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RESEARCH ARTICLE

Genetic analysis of Eclosion hormone action during *Drosophila* larval ecdysis

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ABSTRACT

Insect growth is punctuated by molts, during which the animal produces a new exoskeleton. The molt culminates in ecdysis, an ordered sequence of behaviors that causes the old cuticle to be shed. This sequence is activated by Ecdysis triggering hormone (ETH), which acts on the CNS to activate neurons that produce neuropeptides implicated in ecdysis, including Eclosion hormone (EH), Crustacean cardioactive peptide (CCAP) and Bursicon. Despite more than 40 years of research on ecdysis, our understanding of the precise roles of these neurohormones remains rudimentary. Of particular interest is EH; although it is known to upregulate ETH release, other roles for EH have remained elusive. We isolated an *Eh* null mutant in *Drosophila* and used it to investigate the role of EH in larval ecdysis. We found that null mutant animals invariably died at around the time of ecdysis, revealing an essential role in its control. Further analyses showed that these animals failed to express the preparatory behavior of pre-ecdysis while directly expressing the motor program of ecdysis. Although ETH release could not be detected, the lack of pre-ecdysis could not be rescued by injections of ETH, suggesting that EH is required within the CNS for ETH to trigger the normal ecdysial sequence. Using a genetically encoded calcium probe, we showed that EH configured the response of the CNS to ETH. These findings show that EH plays an essential role in the *Drosophila* CNS in the control of ecdysis, in addition to its known role in the periphery of triggering ETH release.

KEY WORDS: Neuropeptide, Molting, Behavior, Postembryonic development, Insect

INTRODUCTION

In insects, continuous growth and development requires the exoskeleton to be replaced, which occurs during the molt and culminates with the process of ecdysis. During ecdysis, a precisely timed and concatenated series of behaviors causes the remains of the old exoskeleton to be shed and the new one to be inflated, hardened and pigmented. Research conducted during the last 40 years has revealed that a suite of neuropeptides controls the precise sequence of behaviors and physiological events that allow the insect to transition from one stage to the next (for reviews, see Ewer and Reynolds, 2002; Zitnan and Adams, 2012). These neuropeptides include Ecdysis triggering hormone (ETH), which is produced by peripheral endocrine cells, and the centrally produced neuropeptides, Eclosion hormone (EH), Crustacean cardioactive peptide (CCAP) and Bursicon. Evidence from both Lepidoptera (e.g. Zitnan et al., 1996) and *Drosophila* (e.g. Park et al., 2002) indicates that ETH can turn on the entire ecdysial sequence. Direct targets of ETH include neurons that express FMRFamide, EH and CCAP (some of which also express Bursicon and/or the MIP peptide; Kim et al., 2006a,b), and both their timing of activation after ETH release and functional analyses (Lahr et al., 2012; Honegger et al., 2008; Kim et al., 2006a; Gammie and Truman, 1997a) suggest a role in the control of different phases of ecdysis. Thus, FMRFamide is proposed to regulate the early phase of the behavior, EH and the CCAP neurons that express CCAP or CCAP and MIP would regulate ecdysis proper, and neurons that coexpress CCAP, MIP and Bursicon participate in the postecdysial phases of the behavior.

EH has been implicated in the control of ecdysis since its discovery in Lepidoptera more than 40 years ago (Truman and Riddiford, 1970). In *Manduca* (Truman et al., 1980; Copenhaver and Truman, 1982) and *Bombyx* (Fugo and Iwata, 1983), injections of EH into the hemolymph cause premature ecdysis, and addition of EH to an isolated *Manduca* central nervous system (CNS) can induce the ecdysis motor program (Gammie and Truman, 1999; Zitnan and Adams, 2000), indicating that EH is sufficient for turning on ecdysis. In *Tribolium*, injection of EH interfering RNA causes a severe weakening of pre-ecdysis and a complete suppression of ecdysis (Arakane et al., 2008), suggesting that EH is also necessary for ecdysis. Nevertheless, the precise role of EH in *Drosophila* remains elusive. Indeed, flies bearing targeted ablations of EH neurons express relatively minor defects at larval ecdysis (McNabb et al., 1997; Clark et al., 2004), with about a third of animals reaching adulthood (McNabb et al., 1997). In addition, and most perplexingly, flies lacking EH neurons are insensitive to injections of ETH: in contrast to wild-type animals, for which such injections advance the onset of ecdysis, ETH injections do not change the timing of ecdysis of either larvae or adults bearing targeted ablations of EH neurons (McNabb et al., 1997; Clark et al., 2004).

From these observations it is difficult to propose a unified model for the role of EH in the control of ecdysis beyond its well-accepted role in potentiating ETH release (Ewer et al., 1997; Kingan et al., 1997). Furthermore, the majority of the information from *Drosophila* stems from experiments in which the EH neurons were genetically ablated (McNabb et al., 1997; Baker et al., 1999; Clark et al., 2004). Although this approach has provided valuable insights into the possible role of this neuropeptide at ecdysis, the interpretation of the findings is complicated by the fact that such animals lack the EH neurons in addition to the EH peptide, making it impossible to distinguish between functions subserved by the peptide itself from other roles played by the EH neurons.

We report here on the isolation of a null allele of the *Eh* gene and the characterization of the larval ecdysis phenotype of animals

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devoid of EH function. The lack of *Eh* function is completely lethal, with most animals dying during the larval stages, at around the time of ecdysis. We show that these defects are not caused by the accompanying lack of ETH release and report that the response of direct targets of ETH is severely altered in the absence of EH. Thus, our findings reveal that EH plays a key role within the CNS and is required for ETH to cause the expression of normal ecdysis behaviors.

RESULTS

Generation of an *Eh* null allele

We created a null *Eh* allele by excising a *P*-element inserted within the *Eh* gene, downstream of the EH neuropeptide-encoding sequences (Fig. 1A). Potential excision flies were identified by PCR, then screened for progeny that lacked EH immunoreactivity (EH-IR). Larvae from a single excision line (out of ∼500 single male white-eyed excision lines) lacked a diagnostic PCR product and were then found to lack EH-IR (Fig. 1B). Subsequent sequence analyses revealed that this mutant carried a 2.6 kb deletion of *Eh* DNA, which included 1.2 kb downstream of the *Eh* transcription start, including all EH neuropeptide-encoding sequences; it also retained a 1.7 kb fragment of the original *P*-element (Fig. 1A). In addition to the *Eh* gene, this excision also deleted part of the 3′ end of a heme peroxidase gene, *CG5873*, which when mutant causes no apparent defects (FlyBase).

Behavioral defects of *Eh* null mutants

Flies hemizygous for the *Eh* null excision allele [$Eh^{exc}/Df(3)Eh^{-}$] did not survive to adulthood; most (>90%) lethality occurred during larval stages and invariably occurred at around the time of ecdysis, with around 80% lethality occurring at each larval transition. Dead larvae either presented well-pigmented 'double vertical plates', and had therefore failed to ecdyse correctly, or had shed the old cuticle but had then failed to inflate the trachea of the next stage. The few flies that reached the pupal stage showed the hallmarks of animals that had failed to ecdyse properly (Park et al., 2003; Lahr et al., 2012), such as small or absent head and shorter than normal legs and wings.

Larval ecdysis behavior

Ecdysis behaviors of hemizygous *Eh* mutants were examined in most detail at the ecdysis to the third instar. At this ecdysis, larvae switch from locomotion to ecdysial behaviors around 20 min after the appearance of DVP ('double vertical plate', approximately

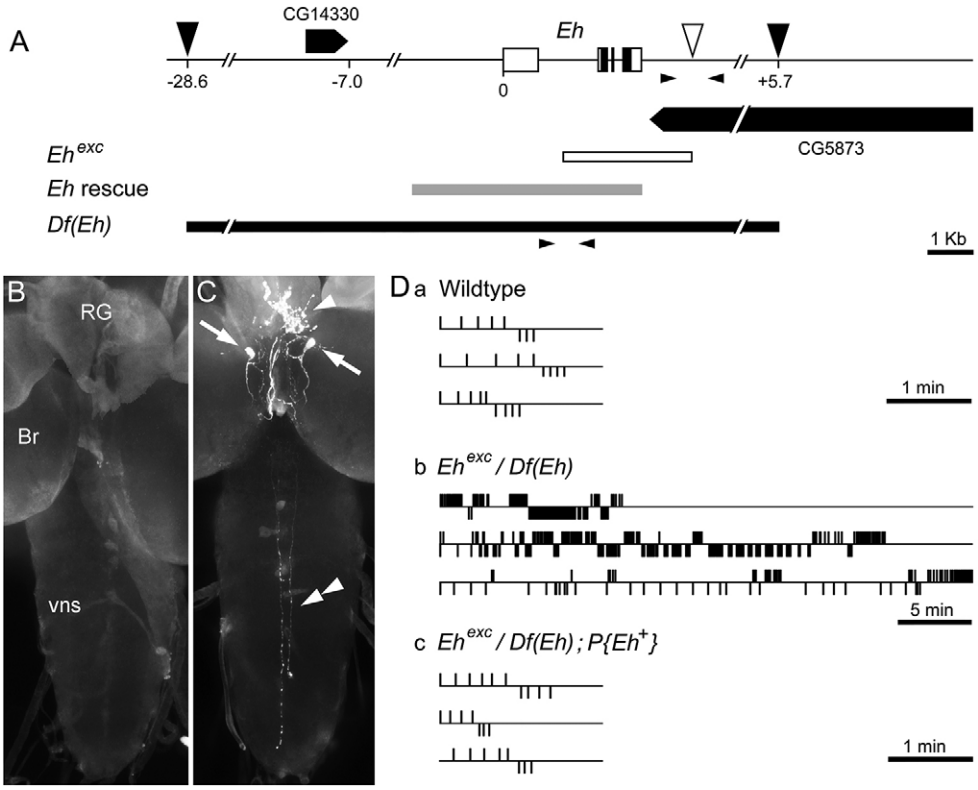

Fig. 1. Basic features of *Eh* null allele. (A) Map of *Eh* region. Filled and open boxes indicate coding and non-coding exons of *Eh* gene, respectively. Open inverted triangle indicates G8594 mobile element used to produce *Eh* null allele [Eh^{exc}]; the deleted fragment is indicated by the open bar below the map; small horizontal triangles indicate position of primers used for the initial screen. Filled inverted triangles indicate mobile elements used to produce genetic deletion that included the *Eh* gene [$Df(3)Eh^{-}$; abbreviated here and in all figures as *Df(Eh)*]; the deleted fragment is indicated by the filled bar below the map; small horizontal triangles indicate the position of primers used for the initial screen. Gray bar below map indicates the extent of the genomic fragment used for transgenic rescue. (B,C) EH immunoreactivity in third instar CNS of (B) *Eh* hemizygous mutant [$Eh^{exc}/Df(3)Eh^{-}$] and of (C) control. In C, arrows point to cell bodies; single arrowhead indicates the neurohemal release site in the corpora cardiaca and double arrowhead points to axons in the vns. Br, brain; RG, ring gland; vns, ventral nervous system. (D) Pictorial representation of ecdysis behavior of three larvae: (a) wild type; (b) *Eh* hemizygous mutant; (c) and transgenic rescue. Each line represents the timecourse of ecdysis behavior, with upward and downward directed lines representing anterior- and posterior-directed ecdysial peristalses, respectively. Mutant larvae expressed long runs of anterior- or posterior-directed peristalses, with no clear temporal order and interspersed with quiescent periods of variable duration. These data are summarized in Fig. 2A-C. Note that the time scale for the record for *Eh* hemizygous mutant larvae (Db) is one-fifth that of control and transgenic rescue animals. Panels B,C are each composites created by combining pictures, taken using identical settings, of the anterior and posterior halves of the CNS.

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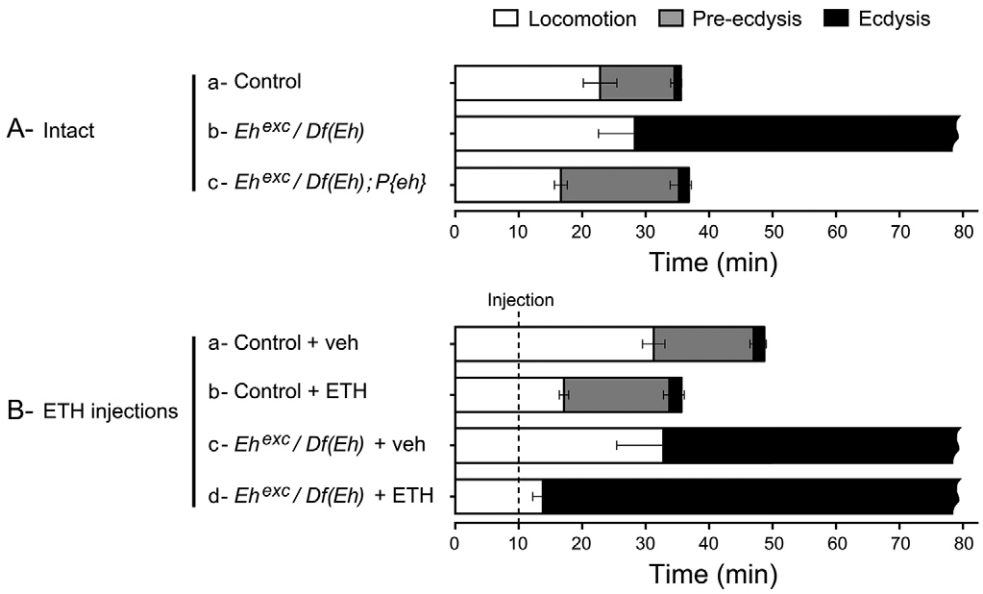

Fig. 2. Summary of larval ecdysis behavior. Ecdysis behavior was assessed in (A) intact and (B) ETH-injected larvae. Each bar represents average (±s.e.m.) duration of locomotion (open bars), pre-ecdysis (gray bars) and ecdysis (black bars). Time zero corresponds to DVP. In B, injections were done at DVP+10 min (vertical dashed line); veh, vehicle injection. (Ab) *Eh* hemizygous mutant larvae did not express pre-ecdysis; ecdysial phase was variable in duration, usually exceeding 80 min (see text); (Ac) these defects were rescued by a transgene containing *Eh* gene. Note that ETH injections accelerated onset of ecdysis of *Eh* hemizygous mutant larvae (compare Bd versus Bc; $P<0.01$), but did not restore a pre-ecdysial phase. Bar associated with ecdysis phase for hemizygous *Eh* null mutant animals has been truncated at 80 min. n=8-11 animals per group.

30 min prior to ecdysis; Park et al., 2002). Ecdysis normally consists of two distinct and concatenated behavioral routines, pre-ecdysis, followed by ecdysis (Fig. 2Aa). Although ecdysial behaviors of hemizygous *Eh* mutant larvae started at the normal time after DVP (start time of hemizygous *Eh* mutant larvae versus control, $P>0.05$), the pre-ecdysis phase was never observed; instead, larvae transitioned directly into expressing ecdysis behaviors (Fig. 2Ab). However, this phase was extremely protracted, generally lasting more than the 90 min observation period. Of 11 animals that were monitored, only five had successfully shed their second instar cuticle when a final inspection was made at 3 h. Of the remaining six, one died within the first hour after the start of the behavior, whereas the other five continued to express ecdysis behavior at 3 h and eventually died. Furthermore, the temporal organization of the behavior was dramatically disrupted. Unlike the normal behavior, which consists of three or four peristaltic waves in the anterior direction followed by two or three in the posterior direction (Fig. 1Da), these larvae expressed long runs of anterior- or posterior-directed peristalses, with no clear temporal order and interspersed with quiescent periods of variable duration (Fig. 1Db); nevertheless, this ecdysis-like behavior was made up of individual contractions that appeared normal in strength and organization. These defects were all rescued by supplying hemizygous animals with a transgene containing a wild-type copy of the *Eh* gene (examples shown in Fig. 1Dc; summary in Fig. 2Ac), indicating that the behavioral defects were specifically a result of the absence of EH; in particular, they were not because of the accompanying lesion in gene *CG5873*.

Eh mutants fail to release ETH and CCAP

To gain greater insight into the bases of the behavioral and physiological defects expressed by Eh^- hemizygotes, we determined the status of ETH and CCAP secretion at ecdysis. In wild-type larvae, ETH secretion is initiated shortly before the onset of pre-ecdysis (Park et al., 2002; Clark et al., 2004) and is complete by the end of ecdysis (Fig. 3C; compare with Fig. 3A; data summarized in Fig. 3G, 'Control': 'pre' versus 'post'), when the remains of the cuticle and the old lining of the trachea have been shed (Fig. 3D; compare with Fig. 3B). In Eh^- hemizygotes, by contrast, we observed no detectable release of ETH after execution of the ecdysis motor program (Fig. 3G, 'Eh^-, post'). In a similar manner, the neuropeptide CCAP is released at the ecdysis of normal larvae (Park et al., 2003; Clark et al., 2004; Fig. 3F; compare with Fig. 3E; data summarized in Fig. 3H, compare 'Control': 'pre' versus 'post') yet no release was detectable in Eh^- hemizygotes at the end of ecdysis (Fig. 3H, 'Eh^-, post'). Secretion of both ETH and CCAP at ecdysis was restored in transgenic rescue animals (Fig. 3G, H, 'Rescue, post', for ETH and CCAP, respectively), indicating that these defects were caused by the lack of EH.

Defects of *Eh* mutants are not rescued by injection of ETH

The explosive release of ETH that occurs at ecdysis is fueled by a reciprocal endocrine relationship between ETH and EH, in which EH triggers ETH release and vice versa (Ewer et al., 1997; Kingan et al., 1997; Clark et al., 2004). Thus, it is possible that the primary reason for the behavioral (Fig. 1Db; Fig. 2Ab) and endocrine (Fig. 3G,H) defects expressed by *Eh* mutants is because of the lack of ETH release (Fig. 3G). To address this possibility, we examined the effects of injecting synthetic ETH into DVP+10 min Eh^- hemizygous larvae. In wild-type larvae, such injections accelerate the onset of the whole ecdysial sequence compared with vehicle-injected control (Fig. 2Bb versus Ba; $P<0.05$). As shown in Fig. 2Bd, such injections did significantly accelerate the onset of ecdysial behaviors of Eh^- hemizygous larvae ($P<0.01$), but, as occurred in the vehicle-injected (Fig. 2Bc) and in intact mutant animals (Fig. 2Ab), these behaviors consisted exclusively of ecdysis behaviors and were never preceded by pre-ecdysis. These injections also failed to cause detectable secretion of ETH (Fig. 3G, 'Eh^-, +ETH, post') or CCAP (Fig. 3H, 'Eh^-, +ETH, post'). Thus, the defects expressed by Eh^- mutants are not solely caused by the failure to release ETH. Furthermore, they show that EH is required for ETH to turn on the pre-ecdysis motor program, not simply to facilitate ETH release. Nevertheless, they do reveal that ETH can trigger the premature onset of the ecdysis motor pattern even in the absence of EH, although the resulting behavior is protracted and generally ineffective in causing the shedding of the old cuticle.

Defects of *Eh* mutants are partly rescued by injection of EH

We next explored the effectiveness of EH injected into the hemolymph in rescuing the defects expressed by *Eh* null mutants.

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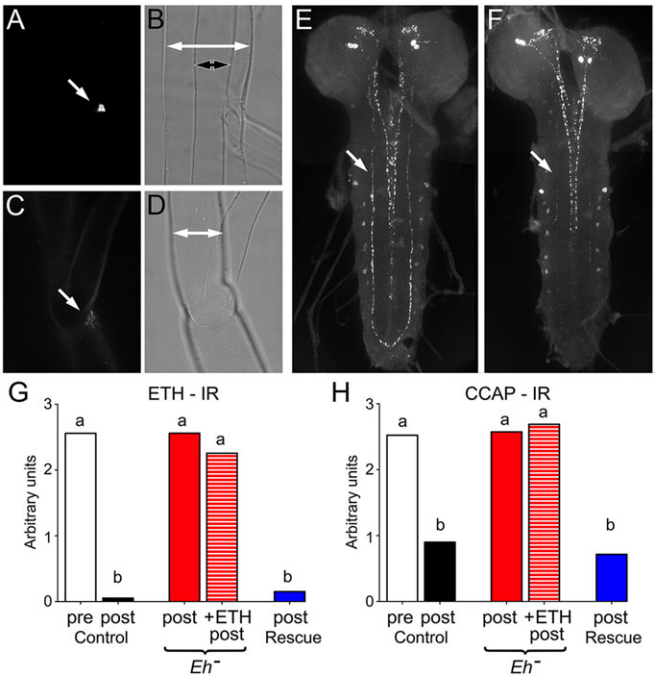

Fig. 3. Status of ETH and CCAP in the absence of EH. (A,C) ETH-IR in ETH cells (arrow) of wild-type larvae (A) before and (C) after ecdysis. Note dramatic loss of immunoreactivity in ETH cells at ecdysis (C versus A). (B,D) Corresponding light-field images of trachea. White double-headed arrow shows the extent of the trachea of third instar, whereas black double-headed arrow (in B) shows that of the lining of second instar trachea, which is shed at ecdysis. (E,F) CCAP-IR in CNS of wild-type larvae (E) before and (F) after ecdysis. The most prominent change after ecdysis is the loss of immunoreactivity of lateral axon (arrow; compare F versus E). (G,H) Quantification of (G) ETHR-IR and (H) CCAP-IR in control larvae before (pre) and after (post) ecdysis in *Eh* hemizygous mutant larvae (Eh^{-}) after expression of ecdysis behaviors in intact animals (post) or following ETH injections (+ETH post). 'Rescue, post' shows immunoreactivity after ecdysis of *Eh* hemizygous mutant larvae carrying wild-type *Eh* transgene. Outcome of Kruskal–Wallis comparisons is indicated, with different letters marking statistically significant differences ($P<0.05$). n=8-11 animals per group. Panels E,F are each composites created by combining pictures, taken using identical settings, of the anterior and posterior halves of the CNS.

The synthetic EH we used consisted of a fusion protein with maltose binding protein (MBP). We were unable to cleave intact EH away from MBP, and thus used the entire fusion protein for our assays; injections of MBP alone were used as the control. The fusion protein used was at a concentration of about 20 µg/µl, but it is unlikely that EH (approximately 8 kDa) is as effective as the native hormone when complexed with MBP (approximately 42 kDa). Thus, rather than relying on the concentration of protein to estimate the dose of EH injected, we 'calibrated' its concentration based on its effectiveness in triggering ecdysis using wild-type larvae. As shown in Fig. 4B-E, injections of increasing amounts of EH-MBP tended to shorten slightly the latency to ecdysis, although this effect was not statistically significant; injections of doses greater than 1× were usually lethal.

Strikingly, and in contrast to what we obtained following ETH injections (Fig. 2Bd), injections of EH-MBP did restore the expression of the preparatory behavior of pre-ecdysis, which was then followed by ecdysis behavior (Fig. 4H). Nevertheless, the duration of pre-ecdysis and ecdysis was longer than that expressed by wild-type larvae injected with the same '1:10' dose (Fig. 4D). In addition, the success of these injections was low, with only four out of ten animals responding; the remaining six animals responded like MBP-injected controls and expressed the characteristic protracted ecdysis-like behavior, which continued for >70 min after injection (data not shown).

Effectiveness of EH when ectopically expressed

As an alternative to injecting EH, we explored the effectiveness of misexpressing EH in the ETH-producing 'Inka' cells in an *Eh* hemizygous mutant background. As shown in Fig. 5D, both the pre-ecdysis and the ecdysis phases of the behavior were rescued in 100% of such animals (n=13).

In order to explore further the effectiveness of EH in rescuing the ecdysis defects caused by the lack of EH, we determined the ability of EH to rescue Eh^{-} hemizygotes when misexpressed in different classes of neurons and cells. In particular, we examined the consequences of expressing EH in CCAP neurons in an *Eh* mutant background. Although rescue was not complete, seven out of ten animals expressed a normal behavioral sequence (Fig. 5E); the remaining three animals expressed a behavior typical of the *Eh* mutant (cf. Fig. 5B). CCAP has been placed downstream of EH in the hierarchy of peptides that controls ecdysis. Yet, contrary to our expectations, rescued animals initiated pre-ecdysis much sooner than normal. In some cases, ecdysis occurred even before the appearance of pigmentation in the mouthplates of the next instar, producing third instars with completely unpigmented mouthparts. This phenotype is unexpected and implies that ecdysis was initiated at least 30 min earlier than normal and that CCAP neurons (or some subpopulation of them) might be active prior to the normal release of EH and ETH; as far as we are aware, this phenotype has only been previously reported for larvae lacking EH and CCAP neurons (Clark et al., 2004).

Response of CCAP network to ETH in the absence of EH

In order to investigate the role of EH in determining the response of the CNS to ETH, we examined the activation of CCAP neurons in CNSs challenged *ex vivo* with ETH. Neuronal activation was monitored using the calcium indicator GCaMP, which was genetically targeted to CCAP neurons. During pupal ecdysis, 600 nM ETH causes the activation of CCAP neurons approximately 20 min after addition of ETH to an isolated CNS, with the exact timing of onset and duration of the response depending on the serial homolog considered (Kim et al., 2006a). In the case of larval ecdysis, we found that activation following addition of 600 nM ETH was first detected approximately 45 min after ETH challenge; this latency was reduced to around 30 min when a higher dose of 1 µM was used, but could not be significantly reduced further by increasing the dose of ETH (not shown). The *ex vivo* response to an ETH challenge started at around 30 min with spikes in CCAP neurons from thoracic ganglion 3 (TN3), which lasted approximately 28 min (Fig. 6A; 28.6±0.9 min, n=7). Shortly afterwards (34.6±5.2 min, n=7) CCAP neurons in abdominal ganglia 1-4 (AN1-4) responded (Fig. 6B), showing a large response followed by a series of spikes of decreasing duration and amplitude. The inset in Fig. 6B shows that the coordination of the response between serial homologs was relatively low. These spikes are likely to correspond to large overshooting calcium action potentials, such as those recorded at ecdysis from homologous neurons in *Manduca sexta* (Gammie and Truman, 1997b).

The response to ETH of *Eh* hemizygous animals differed significantly from the wild-type response in several respects (Fig. 6C,D; Fig. 7). First, the number of neurons that responded was greatly reduced; thus, whereas in wild-type animals 100% of

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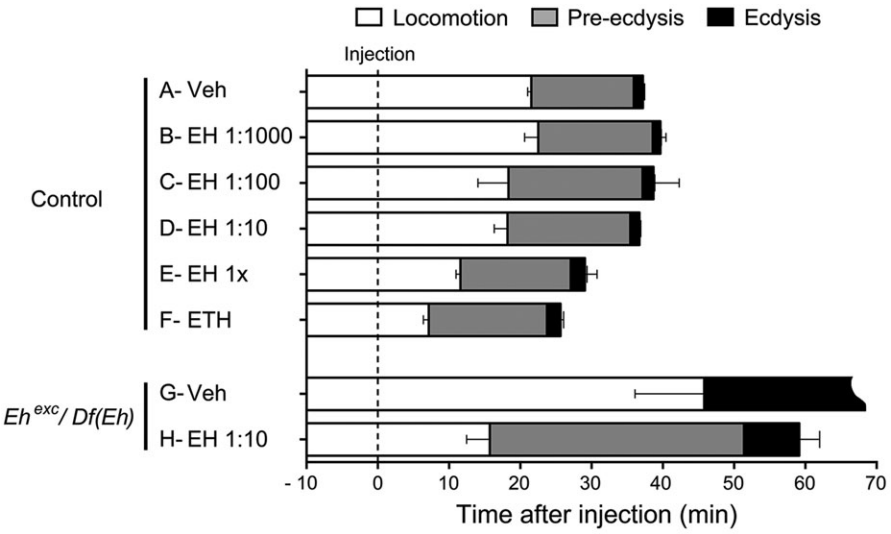

Fig. 4. Injections of synthetic EH can partly rescue pre-ecdysial defects of *Eh* hemizygous mutant larvae. (A-F) Timing of ecdysis behaviors in wild-type larvae injected with (A) vehicle, (B-E) increasing amounts of synthetic EH and (F) ETH. (G,H) Timing of ecdysis behaviors in *Eh* hemizygous mutant larvae injected with (G) vehicle and (H) synthetic EH. Injections of EH caused the expression of a pre-ecdysis phase within the ecdysial sequence. Phases of behavior are indicated as described in Fig. 2; injections were made 10 min after DVP (vertical dashed line). Note that in all cases, injections of vehicle alone caused a delay in the onset of ecdysial behaviors (compare with Fig. 2). *n*=9-11 animals per group, except B-D, for which *n*=4-5.

neurons imaged in ganglia T3 (11 cells) and AN1-4 (52 cells) responded (cf. Fig. 7; *n*=7 preparations), only half of T3 neurons (55%; ten out of 18) and 16% of AN1-4 neurons (ten out of 62) did so in *Eh* hemizygous animals (Fig. 7; *n*=7 preparations). In addition, the amplitude recorded in neurons that responded was significantly attenuated (Fig. 6C,D). This defect was most severe for segments AN1-4, where the average amplitude of the few neurons that responded was only 12% of that recorded in controls (Fig. 7, AN1-4, Eh^{-}).

The defects observed in the response of CCAP neurons of hemizygous mutant larvae were substantially rescued by a single copy of the *Eh* gene in terms of both the number and the amplitude of responding cells (Fig. 6E,F; Fig. 7, Rescue). For example, in the case of AN1-4 neurons, 60% (41 out of 68 neurons) of neurons responded (versus 12% in *Eh* hemizygous animals), and the amplitude of the average response was similar to that of controls (Fig. 7, Rescue). We assume that the partial rescue was because of the presence of a single copy of the *Eh* transgene, which was, nevertheless, sufficient to rescue the behavioral defects to wild-type levels (Fig. 2Ac).

DISCUSSION

A number of neuropeptides have been implicated in the control of insect ecdysis. In *Drosophila*, genetic approaches have been used to characterize the role of ETH (Park et al., 2002), CCAP (Park et al., 2003) and Bursicon (Lahr et al., 2012). Despite the ability of EH to trigger ecdysis in a number of insects, flies bearing targeted ablations of EH neurons express only minor defects at ecdysis (McNabb et al., 1997; Clark et al., 2004) with around 30% reaching adulthood (McNabb et al., 1997), which has suggested that EH plays a relatively minor role in *Drosophila* ecdysis.

The unexpectedly mild and sometimes paradoxical defects of flies lacking EH neurons (e.g. their insensitivity to ETH injections; McNabb et al., 1997; Clark et al., 2004) prompted us to investigate the role of EH using a null allele of the *Eh* gene. In line with expectations based on the ability of EH to induce ecdysis in other insects, we found that the lack of *Eh* function is completely lethal, with most animals dying during the larval stages, at around the time of ecdysis. These animals do not release detectable amounts of ETH; yet, many of their defects could not be rescued by ETH injections, indicating that they are at least in part due to the lack of EH itself. This is the first report to show clearly that EH has a function in the control of *Drosophila* ecdysis in addition to its known role of triggering ETH release (Ewer et al., 1997; Kingan et al., 1997).

Our findings contrast with those reported previously using flies bearing targeted ablations of EH neurons. Although no EH-IR can be detected in cell-ablated animals (McNabb et al., 1997), our results with *Eh* null alleles suggest that some residual EH function might remain in these animals; this scenario would explain their

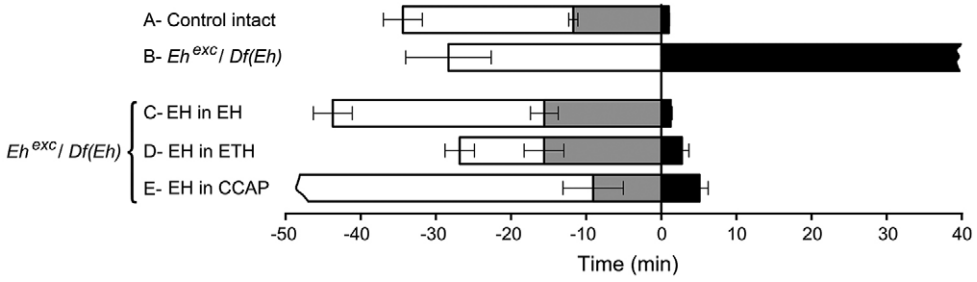

Fig. 5. Rescue of behavior by ectopic expression of EH. Timing of ecdysis behaviors: (A) in control larvae; (B) in *Eh* hemizygous mutant larvae; and (C-E) in *Eh* hemizygous mutant larvae expressing EH (C) in EH neurons, (D) in ETH cells and (E) in CCAP neurons. Phases of behavior are indicated as described in Fig. 2, but because expressing EH in CCAP neurons caused ecdysis to occur before the DVP stage, the records have been aligned relative to the time of onset of ecdysis behavior. The time of the 'locomotion' phase started at DVP, except for E, where animals expressed ecdysial behaviors before pigmentation of vertical plates was apparent; the lack of a DVP stage for this genotype is indicated by the jagged vertical line for the onset of the 'locomotion' period. Bar associated with ecdysis phase in hemizygous *Eh* null mutant animals (B) was truncated at 40 min. *n*=9-13 animals per group.

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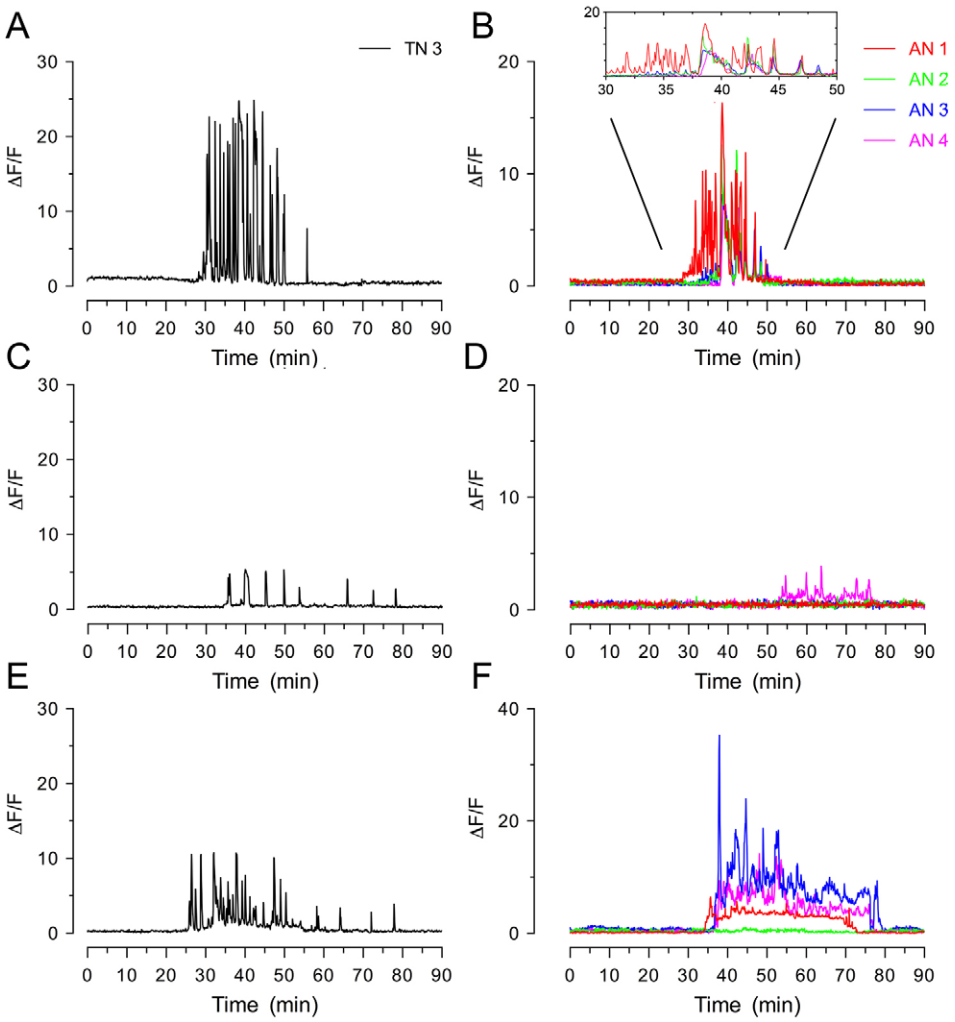

Fig. 6. Pattern of activation of CCAP neurons by ETH in the absence of EH. Representative records of activity of CCAP TN3 (A,C,E) and AN1-4 neurons (B,D,F; each colored line corresponds to the record of a single neuron from each of these neuromeres) from a single preparation induced *ex vivo* by ETH in CNS of (A,B) wild-type (inset above B shows expanded trace of region of record indicated by oblique lines), (C,D) in a *Eh* hemizygous animal, and (E,F) in *Eh* hemizygous larvae bearing wild-type *Eh* transgene. Note that scale in F is half that of B,D. See Fig. 7 for a summary of these data.

comparatively mild defects observed at ecdysis, as well as the observation that ETH release occurs on time prior to larval ecdysis (Clark et al., 2004). The lack of increases in cGMP-IR in ETH cells at this time (Clark et al., 2004) suggests that very little EH function would remain, consistent with it being immunohistochemically undetectable. Another possibility that would explain the differences between the defects expressed by *Eh* mutants and those of flies lacking EH neurons is if EH were expressed by other neurons in addition to the ventromedial EH (Vm) neurons targeted by the transgenic constructs used by McNabb et al. (1997). Such expression would have to be comparatively weak, however, because RNA *in situ* and immunohistochemical localization label only the Vm neurons.

Our findings reveal that the functions of ETH and EH are more complex than previously proposed. Indeed, the prevailing view is that the positive endocrine feedback loop between EH and ETH causes the near complete release of ETH and EH (Ewer et al., 1997; Kingan et al., 1997); ETH then turns on pre-ecdysis, and EH released within the CNS causes CCAP and Bursicon release, which turns on ecdysis and shuts off pre-ecdysis (Gammie and Truman, 1997a; Ewer and Reynolds, 2002; Lahr et al., 2012). Contrary to expectation, we found that the absence of EH caused larvae to lack the pre-ecdysis phase of the ecdysial sequence. Injections of ETH did not rescue this defect, causing only the premature expression of ecdysis behavior. Thus, at least in the larva, ETH is not sufficient to trigger pre-ecdysis; rather this behavioral phase requires EH, either acting alone or in conjunction with ETH. Park et al. (2002) found that *Eth* null animals expressed neither pre-ecdysis nor ecdysis, but the status of EH was not examined in these animals; thus, the behavioral defects could be attributable to a lack of secretion of both ETH and EH. Conversely, our findings show that EH is important for the expression of normal ecdysis behavior. Indeed, although the peristaltic waves of ecdysis themselves appeared normal in *Eh* mutant larvae, the temporal structure of the behavior was severely altered, and rarely resulted in the shedding of the old cuticle. In addition, the fact that ETH release could not be detected in *Eh* null mutant animals indicates that EH is absolutely required for ETH to be released in *Drosophila*; this contrasts with the situation in *Manduca*, where ETH release is initiated by corazonin (Kim et al., 2004). Finally, EH seems to play a role (direct or indirect) in limiting the duration of ecdysis itself, because it is greatly extended in the absence of EH.

The pattern of activation induced by ETH in CCAP neurons provides insights into the role of EH versus that of ETH in the control of ecdysis. The most consistent defect we observed in *Eh* null mutant animals was a significant reduction in the level of responsiveness. Especially for CCAP neurons in abdominal segments AN1-4, we found that only a small percentage of neurons responded, and the few neurons that responded did so with greatly reduced amplitude.

The roles of ETH and EH appear to differ in *Drosophila* compared with their proposed roles in other insects. However, *Drosophila* might not be exceptional. Indeed, the exact function of ecdysial peptides

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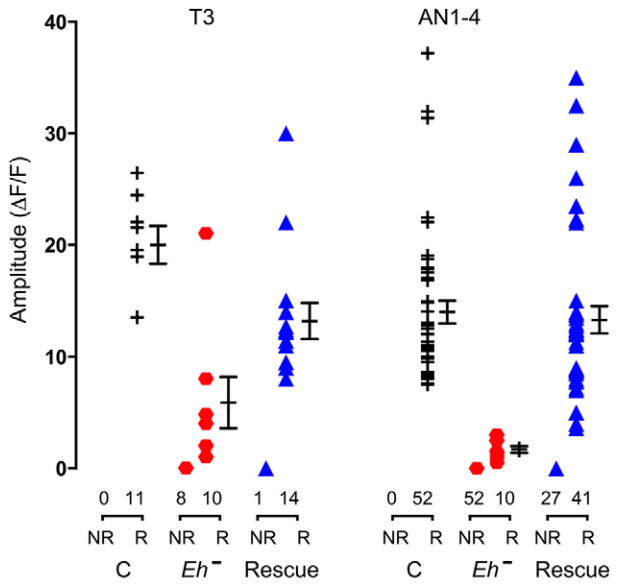

Fig. 7. Summary response of CCAP neurons to ETH in the absence of EH. Each point indicates maximal amplitude of response of CCAP neurons in TN3 and AN1-4 induced *ex vivo* by ETH in control (C; black crosses), *Eh* hemizygous larvae (*Eh*$^{-}$; red polygons) and *Eh* hemizygous larvae bearing wild-type *Eh* transgene (Rescue; blue triangles). Bar next to symbols indicates average (±s.e.m.). NR and R, number of non-responsive and responsive neurons, respectively, out of seven preparations examined for each genotype. Only neurons that were clearly in focus were included in the tally (~80% of total). The absence of EH caused, respectively, 44% (eight out of ten) and 84% (52 out of 62) TN3 and AN1-4 neurons to be unresponsive; it also significantly reduced the amplitude of the response of the neurons that did respond. Both defects were substantially rescued by a single copy of the wild-type *Eh* gene (Rescue).

might be more plastic than previously thought and might differ both between stages and between insect species. For example, the lack of EH eliminates pre-ecdysis in the larva (present study), yet at pupation causes failures at ecdysis and a significant extension in the duration of pre-ecdysis (W.M. and J.E., unpublished observations). This situation is not unique to EH. Indeed, the lack of *Partner of Bursicon* (*Pburs*) gene function (which encodes one of the subunits of the so-called tanning hormone, Bursicon; Luo et al., 2005; Mendive et al., 2005) causes severe defects only at pupal and not at larval ecdysis (Lahr et al., 2012). Yet another change in function occurs at adult ecdysis, where Bursicon is not released until after the adult emerges and is able to spread its wings (Peabody et al., 2009); at this stage, it is required during post-ecdysis to cause wing expansion and cuticle maturation (Honegger et al., 2008). Such changes also occur across species: although ETH, EH, CCAP and Bursicon appear to be associated with ecdysis in many insects and even crustacea (Phlippen et al., 2000; Webster et al., 2013), their exact role might vary. For instance, CCAP plays a minor role in *Drosophila* ecdysis (Lahr et al., 2012), but it is critical for *Tribolium* ecdysis (Arakane et al., 2008). These examples and others (White and Ewer, 2014) suggest that the exact function of ecdysial peptides might change during the development of a single species and across species. The different responses elicited by this highly conserved signaling system are likely to be mediated though changes in the spatial and temporal pattern of receptor expression, allowing the same neuropeptides to trigger behavioral and physiological sequences that are appropriate for that species and stage.

Plasticity mediated by changes in receptor expression also applies to other neuropeptide-controlled behaviors. For example, arginine vasopressin causes different affiliative responses in monogamous versus promiscuous voles (Winslow et al., 1993) owing, at least in part, to the different distribution of arginine vasopressin receptors in the brain (Young et al., 1999). This combination of conserved signals acting on developmentally and evolutionarily different receptor landscapes might provide a general mechanism for creating diversity in peptide action, which is a hallmark of these signaling molecules (Strand, 1999).

Although some of the functions of ETH, EH, CCAP and Bursicon in *Drosophila* ecdysis have been clarified, many questions remain. A deeper understanding of the function of these neuropeptides is needed and will undoubtedly be aided by identifying their neuronal targets and by developing receptor-GAL4 drivers to investigate the role of different neuronal subsets in the control of ecdysis. Furthermore, the widespread utility of the recently described CRISPR/cas9 genome engineering tool (Doudna and Charpentier, 2014) means that an understanding of the control of ecdysis in other insect groups might soon be within reach.

MATERIALS AND METHODS

Fly strains and genetics

Fly strains

Fly stocks were maintained at room temperature (22-25°C) on standard agar-cornmeal-yeast media. Unless noted, they were obtained from the Bloomington *Drosophila* Stock Center (BL; Bloomington, IN, USA; http://flystocks.bio.indiana.edu/). Stocks used included *P*-element insertion G8594 (GenExel, now Aprogen), *PiggyBac* insertions *f01683* and *d00811* (Exelixis Harvard Stock Center), EH-GAL4 (McNabb et al., 1997), CCAP-GAL4 (Park et al., 2003), ETH-GAL4 (Diao et al., 2015) and calcium sensor UAS-GCaMP3.2 (kindly provided by Julie Simpson, HHMI, Janelia Research Campus, Ashburn, VA, USA). Stocks bearing homozygous lethal mutations were maintained heterozygous with GFP-expressing balancer chromosomes (BL#4533 and BL#4534).

Isolation of null allele of *Eh* gene

A null allele of the *Eh* gene (*Eh*^{exc}) was isolated by imprecise excision of *P*-element insertion G8594, located 1.1 kb 3′ of the *Eh* gene (Fig. 1A) using a standard scheme involving the 'Δ2-3' transposase (Robertson et al., 1988). Balanced lines were produced using single white-eyed excision males, and homozygous third instar larvae were screened by PCR using primer pair EH-F1+EH-R1 (see Table 1). Lines that did not produce a PCR product of the expected size were rescreened for EH immunoreactivity, and the *Eh* gene from immunonegative lines was sequenced.

Table 1. Primers used for PCR amplification

Name	Use	Sequence
EH-F1	Screen for excision of G8594	AAGGAAGTGATGGAGAAGTTCG
EH-R1	Screen for excision of G8594	GGAAAGAGCTCTGAAGAAATGG
EH-F2	Screen for *Eh* deletion	CAGAGTAAAGAAGCCCGATACG
EH-R2	Screen for *Eh* deletion	AGTACCGTTCCTACGTCACTGG
EH-F3	Cloning of *Eh* cDNA	CACATCCGTTGGAATCAAAG
EH-R3	Cloning of *Eh* cDNA	gcggccgcAGGCCATAAAAGCACACACC
EH-F4	EH genomic rescue	CTTTCTGATGCTCGGAATCT
EH-R4	EH genomic rescue	CTTAATATTTGTTTATTTAC
EH-F5	*In vitro* EH expression	gaattcTTGCCCGCCATAAGTCATTATACG
EH-R5	*In vitro* EH expression	CGCCTCTTATCGCTTCACTCG

Sequences added to include restriction site are indicated in lower case.

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Creation of genetic deletion that includes *Eh* gene

Exelixis strains *f01683* and *d00811* were used to create a 32 kb genetic deletion that included the *Eh* gene [*Df(3)Eh*$^{-}$; abbreviated in all figures as *Df(Eh)*; cf. Fig. 1A], using the FLP-FRT system as described by Parks et al. (2004). Putative deletion-bearing males were used singly to set up balanced lines; homozygous larvae were then screened by PCR using primer pair EH-F2+EH-R2 (see Table 1) and the limits of resulting deletions verified by PCR. In addition to the *Eh* gene, this deletion also completely removes gene *CG14330* (which encodes a gene of unknown function) and partly removes *CG5873*, a heme peroxidase-encoding gene, which when mutant causes no apparent defects (FlyBase).

Molecular biology

PCR

DNA was obtained from single third instar larvae as described by Gloor et al. (1993), but using 10 µl of 'squish buffer' (0.4 µg/µl proteinase K, 10 mM Tris pH 8, 0.2 mM EDTA and 25 mM NaCl) per fly larva. One microliter of extract was used for each 20 µl PCR, which was run using the following conditions: 94°C (3 min); then 30 cycles of 94°C (45 s), 55°C (0.5 min) and 72°C (1.0 min/kb of product); followed by one cycle at 72°C for 10 min.

Transgenic constructs

UAS-*Eh* construct

Eh cDNA was amplified by RT-PCR from RNA extracted from third instar CNSs following the manufacturer's instructions. The primer pair EH-F3+EH-R3 (Table 1) was used to amplify a 400 bp fragment that includes the entire *Eh* coding region; the 3′ reverse primer included a *Not*I site for subcloning purposes. PCR products obtained from three independent cDNAs were cloned into pGEM-T Easy vector (Promega) and sequenced for verification. The fragment containing the *Eh* cDNA was then cloned into pUAST *P*-element vector (van Roessel and Brand, 2000) and sent to BestGene for germline transformation.

Genomic *Eh* rescue construct

A 4.8 kb fragment of genomic DNA containing the entire *Eh* gene and including 1.9 kb of 5′ regulatory sequences, which is sufficient to drive gene expression faithfully in EH neurons (McNabb et al., 1997), was amplified by PCR from a BAC clone from the RPCI-98 *Drosophila melanogaster* BAC Library (http://bacpac.chori.org/dromel98.htm) using the High Fidelity Expand Long Template PCR system (Roche) following the manufacturer's instructions using primer pair EH-F4+EH-R4 (Table 1). The PCR product was cloned into pGEM-T Easy vector (Promega), subcloned into the *pattB* vector (Venken et al., 2006) by Genewiz and sent to BestGene for germline transformation.

Synthesis of EH

Construction of pMAL-EH

Synthetic EH was produced by *in vitro* expression using the pMAL protein fusion and purification system (pMAL-c2x; New England Biolabs). For this, a 222 bp fragment that encodes the predicted mature EH protein (minus putative leader sequence) was amplified from the EH cDNA (see above) using primer pair EH-F5+EH-R5 (Table 1); forward primer included an *Eco*RI site for subcloning purposes. The PCR product was subcloned into pGEM-T Easy vector (Promega), sequenced for verification, and subcloned in frame into the *Eco*RI site of the pMAL-c2x vector.

EH synthesis

Maltose-binding protein-EH (MBP-EH) fusion protein and MBP alone (control) were expressed following the manufacturer's recommendations in Origami cells (Novagen, Merck) to facilitate disulfide bond formation, which is thought to be a critical component of bioactive EH (Nagasawa et al., 1983; Terzi et al., 1988).

Hormone injections

Synthetic ETH was obtained from Bachem. It was diluted in distilled water and used at a final concentration of 1 mM. EH-MBP and MBP (see above) were diluted in distilled water, and 50-100 nl was injected into pharate second instar larvae using a PV800 pneumatic picospritzer (World Precision Instruments). For ETH, this dose (corresponding to ∼50-100 fmoles) is known to cause suprathreshold responses in pharate larvae (Park et al., 2002; Clark et al., 2004). Control injections consisted of the same volume of distilled water (for ETH) and similar concentration of MBP alone (for EH).

Immunostaining

Immunostaining was carried out as described by Clark et al. (2004) using the following antisera: rabbit anti-CCAP, generously provided by Hans Agricola (Friedrich-Schiller University Jena, Jena, Germany), and used at 1:5000; rabbit anti-EH generously provided by James Truman (HHMI Janelia Research Campus, Ashburn, VA, USA) and used at 1:200; rabbit anti-ETH generously provided by Michael Adams (University of California, Riverside, CA, USA) and used at 1:2000.

Quantification of immunolabeling

CCAP and ETH immunoreactivity were quantified as described by Clark et al. (2004), assigning a subjective score of 0 (no staining) to 3 (strongest staining). The person scoring the preparations did not know the genotype or time at which the tissues had been fixed.

Behavioral analyses

Larvae were collected and their ecdysial behaviors recorded as described by Clark et al. (2004). All analyses involving *Eh* mutants were done using hemizygous *Eh*^{exc}/*Df(3)Eh*$^{-}$ larvae; genetic rescue animals were tested in a similar manner in this genetic background.

Imaging of Ca^{2+} dynamics

Imaging of *ex vivo* Ca^{2+} dynamics was carried out as described by Kim et al. (2006a), using CNSs from second instar larvae at the DVP ('double vertical plate') stage, approximately 30 min prior to ecdysis (Park et al., 2002). Preparations were imaged under an Olympus DSU Spinning Disc microscope using a 40× (NA 0.80) water immersion lens. They were first imaged every 5 s for 5 min, and preparations showing spontaneous activity (∼5% of the preparations) were discarded. They were then stimulated with 1 µM synthetic ETH1 (Bachem) and GFP fluorescence captured every 5 s for 90 min. Resulting recordings were analyzed using Cell^R Olympus Imaging Software (version 2.6).

Statistical analyses

Statistical significance was evaluated using the Prism v. 6.0 (GraphPad Software). Quantitative results were compared by ANOVA followed by Tukey's HSD post hoc analyses. Categorical data based on qualitative measurements were compared by Kruskal–Wallis one-way analysis of variance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.K., W.M. and J.E. planned the experiments; E.K. carried out most experiments; W.M. carried out the calcium imaging; E.C.L. and E.C.J. provided reagents. E.K. and J.E. analyzed the results; J.E. wrote the manuscript. All authors read and commented on the manuscript and approved the final version.

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