein (Nagel et al., 2003), which has been used successfully before in *Drosophila* for acute neuronal activation (Schroll et al., 2006; Suh et al., 2007), proved ineffective in newly emerged adults, perhaps due to poor light penetration through the cuticle or depletion of the retinal-A cofactor during metamorphosis. We therefore sought to develop an alternative, and simpler, system for stimulating neurons in the live animal. To this end, we generated trans-

genic flies that can express the rat TRPM8 gene, which encodes a nonselective cation channel expressed in mammalian sensory neurons (McKemy et al., 2002; Peier et al., 2002). This channel is responsible for the sensation of mild cold (Colburn et al., 2007; Dhaka et al., 2007) and activates in response to decrements in temperature in the range of $\sim 25^\circ\text{C}$ to 10°C . The thermal sensitivity of the TRPM8 channel, which is compatible with the temperatures at which flies are normally active, makes it particularly suitable for manipulating activity in *Drosophila*. Because cold, unlike light, normally attenuates motor activity, neuronal activation using TRPM8 also has an advantage over optical techniques in minimizing the confounding behavioral effects of the activating stimulus.

We made transgenic fly lines that express the TRPM8 channel under the control of Gal4, and tested their efficacy by assaying for abnormal behavior when the channel was expressed pan-neuronally. Several lines expressed the TRPM8 channel at sufficiently high levels to give robust phenotypes (such as seizing, jumping, and eventually falling down) upon transfer to 15°C from room temperature. We combined the strongest inserts to make lines that bore from one to three copies of the UAS-TRPM8 transgene. As shown in Figure 4A, flies expressing UAS-TRPM8 pan-neuronally fell down quickly upon transfer to 15°C , with animals expressing $3 \times$ UAS-TRPM8 displaying a more rapid and robust response. Electrophysiological recordings from flight muscles of these animals revealed sustained action potential generation in motoneurons in response to a downward temperature ramp (Fig. 4B). This effect was readily reversible. Males typically showed greater sensitivity than females to a given temperature shift, as can be seen from the responses of animals expressing $2 \times$ UAS-TRPM8 shifted to 18°C (Fig. 4C). At this temperature, 60% of males eventually fell over and could not right themselves, but none of the females did so, even after 10 min. We also tested the sensitivity of flies expressing UAS-TRPM8 to menthol. Menthol potentiates the activity of TRPM8 by shifting its temperature sensitivity to more positive temperatures (McKemy et al., 2002). Accordingly, saturating menthol vapor administered to flies expressing UAS-TRPM8 pan-neuronally at room temperature (24°C) caused the animals to seize and fall over as they did in response to cold (Fig. 4D). The response was slow and poorly penetrant with a single copy of the transgene, but most animals expressing $2 \times$ UAS-TRPM8 fell down within 5 min of menthol exposure and all were down by 10 min.

Activation of N_{CCAP} using TRPM8 is sufficient to initiate wing expansion

To activate N_{CCAP} with stimuli of various magnitudes, we subjected flies expressing $1 \times$ and $2 \times$ UAS-TRPM8 in N_{CCAP} (see Fig. S3 for anti-TRPM8 immunostaining) to temperatures shifts of various magnitudes. Under conditions similar to our low-perturbation condition, newly eclosed flies were transferred into food vials at room temperature and then placed at temperatures ranging from 18°C to 12°C . Even the mildest stimulus tested gave robust results: $CCAP\text{-Gal4} > 1 \times$ UAS-TRPM8 animals shifted briefly to 18°C immediately after eclosion rapidly expanded their wings relative to controls bearing the $1 \times$ UAS-TRPM8 transgene without the $CCAP\text{-Gal4}$ driver (Fig. 5A, left). Both the speed with which the experimental group expanded their wings and the low variance in expansion times implied strong and uniform stimulation of N_{CCAP} in these animals, effects that were also observed when wing expansion was induced with a 15 min exposure to saturating menthol vapor at room temperature (Fig. 5A, right).

To determine whether strong induction of the perch selection

program attenuated the ability of UAS-TRPM8 to drive wing expansion, we examined the response of flies in the high-perturbation condition. Newly eclosed animals were placed in minichambers and exposed to a 15 min temperature shift from 24°C to 18°C. We found that N_{CCAP} activation by UAS-TRPM8 resulted in a substantial acceleration of wing expansion in all animals (supplemental Movie S4, available at www.jneurosci.org as supplemental material). On average, these animals took 25 ± 3 min ($n = 12$) to expand their wings compared with 219 ± 25 min ($n = 10$) for control flies bearing the 1× UAS-TRPM8 transgene, but lacking the CCAP-Gal4 driver (Fig. 5B). All behavioral phases were typically present in the experimental animals, although phase II was extremely brief. Most animals persisted in walking and digging through most of the 15 min temperature pulse, but then transitioned almost immediately into the expansion phase after perching. Consistent with our observations under other conditions, phase III was relatively uniform in duration and lasted on average ~15 min. All animals remained sedentary during wing expansion, indicating that execution of the expansion program potentially inhibits perch selection behaviors. In addition to performing the expansion motor programs, animals expressing 1× UAS-TRPM8 in the high-perturbation condition displayed normal cuticle tanning after N_{CCAP} activation (Fig. 5C, compare left, right). N_{CCAP} activation thus appears to induce bursicon release into the hemolymph. We confirmed this directly by measuring hormone titers after the temperature shift by Western blot (Fig. 5D).

Thus, under conditions that normally bias the animal to persist in perch selection behaviors, mild activation of N_{CCAP} initiates hormone release and induces both the behavioral and physiological hallmarks of bursicon action. These results indicate that the neural pathways mediating perch selection and/or environmental input regulate the wing expansion program at, or above, the level of N_{CCAP} , and that neurons downstream of N_{CCAP} are not targets of modulation. In addition, our results demonstrate that N_{CCAP} neurons are not only necessary for wing expansion but also sufficient to drive this entire process.

Discussion

The introduction of genetically encoded effectors, which permit the targeted manipulation of neuronal activity *in vivo*, is increasingly facilitating neurobiological studies of behavioral choice *in vivo*, as exemplified by the work presented here. Using a new method for stimulating neurons in live animals, we demonstrate that the N_{CCAP} network, first implicated in governing eclosion, effects the fly's decision to expand its wings after eclosion. This decision is normally coupled to the decision to perch, which we demonstrate is based on evaluation of environmental variables.

Figure 4. UAS-TRPM8 acutely activates neurons in response to temperature decrements. One to three copies of a UAS construct expressing the rat cold-and-menthol receptor, encoded by the *TRPM8* gene, were targeted to neurons using the pan-neuronal elav-Gal4 driver. Flies were tested for aberrant behavior (i.e., falling down) or physiological activity in response to cold or menthol exposure. **A**, Graph of the percentage of flies that have fallen down (typically accompanied by seizing and jumping) in response to a temperature decrement from 24 to 15°C. The percentage of flies lying on their backs was measured at 2.5 min intervals over a 10 min observation period. Experimental animals expressed either one (1×) or three (3×) copies of UAS-TRPM8 and were compared with control animals bearing the 1× UAS-TRPM8 transgene, but no driver. **B**, Intracellular recordings of physiological activity in flight muscles elicited by stimulation of innervating motoneurons. Animals expressing 1× (top trace) or 3× (middle trace) UAS-TRPM8 pan-neuronally were exposed to a 3 min temperature ramp from 23 to 12°C (bottom trace). The time of onset and frequency of the temperature-elicited action potentials increased as a function of UAS-TRPM8 levels, and background activity (arrowhead), presumably deriving from action potentials distal to the recording site, could be seen almost immediately after the onset of the temperature drop. **C**, Males are typically more sensitive to UAS-TRPM8 activation than females as can be seen by the intermediate levels of channel activation. The percentage of elav-Gal4 > 2× UAS-TRPM8 males (solid line) that fall down over time in response to a temperature shift from 24 to 18°C is higher than that of females (dotted line), which are not affected by this manipulation. **D**, Menthol, which potentiates the temperature sensitivity of TRPM8, elicits behavioral effects in flies expressing UAS-TRPM8 at room temperature. The percentage of flies that fall down over time increases very slowly in animals expressing 1× UAS-TRPM8 (dotted line), but the rate is much faster in animals expressing 2× UAS-TRPM8 (solid line), consistent with higher expression levels in these animals.

The postponement of wing expansion by the fly under adverse circumstances is thus a consequence of a value-based choice to prolong search behavior and the delayed activation of a command network responsible for the execution of the wing expansion decision.

Perch selection is environmentally regulated

As indicated in Figure 6, our results support a model in which the motor programs underlying perch selection respond primarily to environmental input. Although the environmental features monitored by the fly and the sensory channels that process them remain to be determined, it is clear from our experiments that flies assess conditions during phase I and assign longer search periods to more adverse environments. The observation that expansion is not deferred indefinitely, even under the high-perturbation condition, suggests that the benefits of continued searching are weighed against the risks of further delay. How these risks, which may include predation and desiccation, are represented physiologically to encode the “value” of different environments is still unclear, but the work described here should facilitate their investigation.

Figure 5. Stimulation of N_{CCAP} using UAS-TRPM8 drives rapid wing expansion and bursicon release in animals in the high-perturbation condition. **A**, Bar graphs show the time taken by flies expressing UAS-TRPM8 in N_{CCAP} to expand their wings after temperature shift to 18°C (Δ Temp) or exposure to saturating menthol vapor (Menthol). In each case, the time taken by CCAP-Gal4 > 1 $\times$ UAS-TRPM8 animals ($n = 31$ and 18 for the Δ Temp and Menthol conditions, respectively) was compared with that of w^{1118} control flies bearing a single copy of the UAS-TRPM8 transgene, but no driver ($n = 30$ and 20 for the Δ Temp and Menthol conditions, respectively). CCAP-Gal4 > 1 $\times$ UAS-TRPM8 animals subjected neither to an 18°C temperature shift nor to menthol took 31 ± 26 min to expand their wings ($n = 20$). **B**, Bar graphs show the average duration of each behavioral phase for animals expressing 1 $\times$ UAS-TRPM8 in N_{CCAP} ($n = 15$) compared with parallel control animals bearing the UAS-TRPM8 transgene, but no driver ($n = 12$). All animals were tested in the high-perturbation condition within 30 min of eclosion. Activation of N_{CCAP} using UAS-TRPM8 significantly shortened both phases I and II, but did not alter the duration of phase III. **C**, N_{CCAP} activation causes rapid tanning, as indicated by these pictures of representative flies expressing 1 $\times$ UAS-TRPM8 in N_{CCAP} , one of which received (right, + Δ Temp), and one of which did not receive (left, - Δ Temp), a 15 min temperature shift to 18°C. Both flies were subjected in parallel to the high-perturbation condition and were photographed 3 h after the time of onset of the temperature pulse delivered to the experimental fly. All flies that received the temperature pulse ($n = 20$) expanded their wings and tanned, as indicated by the generally dark appearance of their cuticles, while control flies ($n = 19$) uniformly failed to expand their wings or tan and retained the whitish cuticle color seen at eclosion. **D**, N_{CCAP} activation also causes rapid release of bursicon into the hemolymph. Hemolymph samples extracted from CCAP-Gal4 > 1 $\times$ UAS-TRPM8 flies that either had (right, +) or had not (left, -) been subjected to a 15 min temperature shift to 18°C were analyzed by Western blot probed with an antibody to the bursicon α -subunit. For each experimental condition, hemolymph was extracted from >40 flies that had been subjected to the high-perturbation condition. Hemolymph was collected 1 h after the onset of the temperature shift or an equivalent time in unshifted controls. Molecular weight markers (in kDa) are shown.

Elucidating which neurons mediate the decision to perch will also require further investigation. Although perching can be induced by the activation of N_{CCAP} using UAS-TRPM8, N_{CCAP} is clearly not normally required for terminating phase I, since its suppression does not affect perching. Also, the perching that does occur when N_{CCAP} is stimulated by UAS-TRPM8 is more tightly linked temporally with the initiation of expansion (i.e., phase III) than with the onset of N_{CCAP} stimulation (i.e., the shift to 18°C), suggesting that neurons downstream of N_{CCAP} mediate phase I termination, most likely at the level of the motor networks underlying perch selection and expansion, as shown in Figure 6. Inhibition of one motor system by another has been demonstrated to explain such behavioral hierarchies in other cases, as in

Figure 6. Proposed model for modulation of N_{CCAP} and posteclosion processes involved in wing expansion. Solid lines indicate interactions supported by the experimental evidence described here and elsewhere. Dotted lines indicate interactions that are either inferred or hypothesized. Our data demonstrate that phase I (i.e., perch selection) is regulated both by environmental factors and by the expansion motor network. Environmental inputs are depicted as positively driving perch selection, but it is equally possible that the perch selection program is endogenously active and inhibited by stimuli that signal a suitable environment for perching. N_{CCAP} activity is necessary and sufficient for activation of the expansion program, and, as indicated, also appears necessary for nonbehavioral processes (such as cuticle plasticization) that are required for wing expansion. Both effects are likely mediated by the release of the hormone bursicon, which is controlled by N_{CCAP} . The mechanisms of N_{CCAP} regulation may include either the perch selection motor network or environmental signals, although these are likely to act indirectly, as described more fully in the Discussion. Eclosion hormone (EH) is also likely to indirectly modulate N_{CCAP} activity, as described in the Discussion. The factors that regulate phase II are unknown, but as mentioned in the Discussion, its duration may be governed in part by bursicon. Both the control and biological significance of this phase remain enigmatic and will have to be addressed by future experiments.

the dominance of feeding over withdrawal in mollusks (Davis, 1979).

N_{CCAP} regulates the expansion program

In contrast to perch selection, the expansion program shows no obvious environmental dependence, but it is strongly dependent on levels of N_{CCAP} activity for its initiation. This is consistent with the known effects of bursicon, which is released by a subset of N_{CCAP} (Luan et al., 2006) and is required for both wing extensibility (Reynolds, 1976, 1977; Dai et al., 2008) and phase III behaviors (Baker and Truman, 2002). The probability of expansion occurring at all is likely to depend on whether the bursicon released into the CNS reaches a critical threshold, while the initiation of expansion most likely depends on the timing and/or rate of bursicon release. The rate of release should decline with N_{CCAP} suppression, which will reduce the excitability of the bursicon-expressing neurons, and may account for both the prolongation of phase II under this condition and the appearance of graded wing expansion deficits. It is also possible that the timing of bursicon release was modulated by our manipulations of N_{CCAP} if delay circuits intrinsic to N_{CCAP} are responsible for initiating its secretion (see below).

Although it remains to be determined whether N_{CCAP} participates in the decision to expand, it is clear that its activation drives all the behavioral and somatic processes necessary for wing expansion (see Fig. 6), placing N_{CCAP} neurons high in the execution pathway of the decision and indicating that their function in wing expansion is command-like. Because this command-like function relies on bursicon and not direct synaptic activation, it differs from familiar command systems that mediate fast behavioral switches, such as those involved in defensive escape (Edwards et al., 1999), and more closely resembles systems in which hor-

mones or neuromodulators elicit profound behavioral transitions such as those responsible for egg-laying in mollusks (Kupferman, 1967), stomatogastric ganglion modulation in crustacea (Hooper and Marder, 1984), and ecdysis in insects (Truman and Riddiford, 1970). Because of the technical difficulty involved, the ability of such systems to drive behavioral programs upon activation has rarely been demonstrated. However, as illustrated by the work presented here, the introduction of genetic techniques for *in vivo* neuronal activation should make such demonstrations increasingly possible.

Coupling perch selection and expansion: insights from ecdysis

The work presented here argues that the motor programs for perch selection and wing expansion have distinct regulatory mechanisms, but leaves unanswered the question of how the decisions to perch and expand are coupled: Specifically, the mechanism by which N_{CCAP} is activated following perching remains to be clarified. As indicated in Figure 6, N_{CCAP} could, in principle, receive input from either the perch selection motor network or from sensory processing pathways. Such input is likely to be indirect since perching and expansion are separated by a delay (i.e., phase II), which varies considerably in duration between individual animals and different conditions (see Figs. 2, 3). This delay period, during which bursicon secretion is initiated, may act, at least in part, to permit bursicon to plasticize the cuticle of the wing and body before expansion.

Behavioral transitions during ecdysis in both *Drosophila* and the hawkmoth *Manduca sexta*, are known to be regulated by inhibitory delay circuits (Zitnan and Adams, 2000; Fuse and Truman, 2002). In the case of eclosion hormone, the delay circuit temporally separates the somatic and behavioral aspects of the hormone's action and has been proposed to act as a control point for environmental modulation by light, which accelerates eclosion, perhaps by suppressing the inhibitory delay circuit (Baker et al., 1999; Truman, 2005). Interestingly, the modulation of eclosion by light is eliminated in animals lacking N_{CCAP} , supporting a role for these neurons in the inhibitory pathway (Park et al., 2003). Because ablation of the EH-expressing neurons causes wing expansion deficits, it is possible that in addition to modulating eclosion, EH may similarly modulate wing expansion by simultaneously upregulating excitability in both the bursicon-expressing neurons and in a delay circuit that inhibits them (McNabb et al., 1997; McNabb and Truman, 2008). By analogy to the action of EH on ecdysis, the latter circuit, which could include non-bursicon-expressing N_{CCAP} neurons, would inhibit bursicon release until inhibition is alleviated by an environmentally mediated signal. Such a model might explain not only the wing expansion deficits observed in animals lacking EH-expressing neurons, but also the eventual wing expansion of animals kept in the high-perturbation condition. In these animals, run down in the delay circuit may trigger bursicon release before the decision to perch, as happens when N_{CCAP} is activated by UAS-TRPM8, and thus also account for their abbreviated phase II (see Fig. 2).

N_{CCAP} function and behavioral adaptation

The merits of this model, and others that invoke delay circuits, remain to be tested. It is interesting to note, however, that the mechanism(s) coupling environmental input to N_{CCAP} is likely to be added during metamorphosis, because the function of this network changes from an intrinsically regulated mediator of pupal ecdysis to an extrinsically modulated mediator of wing expansion (Park et al., 2003; Kim et al., 2006; Luan et al., 2006). Despite

its broad phylogenetic conservation, this network also appears to be differentially regulated in different species, with CCAP-expressing neurons being direct targets of EH in many insects, but not *Drosophila* (Ewer and Truman, 1996). Such differential regulation may underlie, at least in part, species-specific differences in the coordination of wing expansion with adult emergence: In some insects, expansion is initiated at emergence without benefit of environmental input, and in others, like honeybees, it is initiated and completed before emergence. Investigation of the basis of these differences, and of the neural mechanisms that adapt N_{CCAP} function to the individual developmental needs and life histories of different insects, should provide insight into general mechanisms of behavioral adaptation (Katz and Harris-Warrick, 1999).

In general, the work presented here demonstrates that the behavioral programs used by *Drosophila* to achieve wing expansion can serve as a simple and fruitful model for investigating networks underlying behavioral choice. Elucidation of the neuronal pathways that mediate the decisions to perch and expand the wings will shed light on the development and evolution of behavioral networks as well as their architecture and function. Indeed, the decision-making architecture outlined here for wing expansion may prove relevant to the type of instinctive behaviors, first described by Craig (1918), in which an environmentally sensitive "appetitive" phase is coupled to a "consummatory" phase consisting of a stereotyped, motor pattern. Finally, tools that permit one to manipulate decision-making in living, behaving animals, such as UAS-TRPM8, which we introduce here, clearly will play an essential role in mapping the neural networks underlying behavior in *Drosophila* and other animals.

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