

# Chip Is Required for Posteclosion Behavior in *Drosophila*

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Neurons acquire their molecular, neurochemical, and connectional features during development as a result of complex regulatory mechanisms. Here, we show that a ubiquitous, multifunctional protein cofactor, Chip, plays a critical role in a set of neurons in *Drosophila* that control the well described posteclosion behavior. Newly eclosed flies normally expand their wings and display tanning and hardening of their cuticle. Using multiple approaches to interfere with Chip function, we find that these processes do not occur without normal activity of this protein. Furthermore, we identified the nature of the deficit to be an absence of Bursicon in the hemolymph of newly eclosed flies, whereas the responsiveness to Bursicon in these flies remains normal. Chip interacts with transcription factors of the LIM-HD (LIM-homeodomain) family, and we identified one member, *dIslet*, as a potential partner of Chip in this process. Our findings provide the first evidence of transcriptional mechanisms involved in the development of the neuronal circuit that regulates posteclosion behavior in *Drosophila*.

**Key words:** LIM; wing expansion; Bursicon; behavior; neuroendocrine; neurosecretion

## Introduction

Transcriptional mechanisms that regulate neuronal development are the focus of study in many systems. A family of LIM-homeodomain (LIM-HD) transcription factors and their cofactors have been implicated in various aspects of neuronal development in both vertebrates and invertebrates. In the vertebrate spinal cord, LIM-HD protein *Islet1* acts in concert with *Lhx3/4* or *Lhx1* to regulate multiple aspects of motoneuron specification and pathfinding (Shirasaki and Pfaff, 2002; Thaler et al., 2004). In the vertebrate forebrain, *Lhx2* functions as a “cortical selector” gene (Mangale et al., 2008). It is also required for the specification of part of the amygdala (Remedios et al., 2004) and for multiple aspects of olfactory system development, including the regulation of survival, receptor expression, and axon guidance (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Saha et al., 2007). Striking parallels are found in *Drosophila*: *Apterous* (*Ap*), an orthologue of *Lhx2*, acts as a selector gene for dorsal identity in the developing wing disc (Blair et al., 1994) and is required for axonal pathfinding, neurotransmitter identity, and receptor expression in the nervous system (Lundgren et al., 1995; Benveniste et al., 1998; Baumgardt et al., 2007). *dIslet* regulates neurotransmitter identity and pathfinding of specific classes of embryonic neurons and acts in concert with *Lim3* to confer target specificity in motor neurons (Thor and Thomas, 1997; Thor et al., 1999).

None of these functions is possible without a ubiquitous and versatile cofactor, Chip. Each LIM-HD molecule binds to Chip, which dimerizes, forming a functional tetramer (Gill, 2003). In the wing disc, the LIM only gene (*dLmo/Beardex*) is capable of binding Chip with a higher affinity than *Ap* and thus acts as a dominant-negative protein, preventing the formation of *Ap*:Chip tetramers (see Fig. 1) (Milán et al., 1998; Weihe et al., 2001). *Chip*-null mutations result in lethality before third-instar larval stage, whereas embryos derived from *Chip* mutant germ line clones fail to hatch (Morcillo et al., 1997). We predicted that overexpressing *dLmo* in neurons would sequester Chip from its partners and enable us to identify novel functions of Chip in neurons. Pan-neuronal expression of *dLmo* using *ELAV* (embryonic lethal and abnormal vision) *GAL4* resulted in lethality of the progeny at 25°C. When maintained at 18°C, a few flies survived, which failed to expand their wings after eclosion. This directed our interest to the well described posteclosion behavior of *Drosophila*, in which newly eclosed flies expand their wings and the cuticle becomes tanned and hard. The crustacean cardioactive peptide (CCAP)-expressing neurons of the ventral ganglion are known to regulate this behavior by secreting the neurohormone Bursicon (Honegger et al., 2002; Dewey et al., 2004; Luan et al., 2006a). Using multiple approaches to interfere with Chip function, we uncover a novel function of Chip in these neurons in the regulation of posteclosion behavior.

## Materials and Methods

**Fly stocks and culture conditions.** Fly stocks included *CCAPGal4* (16) on II (F. Rob Jackson, Tufts University School of Medicine, Boston, MA), *UAS-Chip* FL, *UAS-Chip-ΔDD*, *UAS-Chip-ΔLLID* (D. van Meyel, McGill University, Montreal, Quebec, Canada), *UAS-lim3*, *UAS-islet* (J. I. Pueyo, University of Sussex, Brighton, UK), EP1306 (U. Heberlein, University of California, San Francisco, San Francisco, CA), *UAS-dimmed* (P. Taghert, Washington University School of Medicine, St.

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Louis, MO), UAS 2x *eGFP* (H. Keshishian, Yale University, New Haven, CT), and Gal80<sup>ts</sup> lines (Bloomington Stock Center). All fly stocks were reared on standard cornmeal medium containing yeast at 25°C unless otherwise stated. UAS-*Chip* RNA interference (RNAi) stock (GD 30454) and UAS-*dicer2* (stock number 60009) were obtained from Vienna *Drosophila* RNAi Centre (VDRC), Austria (Dietzl et al., 2007).

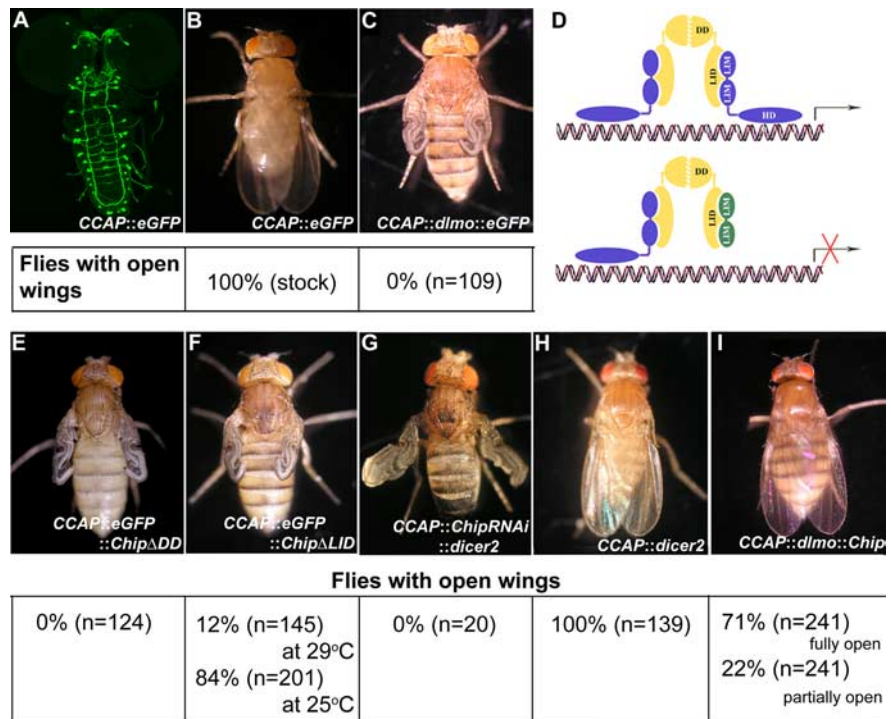
**Staging of pupae.** For developmental studies, white prepupae [0 h after puparium formation (APF)] were collected and allowed to develop on moist filter paper at 25°C unless specified. This stage lasts for an hour; hence the error in staging is  $\pm 30$  min. Ages of pupae grown at other temperatures were normalized with respect to growth at 25°C. Wild-type pupae took  $\sim 100$  h to eclose when grown at 25°C in our laboratory.

**Gal80<sup>ts</sup> experiment.** The cross was grown at 18°C to antagonize Gal4 action; transferring cultures to the restrictive temperature of Gal80<sup>ts</sup> (29°C) resulted in Gal4 being able to regulate genes downstream of UAS. Heat shock was given to the pupae (after the staging) at different time points. Each time point denotes the normalized age of pupae at 25°C when they were shifted to higher temperature (29°C).

**Immunohistochemistry: antibodies.** Rabbit anti-green fluorescent protein (GFP) (1:10,000; Invitrogen; catalog #A11122) and mouse anti-Islet (1:20; DSHB 40.3A4). Secondary antibodies from Invitrogen, Alexa 488 goat anti-rabbit (A11008), and Alexa 555 goat anti-mouse (A-21424) were used were used at 1:400 dilution. Immunohistochemistry was performed as described previously (Jhaveri et al., 2000) and imaged using an Olympus FV1000 confocal microscope. Serial optical frontal sections of the brain were acquired at intervals of 1  $\mu$ m and saved as stacks. Two-dimensional projections were generated by stacking appropriate sections for each channel using Olympus Fluoview 1000 (version 1.4a) viewer software (confocal). Image processing, including pseudocolouring and labeling, were done using Adobe Photoshop 7.0 and WCIF ImageJ (1.37c) software.

**burs (bursicon  $\alpha$ ) and pburs (bursicon  $\beta$ ) probes.** Genes were amplified from fly genomic DNA using primers containing a T7 binding sequence. Primers were as follows: CG13419 (*burs*), forward, 5'-CGCG-GATCCATGCTGCGCCACCTGCTC-3'; reverse, 5'-AATACGACT-CACATATAGGGCTATTGCAGAGCAATGCG-3'; CG15284 (*pburs*), forward, 5'-CGCGGATCCATGCATGTCCAGGAAGT-3'; reverse, 5'-TAATACGACTCACTATAGGGTAAACGTGTGAAATCGCC-3'. *In vitro* transcription was performed using the PCR product as template per the manufacturer's instruction (Roche).

**In situ hybridization.** The tissue was dissected in cold PBS, fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature, and washed with PBS containing 0.3% Triton X-100 (0.3% PTX) with frequent changes for 1 h. The samples were prehybridized at 65°C for 1 h in hybridization buffer (50% formamide, 5 $\times$  SSC, 1% SDS, 0.5 mg/ml salmon sperm DNA, and 5 $\times$  Denhardt's solution) and then hybridized with digoxigenin-labeled RNA probe in hybridization buffer at 65°C for 60 h. After hybridization, the samples were washed with graded dilutions of hybridization buffer in 0.3% PTX at 65°C (75, 50, and 25%). The final wash was with 0.3% PTX at room temperature, followed by incubation with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) diluted at 1:5000 in 0.3% PTX, overnight at 4°C. Samples were washed with several changes of 0.3% PTX for 1 h and further washed overnight. The color reaction was performed using NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Roche) per the manufacturer's instructions. After color development, samples



**Figure 1.** dLmo overexpression in CCAP-expressing neurons produces a wing expansion phenotype. **A**, CCAP::eGFP marks a set of neurons in the brain and ventral ganglia, seen in a third-instar larval preparation. **B**, Flies with this genotype are able to expand their wings normally after eclosion. **C**, Overexpressing dLmo using this driver generates flies that are unable to expand their wings after eclosion, and this phenotype is fully penetrant. **D**, As illustrated in the schematic, dLmo (green) is expected to bind cofactor Chip (yellow) and prevent formation of the active LIM-HD:Chip tetramer. **E, F**, Consistent with this model, flies overexpressing truncated constructs of Chip, such as Chip $\Delta\Delta\Delta$  (**E**) or Chip $\Delta$ LID (**F**) also fail to open their wings. **G, H**, A ChipRNAi line, together with Dicer 2 overexpression, also produces this phenotype (**G**), whereas Dicer 2 alone has no effect (**H**). **I**, Chip is able to rescue the dLmo overexpression phenotype, providing conclusive evidence of the role of Chip in this process.

were transferred through serial grades of glycerol in water and mounted in 70% of glycerol for imaging using an Axiocam attached to a Zeiss Axioplan microscope.

**Drosophila neck ligation and hemolymph transfer assay for Bursicon release.** Newly eclosed adults were collected at 5 min intervals. Flies were immobilized on an ice-chilled Petri plate and ligated at the neck with a thin silk thread, and the head was cut away. They were maintained at room temperature in a humid chamber for 60 min ( $\pm 10$  min). Ligated flies that tanned or expanded their wings during this interval were discarded. For collection of hemolymph, a small hole was made in the thorax and/or abdomen of a fly at 30 min ( $\pm 10$  min) after eclosion with a glass capillary needle and the thorax was compressed using forceps. A drop of hemolymph was collected in another glass capillary needle and was injected into the host neck-ligated fly by connecting the capillary to a glass syringe. The volume of solution injected was  $\sim 65$ –144 nl. Male flies were used as the host because it is easy to observe the cuticular darkening and tanning at abdominal tergites. Injected flies were kept in a moist Petri dish, and cuticular darkening and tanning were examined at 1 h after injection.

For injection of hemolymph/8-bromo-cAMP into intact flies, newly eclosed adults of the mutants (males and females) were collected and immobilized as above. They were injected with PBS or 0.1 M 8-bromo-cAMP or hemolymph from wild-type flies, maintained at room temperature in a humid chamber for 60 min, and examined for wing expansion.

## Results

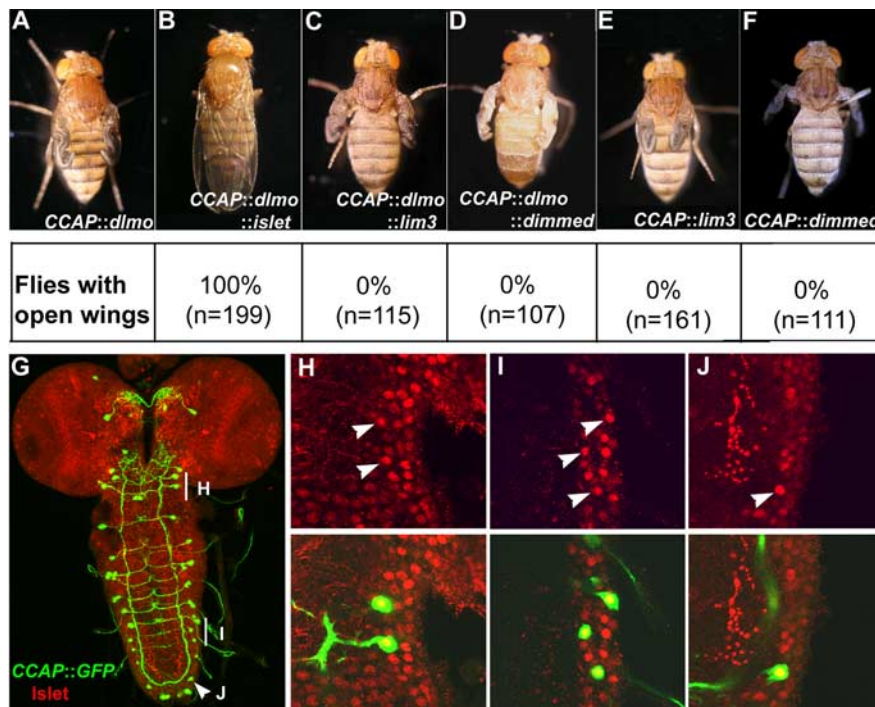
### Functional Chip protein is required in CCAP neurons for posteclosion wing expansion behavior

We used the EP1306 line (Milán et al., 1998; Zeng et al., 1998; Tsai et al., 2004) to drive ectopic expression of dLmo using CCAP-Gal4. This gave rise to flies that failed to expand their wings after

**Table 1. Chip function is required during pupal stages for normal posteclosion wing expansion**

Stage of temperature shift (normalized to growth at 25°C)	Completely folded wings	Partially open wings	Fully open wings
0 hrs APF (n= 24)	87 %	13 %	0 %
40 hrs APF (n= 27)	0	7%	93 %

Flies maintained at 18°C express Gal80<sup>ts</sup>, which interferes with Gal4 function. Consequently, dLmo overexpression does not occur. Raising the temperature to 29°C destabilizes Gal80<sup>ts</sup> and permits the overexpression of dLmo. Such a shift in temperature performed at the beginning of pupal formation (0 h APF) severely affects the posteclosion wing expansion. However, raising the temperature at a stage corresponding to 40 h APF does not affect wing expansion and produces a normal phenotype. Genotype: EP1306/+ or EP1306/Y; CCAP-Gal4/+; tubGal80<sup>ts</sup>.



**Figure 2.** dLslet, but not other partners of Chip, can rescue the wing expansion phenotype. **A–D**, The wing expansion phenotype in dLmo-overexpressing flies (**A**) is rescued by expression of dLslet (**B**) but not dLim3 (**C**) or Dimmed (**D**). **E, F**, dLim3 (**E**) or Dimmed (**F**) expressed alone produce flies with closed wings as expected. **G–J**, Distribution of CCAP-expressing cells (green) and Islet (red) in the third-instar larval brain (**G**), with regions shown at high magnification marked (**H–J**). Single confocal sections reveal that Islet expression overlaps with CCAP-positive neurons in the subesophageal ganglia (**H**) and abdominal segments (**I, J**).

eclosion (Fig. 1). To verify whether the wing expansion defect seen with dLmo overexpression was indeed attributable to impairment of Chip function, we used several different approaches. Truncated forms of Chip can act in a dominant-negative manner when expressed in cells requiring Chip function (Milán and Cohen, 1999; van Meyel et al., 1999). Consistent with this, two such truncated versions independently recapitulated the dLmo overexpression phenotype. Expression of either Chip-ΔDD, a protein that lacks the dimerization domain (DD), or Chip-ΔLID, which

lacks the LIM interaction domain (LID), produced flies that did not open their wings after eclosion (Fig. 1). Chip-ΔDD gave a fully penetrant phenotype at 25°C, whereas Chip-ΔLID gave a stronger phenotype at 29°C than at 25°C (Fig. 1). It is well known that the level of expression from a UAS promoter is dependent on its insertion site in the genome. The ChipΔLID line may require the increased Gal4 levels produced at 29°C, to drive sufficient levels of the transgene to cause a phenotype. However, it is also possible that, compared with Chip-ΔDD, higher levels of Chip-ΔLID are, in fact, required to produce the phenotype. Chip-ΔLID contains the dimerization domain and is therefore able to self-dimerize, leaving fewer molecules free to bind the endogenous Chip. In contrast, Chip-ΔDD lacks the dimerization domain and can be expected to act more efficiently by binding with the partner(s) of Chip.

To confirm the role of Chip in the control of posteclosion wing expansion, a Chip RNAi line was obtained from VDRC, Austria (GD 30454). When expressed together with Dicer 2, flies from this line also fail to open their wings after eclosion, whereas Dicer 2 alone did not cause a phenotype. Finally, we found that the wing expansion defect caused by expression of dLmo can be rescued by coexpressing Chip protein in CCAP neurons (Fig. 1). These results indicate that Chip-mediated functions are required for normal posteclosion behavior.

**Time window of Chip requirement**

Chip is known to be ubiquitously expressed at multiple stages of *Drosophila* development (Morcillo et al., 1997). To identify the stage at which Chip function is required, we used the Gal80<sup>ts</sup> system to control the timing of dLmo overexpression. At the permissive temperature (18°C), the Gal80 protein binds to Gal4, preventing its action in turning on genes downstream of UAS. At restrictive temperatures (29°C), Gal80 is inactivated, leaving Gal4 available to act on UAS-dLmo. As expected, misexpression of dLmo in the CCAP neurons for the entire pupal period (shift up at 0 h APF) led to a fully penetrant

wing expansion defect. Gal4-induced misexpression of dLmo after 40 h (shift up at 40 APF), however, does not affect posteclosion behavior (Table 1).

These experiments indicate that Chip function is likely to be required during the pupal stages to regulate posteclosion behavior. These data do not allow us to accurately pinpoint the time of requirement of Chip function in CCAP neurons because of the lag time for inactivation of Gal80 and restoration of Gal4 function. In addition to this, the time subsequently required for dLmo



induction would cause a delay in the time at which Chip function is compromised. In a different system, the estimate for the lag time of Gal4 function on temperature shift up is a few hours (McGuire et al., 2003). With these caveats, we tentatively conclude that Chip function is necessary in the CCAP neurons in the earlier one-half of pupal life for generation of wing expansion behavior that occurs on eclosion.

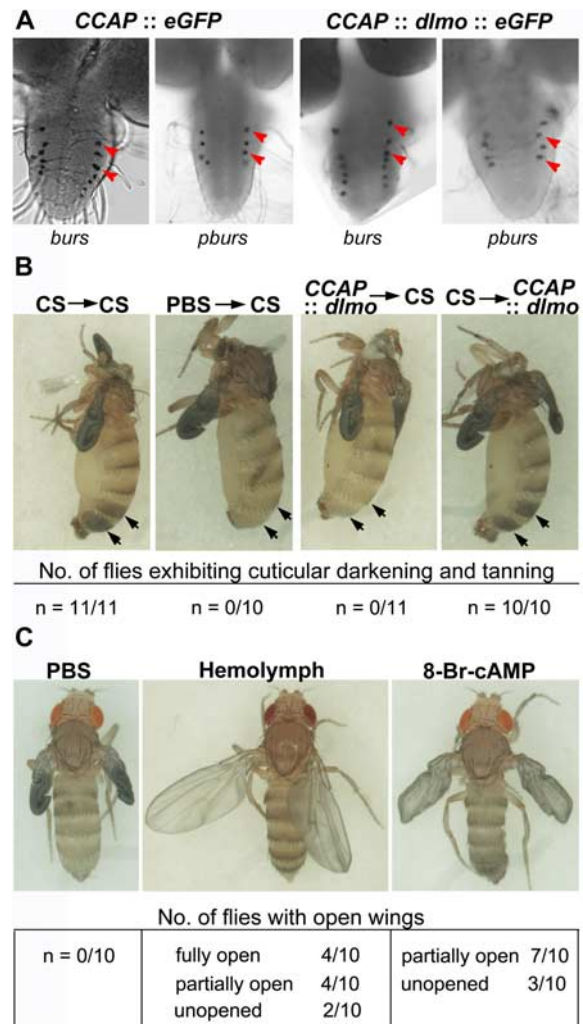
### Islet rescues the dLmo-induced wing expansion defect

To identify potential partners of Chip in regulating posteclosion behavior, we coexpressed three known interactors of Chip (van Meyel et al., 1999; Allan et al., 2005) in CCAP neurons together with dLmo. We hypothesized that the correct partner would be able to overcome the effect of dLmo overexpression. Among the candidates we tested, only dIslet was able to rescue wing expansion, whereas dLim3 (a LIM-HD protein) and Dimmed [a bHLH (basic helix-loop-helix) protein] were not able to do so (Fig. 2). Furthermore, dLim3 or Dimmed expressed alone (i.e., without dLmo) produced a defect in wing expansion when expressed in CCAP neurons (0% open wings;  $n > 100$ ). This is consistent with the expectation that any protein capable of binding Chip can sequester it away from its correct partner(s) and produce phenotypes similar to those seen with dLmo. In contrast, dIslet expressed alone resulted in normal wing opening (100% open wings;  $n = 134$ ). Because dIslet is known to bind Chip (van Meyel et al., 1999), this suggests that Islet may function as the partner of Chip in CCAP neurons in regulating posteclosion behavior.

Additional support for such a function comes from the expression of dIslet in third-instar larval brains. dIslet is widely expressed in the nervous system. In particular, neurons in the subesophageal, thoracic, as well as abdominal ganglia show colocalization of GFP (indicating CCAP-Gal4 expression) with dIslet immunoreactivity ( $n = 5$  animals) (Fig. 2). The subesophageal and thoracic neurons do not express Bursicon but are implicated in regulating its release from the neurons in the abdominal ganglia, which are known to be Bursicon-expressing (Luo et al., 2005). Null mutants of *islet* are embryonic lethal, preventing a direct examination of whether wing expansion can occur in the absence of Islet (Thor and Thomas, 1997). Nonetheless, our findings raise the hypothesis that Islet may interact with Chip to regulate Bursicon production or release, which would then affect posteclosion behavior.

### *burs* (*burs* $\alpha$ )- and *pburs* (*burs* $\beta$ )-expressing neurons are unaffected by dLmo overexpression

The neurohormone Bursicon is expressed in a subset of CCAP neurons and is responsible for initiating wing expansion and tanning and hardening of the cuticle after eclosion (Honegger et al., 2002; Dewey et al., 2004; Luan et al., 2006a). Bursicon is active as a heterodimer (Luo et al., 2005; Mendive et al., 2005), and both subunits are coexpressed in paired neurons in four abdominal segments (A1–A4) of third-instar larval/early pupal CNS. The *burs*  $\alpha$  subunit is expressed in a larger subset of CCAP neurons (seven pairs) (Luo et al., 2005). We analyzed the expression of *burs* or *pburs* transcripts in dLmo-overexpressing flies by *in situ* hybridization. Although this technique does not offer a quantitative measure of the transcripts, the numbers and positions of *burs*- or *pburs*-expressing neurons were normal at this stage of development (Fig. 3). Thus, it seems unlikely that Chip acts directly in controlling Bursicon production.



**Figure 3.** dLmo overexpression interferes with the release, but not the production or the responsiveness to Bursicon in the posteclosion hemolymph. **A**, The *burs* and *pburs* expression appears normal in dLmo-overexpressing third-instar larvae. **B**, Neck ligation of wild-type flies prevents tanning of the cuticle after eclosion. Injecting posteclosion hemolymph from wild-type flies, but not PBS, into such neck-ligated preparations, rescues this tanning phenotype (arrows). Injection of posteclosion hemolymph from dLmo-overexpressing flies fails to rescue the tanning of neck-ligated wild-type flies, indicating a deficiency of Bursicon in the hemolymph. However, injection of wild-type hemolymph into neck-ligated dLmo-overexpressing flies produces normal tanning, indicating that the response to Bursicon is normal in these flies. **C**, Intact dLmo-overexpressing flies do not expand their wings when injected with PBS after eclosion. However, wild-type hemolymph is able to rescue the wing expansion defect. This effect is also mimicked by injection of 8-Br-cAMP, implicating both a signal from the head and a Bursicon-cAMP-mediated pathway in regulating posteclosion wing expansion.

### dLmo overexpression results in an absence of Bursicon in the hemolymph at the time of eclosion

We used a well established assay to test for the presence of Bursicon in hemolymph. Neck ligation of flies immediately after eclosion prevents wing expansion and tanning and hardening of the cuticle. Of these posteclosion behaviors, the cuticle tanning and hardening deficit in neck-ligated flies can be rescued by transfer of hemolymph from a newly eclosed, intact wild-type fly (Cottrell, 1962; Fraenkel and Hsiao, 1965; Kimura and Truman, 1990). This assay is very sensitive to the presence of Bursicon in the hemolymph, because a 30-fold dilution of the hemolymph can also produce tanning (Fraenkel and Hsiao, 1963).

Whereas hemolymph from control Canton S (CS) rescued cuticle tanning when transferred into neck ligated CS flies, hemo-

lymph from dLmo-overexpressing flies failed to do so. In the reverse experiment, using neck-ligated dLmo-overexpressing flies, we found that tanning of the cuticle was rescued by hemolymph from control CS flies (Fig. 3). Both phenomena were highly robust, in that all the flies tested showed consistent results. Together, these data indicate that hemolymph from dLmo-overexpressing flies has inadequate levels of Bursicon but that these flies are able to respond to the Bursicon in CS hemolymph. The lack of Bursicon activity in the hemolymph could be attributable to underproduction of one of the subunits. Also, although we could detect *burs* and *pburs* transcripts in the appropriate neurons at the third-instar larva stage, we cannot rule out a subsequent loss of expression in CCAP neurons during the pupal stages. However, the production of Bursicon could be normal, as our *in situ* experiments suggest, but there could be a defect in release, resulting in inadequate levels of the neurohormone in the hemolymph at the time of eclosion.

Rescue of wing expansion in neck-ligated flies appears to be more complicated, because it is thought to be regulated by an additional inhibitory signal from the head, which appears to act in a highly restricted time window, from 40 min before eclosion to 10 min after eclosion. This inhibition apparently persists in neck-ligated flies, suggesting that the presence of an intact head is required so that the inhibitory control is removed and Bursicon can mediate wing expansion (Baker et al., 1999; Baker and Truman, 2002).

Therefore, we attempted to rescue wing expansion in dLmo-overexpressing intact flies (i.e., without neck ligation). We found that, in CS, hemolymph was indeed capable of rescuing wing expansion in these flies. Eight of 10 flies showed complete or partial rescue. That wing expansion in the remaining flies could not be rescued suggests a higher threshold for Bursicon for this function than for tanning. Moreover, injection of 8-bromocAMP partially mimicked this effect (Fig. 3), consistent with previous reports of Bursicon action via the cAMP pathway downstream of its receptor DLGR2 (Luo et al., 2005; Mendive et al., 2005).

In summary, the dLmo-overexpressing flies appear similar to flies that have inadequate levels of secreted Bursicon, but other aspects such as the response to normal hemolymph or 8-bromocAMP are normal.

## Discussion

In this study, we used a selective overexpression strategy to identify a novel function of Chip in a set of neurons that control a stereotyped behavioral program. Chip is a widely expressed multidomain cofactor molecule that interacts with many transcription factors. It can function both as a transcriptional coactivator and a bridging factor between proteins that bind to distal enhancers and the core transcription machinery (Matthews and Visvader, 2003). Identifying specific functions of such a protein is confounded by the superposition of a multitude of effects. The mutations in *Chip* cause early lethality precluding the examination of later functions. Because the molecule exists as a part of multiple complexes, even simultaneously within the same cell (Ramain et al., 2000), altering the level of one class of interactors can potentially disrupt several functions. We have elucidated a highly specific role of Chip in a particular class of neurons in *Drosophila* and implicated a known LIM-HD partner of Chip, Islet, in this function.

We propose that the defect is attributable to a failure in the release rather than in the production or the responsiveness to the neurohormone Bursicon. How might Bursicon release be con-

trolled as a result of Chip function in development? The hemolymph transfer experiments provide a unique insight into this puzzle. The literature describes a model wherein posteclosion wing expansion requires a combination of a neural signal from the brain as well as Bursicon release. Our results extend our understanding of how this interplay of activity and secreted factors is set up in development. It appears that, several days before eclosion, Chip is able to regulate an as-yet-unidentified event in the CCAP neurons, such that the hemolymph contains adequate levels of Bursicon after eclosion. The CCAP-expressing neurons are divided into at least two interacting subpopulations, only one of which secretes Bursicon. The other subpopulation does not secrete Bursicon but is implicated in regulating its release (Luan et al., 2006a,b). Chip may therefore mediate the formation of proper connectivity among CCAP neurons, which eventually ensures timely Bursicon release several days later. Supporting this scenario, Chip has been reported to regulate axon pathfinding and proper innervation of targets in other systems (van Meyel et al., 2000; Komiyama and Luo, 2007). Our data are suggestive of Chip requirement in the early period of puparium formation, which fits well with a report that CCAP neurons undergo extensive remodeling in this period of metamorphosis (Zhao et al., 2008). The importance of this connectivity is underscored by the identification of several other genes in a gain-of-function screen, which displayed a simultaneous disruption of both posteclosion wing expansion and the pattern of CCAP neuron innervation (Zhao et al., 2008). Therefore, our findings motivate an examination of Chip function in regulating the connectivity of CCAP neurons, a role that directly links this key aspect of neuronal development with the control of posteclosion behavior in *Drosophila*.

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