

# Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior

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## SUMMARY

Insect growth and metamorphosis is punctuated by molts, during which a new cuticle is produced. Every molt culminates in ecdysis, the shedding of the remains of the old cuticle. Both the timing of ecdysis relative to the molt and the actual execution of this vital insect behavior are under peptidergic neuronal control. Based on studies in the moth, *Manduca sexta*, it has been postulated that the neuropeptide Crustacean cardioactive peptide (CCAP) plays a key role in the initiation of the ecdysis motor program. We have used *Drosophila* bearing targeted ablations of CCAP neurons (CCAP KO animals) to investigate the role of CCAP in the execution and circadian regulation of ecdysis. CCAP KO animals showed specific defects at ecdysis, yet the severity and nature of the defects varied at different developmental stages. The majority of CCAP KO animals died at the pupal stage from the failure

of pupal ecdysis, whereas larval ecdysis and adult eclosion behaviors showed only subtle defects. Interestingly, the most severe failure seen at eclosion appeared to be in a function required for abdominal inflation, which could be cardioactive in nature. Although CCAP KO populations exhibited circadian eclosion rhythms, the daily distribution of eclosion events (i.e., gating) was abnormal. Effects on the execution of ecdysis and its circadian regulation indicate that CCAP is a key regulator of the behavior. Nevertheless, an unexpected finding of this work is that the primary functions of CCAP as well as its importance in the control of ecdysis behaviors may change during the postembryonic development of *Drosophila*.

Key words: Molting, Neurohormone, Behavior, Pupation, Eclosion, *Drosophila melanogaster*

## INTRODUCTION

Insect growth and development occurs through multiple stages. At the end of each stage insects molt to produce a new cuticle for the next stage. During this process, the new cuticle develops beneath the old one, while much of the old cuticle is resorbed. The final, vital, step of this developmental process is ecdysis, the shedding of the remaining old cuticle. Ecdysis is a complex yet stereotyped behavior whose timing must be precisely coordinated with the molting cycle such that it is turned on only when the old cuticle is sufficiently resorbed that it can successfully be shed. In addition, the timing of some ecdyses, typically that to the adult (adult ecdysis or eclosion), can be under the control of the circadian clock.

While molting (the production of the new cuticle) is regulated by the ecdysteroid class of steroid hormones, the timing as well as the execution of ecdysis behavior is controlled by the neuropeptides, Eclosion hormone (EH), Ecdysis triggering hormone (ETH, and associated Pre-ecdysis triggering hormone, PETH), and Crustacean cardioactive peptide (CCAP) (reviewed

by Ewer and Reynolds, 2002). Of these, CCAP is believed to be the neuropeptide that turns on the ecdysis motor program. In addition to a role in the execution of ecdysis, strong circumstantial evidence suggests that CCAP may be one of the factors that regulate the circadian timing of adult ecdysis (eclosion). For example, the LARK RNA-binding protein has been implicated in the circadian control of *Drosophila* eclosion (Newby and Jackson, 1993), and it is localized preferentially in the cytoplasm of CCAP neurons (McNeil et al., 1998; Zhang et al., 2000).

Although our model for the hormonal control of ecdysis is consistent with most of the available data, a number of observations suggest that the control of this behavior occurs via a more complicated mechanism. For instance, adult ecdysis still occurs in *Drosophila* lacking EH neurons (McNabb et al., 1997). Likewise, although the genetic deletion of the gene encoding ETH causes most animals to die at the first larval ecdysis, many of these animals still display ecdysis-like behavior at the end of this molt (Park et al., 2002a).

The complex phenotypes of these variants also raises the

possibility that the role of CCAP in the control of ecdysis may not be as simple as currently proposed. Here we have used *Drosophila* to investigate the roles of CCAP in the control and circadian regulation of ecdysis. We find that the genetic ablation of the CCAP neurons causes defects at ecdysis. However, the type of defects observed at the ecdyses to different developmental stages as well as the severity of these defects suggest that the role of CCAP in the control of ecdysis varies during postembryonic development. In addition, although populations of flies lacking CCAP neurons exhibited a circadian rhythmicity of eclosion, the daily timing of eclosion events was abnormal in these animals, implying a modulatory role for CCAP in the circadian control of this behavior.

## MATERIALS AND METHODS

### Cloning of *Drosophila* CCAP gene

We used RACE to clone and define the 5' and 3' end of the *Drosophila* CCAP (*DmCCAP*; *Ccap - FlyBase*) cDNA using total RNA from adult heads. The degenerate primers used were designed on the basis of the amino acid sequences of the CCAPs identified in other species (reviewed by Dirksen, 1998), and PCR reactions were carried out as described previously (Park and Hall, 1998). The sequences obtained from 3'- and 5'-RACE were assembled to construct a complete *DmCCAP* cDNA sequence. Primers corresponding to the 5' and 3' ends of the *DmCCAP* cDNA were then used to obtain full-length *DmCCAP* cDNA by RT-PCR. These primers were also used to obtain the sequence of the genomic DNA, using DNA isolated from wild-type Canton-S adult flies.

### Generation of CCAP-GAL4 driver

We used the GAL4 system (Brand and Perrimon, 1993) to drive gene expression in CCAP neurons. DNA immediately 5' of the *DmCCAP* coding region and extending from -516 to +39 bp was obtained by PCR using wild-type genomic DNA as a template. The PCR products were inserted into the pPTGAL transformation vector (Sharma et al., 2002). The recombinant *DmCCAP* promoter-GAL4 fusion (CCAP-GAL4) construct was introduced into the germline using standard methods. Several independent transformant lines were obtained.

### Immunohistochemistry

Immunohistochemistry was performed using standard techniques (cf. Ewer and Truman, 1996). Primary antibodies used were rabbit anti-CCAP (used at 1:5,000; a kind gift from Dr Hans-Jürgen Agricola, U. Jena, Germany), rabbit anti-ETH1 (used at 1:2000; a kind gift from M. Adams and Y. Park), and mouse anti- $\beta$ -galactosidase (anti- $\beta$ -gal; used at 1:2000; Promega). Secondary antibodies were obtained from Jackson ImmunoResearch and Molecular Probes. Fluorescent preparations were viewed under a conventional fluorescent microscope as well as under a confocal microscope (Biorad MRC600 with Zeiss Axiovert inverted microscope, or a Leica DMR system).

### Quantitation of immunolabeling

Fluorescently labeled tissues to be quantitated were all processed and stained in parallel and under the same conditions. In order to quantitate CCAP immunostaining, Z-series of confocal sections were collected at non-saturated settings, then collapsed keeping the maximum intensity pixels. These images were then analyzed using NIH Image. First, the background signal was subtracted and the resulting image was smoothed. A threshold was then set such that only the intensely stained varicosities were visible, and the number of varicosities was counted using the same threshold for all preparations. A 'Varicosity index' was defined, based on the number of varicosities per unit of axon length. The intensity of ETH-IR was scored

qualitatively by assigning a subjective score of 3 (strong staining) to 0 (no staining) to Inka cells. The person scoring the preparations did not know the timepoints at which the tissues had been fixed.

### In situ hybridization

RNA in situ hybridization was carried out using standard methods (cf. Patel, 1996). CCAP RNA probes were labeled with DIG and visualized using either NBT/BCIP (blue reaction product) or Fast Red (fluorescent red label; Sigma Chemical Co.). Tissues labeled for both CCAP RNA and CCAP-IR were processed sequentially, first for RNA in situ hybridization and reacted with NBT/BCIP, and then processed for CCAP-IR and reacted using DAB and H<sub>2</sub>O<sub>2</sub>.

### Fly strains and genetics

A UAS-*lacZ* line that produced cytoplasmic  $\beta$ -gal expression was obtained from the Bloomington *Drosophila* stock center; the UAS-*rpr* strain used was obtained from H. Steller. Since driving *lacZ* expression in the CCAP neurons produced  $\beta$ -gal immunoreactivity (-IR) that was stronger than was the normal CCAP-IR, we used  $\beta$ -gal as an independent marker for the presence of the CCAP neurons. Thus, for experiments involving the targeted ablation of CCAP neurons, we used flies bearing both UAS-*rpr* and UAS-*lacZ* inserts, which were generated by standard recombination, and are referred to as UAS-*rpr* + *lacZ*. Targeted ablation of CCAP neurons was produced by crossing CCAP-GAL4 flies (males were typically used) to flies bearing UAS-*rpr* + *lacZ*. Flies used in the experiments shown in Fig. 7A were generated by crossing UAS-GFP flies (carrying a P{UAS-GFP.S65T} insert) to *tim*-GAL4 flies (Kaneko and Hall, 2000); *pdf*-GAL4 transgenics have been described previously (Park et al., 2000); UAS-*shibire*<sup>ts</sup> transgenics were kindly provided by Toshi Kitamoto (Beckman Research Institute of the City of Hope). All flies were raised at 25°C on standard fly food under a 12 hour:12 hour light:dark regime unless otherwise indicated.

### Behavioral analyses

#### Larval ecdysis

Approximately 20 males and 80 females were placed in a population cage. For controls, CCAP-GAL4 males were crossed to UAS-*lacZ* flies. Eggs were collected daily on standard agar/apple juice plates (Wieschaus and Nüsslein-Volhard, 1998). Zero- to 12-hour old larvae were then collected and transferred to plates containing standard fly food. These plates were kept at 25°C. At the end of day 3, larvae approaching the ecdysis from the 2nd to 3rd instar were identified based on the appearance of 'double mouth plates' [DMP; approx. 30 minutes prior to ecdysis (Park et al., 2002a)]. Animals at an early DMP stage were individually transferred to agar/apple juice plates and their behavior recorded. All behavioral observations were done at 25°C. Recordings were done under a Leica dissecting microscope using an Optronix 750DE camera attached to a Panasonic AG-6040 time-lapse video recorder (used at normal speed).

#### Pupal ecdysis

First instar larvae were collected as described above, placed in vials containing standard fly medium, and transferred to 20°C. Animals that had recently pupariated were examined, and those containing a bubble in mid-region of the puparium [late stage P4(i) (Bainbridge and Bownes, 1981)] were selected, placed on their side on a microscope slide, and filmed at room temperature (approx. 22°C) under dim transmitted light using a Leica DMLB microscope (10 $\times$  magnification). One experimental and one control animal was filmed simultaneously, at one-sixth the normal speed.

#### Adult ecdysis (eclosion)

Pharate adults that had reached the 'grainy' stage [approx. 3 hours before eclosion (Kimura and Truman, 1990)] were placed on a microscope slide, the operculum carefully removed in order to more clearly visualize the head movements, and the slide placed in a humidified chamber. For consistency, only females were used.

Eclosion behavior was recorded at room temperature under a Leica dissecting microscope at 1/6 the normal speed.

Analysis of eclosion rhythms

Crosses consisting of at least 20 males and 20 virgin females were set up in culture bottles. As control populations, either *y w; CCAP-GAL4* or *UAS-rpr* homozygous flies were crossed to *w<sup>1118</sup>* flies. Because of the low percentage of surviving CCAP KO adults, 20-35 bottles were set up for this genotype, whereas between eight and 10 bottles were used for each control cross. Cultures were reared at 25°C for 5-7 days and then shifted to 18°C for the remainder of development. Developing progeny were entrained for at least 5 days to a 12 hours light:12 hours dark (LD 12:12) lighting schedule. Once adult eclosion commenced, bottles were cleared every 2 hours over a 48-hour period and newly

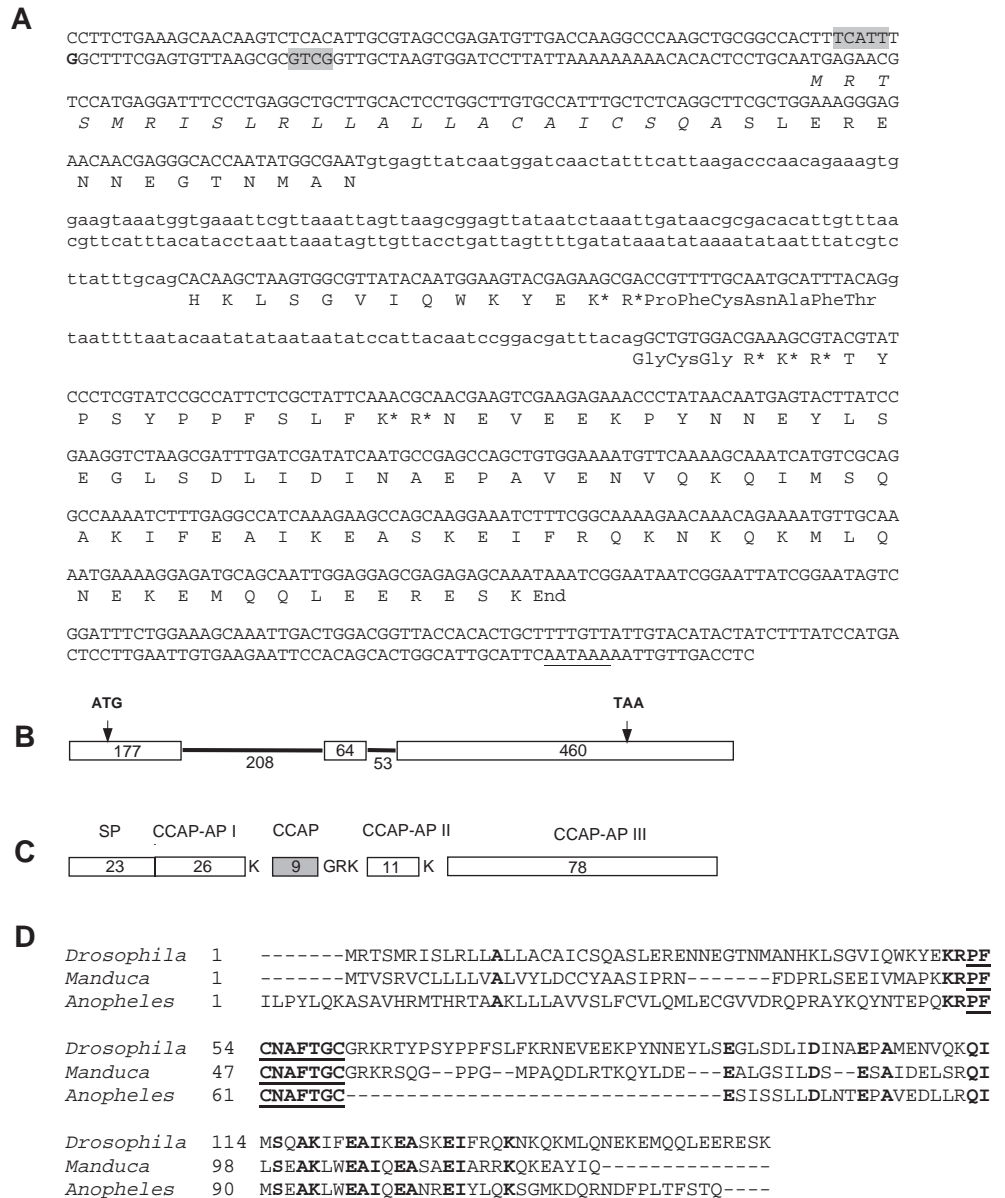
emerged adults were counted. After this 48-hour LD collection was completed, the lights were turned off and the populations were allowed to free run in constant darkness (DD). After 4-5 days in DD, eclosion was then monitored (in DD) for 24 or 48 hours.

RESULTS

Characterization of the *Drosophila* CCAP (*DmCCAP*) gene

We used RACE to isolate the 701-nucleotide-long *Drosophila* CCAP (*DmCCAP*) cDNA, which encodes a 155 amino acid precursor (Fig. 1A). Conceptual translation of this precursor

**Fig. 1.** Nucleotide and deduced amino-acid sequence of the *DmCCAP* gene. (A) Genomic sequence of *DmCCAP*. Sequences for the partial 5'-upstream region and for the cDNA are indicated in upper case, and the intervening sequences are presented in lower case. A consensus polyadenylation signal (AATAAA) is underlined, and the transcription initiation site is designated by a bold-faced letter. A putative arthropod initiator (TCATT) and a downstream promoter element (GTCG) are shaded gray. A putative signal peptide is indicated by italics; amino acids represent the predicted pre-pro-DmCCAP peptide. Potential endoproteolytic cleavage sites are designated by asterisks. (B) Schematic diagram of the genomic organization of *DmCCAP*. Open boxes represent exons and solid lines represent introns. Numbers indicate the nucleotide length for the corresponding exons and introns. Approximate positions for the start (ATG) and stop (TAA) codons are indicated by arrows. (C) Reconstruction of the pre-pro-DmCCAP structure. SP, signal peptide; CCAP-AP I, II, and III: CCAP-associated peptides I, II and III, respectively. CCAP and the other domains are represented by a shaded box and by open boxes, respectively. The number in each box indicates the amino acid length for each domain. The consensus endoproteolytic cleavage sites are also shown between the boxes. (D) Comparison of the amino acid sequences of CCAP precursors. *Manduca sexta* sequence from Loi et al. (Loi et al., 2001); mosquito (*Anopheles gambiae*) CCAP gene sequence was obtained from



mosquito genome project database (agCG50022: accession no. EAA14174). Identical amino acids are highlighted in bold; there is a perfect match between sequences for the CCAP peptide (underlined). In addition, a significant homology was observed for the CCAP-AP III predicted peptide. Consensus proteolytic cleavage site between DmCCAP-AP II and III was not found in *Manduca* CCAP precursor structure. Surprisingly, the amidation signal (GRKR) was absent from the mosquito sequence, suggesting that CCAP in this insect may not be modified at its C terminus, resulting in much longer CCAP-like peptide. More careful characterization of the corresponding cDNA will be necessary to confirm this result.

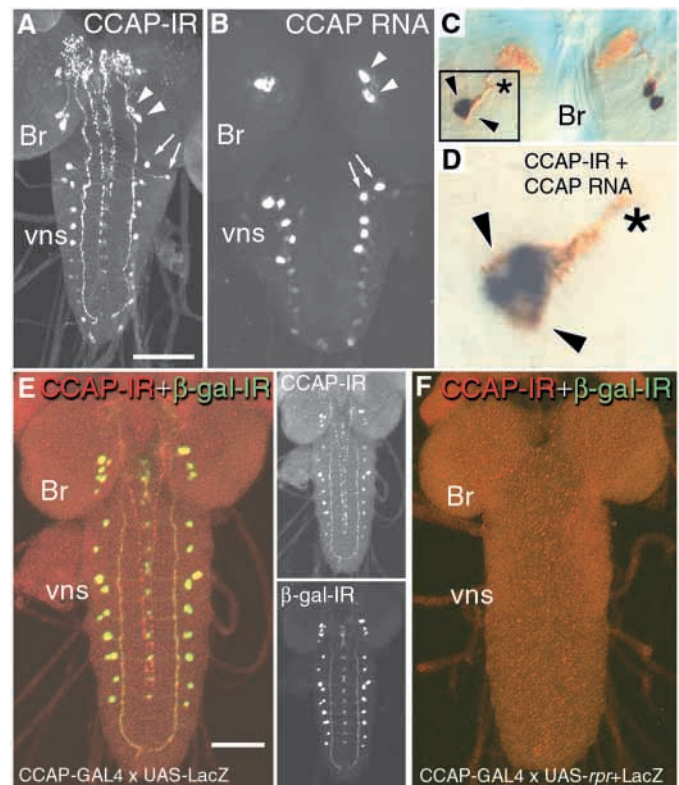
indicates that several peptides may be derived from the *DmCCAP* gene via post-translational processing. The N-terminal 23 amino acid residues are characteristic of a signal peptide [(Nielsen et al., 1997) and see <http://www.cbs.dtu.dk>]. In addition, the presence of three consensus endoproteolytic cleavage sites [double or triple basic amino acids (Sossin and Scheller, 1991)] suggests that four peptides could be produced from this precursor (Fig. 1A,C). The amino acid sequence of one of these is identical to that of CCAP, which is, so far, 100% invariant among a number of crustacean and insect species (reviewed by Dirksen, 1998). The presence of the consensus modification site (GRKR; Fig. 1A,C) suggests that CCAP is likely amidated at the C terminus in this fly (see Kolhekar et al., 1997) as it is in other arthropods. The other 3 putative peptides are called here CCAP-associated peptides (CCAP-AP) I, II and III. Comparisons between the conceptually translated products of *DmCCAP* and those of other sequenced CCAP genes showed that, of the associated peptides, only CCAP-AP III exhibits any significant homology among CCAP precursors (Fig. 1D) (see Loi et al., 2001). The *DmCCAP* gene includes three exons separated by two introns, 208 and 53 nucleotides long (Fig. 1A,B). The second intron occurs within the sequence encoding the CCAP peptide, as is also seen in the *Manduca CCAP* gene (Loi et al., 2001).

We used DIG-labeled antisense probes synthesized using the *DmCCAP* cDNA to determine the in situ pattern of expression of the gene corresponding to this cDNA in the CNS of 3rd instar larvae. The observed pattern of RNA (Fig. 2B) expression matched that of the known patterns of CCAP-immunoreactivity [CCAP-IR; Fig. 2A (Ewer and Truman, 1996)]. Furthermore, processing these tissues simultaneously for both RNA expression and immunoreactivity revealed that CCAP immunoreactivity and *DmCCAP* mRNA were always co-localized (Fig. 2C,D). This complete concordance between the two signals, coupled with the sequence information of the *DmCCAP* gene, demonstrate that the cloned sequence encodes the CCAP peptide. BLAST searches against the sequence of the *Drosophila* genome (Adams et al., 2000) produced the gene CG4910 as the only hit.

Analysis of the 5' regulatory region of *DmCCAP* showed that it is devoid of a canonical TATA box; however, the region immediately upstream of transcription start does contain a putative arthropod initiator element (Cherbas and Cherbas, 1993) and a downstream promoter element (Kutach and Kadonaga, 2000), as potential core regulatory elements (Fig. 1A). A potential TATA box is found 367 bp 5' of this putative arthropod initiator element and preliminary results suggest that an additional stage-specific transcript may be initiated from this upstream location.

### Defining the 5' regulatory region of the CCAP gene

In order to establish that the *CCAP-GAL4* transgene accurately reproduced the expression of the *DmCCAP* gene, we first used it to drive expression of the reporter *lacZ*, and compared the spatial expression of the reporter to that of CCAP. In *Drosophila* the CCAP peptide is consistently expressed in 2 pairs of neurons in the brain, 5 pairs in the subesophageal ganglion, 1-2 pairs in at least 8 ganglia of the ventral nervous system (vns) (Fig. 2A,E), as well as in 2 pairs of strongly immunoreactive descending axons, one lateral and one medial (see Ewer and Truman, 1996). We found no evidence of changes in the number



**Fig. 2.** Expression of CCAP RNA in CCAP neurons, and use of *CCAP-GAL4* fusion for targeted ablation of CCAP neurons. (A,B) Expression of CCAP-IR (A) and of CCAP RNA (B), in 3rd instar larva CNS. Neurons located in similar positions are indicated by the same symbols, emphasizing the similarity of the two patterns of expression. (C,D) CCAP-IR (brown) in combination with CCAP RNA expression (blue), illustrating co-localization of these two signals in the 2 pairs of CCAP-immunoreactive neurons in the brain (arrowheads in A and B). (D) Higher magnification of boxed pair of neurons in C; the 2 neurons are very close to each other. Arrowheads point to (clear) nuclei; blue staining is due to RNA expression in the cell bodies, while brown is CCAP-IR, and is especially visible in the neuronal processes (asterisk in C and D). (E) Pattern of CCAP-IR (red and upper right panel) and  $\beta$ -gal-IR (green and lower right panel) in late 2nd instar CNS of *CCAP-GAL4*  $\times$  *UAS-lacZ* progeny. All CCAP-immunoreactive neurons were  $\beta$ -gal immunoreactive, and vice versa. (F) Targeted ablation of CCAP neurons. Pattern of CCAP-IR (red) and  $\beta$ -gal-IR (green) in CNS of *CCAP-GAL4*; *UAS-rpr* + *lacZ* late 2nd instar. Br, brain; vns, ventral nervous system. Scale bar: (A) 80  $\mu$ m, (E) 40  $\mu$ m.

of neurons that expressed CCAP-IR during postembryonic development except following adult eclosion, when there is a precipitous decrease in the number of CCAP neurons due to their elimination by programmed cell death (Draizen et al., 1999). Thus, unlike the situation in *Manduca* (Davis et al., 1993; Loi et al., 2001), no CCAP immunoreactive neurons appear to be added to the pattern that is established by the 1st instar larval stage.

We found that the *GAL4* fusions bearing the -516 to +39 bp fragment of 5' *DmCCAP* DNA faithfully reproduced the temporal and spatial pattern of *DmCCAP* expression. Thus, in all cases examined, neurons that were CCAP immunoreactive were also  $\beta$ -gal immunoreactive, and vice versa. The stages

examined included 1st instar (0- to 2-hour, 6- to 8-hour and 21- to 24-hour-old 1st instars), mid- and late-2nd instar, pharate and wandering 3rd instar larvae, pharate pupae, pharate adults, and 6-day-old adults (late 2nd instar: Fig. 2E; other stages not shown;  $n > 10$  for each stage). All three independent transformant lines bearing this construct showed indistinguishable patterns of expression. All experiments reported here were carried out using line no. 16, hereafter referred to as *CCAP-GAL4*.

### Targeted ablation of CCAP neurons

To produce animals lacking CCAP neurons, we drove expression of the cell death gene *reaper* (*rpr*) (White et al., 1994; White et al., 1996) in these neurons, using the *CCAP-GAL4* transgenic strain. A similar approach has been successfully used to study the function of other *Drosophila* neuropeptides and hormones (e.g. McNabb et al., 1997; Renn et al., 1999; Rulifson et al., 2002).

To investigate the consequences of loss of CCAP neurons on larval ecdysis, CCAP neurons had to be absent, at the latest, prior to the last larval ecdysis, that from 2nd to 3rd larval instar. To determine the extent to which targeted expression of *rpr* in the CCAP neurons caused their ablation prior to the end of the 2nd instar molt, we dissected the CNSs from mid-2nd instar progeny of a *CCAP-GAL4* × *UAS-rpr* + *lacZ* cross, and processed them simultaneously for CCAP- and  $\beta$ -gal-IR (the cytoplasmic *lacZ* reporter used acting as an independent and robust marker for CCAP neuronal cell bodies and processes, see Materials and Methods). In control *CCAP-GAL4* × *UAS-lacZ* animals, CCAP- and  $\beta$ -gal-IR was detectable in two pairs of neurons in the brain, around 15 pairs in the vns (average:  $32.2 \pm 0.7$  neurons;  $n = 11$ ), as well as in strongly immunoreactive descending axons (Fig. 2E). In contrast, out of 32 *CCAP-GAL4* × *UAS-rpr* + *lacZ* CNSs processed, 29 had no detectable CCAP- or  $\beta$ -gal-immunoreactive neurons or processes (Fig. 2F), while the remaining three CNSs had only one weakly stained neuron each but no visible stained axonal processes (not shown). Thus, the targeted expression of *rpr* using the *CCAP-GAL4* driver produced late 2nd instar larvae that are probably entirely devoid of CCAP function.

In certain experiments that examined post-larval ecdyses, animals were transferred to 20°C after collection as first instar larvae and raised at this temperature until pupation or eclosion (see below). At this lower temperature the vast majority of the CNSs were also mostly devoid of CCAP neurons by the end of the 3rd instar (at wandering). Thus, of 22 CNSs examined at this time, 15 showed no CCAP- or  $\beta$ -gal-immunoreactive neurons or processes, while four, two and one CNSs had one, two and four weakly stained neurons, respectively, and none of these CNSs had visible immunoreactive processes (not shown). When the CNS of animals raised using the eclosion rhythm paradigm (25°C to 18°C; see Materials and Methods) was processed for CCAP- and  $\beta$ -gal-IR immediately after adult eclosion, 25 of 28 CNSs showed no immunoreactivity, while two and one CNSs had one and two weakly staining neurons, respectively, lacking visible processes (not shown).

### Behavioral and developmental defects caused by the targeted ablation of CCAP neurons

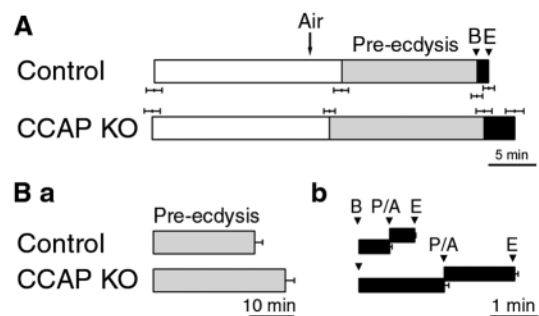
#### Larval ecdysis

In the moth *Manduca sexta*, addition of CCAP to an isolated

larval abdominal CNS turns on the ecdysis motor program (Gammie and Truman, 1997b; Zitnan and Adams, 2000). This, and other evidence (reviewed by Ewer and Reynolds, 2002), strongly implicates the CCAP neuropeptide in the control of ecdysis behavior in this moth. In the CNS of *Drosophila* larvae, CCAP-IR decreases shortly before the onset of larval ecdysis (A. C. Clark, M. del Campo and J.E., unpublished data), suggesting that CCAP is similarly important for the control of ecdysis in this insect.

To investigate directly the role of CCAP in larval ecdysis, we characterized animals lacking the CCAP neuronal population. Surprisingly, we found that genetic ablation of the CCAP neurons was not lethal during the larval stages. Indeed, the survival rate of CCAP KO from 1st instar to the end of the 3rd (last) instar was indistinguishable from that of the control population (97% vs. 95%, respectively;  $n = 400$  for each group). This indicates that CCAP is not essential for viability during (at least) the latter part of the 2nd larval intermolt period and the entire 3rd larval instar. Most significantly, animals lacking CCAP neurons were able to shed their old cuticle at the end of molt to the 3rd instar. Independent studies show that animals homozygous for small chromosomal deletions including *CCAP* (and 14 other genes) survive until the 3rd instar (J.E., unpublished data). Thus, survival of CCAP KO larvae until this stage is not due to persisting (but immunohistochemically undetectable) CCAP peptide.

To determine whether ecdysis behavior was normal in KO animals, we examined the sequence and timing of ecdysis behavior from the 2nd to the 3rd instar. Markers for the completion of a larval molt have been described previously (Park et al., 2002a) (see Fig. 3 and Table 1). The earliest obvious marker for the impending ecdysis is the appearance of pigmentation in the mouth plates of the future 3rd instar (double mouth plates stage; DMP), which occurs about 30 minutes before ecdysis. Approximately 16 minutes after the DMP stage, air enters the new trachea, which is followed shortly by the onset of the preparatory behavior called pre-



**Fig. 3.** Larval ecdysis behavior in KO animals. (A) Timing of morphological and behavioral markers at ecdysis from the 2nd to the 3rd larval instar. (B) Direct comparison of the duration of pre-ecdysis (a) and of the components of ecdysis (b). Open bars show the period between DMP and onset of pre-ecdysis (gray bar). 'Air' marks the time of entry of air into the 3rd instar trachea. Pre-ecdysis terminates with the occurrence of 'biting' behavior (B), which is then followed by the expression of posterior-to-anterior peristalses (P/A) and the ensuing shedding of the old cuticle (E, ecdysis). Records were aligned relative to the time of tracheal air filling ('Air') and are averages  $\pm$  s.e.m.;  $n = 8$  for each group. These data and the statistical significance of the observed differences are given in Table 1.

**Table 1. Timing of developmental and behavioral events at ecdysis from 2nd to 3rd instar larva in KO animals**

| Event  | KO <sup>†</sup> | Control                      |
|--|-----------------|------------------------------|
| DMP <sup>‡</sup> – cuticle shed                | 36.29±0.91      | 31.89±1.01 (*)               |
| DMP – tracheal air entry                       | 16.71±0.97      | 16.41±0.81 (ns) <sup>§</sup> |
| Tracheal air entry – pre-ecdysis start         | 2.09±0.61       | 3.33±0.77 (ns)               |
| Pre-ecdysis start – end                        | 14.25±1.01      | 11.00±0.86 (*)               |
| No. of pre-ecdysis contractions                | 99.1±5.7        | 80.6±7.1 (**)                |
| Frequency of pre-ecdysis contractions          | 7.11±0.49       | 7.40±0.43 (ns)               |
| Ecdysis ‘bites’ – cuticle shed                 | 3.24±0.53       | 1.15±0.07 (**)               |
| Ecdysis ‘bites’ – P/A peristalses <sup>¶</sup> | 1.78±0.39       | 0.64±0.08 (**)               |
| P/A peristalses – cuticle shed                 | 1.46±0.42       | 0.51±0.06 (*)                |
| No. of ecdysis ‘bites’                         | 64.9±12.0       | 42.2±4.1 (*)                 |
| No. of A/P peristalses <sup>††</sup>           | 4.4±1.6         | 0.0±0.0 (**)                 |
| No. of P/A peristalses                         | 8.7±0.9         | 3.7±0.2 (**)                 |

<sup>†</sup>Values are averages±s.e.m.; *n*=8 for each group. Units are minutes or number, depending on the event.

<sup>‡</sup>Double Mouth Plate stage.

<sup>§</sup>(ns), *P*>0.05; (\*) 0.01<*P*<0.05; (\*\*) *P*<0.01. Student's *t*-test.

<sup>¶</sup>P/A peristalses: Anteriorly directed peristaltic waves.

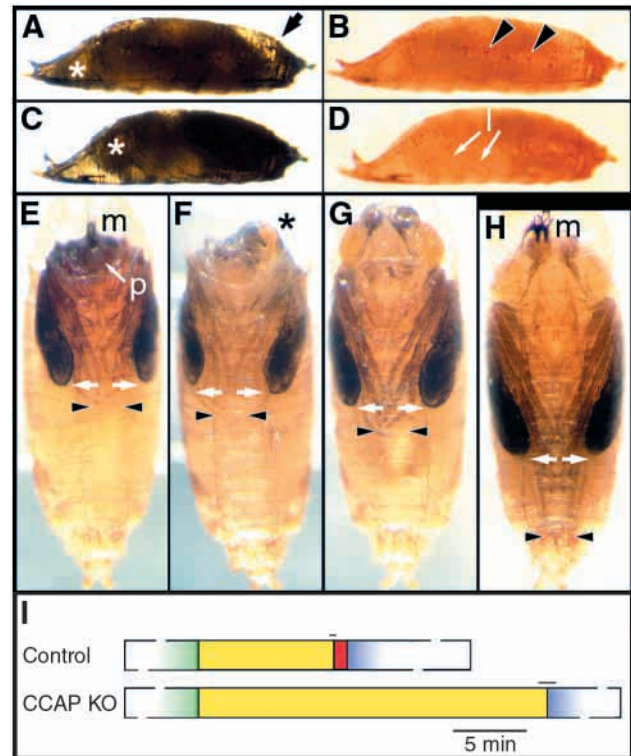
<sup>††</sup>A/P peristalses: Posteriorly directed peristaltic waves.

ecdysis (see Park et al., 2002a). Approximately 15 minutes after air filling, pre-ecdysis stops and the animal executes a characteristic ‘biting’ behavior during which it appears to be attempting to tear the anterior region of the old cuticle. This period is then followed by the onset of ecdysis proper, which is characterized by vigorous peristaltic waves sweeping along the animal in a posterior-to-anterior direction. Typically after three to four waves, the anterior cuticle breaks, freeing the 3rd instar of its 2nd instar cuticle. After a period of a few minutes the animal resumes feeding and locomotory behavior.

Fig. 3 and Table 1 summarize the larval ecdysis phenotype of CCAP KO animals compared to that of the appropriate controls. Although the CCAP KO larvae were clearly able to initiate ecdysis behavior and use this behavior to free themselves from the 2nd instar cuticle, there were subtle but significant differences between the behavior of CCAP KO and control animals. The duration of events up to the onset of pre-ecdysis was indistinguishable for these two groups of larvae. The first notable difference between CCAP KOs and control larvae was a modest but significant lengthening of pre-ecdysis behavior (Fig. 3A,Ba, Table 1), although pre-ecdysis behavior itself appeared normal (for instance, the frequency of pre-ecdysis contractions was no different from that of the controls; Table 1).

Additionally, the subsequent events that led to cuticle shedding took approx. three times longer in CCAP KO larvae than in controls (Fig. 3A,Bb, Table 1). Both the biting period, which occurs between the end of pre-ecdysis and ecdysis onset, and the duration of ecdysis itself, were significantly extended in CCAP KO animals. Interestingly, CCAP KO larvae exhibited anterior to posterior peristaltic waves interspersed with the typically occurring posterior to anterior waves, a behavior never observed in control animals (Table 1). Because the waves moving in the anterior to posterior direction do not aid in breaking the old cuticle, the time to successful shedding of the old cuticle was lengthened.

These results reveal that the ablation of CCAP neurons is associated with defects that are strictly confined to the execution of ecdysis itself. Thus, while the entire duration of the period between DMP and ecdysis was increased by only 14%, from the normal 31.9±1.0 minutes (*n*=8) to 36.3±0.9



**Fig. 4.** Targeted ablation of CCAP neurons causes failures of pupation. (A–D) Early KO pupa (A, B) and corresponding control (C, D). KO animals show defects such as incomplete head eversion, as evidenced by the anterior position of head (white asterisk indicates the position of the eye); retracted posterior cuticle due to failure to transition from pre-ecdysis to ecdysis (arrow in A), and incomplete shedding of tracheal lining (arrowheads in B point to scars left on the larval cuticle). White arrows in D indicate properly everted legs (l) in control animal. (E–H) KO (E–G) and control (H) pharate adults. The failure at pupation in KO animals results in defects in adult head formation and in leg and wing extension. Black asterisk: partial adult head; m, larval mouthhooks; p, proboscis of adult. White arrows and black arrowheads in E–H indicate the posterior extent of the wings and prothoracic set of legs, respectively. (I) Timecourse of morphological and behavioral events at pupation. Pharate pupae collected at late stage P4(i) (Bainbridge and Bownes, 1981) were first quiescent (green gradient), then went into pre-ecdysis (yellow), followed by ecdysis (head eversion; red segment), which was specifically absent in KO animals. Ecdysis (in the control) and the extended pre-ecdysis (of KO animals) were followed by a long period of abdominal movements (blue gradient). Records were aligned relative to the onset of pre-ecdysis, and the duration of each interval is indicated as average±s.e.m.; times prior to pre-ecdysis were not tabulated. These data and the statistical significance of the observed differences are tabulated in Table 2.

minutes (*n*=8), the timing and organization of ecdysis behavior itself was quite severely disrupted in the KO animals.

#### Pupal ecdysis

In higher Diptera such as *Drosophila*, pupal ecdysis (pupation) corresponds to the behavior referred to as ‘head eversion’ (Zdarek and Friedman, 1986). During head eversion, the brain, which in the larva is located behind the mouthparts, is pushed anteriorly to become positioned in front of the thorax and the mouthparts. At the same time, the appendages, which were

**Table 2. Timing of developmental and behavioral events at pupal ecdysis in KO animals**

| Event  | KO <sup>†</sup> | Control                     |
|--|-----------------|-----------------------------|
| Pre-ecdysis  | 24.1±1.3        | 9.35±0.49 (**) <sup>‡</sup> |
| No. of pre-ecdysis sweeps                                | 20.8±1.4        | 9.4±1.0 (**)                |
| Frequency of pre-ecdysis sweeps                          | 0.88±0.06       | 1.02±0.13 (ns)              |
| Ecdysis  | — <sup>§</sup>  | 0.94±0.21                   |
| No. of ecdysis sweeps                                    | —               | 5.1±0.5                     |
| Post ecdysis period <sup>¶</sup>                         | 75.8±5.9        | 125.9±7.5 (**)              |
| No. of postecdysis contractions, first 30 minutes        | 17.3±0.7        | 20.2±1.0 (*)                |
| Average length of prothoracic pair of legs <sup>††</sup> | 1.09±0.01       | 1.53±0.01 (**)              |
| Average length of wings <sup>‡‡</sup>                    | 0.95±0.01       | 1.12±0.01 (**)              |

<sup>†</sup>Values are averages±s.e.m.; n=10 for each group. Units are minutes or number, or mm, depending on the measurement.  
<sup>‡</sup>(ns) P>0.05; (\*) 0.01<P<0.05; (\*\*) P<0.01. Student's t-test.  
<sup>§</sup>Behaviors resembling ecdysis were not observed.  
<sup>¶</sup>Period between the end of head eversion [ecdysis (for controls) or pre-ecdysis sweeps (for KO larvae)] and the end of body contractions.  
<sup>††</sup>Measured on pharate adults, from joint at the level of the neck; one prothoracic leg was measured per animal; n=23 per group.  
<sup>‡‡</sup>Measured on pharate adults, one wing measured per group. n=20 per group.

formed from the eversion of the imaginal discs at pupariation, are extended to attain their final size and shape.

In contrast to the situation observed in the larva, most CCAP KO animals died during the pupal stage. Furthermore, the appearance of KO animals at the end of pupation (Fig. 4A,B) and of metamorphosis (Fig. 4E-G) suggests that the primary cause of their death was a specific failure of pupal ecdysis. Indeed, in KO pupae and pharate adults, the head was located much more anteriorly than normal (Fig. 4A,G) or was only partially everted (Fig. 4E,F); the larval tracheae were not completely shed (Fig. 4B); and the appendages were not properly extended, resulting in a pharate adult that had abnormally short wings and legs (compare Fig. 4 E-G with 4H; Table 2).

In order to determine the bases for these defects, we examined the timecourse of pupal ecdysis in KO animals. In *Drosophila*, pupation occurs approx. 12 hours after pupariation (Bainbridge and Bownes, 1981). The timecourse of events normally seen at pupal ecdysis is shown in Fig. 4I and is quantitated in Table 2. Pupal ecdysis is preceded by a preparatory behavior (termed here pre-ecdysis by analogy to

the corresponding larval behavior), during which the posterior part of the animal rhythmically retracts from the puparium. About 9-10 minutes after the onset of pre-ecdysis, a short succession of peristaltic waves sweeps from the posterior to the anterior of the animal and causes the eversion of the head, the shedding of the larval tracheae, and a rapid extension of the appendages. Head eversion is followed by a long post-ecdysis period of several hours during which regular contractions, primarily of the abdomen, occur; this presumably aids in giving the insect its final form.

As shown in Fig. 4I and Table 2, CCAP KO animals initiated normal pre-ecdysis behavior (for instance the frequency of abdominal 'sweeps' was the same as in controls). However, this behavior lasted significantly longer than in controls and was not followed by head eversion. Instead, abdominal pre-ecdysis movements eventually ceased during a final retraction (Fig. 4A) and were followed by a period that resembled the postecdysis period seen in the control (but which was significantly shorter in CCAP KO animals; Table 2).

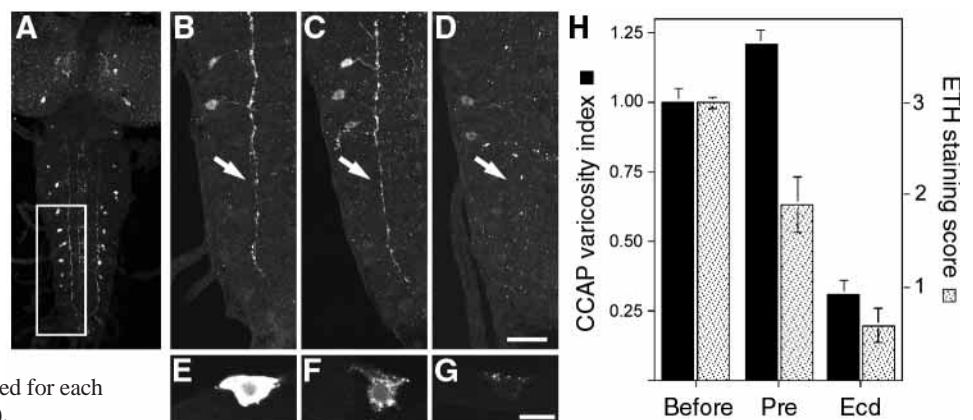
Although the KO pre-pupae all lacked CCAP neurons, the morphological phenotype seen at the end of metamorphosis was somewhat variable, with, for instance, a variable amount of the adult head visible at the end of adult development (Fig. 4E-G, Table 2). However, all flies showed shortened appendages (Fig. 4E-G, Table 2), and all animals whose pupation behavior we observed in detail showed no pupal ecdysis (n=10). The basis for this variable phenotype is currently unknown.

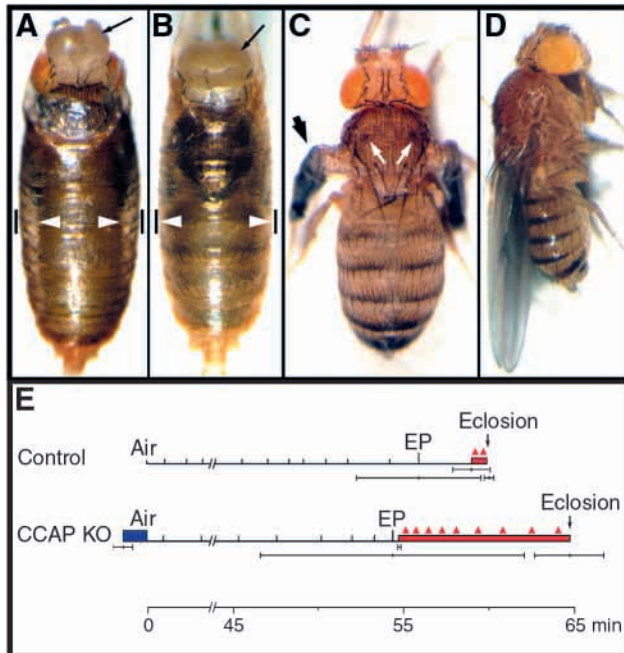
If head eversion is stimulated by CCAP, the neuropeptide should be released at this time. As shown in Fig. 5, a substantial decrease in CCAP-IR was indeed detected following pupation in descending CCAP immunopositive axons. The slight increase in the number of CCAP-immunoreactive varicosities that is apparent at the start of pre-ecdysis is a reflection of a subtle fragmentation in the pattern of CCAP-IR that is seen at this time, and may be the first sign that CCAP has started to be released. The ETH peptides are known to be essential for larval pre-ecdysis in *Drosophila* (Park et al., 2002a), and the drop in ETH-IR that was observed at the onset of pupal pre-ecdysis (Fig. 5F) suggests that these peptides may also control this behavior at pupation.

#### Adult ecdysis (eclosion)

The KO animals that formed relatively normal heads at the end of metamorphosis (~10%; e.g., Fig. 4G) were usually able to

**Fig. 5.** CCAP and ETH are released at pupation. (A) Pattern of CCAP-IR in the CNS of a pre-pupa. (B-G) Enlargement of boxed area in A, showing (B-D) CCAP-IR in descending axons (arrows) and (E-G) ETH-IR in Inka cell before pre-ecdysis (B,E), at start of pre-ecdysis (C,F) and immediately after (D,G) pupal ecdysis. (H) Quantitation of the intensity of CCAP-IR in descending axon (arrow in B-D) and of ETH-IR. Before, before pre-ecdysis (as in B,E); Pre, at the start of pre-ecdysis (as in C,F); Ecd, immediately after pupal ecdysis (as in D,G). Values are averages±s.e.m.; 8-10 preparations were scored for each time point. Scale bars: 40 µm (D); 10 µm (G).





**Fig. 6.** Targeted ablation of CCAP neurons causes defects at eclosion and in post-eclosion events. (A,B) Pharate KO (A) and control (B) animals at eclosion. While both flies inflated their ptilinum (arrow), the abdomen of KO pharate adults failed to distend and exert traction on the internal surface of the puparium. White arrowheads indicate outer limits of abdomen; black bars indicate width of puparium. (C) Adult KO animals did not inflate wings (arrow) or correctly tan the cuticle. White arrows indicate dimples in dorsal thorax at insertion point of thoracic musculature. (D) Control adult of similar age (2- to 4-day old). (E) Eclosion in control and KO animals. Eclosion behaviors (red bars; triangles represent 10 bouts of eclosion peristalses) were preceded by the entry of air into the trachea (Air) and by the deployment of the ptilinum (EP). Successful extrication from the puparium is indicated (Eclosion). Times represent the average length of each interval $\pm$ s.e.m.;  $n=10$  for each group, except for eclosion events themselves where  $n=9$  for KO animals (one animal of the 10 animals monitored failed to escape the puparium). The most noticeable difference between KO and control pharate adults was that the former showed many more bouts of eclosion behavior before emerging from the puparium, due primarily the poor traction exerted by the abdomen on the inside walls of the puparium as seen in A.

exit from the pupal case. A careful examination of eclosion showed that the developmental and behavioral events that take place at this time occurred in the correct sequence in CCAP KO animals, although some quantitative differences in the duration or number of events were observed (Fig. 6E, Table 3). Thus, while tracheal filling occurred before the start of the eclosion behaviors, in CCAP KO animals it took longer than in controls. However, the ptilinum, which is used to rupture the anterior of the pupal case, was deployed normally (Fig. 6A) at the expected time (Fig. 6E, Table 3). Finally, and most significantly, the bouts of rapid anteriorly directed peristalses of eclosion proper occurred in the KO animals. Interestingly, however, these bouts were relatively ineffective at propelling the animal forward. This was not due to a difference in the characteristics of the bouts themselves, which careful cinematographic analyses showed were very similar to those

**Table 3.** Timing of developmental and behavioral events at adult eclysis (eclosion) in KO animals

| Event   | KO <sup>†</sup>             | Control                           |
|---|-----------------------------|-----------------------------------|
| Tracheal filling start – eclosion end                       | 73.6 $\pm$ 8.1              | 67.2 $\pm$ 2.1 (ns)               |
| Tracheal filling start – end                                | 1.4 $\pm$ 0.6               | (0) <sup>‡</sup> (*) <sup>§</sup> |
| Tracheal filling end – EP <sup>¶</sup>                      | 59.4 $\pm$ 7.7              | 60.9 $\pm$ 3.7 (ns)               |
| Tracheal filling end – eclosion start                       | 59.8 $\pm$ 7.7              | 66.3 $\pm$ 1.1 (ns)               |
| EP – eclosion start   | 0.36 $\pm$ 0.1              | 3.1 $\pm$ 2.4 (*)                 |
| No of head movements prior to EP                            | 18.1 $\pm$ 9.3              | 33.6 $\pm$ 4.3 (ns)               |
| Eclosion start – eclosion end                               | 10.0 $\pm$ 2 <sup>††</sup>  | 0.91 $\pm$ 0.3 (**)               |
| No. of eclysis peristalses until eclosion                   | 85 $\pm$ 14 <sup>††</sup>   | 26 $\pm$ 2 (**)                   |
| No. of eclysis peristalses during 1st minute <sup>†††</sup> | 9.8 $\pm$ 0.9 <sup>††</sup> | 26 $\pm$ 2 (**)                   |

<sup>†</sup> Values are averages $\pm$ s.e.m.;  $n=10$  for each group unless noted. Units are minutes or number, depending on the event.

<sup>‡</sup> Air filling occurred in a few seconds in the controls and could not be measured precisely.

<sup>§</sup> (ns)  $P>0.05$ ; (\*)  $0.01<P<0.05$ ; (\*\*)  $P<0.01$ . Student's  $t$ -test.

<sup>¶</sup> EP, extended ptilinum (see Fig. 6A,B).

<sup>††</sup>  $n=9$ ; one animal failed to eclose.

<sup>†††</sup> Approximate time for control to eclose (0.91 $\pm$ 0.3 minutes; see ‘‘Eclosion start – eclosion end’’, above).

of control animals (not shown). Instead, this failure occurred because the abdomen of KO animals was not distended at this time (Fig. 6A), severely reducing the traction exerted by the body on the inner walls of the pupal case, which is needed in order for the abdominal peristalses to cause the rapid net forward movement that is seen in control animals. Although most KO animals (nine out of 10 examined) eventually succeeded in eclosing, extrication took much longer than normal (Fig. 6E, Table 3). Thus, unlike larval and pupal eclysis, the actual eclysis motor program of the adult appears to be relatively normal in KO animals (however, the frequency of peristalses was lower than in controls, even when the two groups were compared during the first minute after the onset of eclosion, which corresponds approximately to the duration of eclosion in controls; Table 3).

Therefore, the most dramatic deficiency of CCAP KO animals at eclosion appears to be due to the absence of a function required to expand the body rather than to a failure in the adult eclysis motor program itself.

### Adult phenotype

The phenotypes of adult CCAP KO flies suggest that CCAP neurons play some role in post-eclosion events. KO adults do not inflate their wings, and their cuticle appears to remain soft and untanned, as evidenced by the dimpling that is observed on the dorsal thorax at sites of thoracic muscle insertion (Fig. 6C). The defect in wing expansion may be due, in part, to the failure in wing extension at the time of pupation (see above). The tanning defect of the KO flies may occur because a subset of the CCAP neurons expresses the gene encoding the tanning hormone, bursicon (E. Dewey and H. W. Honegger, personal communication).

In another experiment, we employed the *CCAP-GAL4* driver to overexpress a temperature-sensitive form of *shibire* (*shi*; the fly dynamin homolog) in the CCAP cell population (using a *UAS-shi<sup>ts</sup>* transgene). When reared at 29°C, progeny carrying both the *CCAP-GAL4* and *UAS-shi<sup>ts</sup>* transgenes exhibited defects in wing expansion (~80–100% of the populations), whereas control progeny (with only one

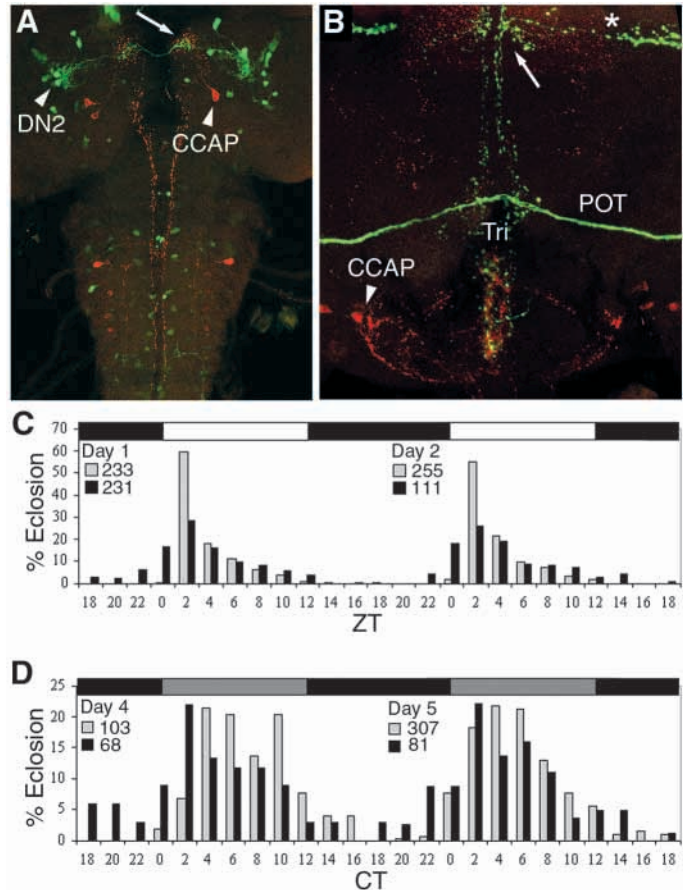


transgene) had normal wings (data not shown). At 25°C, both types of progeny had normal wings, indicative of a temperature-sensitive effect.

#### Eclosion rhythms in the absence of CCAP neurons

In *Drosophila*, a circadian clock controls the timing of adult emergence, with most adults eclosing between subjective dawn and late subjective morning (Saunders, 1982). Although much is known about the circadian clock mechanism (reviewed by Allada et al., 2001; Young and Kay, 2001), comparatively little is known about how the clock regulates the expression of overt rhythmicity (reviewed by Jackson et al., 2001; Taghert, 2001; Wang and Sehgal, 2002; Park, 2002). The co-localization of LARK and CCAP (McNeil et al., 1998; Zhang et al., 2000) suggests that CCAP neurons could mediate the circadian control of ecdysis, independent of its possible role in the execution of the behavior itself. To determine whether the clock directly regulates the CCAP cells, we examined the relative locations of the clock and CCAP neuronal populations in larval and pharate adult brains. This was accomplished by examining CCAP immunoreactivity in brains expressing green fluorescent protein (GFP) in the clock cell population. Using a *timeless*-GAL4 driver (Kaneko and Hall, 2000) we observed that projections from the TIM-containing DN2 neurons (Kaneko et al., 1997) overlapped with CCAP-immunoreactive synaptic endings in the dorsal aspect of the larval and pharate adult brains (Fig. 7A arrow, and data not shown). Interestingly, DN2 neurons are postulated to be targets of the pigment dispersing factor (PDF)-containing small ventral lateral neurons (LN<sub>v</sub>), and they have been implicated in the circadian control of locomotor activity (Helfrich-Förster et al., 2000). In a separate experiment using a *pdf*-GAL4 driver (see Park et al., 2000), we demonstrated overlap between the processes of CCAP neurons and those of tritocerebral PDF neurons (Fig. 7B, arrow). The latter population arises post-embryonically at the mid-pupal stage, and it has been suggested that it might be involved with the circadian control of adult eclosion (Helfrich-Förster, 1997).

To examine circadian rhythms of eclosion, CCAP KO and control animals were reared under conditions that produced the maximal number of pharate adults (see above and Materials and Methods), and then adult emergence was scored at two-hour intervals over the course of several days, both under a light:dark cycle (LD 12:12) and in constant darkness (DD). In three separate experiments using *CCAP*-GAL4 line no. 16 (Fig. 7C,D) and in two separate experiments using the independent transgenic line no. 9 (not shown), a clear rhythmicity was observed under both LD and DD conditions, with most of the animals eclosing in the dawn-early morning (or subjective dawn-early morning) interval (LD, Fig. 7C; DD, Fig. 7D). Nevertheless, there were differences between the eclosion profiles of KO and control populations. Most notably, the temporal gate of eclosion was lengthened in KO animals, with significant emergence occurring in the late night/predawn period (Fig. 7C,D; black bars). Coupled with this wider eclosion 'gate', we also observed a significant diminution in the amplitude of the eclosion burst that occurs immediately following lights-on (Fig. 7C), which in control populations constitutes approximately 40% of the flies that emerge on any given day. No consistent difference in the peak time of eclosion was observed between KO and control populations in LD or DD conditions.



**Fig. 7.** Anatomy of CCAP neurons and their role in the clock control of eclosion. (A,B) overlap among the projections and arborizations of CCAP-, *timeless*-, and PDF-containing neurons. (A) Overlap between projections (arrow) of protocerebral CCAP neurons and *tim*-containing DN2 neurons of the 3rd instar larval brain. (B) Overlap of the synaptic fields (arrow) of tritocerebral PDF (green) and subsophageal CCAP neurons of the pharate adult brain. Asterisk shows ending of the LN[v] clock cells. DN2, dorsal neuron 2; Tri, tritocerebrum. POT, posterior optic tract (containing projections of the LN[v] clock cells). (C,D) Eclosion profiles for KO (black bars) and control (gray bars) populations under (C) LD (12 hours light:12 hours dark) and (D) DD (continuous darkness). The profile for subjective days 4 and 5 is shown. Height of each bar represents the percentage of flies that eclosed within a 2 hour window normalized to the total number of flies that eclosed during that day (indicated separately for each genotype and day). The open and closed horizontal black rectangles (C) show the light and dark phases of the LD schedule, respectively, while the black and gray rectangles in D show those for the LD regime prior to the shift to DD. Collections were performed at the zeitgeber (ZT) or circadian (CT) times indicated.

## DISCUSSION

### Role of CCAP in the execution of ecdysis

Strong circumstantial evidence implicates the neuropeptide CCAP in the control of ecdysis behavior. In the moth *Manduca*, *in vitro* experiments using isolated abdominal CNSs suggest that CCAP is required for turning off the pre-eclosion motor program (Gammie and Truman, 1997b) and turning on that for ecdysis (Gammie and Truman, 1997b; Zitnan and Adams,

2000). The phenotypes caused by ablating CCAP neurons in *Drosophila* are, overall, consistent with this model. Indeed, both larval and pupal pre-ecdysis are longer than normal in KO animals, in agreement with an inhibitory action of CCAP on the expression of this preparatory behavior. In addition, the lack of CCAP neurons causes the complete failure of pupal ecdysis, strongly suggesting that the CCAP neuropeptide is essential for turning on ecdysis behavior.

### Compensatory mechanisms in the neural bases of ecdysis

Although CCAP neurons are essential for pupation, the ecdysis motor program of both larval and adult KO animals appears qualitatively normal, implicating additional mechanisms in the control of these behaviors. CCAP may play a minor role at these times or, alternatively, other neuropeptides may compensate for the loss of CCAP. Irrespective of the exact mechanism, our results strongly suggest that other pathways, independent or compensatory, exist, which control the expression of these motor programs. To date the only gene that is known to be essential for ecdysis is the *ETH* gene, and flies carrying the null *ETH* alleles die at the first larval ecdysis (Park et al., 2002a). However, the ETH peptides are believed to act upstream of CCAP (reviewed by Ewer and Reynolds, 2002), and in *Drosophila* ETH is released before CCAP at larval (M. del Campo, A. Clark and J.E., unpublished data) and pupal (this work) ecdysis, consistent with this hypothesis. Thus, it is unlikely that the ETH peptides act in parallel with CCAP or can compensate for its absence. In addition, our findings that the lack of CCAP does not cause larval lethality argue against a simple linear pathway in which the essential function of ETH is to cause release of CCAP leading to the initiation of the ecdysis motor program. EH is also believed to act upstream of CCAP (reviewed by Ewer and Reynolds, 2002). However, the exact role of EH in the control of ecdysis is currently unclear, as EH KO animals are usually able to ecdyse, although their behavior is somewhat disorganized (McNabb et al., 1997). An examination of the ecdysis of animals lacking both EH and CCAP neurons compared with that of CCAP KO and EH KO animals will reveal the extent to which EH can compensate for the lack of CCAP, and vice versa.

### Bases for the changing roles for CCAP during postembryonic development

In addition to compensatory mechanisms, other mechanisms may contribute to the varying importance of CCAP at different ecdyses. For instance, subsets of CCAP neurons may participate at some ecdyses but not at others. In the abdominal CNS of the *Manduca* for example, 2 pairs of CCAP-immunoreactive neurons up-regulate the second messenger cGMP at larval ecdysis, whereas only one pair does so at pupal and adult ecdysis (Ewer and Truman, 1997). Since this cGMP response likely increases the excitability of the CCAP neurons [it is known to do so for the thoracic set (Gammie and Truman, 1997a)], this change in the pattern of cGMP expression could change the relative participation of the different CCAP neurons at each ecdysis. It is not known if this sort of mechanism applies to *Drosophila*, since no cGMP response is detected in CCAP neurons at any ecdysis in this species (Ewer and Truman, 1996; Baker et al., 1999). Nevertheless, the differential activation of a subset of peptidergic neurons at

different times in development could provide a mechanism for modifying the extent of the participation of these neurons in different behavioral or developmental contexts. Alternatively, the role of CCAP may change during postembryonic development because of changes in the expression of CCAP receptors. Although the CCAP receptor has not been conclusively identified (but see Park et al., 2002b), the completion of the *Drosophila* genome sequence and its subsequent analyses has produced a list of potential candidates (Brody and Cravchik, 2000; Hewes and Taghert, 2001).

### Other roles for CCAP at ecdysis

The most dramatic feature of KO animals at adult eclosion is not in the expression of the ecdysis motor program itself, but a function that may be cardioactive in nature. It may be that the CCAP neurons are important for increasing hemolymph pressure, and CCAP is known to be a cardioactive peptide in insects (see Dirksen, 1998) including *Drosophila* (Nichols et al., 1999), and to be released at eclosion in *Manduca* (Tublitz and Truman, 1985). Alternatively, the defect may be in fluid homeostasis. In crabs, for instance, the shedding of the old carapace is preceded by a massive release of hyperglycemic hormone (HH) which causes a swelling of the body via an anti-diuretic mechanism (Chung et al., 1999). CCAP is also released at this time (Phlippen et al., 2000) and could regulate HH release. Regardless of the bases for the defects observed in eclosing KO animals, their phenotype suggests that maintaining a high internal body pressure is critical for adult eclosion, and implicates the CCAP neurons in this process.

### Role of CCAP in the circadian timing of adult eclosion

Features of *lark* gene expression in the CCAP neurons, as well as the potential for synaptic contact between CCAP and clock neurons suggests that CCAP may play a role in mediating the circadian control of adult eclosion. Although the rhythmic eclosion profile of CCAP KO populations shows that CCAP neurons are not essential for the circadian gating of eclosion, the distribution of eclosion events in this population indicates that these neurons modulate the gating process. This modulation may occur via a direct connection with clock neurons or other peptidergic neurons (e.g., those expressing PDF), and the anatomy of CCAP neurons in the brain is consistent with this hypothesis. The robust circadian rhythmicity of CCAP KO populations indicates that there are multiple (and potentially redundant) cellular pathways mediating the output of the clock.

Several lines of evidence suggest that CCAP neurons mediate the effects of light on eclosion, indirectly via the EH neurons. In *Manduca*, strong circumstantial evidence suggests that CCAP acts downstream of EH (reviewed by Ewer and Reynolds, 2002). In *Drosophila*, CCAP release occurs after EH release at larval ecdysis (A. C. Clark, M. del Campo, and J. Ewer, unpublished data), suggesting that the same relationship may exist in the fly. Importantly, EH KO and CCAP KO animals both show an altered response to the light-on signal (McNabb et al., 1997) (this paper), and recent evidence suggests that light can cause a premature release of EH (S. McNabb and J. W. Truman, personal communication). Thus, it is possible that certain CCAP neurons mediate the light-on response that is channeled through the EH neurons.

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