# Supporting Information

## Veverytsa and Allan 10.1073/pnas.1114710109

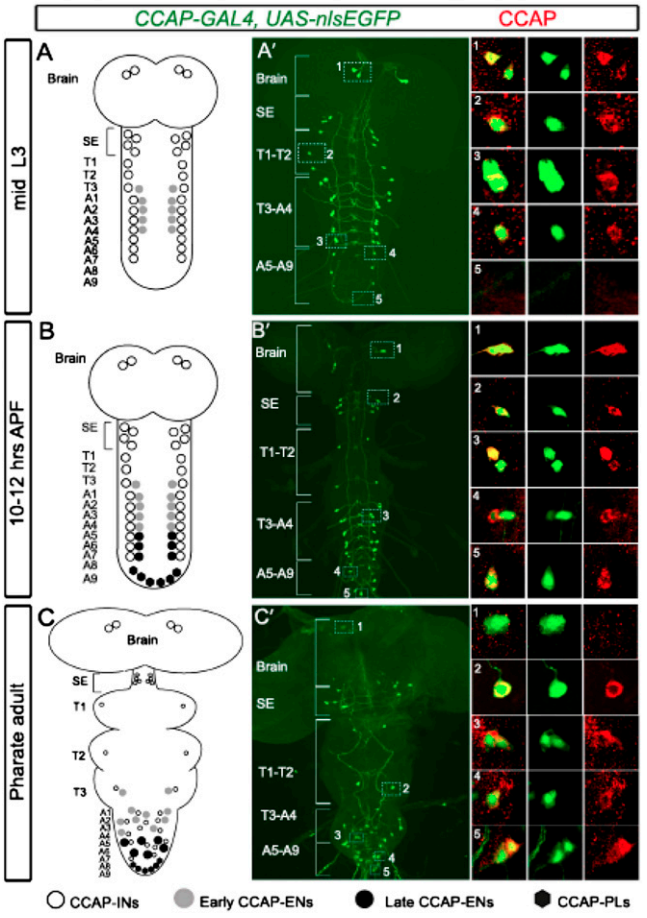


**Fig. S1.** A set of late CCAP neurons emerges at pupariation. (*A*–*C*) Cartoons summarizing the distribution of crustacean cardioactive peptide (CCAP) neurons in the CNS in mid-L3 larvae (*A*), in pupae 10–12 h after puparium formation (APF) (*B*), and in pharate adults (*C*). To visualize the distribution of CCAP neurons within the CNS at different ages, we used *CCAP-GAL4* to drive the expression of *UAS-nlsEGFP* (green) (*A*′, *B*′, and *C*′). We also show magnified views of the numbered boxed regions from *A*′, *B*′, and *C*′. These images are double-labeled and fluorophore splits for *CCAP-GAL4*;*UASnlsEGFP* (green) and CCAP immunoreactivity (red) in representative subsets of CCAP neurons. The number of CCAP neurons in the brain and in the subesophageal and thoracic regions remained constant throughout. However, we observed the emergence of additional neurons in abdominal segments A5–A9 in pupae 10–12 h APF and in pharate adults (boxes 4 and 5 in *B*′ and *C*′). Genotype: *CCAP-GAL4,UAS-nEGFP*/+.

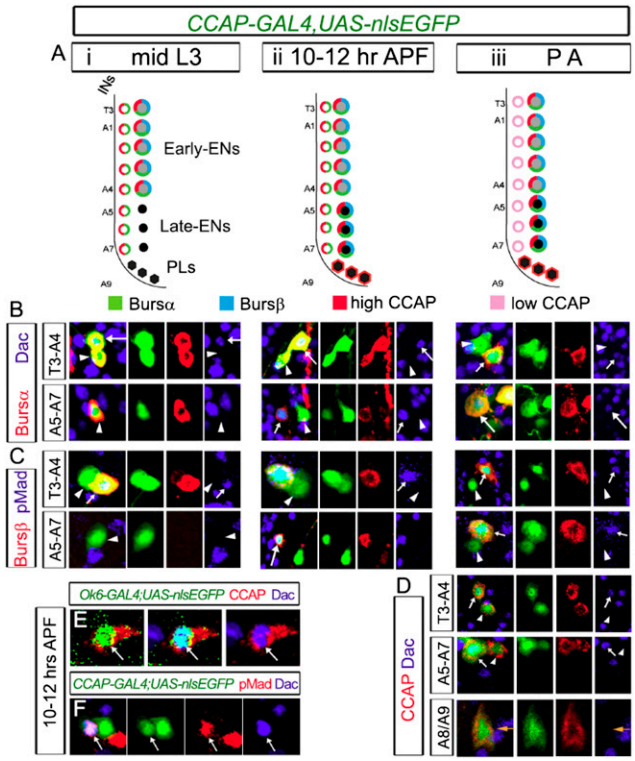


**Fig. S2.** Late CCAP neurons start to differentiate by late L3. Late CCAP neurons started to differentiate by late L3 and through early pupariation up to the time of pupal ecdysis. (*A*) Cartoon summaries of gene expression in ventral nerve cord (VNC) CCAP neurons from mid-L3 to pharate adults. (*B* and *C*) Expression of Bursα (*B*) and Bursβ (*C*) in CCAP neurons is shown at mid-L3 (*i*) before late CCAP neuron differentiation, (*ii*) at 10–12 h APF immediately before pupal ecdysis, and (*iii*) in pharate adults (PA). In *i–iii* T3–A4 hemisegments had a CCAP neuron doublet from mid-L3 to PA, including a $Dac^+$ CCAP efferent (CCAP-EN) (arrows) and a $Dac^-$ CCAP interneuron (CCAP-IN) (arrowheads). In contrast, in A5–A7 hemisegments there was only a single CCAP neuron (a $Dac^-$ CCAP-IN, arrowhead) at mid-L3. However, in pupae 10–12 h APF and in pharate adults, a doublet of CCAP neurons was observed in hemisegments A5–A7, namely, a $Dac^+$ CCAP-EN (arrows) and a $Dac^-$ CCAP-IN (arrowheads). (*B*, *i–iii*) Bursα expression (red) was lost in the CCAP-IN by pharate adults (arrowhead in *B*, *iii*). In hemisegments A5–A7, the CCAP-IN expressed Bursα at mid-L3. By 10–12 h APF and thereafter, a late CCAP-EN had differentiated and expressed Bursα (arrow in *B*, *ii* and *iii*). (*C*, *i–iii*) Expression of Bursβ (red) in hemisegments T3–A4 and A5–A7. Bursβ was expressed only in early CCAP-ENs ($CCAP^+$, $pMad^+$) from mid-L3 to PA in hemisegments T3–A4. Bursβ expression then was induced by 10–12 h APF (arrow in *C, ii*) and retained thereafter in all CCAP-ENs up to the pharate adult stage (arrow in *C*, *iii*). (*D*, *iii*) Expression of CCAP (red) is shown in CCAP neurons in hemisegments T3–A4, A5–A7, and A8/A9 in pharate adults. Dac expression (blue) denotes CCAP-ENs in hemisegments T3–A7. CCAP expression in all CCAP-INs and early CCAP-ENs continued to the pharate adult stage. CCAP expression in late CCAP-ENs (white arrow in A5–A7) and late posterior lateral CCAP neurons (CCAP-PLs) (orange arrows in A8/A9) is observed. (*E* and *F*) Triple-labeled images and fluorophore splits of late CCAP-ENs (arrow) at 10–12 h APF that express *OK6-GAL4,UAS-nEGFP* (green), CCAP (red), and Dac (blue) (*E*), as well as pMad (red) and *CCAP-GAL4,UAS-nEGFP* (green) (*F*). Genotypes: *CCAP-GAL4,UAS-nlsEGFP/+* (*B–D* and *F*); *OK6-GAL4,UAS-nEGFP/+* (*E*).

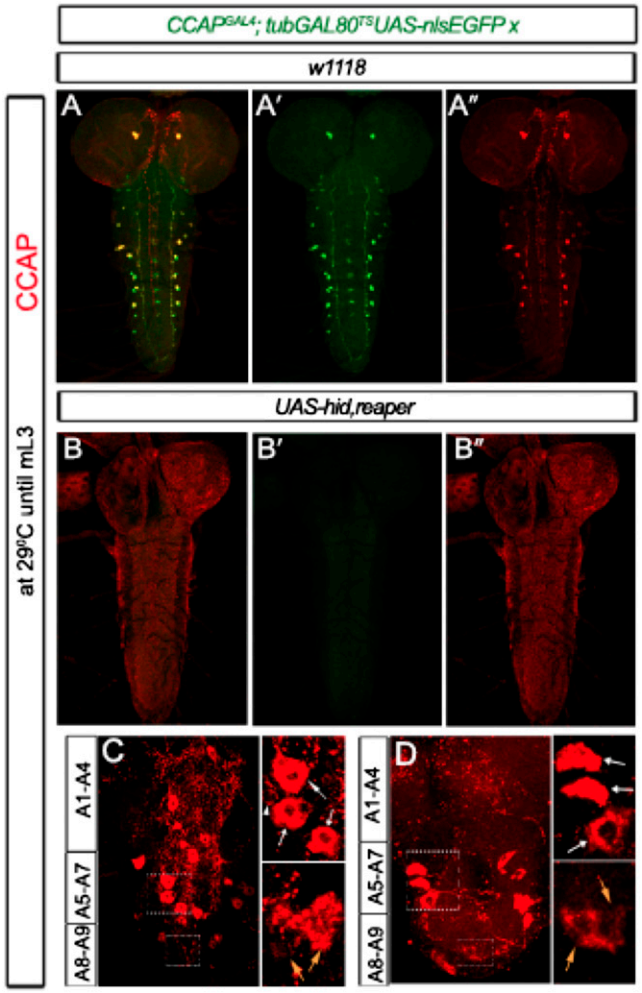


**Fig. S3.** Temporally regulated expression of cell death genes *hid* and *reaper* during larval development kills all early CCAP neurons but leaves late CCAP neurons intact. Expressing cell death genes up until mid-L3 in CCAP neurons using *CCAP-GAL4,UASnlsEGFP* selectively ablates all early CCAP neurons by mid L3 (*B*–*B*′′) compared with control animals in which cell death genes were not expressed (*A*–*A*′′). (*C* and *D*, *Left*) In controls, the full complement of CCAP neurons was observed in newly eclosed adults. (*C*, *Right*) Early CCAP-ENs (long white arrow), late CCAP-ENs (short white arrows), and CCAP-PLs (orange arrows) are indicated in magnified views. (*D*, *Right*) After selective ablation of early CCAP neurons, only late CCAP neurons (white arrows) and CCAP-PLs (orange arrows) remained. Genotypes: (*A* and *C*) w/w; CCAP-GAL4/+; tubP-GAL80$^{TS}$,UAS-nlsEGFP/+; (*B* and *D*) UAS-hid,UASreaper/w or Y; CCAP-GAL4; tubP-GAL80$^{TS}$, UAS-nlsEGFP/+.

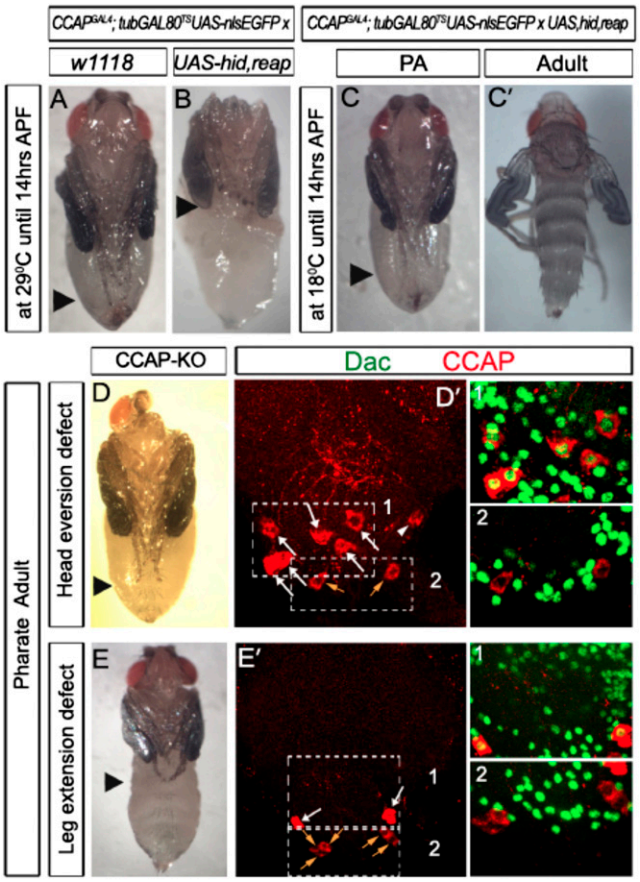


**Fig. S4.** Head eversion and leg extension are directed by different late CCAP neuron subsets. Subset-selective ablation of late CCAP neurons provides evidence that late CCAP-ENs direct leg extension, whereas CCAP-PLs direct head eversion. (*A*) Control pharate adults (PA) had wild-type leg extension (arrowhead) and head eversion. (*B*) Animals were raised at 29 °C to 14 h APF to inactivate GAL80$^{TS}$ so that *CCAP-GAL4* drives *Hid*/*Reaper* expression. This treatment resulted in ablation of all CCAP neurons and blocked leg extension and head eversion. (*C* and *C′*) Ablation of all CCAP neurons immediately after pupal ecdysis (at 14 h APF) resulted in wild-type leg extension and head eversion (*C*). One hundred per cent of these animals eclosed, but their wings failed to inflate (*C′*). (*D* and *E*) Switching animals from 29 °C to 18 °C around the time of pupariation onset (to deactivate *CCAP-GAL4* and cell death genes) resulted in pharate adult animals with (*D*) failed head eversion but normal leg extension or (*E*) normal head eversion but failed leg extension. (*D′* and *E′*) Examination of CCAP neuron distribution in these two sets of animals showed that all early CCAP neurons were ablated, but different subsets of late CCAP neurons remained. (*D′*, *Left*) Animals with head-eversion defect/wild-type leg extension retained most late CCAP-ENs (white arrows; Dac$^{+}$ shown in green) but lost most CCAP-PLs (orange arrows; Dac$^{-}$). (*E′*, *Left*) Animals with wild-type head eversion/leg-extension defect retained CCAP-PLs (orange arrows) but lost most CCAP-ENs (white arrows). (*D′* and *E′*, *Right*) Magnified views of boxed areas in *D′* and *E′*, *Left*. Genotypes: (*A*) w/w; CCAP-GAL4/+; tubP-GAL80$^{TS}$,UAS- nlsEGFP/+; (*B*–*E′*) UAS-hid,UASreaper/w or Y; CCAP-GAL4; tubPGAL80$^{TS}$,UAS-nlsEGFP/+.

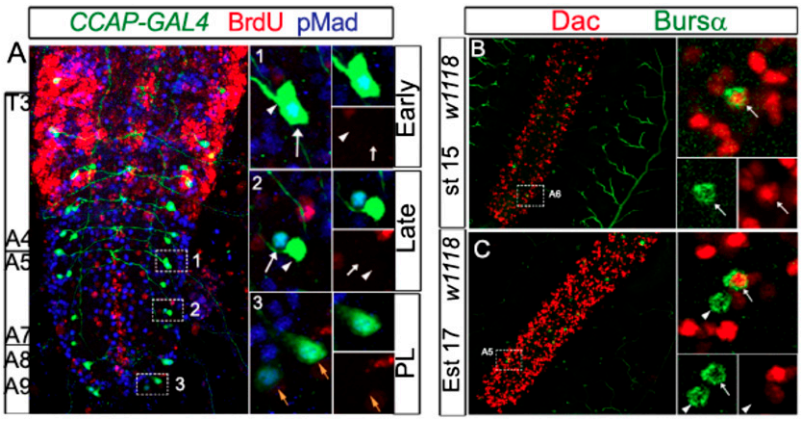


**Fig. S5.** Absence of BrdU incorporation in all late CCAP neurons and transient Bursα expression at embryonic stages point toward embryonic origin of late CCAP neurons. (*A*) Animals were fed 1 mg/mL BrdU-supplemented yeast paste from early L1 until pupariation. We show images and fluorophore splits of 2-h APF pupal VNCs that were triple labeled for *CCAPGAL4* (green), BrdU (red), and pMad (blue). BrdU labeling is extensive throughout the VNC. However, no CCAP neuron exhibited BrdU labeling. Magnified views of the numbered boxed regions show the lack of BrdU incorporation into CCAP-INs (arrowhead) and early CCAP-ENs (white arrow) (Early), into late CCAP-ENs (white arrow) (Late), or into CCAP-PLs (orange arrows) (PL). (*B* and *C*) Images and fluorophone splits of the VNC in embryonic stage 15 (st 15) (*B*) and early stage 17 (st 17) (*C*) $w^{1118}$ flies double-labeled for Bursα (green) and Dac (red). Bursα expression can be observed in a $Dac^{+}$ CCAP neuron in hemisegments A5–A7. This expression typically is adjacent to a $Dac^{-}$ CCAP neuron (arrow) in the same hemisegment, which is a CCAP-IN (arrowhead). Thus, late CCAP-ENs appear to become postmitotic and transiently express the differentiation marker Bursα in stage 15/17 embryos before larval stages. Genotypes: (*A*) CCAP-GAL4,UAS-nEGFP/+; (*B* and *C*) $w^{1118}$.

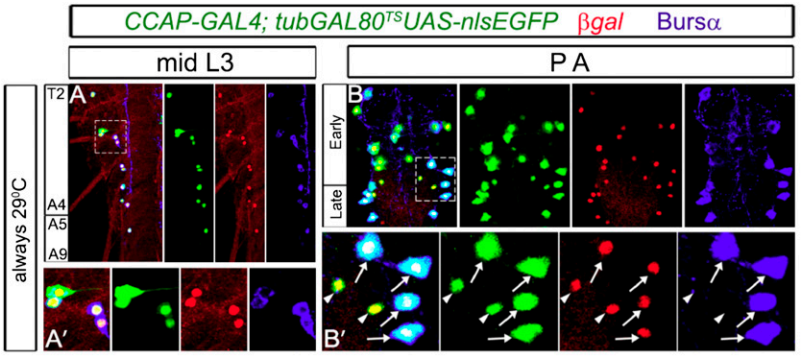


**Fig. S6.** The Flp/FRT system functions to Flp-in β-Gal into all CCAP neurons. Combining the TARGET and Flp/FRT systems, we permanently β-Gal–marked neurons that expressed *CCAP-GAL4* at specific time points. We maintained animals at 29 °C throughout all development up to pharate adults. (*A* and *A′*) A mid-L3 hemi-VNC (T2–A9) including triple-labeling and fluorophore splits. *CCAP-GAL4* (green) and anti-Bursα (blue) identify early CCAP neurons. β-Gal (red) labeled all differentiated early CCAP neurons but no late CCAP-ENs. (*B* and *B′*) In pharate adults (PA), *CCAP-GAL4* (green) and β-Gal (red) labeled all CCAP neurons, but Bursα (blue) did not. (*B*) Early and late CCAP-EN subsets are demarcated, and both subsets express β-Gal. (*B′*) Magnification of boxed area in *B* showing that CCAP-ENs (arrows) express *CCAP-GAL4* (green) and Bursα (blue), whereas CCAP-INs (arrowhead) express only *CCAP-GAL4*. However, both subsets express β-Gal (red). Genotypes: CCAP-GAL4 /Act-FRT > STOP > FRT-nlsLacZ; tubP-GAL80$^{TS}$, UAS-nlsEGFP /UAS-Flp.

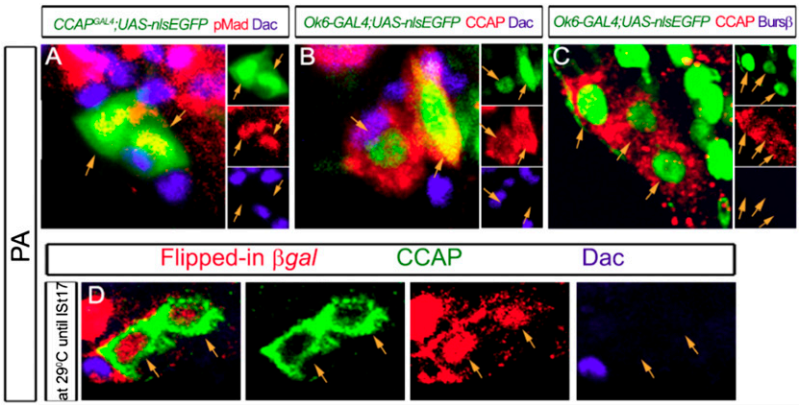


**Fig. S7.** Molecular markers expressed in CCAP-PL neurons in pharate adults. (*A*–*C*) Triple-labeled images and fluorophore splits showing hemisegments A8/A9 in pharate adult (PA) VNCs. CCAP-PLs (orange arrows) can be distinguished from other CCAP neurons by their position at the posterior lateral edges of the ventral nerve cord in A8/A9 and by their irregular shape, compared with the rounder CCAP-ENs and CCAP-INs. (*A*) CCAP-PLs express *CCAP-GAL4,UASnlsEGFP* (green) and anti-pMad (red) but do not express Dac (blue). (*B*) CCAP-PLs express *OK6-GAL4,UAS-nlsEGFP* (green) and the CCAP neuropeptide (red). (*C*) CCAP-PLs do not express Bursβ or Bursα. (*D*) Using *OK6-GAL4* with TARGET and Flp/FRT systems, we raised animals at 29 °C to the end of embryogenesis at late stage 17 and switched to 18 °C thereafter. In pharate adults, anti–β-Gal was observed in all CCAP-PLs (orange arrows). Thus, all CCAP-PLs expressed *OK6-GAL4* before late stage 17. Genotypes: (*A*) CCAP-GAL4,UAS-nlsEGFP/+; (*B* and *C*) OK6-GAL4,UAS-nlsEGFP/+; (*D*) $OK6^{GAL4}$/Act-FRT > STOP > FRT-nlsLacZ; $tubP\text{-}GAL80^{TS}$, UASnlsEGFP /UAS-Flp.