Temporally tuned neuronal differentiation supports the functional remodeling of a neuronal network in *Drosophila*

Lyubov Veverytsa and Douglas W. Allan[1]

Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

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During insect metamorphosis, neuronal networks undergo extensive remodeling by restructuring their connectivity and recruiting newborn neurons from postembryonic lineages. The neuronal network that directs the essential behavior, ecdysis, generates a distinct behavioral sequence at each developmental transition. Larval ecdysis replaces the cuticle between larval stages, and pupal ecdysis externalizes and expands the head and appendages to their adult position. However, the network changes that support these differences are unknown. Crustacean cardioactive peptide (CCAP) neurons and the peptide hormones they secrete are critical for ecdysis; their targeted ablation alters larval ecdysis progression and results in a failure of pupal ecdysis. In this study, we demonstrate that the CCAP neuron network is remodeled immediately before pupal ecdysis by the emergence of 12 late CCAP neurons. All 12 are CCAP efferents that exit the central nervous system. Importantly, these late CCAP neurons were found to be entirely sufficient for wild-type pupal ecdysis, even after targeted ablation of all other 42 CCAP neurons. Our evidence indicates that late CCAP neurons are derived from early, likely embryonic, lineages. However, they do not differentiate to express their peptide hormone battery, nor do they project an axon via lateral nerve trunks until pupariation, both of which are believed to be critical for the function of CCAP efferent neurons in ecdysis. Further analysis implicated ecdysone signaling via ecdysone receptors A/B1 and the nuclear receptor ftz-f1 as the differentiation trigger. These results demonstrate the utility of temporally tuned neuronal differentiation as a hard-wired developmental mechanism to remodel a neuronal network to generate a scheduled change in behavior.

neuronal identity | neuropeptide | neuronal plasticity

Neuronal remodeling is a pervasive mechanism that matches network output to the physiological and behavioral demands of the organism throughout development and in response to changing input or injury (1, 2) via morphological remodeling (3, 4), neuronal addition through neurogenesis (5), and functionally significant changes in gene expression (6–8). *Drosophila* metamorphosis has provided many striking and informative examples. Most larval neurons undergo morphological restructuring to rewire into adult networks. Also, postembryonic waves of neurogenesis and programmed cell death combine to remodel existing or adult-specific networks through the addition of newly born neurons or subtraction of larval-specific neurons (9–13).

The network of neurosecretory neurons that directs ecdysis must function at each major developmental transition in insects; however, the behavioral sequence generated by this network differs at each stage (14). Larval ecdysis sheds the old cuticle between larval stages, pupal ecdysis everts and extends the head and appendages to their adult position in 12-h pupae, and adult ecdysis ecloses the adult from the pupal case and inflates the wings (15). The crustacean cardioactive peptide (CCAP) neuron population plays a critical role; targeted ablation of *Drosophila* CCAP neurons disrupts the timing of larval ecdysis and results in a lethal failure of pupal ecdysis (16).

Previous work had shown that CCAP neurons undergo morphological remodeling after pupal ecdysis (17). Here, we find that the CCAP neuronal network is functionally remodeled between larval and pupal ecdysis by the delayed differentiation of a subset of CCAP neurons. We find that 12 late CCAP neurons differentiate to express the functionally critical peptide hormones CCAP, Bursα, and Bursβ immediately before pupal ecdysis and at the same time project axons out of the central nervous system. Notably, these late CCAP neurons are sufficient for pupal ecdysis; in genetic ablation studies wherein only the late-differentiating subset of CCAP neurons survived, pupal ecdysis was entirely wild type. Further analysis showed that late CCAP neurons become postmitotic in the embryo in segments A5–A9 (segment designations are shown in Fig. S1), but, unlike the other CCAP neurons, their terminal differentiation is triggered at pupariation by ecdysone pathway signaling. These data provide evidence for temporally tuned terminal differentiation as a mechanism to remodel a functioning neuronal network at a specific time point to enable a switch in behavioral output. Evidence of similar delayed differentiation within other insect neuronal subsets supports our proposal that this mechanism is used by a number of neuronal networks (18–23).

Results

CCAP neurons in *Drosophila* can be identified robustly using *CCAP-GAL4* (Fig. S1) (16, 24). In the ventral nerve cord (VNC), there is a single CCAP interneuron (CCAP-IN) in each hemisegment T1–A7 as well as a single CCAP efferent (CCAP-EN), which exits the VNC along the lateral segmental nerve trunk, in each hemisegment T3–A4 (Fig. 1*B* and Fig. S1 *A* and *A′*) (25). Here, we examined CCAP neuron subsets in pupae (Fig. 1 and Fig. S1) and identified 12 late-emerging CCAP neurons restricted to segments A5–A9. These late CCAP neurons emerge during early pupariation before pupal ecdysis (APF). A single late CCAP neuron emerges adjacent to each CCAP-IN in hemisegments A5–A7, and six late CCAP neurons emerge in the A8/A9 segments (Fig. 1 *C–E* and Fig. S1 *B* and *B′*). Previously, we found that CCAP-ENs can be discriminated from CCAP-INs by their coexpression of Dachshund (Dac), *OK6-GAL4*, and nuclear phosphorylated Mad (pMad) (25). Nuclear pMad is indicative of active bone morphogenetic protein (BMP) signaling and is exclusive, in the *Drosophila* VNC, to efferent neurons (26, 27). Applying these markers to late CCAP neurons, we found that A5–A7 CCAP neurons expressed Dac, *OK6-GAL4*, and pMad

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[1]To whom correspondence should be addressed. E-mail: doug.allan@ubc.ca.

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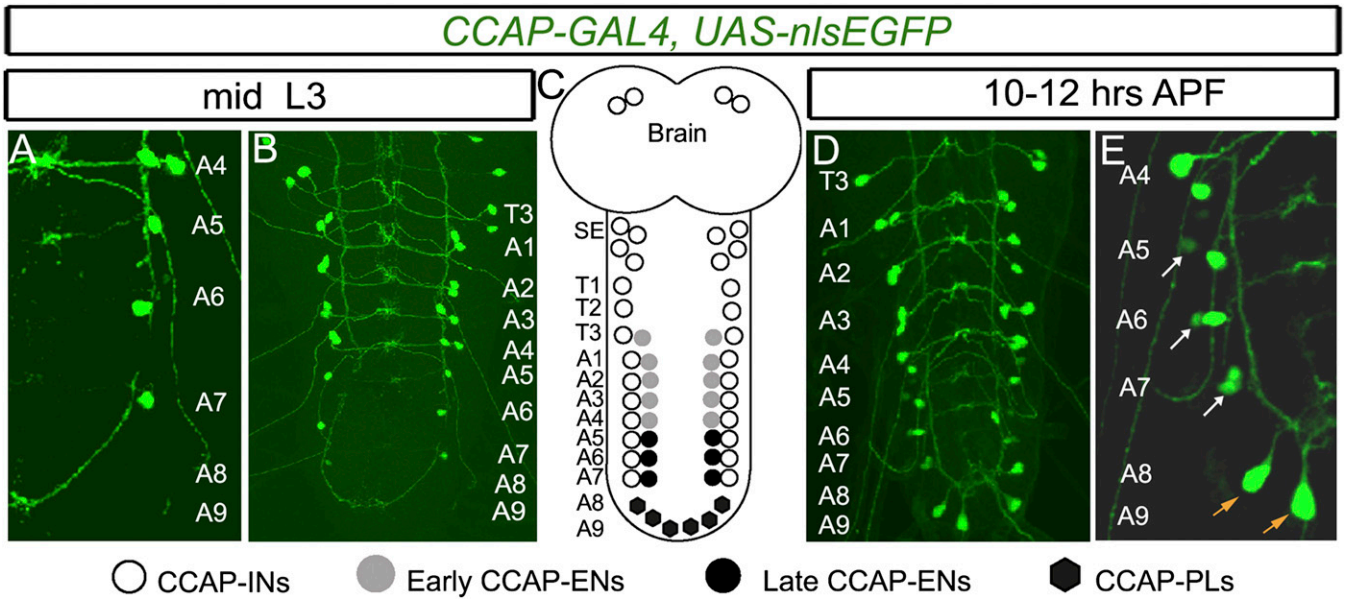

Fig. 1. Emergence of late CCAP neurons in the A5–A9 abdominal VNC at pupariation. Expression of *CCAP-GAL4,UAS-nlsEGFP* (green) in the T3–A9 hemisegments in mid-L3 larvae (*A* and *B*) and in pupae 10–12 h APF (*D* and *E*) is shown with a summary depicting all CCAP neuronal subsets (*C*). Within each segment (T1–A7), CCAP neurons project across the midline forming a ladder-like structure that we used to confirm the segment identity of every CCAP neuron (see also Fig. S1). (*A* and *B*) At mid-L3, there is a CCAP neuron doublet in the T3–A4 hemisegments. In A5–A7 hemisegments, there is only a single CCAP neuron. (*D* and *E*) By 10–12 h APF, a second CCAP neuron has emerged in each A5–A7 hemisegment (white arrow). Also, six CCAP neurons emerge within hemisegments A8 and A9 (orange arrow). (*C*) Cartoon summary of CCAP neurons in the CNS. Based on previous work (25) and the identification of late CCAP-ENs and CCAP-PLs in Fig. 2, we summarize the identity of each CCAP neuron subtype here. Genotype: *CCAP-GAL4,UAS-nlsEGFP/+*.

(Fig. 2 *A*, *ii* and Fig. S2 *A*, *C*, and *E*). We refer to these neurons as "late CCAP-ENs." Late A8/A9 CCAP neurons expressed *OK6-GAL4* and pMad (see Fig. S7 *A*–*C*) but not Dac (see Fig. S7 *A* and *B*). We refer to these neurons as "posterior lateral CCAP neurons" (CCAP-PLs) to discriminate them from the A5–A7 late CCAP-ENs. The late CCAP-ENs and CCAP-PLs reported here correspond to CCAP neurons that had been observed in certain previous reports, but their origin and subtype identity had not been established. The late CCAP-ENs correspond to the large dorsal CCAP neurons in A5–A7 segments that express CCAP, Bursα, and Bursβ reported in pharate adults (immediately before eclosion of adults) (17, 24, 28). This correspondence is verified below (see Fig. 4). Additionally, a set of A8/A9 CCAP neurons observed at the pupal ecdysis stage (29) likely corresponds to the CCAP-PLs characterized here.

We examined whether the late CCAP neurons activated *CCAP-GAL4* expression late or actually underwent terminal differentiation at pupariation. No peptide hormone expression could be detected in late A5–A7 CCAP-ENs and A8/A9 CCAP-PLs by mid-L3 (Fig. 2 *A*, *i* and *B* and Figs. S1*A* and S2 *B*, *i* and *C*, *i*). However, by 10–12 h APF, late CCAP-ENs robustly expressed CCAP, Bursα, and Bursβ (Fig. 2 *A*, *ii* and *B* and Fig. S2 *A*, *ii*; *B*, *ii*; and *C*, *ii*), and CCAP-PLs expressed CCAP but not Bursα and Bursβ (Fig. 2 *A*, *ii*). Expression of these peptide hormones was retained in late CCAP neurons up to the pharate adult stage (Figs. S1 and S2 *B*, *iii*; *C*, *iii*; and *D*, *iii*). We also found that late CCAP neurons do not extend axons out of the VNC until pupariation (detailed below; see Fig. 6).

Late CCAP Neurons Are Sufficient for Pupal Ecdysis. We postulated that late CCAP neurons function in pupal ecdysis. Previously, ablation of CCAP neurons (*CCAP-GAL4* expressing the proapoptotic genes *hid* and *reaper*) resulted in a failure of head eversion and leg extension at pupal ecdysis (16). Here, we adapted this approach to examine pupal ecdysis after selective ablation of early CCAP neurons, leaving late CCAP neurons intact. To do so, we used a temperature-sensitive variant of GAL80 ($GAL80^{TS}$), a potent GAL4 repressor (30). Larvae were raised at 29 °C up to mid-L3 and were switched to 18 °C thereafter. At 29 °C, $GAL80^{TS}$ is inactivated, and *CCAP-GAL4* can drive *UAS-hid/reaper* expression, killing 100% of early CCAP neurons by mid-L3 (Fig. S3*B*). By subsequently shifting larvae to 18 °C, active $GAL80^{TS}$ then could block *CCAP-GAL4* function upon its expression in late CCAP neurons at pupariation. This protocol selectively spared late CCAP neurons from cell death ($n = 24$ animals) (Fig. 3 *F* and *F′* and Fig. S3*D*).

Remarkably, we found that the 12 late CCAP neurons were entirely sufficient to support wild-type pupal ecdysis. In 96% of these animals (22/23), head eversion and leg extension were as in wild-type animals, and 100% of the animals eclosed ($n = 22$) (Fig. 3 *D* and *E*). The only observable phenotype was a failure of wing inflation (Fig. 3*E*), which is known to require a subset of (ablated) early suboesophageal CCAP-INs (28, 31). As a control, we shifted animals from 29 °C to 18 °C at 14 h APF to repress *UAS-hid/reaper* expression after pupal ecdysis. This protocol resulted in a 100% failure of head eversion and leg extension ($n = 15$) (Fig. S4*B*). In a reciprocal test, we killed CCAP neurons only after pupal ecdysis by shifting animals from 18 °C to 29 °C at 14 h APF; pupal ecdysis and eclosion were as in wild-type animals, but wing inflation failed in 100% of animals ($n = 12$) (Fig. S4 *C* and *C′*).

We postulated that *UAS-hid/reaper* expression could be timed to kill different subsets of late CCAP neurons stochastically. We raised larvae at 29 °C until puparium formation, when CCAP neurons start to express *CCAP-GAL4*, and shifted the pupae to 18 °C thereafter. Intriguingly, we observed four primary pupal ecdysis phenotypic categories. Category 1 had a lethal failure of head eversion and leg extension ($n = 36$; 100% CCAP neuron lethality). Category 2 had wild-type pupal ecdysis and eclosion ($n = 27$; 5.7 ± 0.5 late CCAP-ENs; 5.6 ± 0.7 CCAP-PLs). Category 3 had wild-type leg extension but a failure of normal head eversion ($n = 9$; 6 ± 0 late CCAP-ENs; 1.8 ± 1.0 CCAP-PLs) (Fig. S4 *D* and *D′*). Category 4 had failure of leg extension but wild-type head eversion ($n = 20$; 2.7 ± 1.0 late CCAP-ENs; 5.9 ± 0.4 CCAP-PLs) (Fig. S4 *E* and *E′*). In each category, we examined the number and subset of remaining

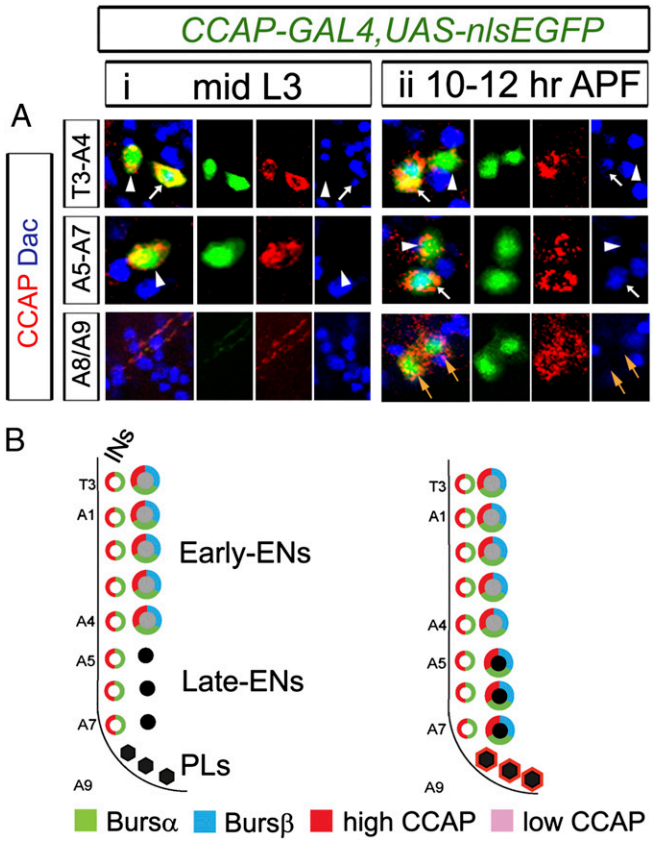

Fig. 2. Late CCAP neurons differentiate at pupariation. Differentiation of peptide hormone-expressing late CCAP neurons occurs between late wandering L3 larvae and pupal ecdysis (at 12 h APF). (*A*) Representative images of CCAP neurons in hemisegments T3–A4, A5–A7, and A8 and A9 showing expression of *CCAP-GAL4* (green) and immunoreactivity to CCAP (red) and Dac (blue) at mid-L3 (*i*) and at 10–12 h APF (*ii*). CCAP-ENs were identified by Dac expression. In segments T3–A4, CCAP is expressed in CCAP-INs (arrowheads) and early CCAP-ENs (arrows) from mid-L3 to pupal ecdysis. In segments A5–A7 and A8/A9, *CCAP-GAL4* and CCAP immunoreactivity started after mid-L3 (arrows) in late CCAP-ENs (A5–A7) and in CCAP-PLs (A8/A9). CCAP-PLs did not express Dac. (*B*) Cartoon summaries of gene expression in VNC CCAP neurons at mid-L3 and 10–12 h APF. (See Fig. S2 for further details.) Genotypes: *CCAP-GAL4,UAS-nlsEGFP/+*.

CCAP-immunoreactive late CCAP neurons (all early CCAP neurons were eliminated in all categories; Fig. S3 *D′* and *E′*). The unexpected observation that head-eversion and leg-extension phenotypes could be uncoupled suggested their regulation by distinct CCAP neuronal subsets. In correspondence with this phenotypic data, ablation of most A5–A7 CCAP-ENs correlated only with failed leg extension. Conversely, ablation of most A8/A9 CCAP-PLs correlated only with head-eversion defects. These data were substantiated by previous observations that a group of A8/A9 CCAP neurons exhibit heightened Ca^{2+} activity during early fictive pupal ecdysis (corresponding to the time of head eversion) (29). These data led us to propose that late CCAP-ENs are required for leg extension, whereas late CCAP-PLs are required for head eversion.

Addition of Late CCAP Neurons. Postembryonic neuroblast lineages generate newly born neurons in larvae that are recruited into existing or adult-specific circuits (11). We tested whether CCAP-neurons derived from post-embryonic lineages by feeding larvae BrdU from early L1 to late L3. However, despite very robust BrdU incorporation into neurons that reproduced the reported total BrdU larval incorporation pattern (32, 33), we never observed BrdU incorporation into late CCAP neurons (Fig. S5*A*). We also tracked early CCAP neurons with a permanent marker to test whether late CCAP neurons derived from early CCAP neurons. We raised animals of the genotype *Act-FRT > STOP > FRT-nLacZ, UAS-Flp; CCAP-GAL4, UAS-nlsEGFP; tubP-GAL80^{TS}*. Here, Flp-mediated *cis-FRT* recombination brings lacZ under the actin promoter in neurons expressing *CCAP-GAL4* but only at 29 °C when GAL80^{TS} is inactive. Animals were raised at 29 °C to mid-L3 and then were switched to 18 °C. We verified that β-Gal was expressed in all early CCAP neurons by mid-L3 (Fig. S6 *A* and *B′*). However, by the pharate adult stage, β-Gal was not expressed in A5–A7 CCAP-ENs or in the CCAP-PLs but was expressed in CCAP-INs and in T3–A4 CCAP-ENs (Fig. 4 *A* and *B*). In control animals raised at 29 °C up to the pharate adult stage, we found that β-Gal expression could be induced in late CCAP neurons (Fig. S6 *B* and *B′*).

Late CCAP Neurons Derive from Early Lineages but Terminally Differentiate at Pupariation. The lack of evidence supporting the emergence of late CCAP neurons from postembryonic lineages or early CCAP neuron transfating led us to test whether late CCAP neurons are embryonic but undergo delayed differentiation at pupariation. We previously reported that coexpression of Dac with either pMad or *OK6-GAL4* could discriminate T3–A4 CCAP-ENs from all other cells in the dorsal VNC in embryos and larvae (25). Because late CCAP-ENs coexpressed these markers (Fig. 2 *A*, *ii* and Fig. S2 *C*, *ii* and *E*), we examined their coexpression in A5–A7 segments from stage 17 to mid-L3. At all ages, we identified a single neuron per A5–A7 hemisegment with the appropriate axial position to be an undifferentiated CCAP-EN (stage 17; Fig. 5*A*). By 10–12 h APF, this single neuron expressed CCAP, Bursα, and Bursβ (Fig. S2 *C*, *ii* and *E*). Strikingly, in stage 15–17 embryos we frequently observed precocious Bursα expression in an A5–A7 neuron that expressed Dac adjacent to a CCAP-IN (Fig. S5 *B* and *C*).

To show more directly that late CCAP-ENs are born in the embryo, we used *Dac^{GAL4}* to Flp-in a lacZ reporter conditionally only in embryos. *Act-FRT > STOP > FRT-nLacZ, UAS-Flp; dac-GAL4,UAS-nlsEGFP; tubP-GAL80^{TS}* animals were raised at 29 °C up to the end of embryogenesis at late stage 17 and were switched to 18 °C thereafter. In pharates, we observed β-Gal immunoreactivity in all CCAP-ENs, including the six late CCAP-ENs in A5–A7 (Fig. 5*B*). Lack of BrdU incorporation into CCAP-PLs in larvae suggested that CCAP-PLs also are born in the embryo (Fig. S5*A*). However, the absence of Dac in CCAP-PLs precluded their direct identification by marker coexpression. However, we took advantage of their *OK6-GAL4* expression to Flp-in lacZ selectively in the embryo. Animals of genotype *Act-FRT > STOP > FRT-nLacZ, UAS-Flp; OK6-GAL4,UAS-nlsEGFP; tubP-GAL80^{TS}* were raised at 29 °C to late stage 17 and then were switched to 18 °C thereafter. In pharate adults, we observed β-Gal immunoreactivity in most CCAP-PLs (Fig. S7*D*), showing that CCAP-PLs are born and express *OK6-GAL4* in the embryo. These results indicate that late CCAP neurons most likely are generated in the embryo in segments T3–A9 and that a mechanism exists to delay their peptide hormone differentiation in segments A5–A9.

We took advantage of early *dac* expression in CCAP-ENs to determine whether late CCAP-ENs morphologically differentiate at pupariation. Our previous work (25) demonstrated that T3–A4 CCAP-ENs exit the VNC via A1–A5 lateral segmental nerves, and that they are the only axons to express *dac^{GAL4}, UAS-CD8-GFP* within those nerve trunks. Here we found that *dac^{GAL4},UAS-CD8-GFP*–expressing axons are not observed in A6 and A7 lateral segmental nerve trunks in larvae up to mid-L3 (Fig. 6*A*). The fused A8/A9 nerve trunk carries other *dac*-expressing efferents, such as dMP2 neurons (34); thus we could not discriminate CCAP efferents by *dac* expression. However, by 4–5 h APF, we observed a single *dac^{GAL4},UAS-CD8-GFP*–

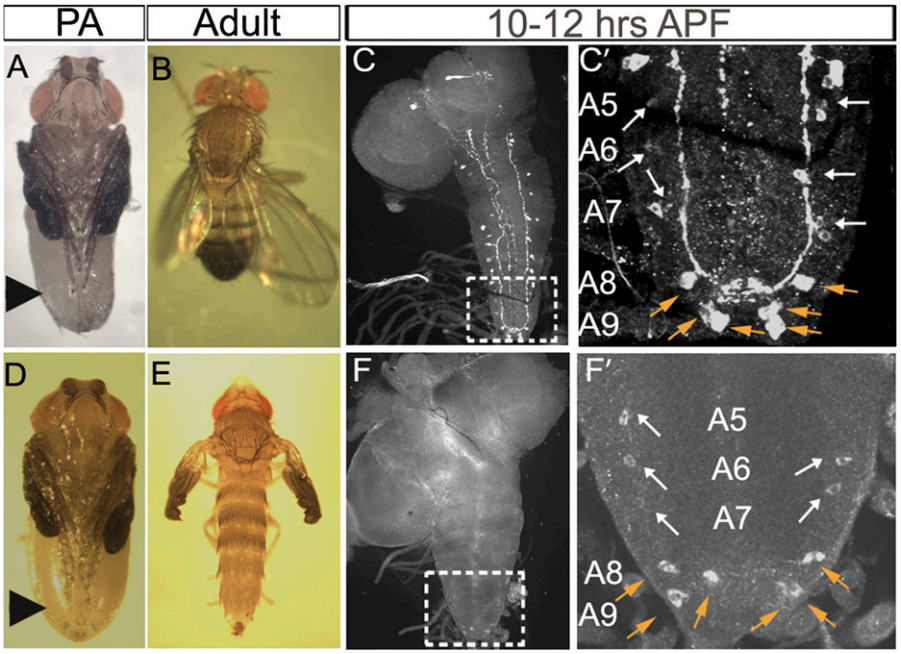

Fig. 3. Late CCAP neurons are sufficient for pupal ecdysis. Early CCAP neurons were ablated selectively using temporally controlled expression of the cell death gene in larval CCAP neurons. (*A* and *B*) Control animals exhibit wild-type leg extension (arrowhead) and head eversion in pharate adults (PA) (*A*), and adults have wild-type wing inflation (*B*). (*D* and *E*) Selective ablation of early CCAP neurons results in wild-type leg extension (arrowhead) and head eversion in pharate adults (*D*), but adults have failed wing inflation (*E*). (*C* and *F*) CCAP immunoreactivity in whole CNS at 10–12 h APF. (*C′* and *F′*) Magnified views of boxed areas in *C* and *F*. (*C* and *C′*) In controls, the full complement of CCAP neurons was seen, including A5–A7 late CCAP-ENs (white arrows in *C′*) and CCAP-PLs (orange arrows in *C′*). (*F* and *F′*) All early CCAP-ENs and CCAP-INs were ablated. Only late CCAP-ENs (white arrows in *F′*) and CCAP-PLs (orange arrows in *F′*) remained. Genotypes: (*A–C′*) *CCAP-GAL4/+; tubP-GAL80^{TS}, UAS-nlsEGFP/+*; (*D–F′*) *UAS-hid,UAS-reaper/w* or Y; *CCAP-GAL4; tubP-GAL80^{TS},UAS-nlsEGFP/+*.

expressing axon in each of the A6 and A7 nerve trunks (Fig. 6*B*). Moreover, Bursα immunoreactivity was first observed in the A6, A7, and A8/A9 nerve trunks at pupariation and always within *dac^{GAL4},UAS-CD8-GFP*–expressing axons. These data indicate that late CCAP-EN axons do not extend an axon out of the VNC until pupariation. Thus, we conclude that late CCAP-EN axons do not morphologically differentiate until that time.

Ecdysone Cascade Drives Late CCAP Neuron Differentiation. The ecdysone-induced nuclear hormone receptor cascade plays a critical role in the metamorphic changes that take place during pupariation. This cascade promotes postembryonic neurogenesis, remodeling of neuronal morphology, and, in certain cases, changes in neuronal gene expression (9, 35). We tested a cell-autonomous role for this cascade in temporally inducing late CCAP neuron differentiation. We used *CCAP-GAL4* to drive isoform-specific blockers of the ecdysone receptor cell specifically and, as expected, found that dominant-negative transgenes to EcR-A (EcR-A^{DN}) or EcR-B1 (EcR-B1^{DN}) blocked the induction of late CCAP-EN peptide hormone (Fig. 7 *B′* and *C′*). EcR-B2 expression had no effect (Fig. 7*D′*). In controls, CCAP immunoreactivity was observed in 14.7 ± 1.7 CCAP-ENs per VNC, Bursα in 14.7 ± 1.7 CCAP-ENs per VNC (n = 10 VNCs), and Bursβ in 14.1 ± 1.5 CCAP-ENs per VNC (n = 10 VNCs). EcR-A^{DN} reduced CCAP expression to 6.4 ± 1.1 CCAP-ENs (n = 9, $P < 0.0001$), Bursα to 7.3 ± 1.2 CCAP-ENs (n = 6, $P < 0.0001$), and Bursβ to 7.7 ± 3.0 CCAP-ENs (n = 10, $P < 0.0001$). Similarly, EcR-B1^{DN} reduced CCAP expression to 5.8 ± 2.4 CCAP-ENs (n = 8, $P < 0.0001$), Bursα to 6.7 ± 1.2 CCAP-ENs (n = 6, $P < 0.0001$), and Bursβ to 6.7 ± 1.8 CCAP-ENs (n = 9, $P < 0.0001$). Importantly, in all cases peptide hormone expression was eliminated in the A5–A7 late CCAP-EN subset. These data correspond well with pupal ecdysis phenotypes observed in these animals; expression of either *UAS-EcR-A^{DN}* or *UAS-EcR-B1^{DN}* (Fig. 7 *B* and *C*) resulted in a failure of leg extension, whereas *UAS-EcR-B2^{DN}* had no effect (Fig. 7*D*).

We also examined the effect of expressing dsRNAi to the common isoform of EcR (*UAS-EcR-C^{dsRNAi}*) under the control of *dac^{GAL4}* to knock down EcR expression in all CCAP-ENs. In correspondence

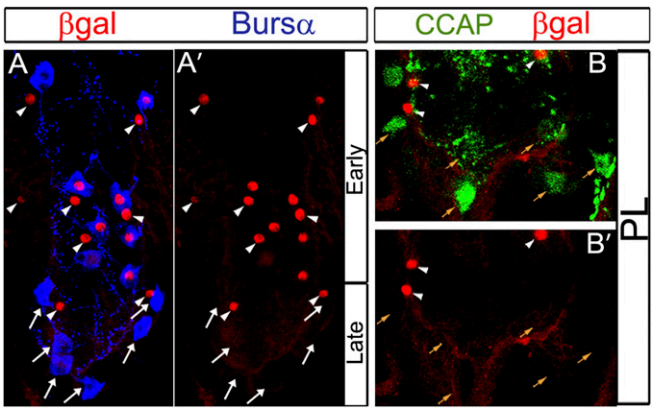

Fig. 4. Late subsets of CCAP neurons emerge at late L3. Combining the TARGET and Flp/FRT systems, we permanently β-Gal–marked neurons that expressed *CCAP-GAL4* in larvae. Animals were raised at 29 °C until mid-L3, and at 18 °C thereafter. Representative images of pharate adult VNC, showing anti-Bursα to identify CCAP-ENs (*A*) and anti-CCAP to identify CCAP-PLs (*B*). (*A* and *A′*) β-Gal immunoreactivity (red) was not observed in the six A5–A7 late CCAP-ENs (arrows) but was seen in CCAP-INs (arrowheads) and early CCAP-ENs (Bursα and β-Gal are colabeled). (*B* and *B′*) Posterior abdominal VNC showing that CCAP-PLs (orange arrows) expressed anti-CCAP (green) but did not express β-Gal (red). CCAP-INs (arrowheads) expressed anti-CCAP and β-Gal. Genotypes: *CCAP-GAL4 /Act-FRT > STOP > FRT-nLacZ; tubP-GAL80^{TS}, UAS-nlsEGFP/UAS-Flp*.

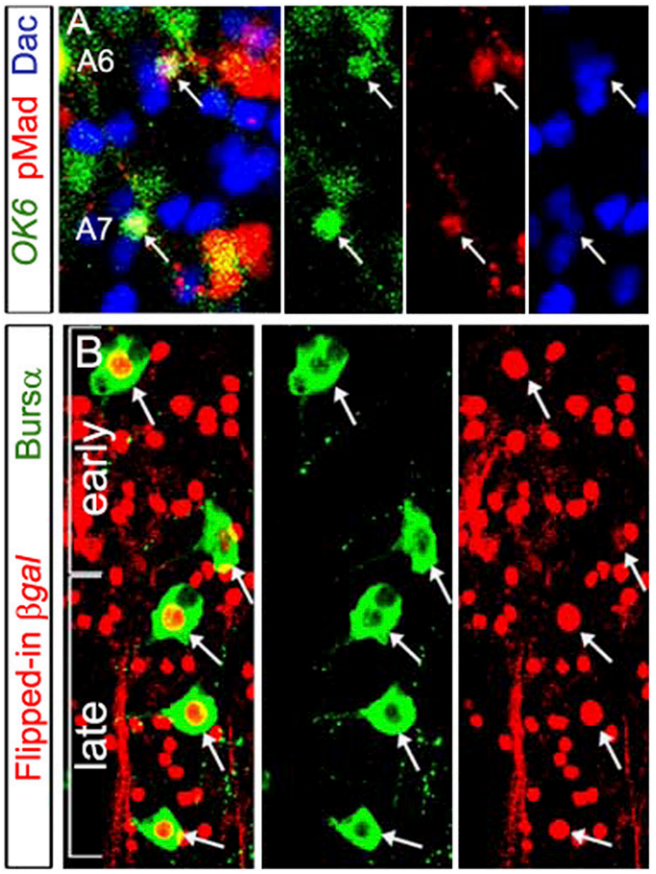

Fig. 5. Late CCAP-ENs derive from an embryonic lineage. Postmitotic late CCAP-ENs can be observed at late embryonic stages. (*A*) Representative triple-labeled images and fluorophore splits of VNC hemisegments A6 and A7 at embryonic stage 17. In the dorsal-half VNC, only a single neuron (arrows) coexpressed pMad (red), *OK6-GAL4* (green), and Dac (blue). In previous work (25), this overlap was unique to CCAP-ENs in dorsal hemisegments T3–A4. In Fig. 2 and Fig. S2, we show that this overlap is unique to A5–A7 late CCAP-ENs by 10–12 h APF. (*B*) Abdominal VNC of a pharate adult showing Bursα (green) and β-Gal (red) (with fluorophore splits). Dac^{GAL4} was used to Flp-in constitutive lacZ expression only in embryos, using $GAL80^{TS}$ to control the time of *UAS-Flp* expression. By the pharate adult stage, β-Gal was observed in all CCAP-ENs including T3–A4 early CCAP-ENs and A5–7 late CCAP-ENs. Thus, all six late CCAP-ENs in A5–A7 expressed Dac before late stage 17. Genotypes: (*A*) *OK6-GAL4, UAS-nlsEGFP/+*; (*B*) dac^{GAL4}*/Act-FRT > STOP > FRT-nLacZ; tubP-*$GAL80^{TS}$*, UAS-nlsEGFP /UAS-Flp*.

with the EcR^{DN} data above, we found that EcR^{dsRNAi} significantly reduced CCAP and Bursα expression in late CCAP-ENs by 10 h APF, immediately before pupal ecdysis. The intensity of CCAP immunoreactivity per CCAP-EN neuron (normalized to the mean of the control) was reduced from 100.0 ± 61.7% (n = 55 late CCAP-ENs) to 50.0 ± 31.9% (n = 55 late CCAP-ENs) by EcR^{dsRNAi} ($P < 0.0001$). Similarly, in late CCAP-ENs the intensity of Bursα immunoreactivity was reduced from 100.0 ± 59.3% (n = 50 late CCAP-ENs) to 30.0 ± 27.8% (n = 47 late CCAP-ENs) ($P < 0.0001$). These data confirm that peptide hormone expression in late CCAP-ENs requires EcR function. In contrast, EcR manipulation had little effect on CCAP expression in late CCAP-PLs. These data may explain the weak head-eversion phenotype upon EcR-$B1^{DN}$/A^{DN} expression using *CCAP-GAL4* (Fig. 7 *B–D*). This lack of effect likely reflects EcR isoform and/or nuclear hormone receptor redundancy in CCAP-PLs or may reflect the significantly weaker expression of *CCAP-GAL4* (and thus the lowered expression of the EcR-DN constructs) observed in those neurons compared with CCAP-ENs.

We examined whether late CCAP neurons are poised for ecdysone pathway-induced differentiation throughout larval stages. To test this hypothesis, we tested the effect of early induction of *ftz-f1*, a central player in the ecdysone-induced nuclear hormone receptor cascade (36, 37). Remarkably, we found that 1-h (37 °C) induction of a heat shock-inducible *ftz-f1* transgene (36) in late L1 larvae resulted in precocious differentiation of late CCAP-ENs and CCAP-PLs within 4 h. We observed precocious Bursα immunoreactivity in late CCAP-ENs in 54% of animals (n = 13 animals), Bursβ immunoreactivity in 40% of animals (n = 20), and CCAP immunoreactivity in 38% of animals (n = 24) (results for Bursα are shown in Fig. 7*F*). Precocious CCAP expression also was observed in CCAP-PLs, but, appropriately for CCAP-PLs, neither Bursα nor Bursβ was observed. Although the results were not as robust, we also found that a 1-h heat shock in late stage 17 embryos also could induce some precocious peptide hormone expression in late CCAP neurons in L1 larvae. Thus, throughout larval development late CCAP neurons appear to be poised for an inductive ecdysone signal to undergo terminal differentiation into a peptide hormone-expressing CCAP neuron that can contribute to the execution of pupal ecdysis.

Discussion

We show that two additional subsets of CCAP neurons differentiate to express their mature peptide hormone battery and extend their axon into the periphery during the first 12 h of pupariation. Moreover, we show that these late CCAP neurons are sufficient for pupal ecdysis occurring 12 h after pupariation. We conclude that the CCAP neuronal network functionally recruits these late CCAP neurons to switch network output from a larval ecdysis mode to a pupal ecdysis mode. In other well-established models, postembryonic neurogenesis adds neurons to existing networks at metamorphosis to change network function. Here, we propose that temporally tuned terminal differentiation provides an alternate mechanism of functional recruitment to an existing neuronal network and show that this mechanism can support a change in network output. The extension of an axon into the periphery and the induction of peptide hormone expression in late CCAP neurons at pupariation are of critical relevance, because these peptide hormones are the primary output of CCAP neurons that direct ecdysis (14, 25, 38). Together, these data highlight an unanticipated functional heterogeneity in the CCAP neuronal network (24) and outline the regulatory mechanisms and functional relevance of temporally tuned neuronal differentiation in network remodeling.

A neuron typically is not considered to have terminally differentiated until it expresses the terminal differentiation genes and full morphology required for its function (39, 40). However, neuronal differentiation is not a singular event. In many neurons, the expression of genes critical for mature function (such as neuropeptides, neurotransmitter biosynthetic enzymes, or ion channels) or mature axon or dendritic branching patterns are induced only after many other aspects of neuronal identity are established. In many cases, these late-expressed genes also depend upon extrinsic input for their expression (26, 41, 42). Thus, here we refer to "terminal differentiation" as the completion of a protracted differentiation process required for mature function. In this context, we refer to terminal differentiation of late CCAP neurons as the completed induction of all genes critical for function, which requires the expression of their peptide hormone battery and the extension of their axon into the periphery (14, 25, 38). The development of tools to image late CCAP neuron morphology and other markers of CCAP neuron differentiation before pupariation would allow us to understand better how the entire differentiation program of late CCAP neurons proceeds from their birth in the embryo to pupariation. This understanding would be an important step toward resolving the extent of late CCAP neuron developmental stalling before pupariation. For

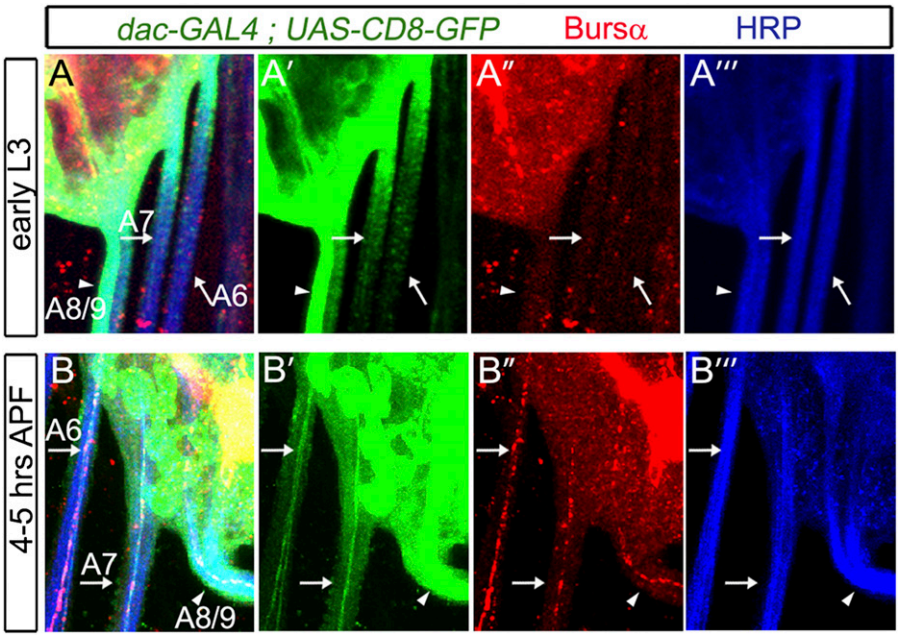

Fig. 6. Late CCAP neurons exit the VNC during pupariation. We used dac^{GAL4},*UAS-CD8-GFP* expression in CCAP-ENs to show that late CCAP-ENs differentiate morphologically at pupariation. We show representative overlapping and split-fluorescence images of the posterior nerve trunks that exit the VNC in early L3 larvae and in pupae 4–5 h APF, triple-labeled for dac^{GAL4},*UAS-CD8-GFP* (green), Bursα (red), and anti-HRP (blue), which stains all neuronal membranes in *Drosophila* and outlines the nerve trunk. Arrows indicate nerve trunks in A6 and A7; the arrowhead indicates the fused A8/A9 nerve. (*A*–*A*′′′) In L3 larvae, dac^{GAL4}, *UAS-CD8-GFP*–expressing and Bursα-expressing axons were not observed in A6 and A7 lateral nerve trunks (arrows). The A8/A9 nerve trunk (arrowhead) carries other *dac*-expressing efferents (see text for details) but did not carry Bursα-expressing axons. (*B*–*B*′′′) By 4–5 h APF, a single dac^{GAL4},*UAS-CD8-GFP*–expressing axon was detected in each of the A6 and A7 nerve trunks (arrows). This axon coexpressed Bursα. Bursα also was observed in the A8/A9 nerve trunk at this age. Genotypes: dac^{GAL4}/*UAS-CD8-GFP*.

example, it is intriguing that late CCAP neurons exhibit pMad signaling throughout larval development. Previously, only neurons that exit the VNC were found to exhibit nuclear pMad expression (26, 27). We were unable to resolve the morphology of late CCAP neurons at these early time points, but it would be interesting to determine whether these neurons abut the exit point for peripheral nerve trunks and can access BMP ligand there.

Most embryonic *Drosophila* neurons differentiate fully by early larval stages (43), but the literature does provide examples of delayed differentiation. Most motoneurons are born in the embryo and function in larvae and then are remodeled during metamorphosis and reused to innervate adult muscles (43). However, the MN5 mesothoracic motoneuron projects short, immature processes during embryogenesis but then arrests developmentally until metamorphosis, whereupon it elaborates mature arbors and innervates its muscle target (20). The functional relevance of this delayed-onset morphological differentiation is evident, but the underlying gene-regulatory mechanisms are unknown. Also, Tv2/3 neurons are born during embryogenesis but do not differentiate into FMRFamide-expressing neurons until metamorphosis (19, 21). Work in *Manduca sexta* has reported an increase in the number of CCAP-immunoreactive neurons in abdominal segments A2–A7 by day 1 of the fifth instar, before pupal ecdysis. Backfill experiments suggested that these neurons were present in the third and fourth instars but did not differentiate until the fifth instar (18, 22, 23). Our data suggest that those neurons are homologs of the late CCAP neurons that we identify in this work.

The role of ecdysone signaling in the metamorphic changes in the insect nervous system (e.g., in neuronal programmed cell death, postembryonic neurogenesis, and morphological remodeling) is well established (35, 44, 45). Ftz-f1 is an important downstream regulator of the ecdysone nuclear hormone cascade (46), but its role in neurons is less well studied. Previous studies demonstrated that *ftz-f1* dictates the temporal specificity of ecdysone signaling by integrating messages between numerous nuclear receptors (E75, DHR3, DHR4, and potentially also E78 and DHR39) (36, 37). Despite its long-known expression in the nervous system, the only reported role for *ftz-f1* in nervous system metamorphosis, to our knowledge, is in the remodeling of mushroom body neuron morphology (47–50). Here, we describe a role for *ftz-f1* in triggering timed neuronal differentiation. It will be illuminating in the future to determine how EcR and Ftz-f1 coordinate peptide hormone differentiation in late CCAP neurons.

Why late CCAP neurons would undergo delayed terminal differentiation is unclear. Previously, we found that CCAP-ENs regulate both larval and pupal ecdysis (25). However, larval and pupal ecdyses are behaviorally distinct, and here we found that late CCAP-ENs had not differentiated in time for larval ecdysis. We postulate that the emergence of late CCAP-ENs immediately before pupal ecdysis offers an elegant means to meet the changing functional role of the CCAP neuron network from larval to pupal ecdysis. From this network perspective, we could regard late CCAP neuron terminal differentiation and the role of these neurons in pupal ecdysis in two ways. First, late CCAP neurons may be recruited functionally to the existing CCAP neuronal network to switch its ecdysis program from larval to pupal. Second, late CCAP neurons may constitute an independent subnetwork of CCAP neurons that is responsible for pupal ecdysis and supersedes a subnetwork of early CCAP neurons responsible larval ecdysis. Two lines of evidence led us to favor the first hypothesis. Adams and colleagues (29) imaged CCAP neuron activity during fictive pupal ecdysis using a *CCAP::GcaMP* transgene. They observed activity of CCAP neurons within segments T3–A4, which have only early CCAP neurons (29), and in segments A7–A9, which have late CCAP neurons. Thus, although we demonstrate here that T3–A4 early CCAP neurons are not es-

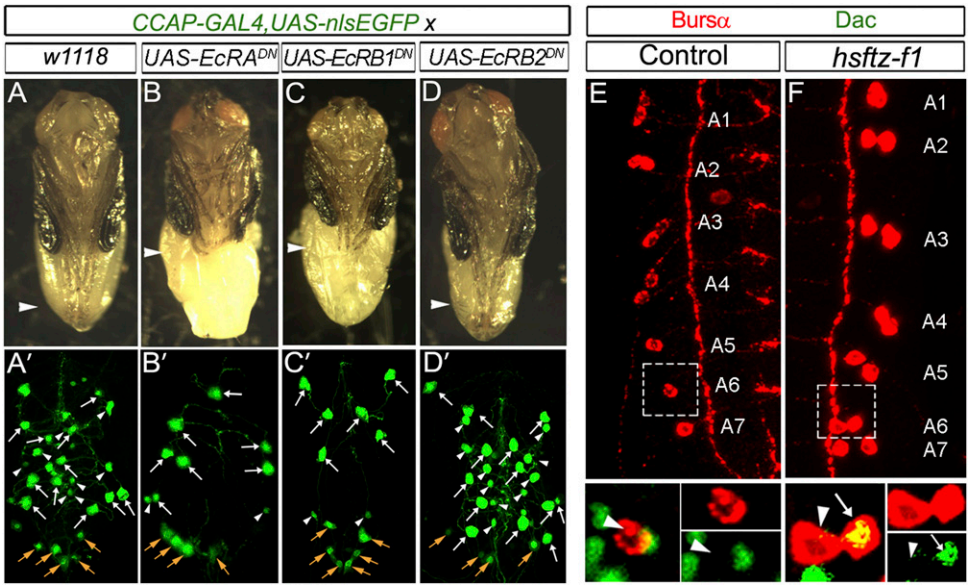

Fig. 7. Ecdysone signaling is required for late CCAP-EN differentiation. *CCAP-GAL4* was used to overexpress dominant-negative EcR-B1/A in CCAP neurons. This overexpression prevented further differentiation of late CCAP-ENs and resulted in a failure of leg extension. (*A*–*D*) Leg extension (arrowhead) in pharate adults for each dominant-negative EcR isoform. Control (*A*) and *EcR-B2*^{DN} flies (*D*) had wild-type leg extension, but *EcR-A*^{DN} (*B*) or *EcR–B1*^{DN} (*C*) expression resulted in failed leg extension. (*A′*–*D′*) Pharate adult abdominal VNC showing distribution of all *CCAP-GAL4,UAS-nlsEGFP*–labeled neurons (green). In control (*A′*) and in *EcR-B2*^{DN} (*D′*) flies, all CCAP neurons expressed *CCAP-GAL4,* including *CCAP*-ENs (white arrows), CCAP-INs (arrowheads), and CCAP-PLs (orange arrows). In *EcR-A*^{DN} (*B′*) or *EcR–B1*^{DN} (*C′*) flies, late CCAP-ENs lose *CCAP-GAL4* expression. Early CCAP-ENs (white arrows) and CCAP-PLs (orange arrows) are unaffected. Segmental midline CCAP projections (Fig.1 and Fig. S1) were used as a reference to confirm that all remaining CCAP-ENs were within T3–A4 segments. (*E* and *F*) Early L2 VNCs double-labeled (with fluorophore splits of boxed area) for Bursα (red) and Dac (green), showing A1–A7 hemisegments. (*E*) (*Upper*) In hemisegments A5–A7 there was only a single CCAP neuron expressing Bursα. (*Lower*) Magnified view of boxed region in *E* shows that those neurons lack Dac expression (arrowhead), confirming them to be CCAP-IN. (*F*) (*Upper*) *hs-ftz-f1* animals were heat shocked at late L1. CCAP neuron doublets expressing Bursα were observed in every hemisegment from T3–A7. (*Lower*) Magnified view of boxed region shows close-up of A6 hemisegment. The additional Bursα-expressing CCAP neuron (arrow) expressed Dac, indicative of a late CCAP-EN that had differentiated precociously. Genotypes: (*A* and *A′*) *CCAP-GAL4, UAS-nlsEGFP/+*; (*B* and *B′*) *CCAP-GAL4, UAS-nlsEGFP/UAS-EcR-A*^{DN} ; (*C* and *C′*) *CCAP-GAL4, UAS-nlsEGFP/UAS-EcR-B1*^{DN}; (*D* and *D′*) *CCAP-GAL4, UAS-nlsEGFP/UAS-EcR-B2*^{DN} ; (*E*) *CCAP-GAL4,UAS-nlsEGFP/+*; (*F*) *hs-ftz-f1/hs-ftz-f1*.

sential for pupal ecdysis, this previous study shows that they are active and presumably perform some function. Also, CCAP neurons in the suboesophageal region are required in combination with abdominal CCAP-ENs for wing inflation in young adults. Thus, components of the CCAP network, in addition to the late CCAP neurons, continue to function beyond larval stages.

It is unclear why late CCAP-ENs do not differentiate into peptide hormone-expressing CCAP neurons until pupal ecdysis, but we propose three potential explanations. (*i*) Late CCAP-ENs may have distinct central connectivity or receptor expression that may mediate a difference in the sequence or activation of CCAP neuron activity that is specific to pupal ecdysis and perhaps detrimental to larval ecdysis. (*ii*) Early CCAP-ENs on their own may not secrete sufficient peptide hormone levels into the hemolymph for robust pupal ecdysis. Thus, late CCAP-ENs may be required to boost levels of secreted peptide hormones required for pupal ecdysis. It is possible that elevated peptide hormone expression may be detrimental to larval ecdysis. (*iii*) The temporally tuned differentiation mechanism may have evolved simply to put off the demands of secreting high levels of circulating peptide hormones until the time point at which such secretion is absolutely required.

The early neurogenesis of neurons that do not undergo terminal differentiation until pupariation was unexpected. However, we propose that such early neurogenesis offers a mechanism to generate late CCAP neurons that is simpler than postembryonic neurogenesis. The process of neuronal specification and differentiation is a highly orchestrated process through which complex cascades of spatially and temporally patterned transcription factors direct the emergence of specific neuronal subsets. Moreover, the *Drosophila* nervous system is segmented, with abdominal segments A1–A7 giving rise to essentially the same neuroblasts and mostly to the same neurons in each segment. Thus, generating a single CCAP-EN in each segment from the same neuroblast lineage would be an economical way to produce late CCAP-ENs with much the same terminal gene-expression profile as early CCAP-ENs. Additional segment-specific mechanisms then could be superimposed to delay late CCAP-EN differentiation until it received its ecdysone trigger. Recent work has described mechanisms to diversify the same postmitotic neuron in a segment-specific manner. The best described of these mechanisms is the segment-specific programmed cell death of dMP2 and Va neurons in the *Drosophila* VNC (51, 52). These studies indicate that segment-specific differences in Hox gene expression within these postmitotic neurons determine their survival versus apoptosis during embryogenesis. Here, we find that segment-specific differences in the timing of neuronal differentiation provide a mechanism for neuronal diversity. It will be intriguing in the future to investigate the potential role of segment-specific Hox gene expression in directing early versus late CCAP neuron differentiation.

Materials and Methods

Fly Stocks. We used fly stocks *dac*^{GAL4} (53); *CCAP-GAL4* (16); *OK6-GAL4* (54); *tubP-GAL80*^{TS} (55); *UAS-nlsEGFP*; *UAS-CD8-GFP*; *UAS-EcR-A*^{DN} (BL9451) ; *UAS-EcR-B1*^{DN} (BL6872); *UAS-EcR-B2*^{DN} (BL9449); *UAS-EcR-dsRNAi* (BL9327), *UAS-CD8-EGFP*^{LL5} (Bloomington *Drosophila* Stock Center); *Act-FRT > STOP > FRT. nlacZ, UAS-Flp* (56); and *hs-ftz-f1* (36). Lethal alleles were maintained over CyO,Act-EGFP or TM3,Ser,Act-EGFP balancer chromosomes. The control genotype was w^{1118}. Flies were maintained on standard cornmeal food (25 °C, 70% humidity).

Immunohistochemistry. Standard immunohistochemical protocols were used, as previously described (57). Primary antibodies were rabbit anti-CCAP (code 2TB; 1:2,000), a gift from H. Dircksen (Stockholm University, Stockholm, Sweden) (58); rabbit anti-Bursα (1:5,000), a gift from B. White (NIH, NIMH, Bethesda, MD) (24); mouse anti-Bursβ (1:2,000), a gift from C. Klein (Stanford University, Palo Alto, CA) (59); mouse anti-Dac (1:25; clone dac2-3) and mouse anti-BrdU (1:20; clone G3G4) (Developmental Studies Hybridoma Bank); rabbit anti-pMad (1:100) (41D10; Cell Signaling Technology); chicken anti–ß-Gal (1:1,000) (ab9361; Abcam); and goat anti-HRP-Cy5 (1:100) (Jackson ImmunoResearch). Secondary antibodies were anti-mouse, anti-chicken, anti-rabbit IgG (H+L) conjugated to either DyLight 488, Cy3, and Cy5 (1:200) (Jackson ImmunoResearch).

Image Analysis. Images were acquired on an Olympus FV1000 confocal microscope and analyzed with Image J (National Institutes of Health). Images for comparison were processed identically, and image capture was set to avoid fluorescent saturation. Analysis was performed as described (13). Data are presented as the percentage intensity relative to the mean of the control.

Statistics. Statistical analysis was performed using Graphpad Prism 4. Data for immunofluorescence and cell number underwent D'Agostino and Pearson Omnibus normality testing. Normally distributed data were compared using a two-tailed *t* test assuming equal variance. Non-normally distributed data were compared using a nonparametric Mann–Whitney test. Statistical data are presented to the exact *P* value to $P < 0.0001$.

Heat Shock Ftz-F1 Induction. Larvae were heat shocked at 37 °C for 1 h and then were allowed to recover at 25 °C for 2–4 h before dissection and analysis.

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