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Light and peptidergic eclosion hormone neurons stimulate a rapid eclosion response that masks circadian emergence in *Drosophila*

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SUMMARY

Light signals can entrain circadian clocks, but they can also mask aspects of the circadian output. We have analyzed the masking effects of a lights-on (LOn) signal on *Drosophila* eclosion. The LOn response results in 12–21% of the flies that emerge on a given day eclosing within 10 min of the LOn signal. Flies that lack the neuropeptide eclosion hormone (EH), or in which its release is inhibited by the tetanus toxin light chain, lack the response. Optic photoreceptors in both the ocelli and the compound eyes appear to be required for the response. The LOn signal has two effects: (1) it drastically reduces the interval between EH release and eclosion, presumably by suppressing a transient descending inhibition that immediately follows EH release, and (2) it stimulates premature EH release. The LOn signal does not influence the latency of wing spreading, an EH-regulated post-ecdysis behavior.

INTRODUCTION

Light can play a number of roles in coordinating the temporal activity of an organism. It has been most extensively studied as a signal for entraining circadian rhythms. However, these same light signals often act to modify or mask aspects of circadian output (Aschoff, 1960; Mrosovsky, 1999). Classic examples of masking by changes in light include the initiation of singing in sparrows at lights-on (Binkley et al., 1983) and the onset of hamster running behavior at lights-off (Redlin and Mrosovsky, 1999). Although numerous examples of masking have been identified, their underlying cellular and molecular mechanisms and how they interact with circadian systems are not well understood.

For many insects, the emergence of the adult (eclosion) is under circadian regulation (Helfrich-Förster, 2006; Saunders, 1976; Saunders, 1982; Saunders, 2002). In *Drosophila melanogaster* Meigen, the circadian rhythm of eclosion is based on a central clock that depends on the interaction of a number of proteins encoded by genes including *Clock*, *cycle*, *period* and *timeless* (reviewed by Helfrich-Förster, 2006). The circadian clock restricts the timing of *Drosophila* eclosion to a 'gate' that occurs during the early part of the day (Engelmann and Honegger, 1966; Jackson, 1983; Lorenz et al., 1989). Gating results in a discontinuous eclosion pattern in which entrained pharate adults that complete adult development at night wait for the opening of the gate at around dawn in order to eclose (Pittendrigh and Skopik, 1970). Release of the neuropeptide eclosion hormone (EH), a key regulator of ecdysis (reviewed by Truman, 2005), is gated in the moth *Manduca sexta*, suggesting that eclosion gating may result from circadian regulation of EH release. This could occur directly as the result of declining ecdysone titers that are associated with the activation of ecdysis (Hewes and Truman, 1991). Alternatively, it could result from the ecdysone-responsive release of ecdysis triggering hormone (ETH) (Kingan and Adams, 2000; Zitnan et al., 1999; Zitnanova et al., 2001), which stimulates EH release (Ewer et al., 1997; Kingan et al., 1997; Zitnan et al., 1996). Pigment dispersing factor (PDF) produced by the lateral neurons may influence the gate by modulating ecdysteroid release from the prothoracic gland (Myers et al., 2003).

The circadian pattern of *Drosophila* eclosion can be masked by light. When administered close to the eclosion gate, a lights-on (LOn) signal shifts the distribution of flies emerging within the gate. This is manifest as a burst of eclosion soon after the LOn signal. The pathway of light reception for this LOn response is distinct from those utilized for circadian entrainment. *Drosophila* mutants that lack both the ocelli and compound eyes lack the lights-on response but nevertheless show normal circadian entrainment of their ecdysis clock (Engelmann and Honegger, 1966). For circadian regulation of locomotion and eclosion, the central clock resides in lateral neurons of the brain that express central clock proteins, the neuropeptide pigment-dispersing factor (PDF) and a cryptochrome photoreceptor (reviewed by Helfrich-Förster, 2006; Nitabach and Taghert, 2008).

Eclosion is regulated by a cascade of peptide hormones. These peptides include EH from the brain, pre-ecdysis triggering hormone (PETH) and ecdysis triggering hormone (ETH) from the epitracheal glands (Park et al., 2002; Zitnan et al., 1996), and crustacean cardioactive peptide (CCAP) from the ventral central nervous system (CNS) (reviewed by Truman, 2005). To test the requirement for EH in eclosion, molecular genetic tools were used to target the ablation of the EH-expressing neurons in *Drosophila* (McNabb et al., 1997). Surprisingly, these experiments showed that EH is not strictly required for eclosion. However, the EH cell knockouts had significant defects. A third died at larval ecdyses with defects in tracheal filling, and the two thirds that eclosed as adults had defects in eclosion and post-eclosion behaviors. Interestingly, the EH cell knockouts had normal circadian eclosion rhythms but lacked the LOn response. Thus, like the retinal photoreceptors, the EH neurons appear to be components of the LOn pathway. In this paper, we define the basic characteristics of the LOn response and examine the way this signal interacts with EH release, eclosion, and post-ecdysial wing spreading.

MATERIALS AND METHODS

Drosophila strains

The w^{1118}×*UAS-reaper* (*UAS-rpr*; see below) control flies were generated by crossing males from the w^{1118} strain to females of the *UAS-rpr* strain (McNabb et al., 1997). The w^{1118} strain was the recipient strain for the transposon that contains the *GAL4* transcription factor under the regulation of EH gene upstream sequences (see below). When crossed to the *UAS-rpr* strain, it is the control for the *EHups-Gal4*×*UAS-rpr* flies. Since the *UAS-rpr* insertion is carried on a *y* w^{67} c^{23} X chromosome, the hemizygous male progeny of this cross have yellow cuticle and pale apricot eyes. Female progeny are heterozygous w^{1118}/*y* w^{67} c^{2} *UAS-rpr*, with wild-type cuticle and eyes that are slightly paler than those of the males. Despite these differences, no difference in LOn responsiveness or wing spreading latency (see below) was detected between these males and females.

The EH cell knockout flies (McNabb et al., 1997) were generated by using the *GAL4-UAS* system (Brand and Perrimon, 1993). EH gene upstream sequences (*EHups*) fused to *Gal4* (*EHups-Gal4;P{GAL4-Eh2.4}*) were used to drive expression of the cell death gene *reaper* (*rpr*) in the neurons that produce EH. Male flies from the C21 *EHups-Gal4* strain were crossed to females of the *UAS-rpr* strain. Their progeny are referred to as *EHups*×*UAS-rpr* throughout this paper. Since the C21 transposon is located on the second chromosome, the progeny possess the X chromosome genotype described for the w^{1118}×*UAS-rpr* strain above, which results in male progeny with yellow cuticle and females with wild-type cuticle. All knockouts were heterozygous for the second chromosome, i.e. C21/+. The *EHups* transposon conferred bright orange-red eyes on all progeny, although the eyes of females were slightly paler than those of the males. Despite differences in cuticle and eye pigmentation, no differences between males and females were detected in the LOn response or wing spreading latency.

Canton-S (CS), a standard wild-type lab strain, was used as a control for the ocelliless and eyeless strains. Although the progenitor strains for these mutants is unknown, it is likely to be CS. Strains that lacked ocelli were *ocelliless* (oc^{1}) (Flybase, 1999; Lindsley and Zimm, 1992) and *sine oculis* (so^{+2}). Strains that lacked compound eyes were alleles of *eyes absent* [eya^{2}; also called $clift^{eya\text{-}2}$ (Bonini et al., 1993)] and eya^{1} [also called $cli^{eya\text{-}1}$ (Eissenberg and Ryerse, 1991; Sved, 1986)]. To ensure that the lack of a LOn response observed for the ocelliless and eyeless strains were not due to locomotor defects, they were tested for geotaxis using a countercurrent assay (Benzer, 1967). All exhibited positive geotaxis. These strains were also tested by immunocytochemistry for normal levels and release of EH. The CNS of flies that were staged at approximately 6 h prior to eclosion and those that had just eclosed were labeled with anti-EH and analyzed as described below. These strains appeared to synthesize normal levels of EH and to release it at eclosion as expected.

The w^{1118}×*UAS-TNT-L* and *EHups*×*UAS-TNT-L* flies were generated by crossing males from the w^{1118} strain or the C21 *EHups-Gal4* strain, respectively, to females of the *UAS-TNT-L* strain (Sweeney et al., 1995). We detected no sex-specific differences in cuticle or eye pigmentation, LOn response or wing spreading latency.

Lights-on (LOn) assays

Flies were cultured in half-pint culture bottles containing cornmeal agar food, at 25°C. Cultures were raised continuously under a 14 h:10 h light:dark (14L:10D) cycle at approximately 750 lx. Embryos were collected over 24 h to an experimentally determined optimum density that depended on both parental strain fecundity and mortality of the progeny. Food was removed from the bottles on the afternoon prior to assay. Five bottles of flies were reared for each assay condition and the eclosing flies for each treatment were pooled upon collection. Flies that emerged before the day of assay were removed just prior to lights-off of the preceding night. Emerging flies were collected at 10 min intervals, except as noted, and subsequently counted. One final collection of adults was made just prior to the end of the photoperiod and the total number of flies that emerged between the first and last collection periods used to generate eclosion rates as a percentage of the day's total eclosion. Each treatment group was reared and tested in parallel.

For the LOn shift paradigm, entrained adults were subjected to normal lighting or to a LOn that was shifted either earlier (–1 h or –2 h) or later (+2 h) than normal. Each strain was tested at least twice and each test yielded qualitatively similar results.

Immunocytochemistry

Fly CNSs were dissected into Ca^{2+}-free Ikeda's saline (Ashburner, 1989) then fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Samples were washed extensively (5×10 min) with PBS containing 0.3% Triton X-100 (PBST) and blocked in 1% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA). They were then incubated overnight at 4°C in a rabbit anti-EH antiserum (Copenhaver and Truman, 1986) at 1:100 in PBST. Samples were washed extensively in PBST, and then incubated in donkey anti-rabbit Cy5 (Jackson ImmunoResearch Laboratories, Inc.) at 1:1000 overnight at 4°C. Samples were then washed extensively, mounted on poly-lysine-coated coverslips, passed through an ethanol dehydration series, cleared in xylene, and mounted in DPX histological mountant (Fluka BioChemika, Sigma Aldrich Chemie, Steinheim, Germany). Preparations were examined on a Bio-Rad MRC-600 (Hercules, California, USA) confocal microscope. Image stacks were scored visually and representative *z*-series were collapsed to provide two-dimensional images.

To examine EH release in w^{1118}×*UAS-TNT-L* and *EHups*×*UAS-TNT-L* strains, CNSs were collected either 8–11 h prior to eclosion before meconium transport (Kimura and Truman, 1990) or within 1 min post-eclosion.

To examine the effects of light on EH release, we used w^{1118}×*UAS-rpr* flies. On the night before eclosion, 0–2 h before lights-off, late pharate adults with anterior meconia were removed from their puparial cases. One group was dissected immediately as a no-treatment control and the rest were returned to 14L:10D conditions. One group was dissected 1 h before normal LOn to verify that EH release had not occurred; another was dissected 10–20 min after LOn. Two groups served as controls: one was maintained in darkness and dissected at the same time as the post-LOn set, the other was observed for time of eclosion. The latter control showed that the flies selected at this stage emerged approximately 1 h after the normal LOn signal. The CNSs of flies in the no-treatment control group were dissected into saline and fixed overnight. All other groups were dissected directly into fix to preserve their *in vivo* release state and maintained in fix overnight at 4°C. They were subsequently treated as described above. Samples were examined on a confocal microscope (as above) and graded according to intensity of labeling, on a scale of 0–4. *Z*-series were collapsed to provide two-dimensional images.

Wing spreading assay

To determine if light influences the interval between eclosion and the completion of wing spreading (referred to as the wing spreading

latency; WSL), newly emerged flies from LOn shift experiments were collected, placed four to a vial, held in the light at 25°C and scored for wing spreading at 10 min intervals. It was important to avoid disturbing the flies during this time as physical agitation delayed the wing spreading process. For assessing the effect of the LOn signal, we examined only the flies that eclosed within the first 20 min after LOn. Each experiment was performed at least twice.

RESULTS

The lights-on eclosion response is independent of the rate of development

Populations of newly emerging adult *Drosophila* exhibit a LOn response when entrained to a 14L:10D cycle. As shown in Fig. 1, the LOn response is independent of the development rate of these flies. This was demonstrated using the $w^{1118}\times$*UAS-rpr* strain, a strain that showed a robust LOn response in previous studies (McNabb et al., 1997). Eggs collected over a 24 h period and subsequently maintained under a 14L:10D cycle emerged as adults over a 3–4 day period. Since previous experiments demonstrated that the largest peak of the day's eclosion takes place within 1 h of LOn, we focused on the distribution of eclosion around the beginning of the LOn signal, collecting newly emerged flies at 10 min intervals. We also counted the total number of flies that emerged each day and have expressed the emergence observed within a particular time bin as a percentage of the day's total eclosion. This experiment was repeated three times and yielded very similar results each time. Very few flies eclosed during the 9 h of darkness between lights-off and –1 h, on average 7±2% of the day's eclosion whereas 42±4% of the day's eclosion took place within 2 h of light onset on each day sampled.

Most of the adults emerged during the first 2 days of the distribution, then eclosion declined on days 3 and 4. A few flies from this experiment (85 of 2654; 3%) had previously eclosed during the afternoon of day 0, within the distribution expected for normal entrained eclosion. A strong LOn response was observed for each day's eclosion irrespective of the numbers eclosing. This response resulted in 12–21% of the flies that eclosed throughout the entire day emerging in the 10 min interval immediately following LOn. The effect of the LOn signal was short-lived; in the second 10 min following LOn, there was a 53–78% decline in eclosion rate, and often it decreased to the level seen for the next few hours.

The effects of a light pulse on stimulating eclosion

To separate the effects of light as a LOn signal from its role as an entraining signal, we used a pulse as a LOn signal. Entrained $w^{1118}\times$*UAS-rpr* flies were subjected in parallel to either light at the normal time, extended darkness or extended darkness except for a brief (20 min) pulse of light. Fig. 2 summarizes the results of four independent replicate experiments for which sample sizes were between 993 and 1316. For flies that received light at the normal LOn time, 14% of the day's total eclosion occurred within 20 min of the signal (Fig. 2A). This 'burst' of eclosion was not seen for flies maintained in darkness (Fig. 2B). Instead, those flies showed a normal distribution of eclosion that ranged up to a maximum of 5% of the daily emergence per 10 min collection window. When a 20 min light pulse was delayed to 1 h after the expected LOn signal (Fig. 2C), it also resulted in a massive eclosion burst (25% of the day's eclosions in 20 min) that rapidly tailed off. Despite the differences in eclosion distributions, the proportion of the day's total eclosion that ensued in the first 4 h after normal LOn was very similar under each of the three conditions: 60% for flies that received the normal LOn, 62% for flies that received the pulse, and 59% for flies held in the dark.

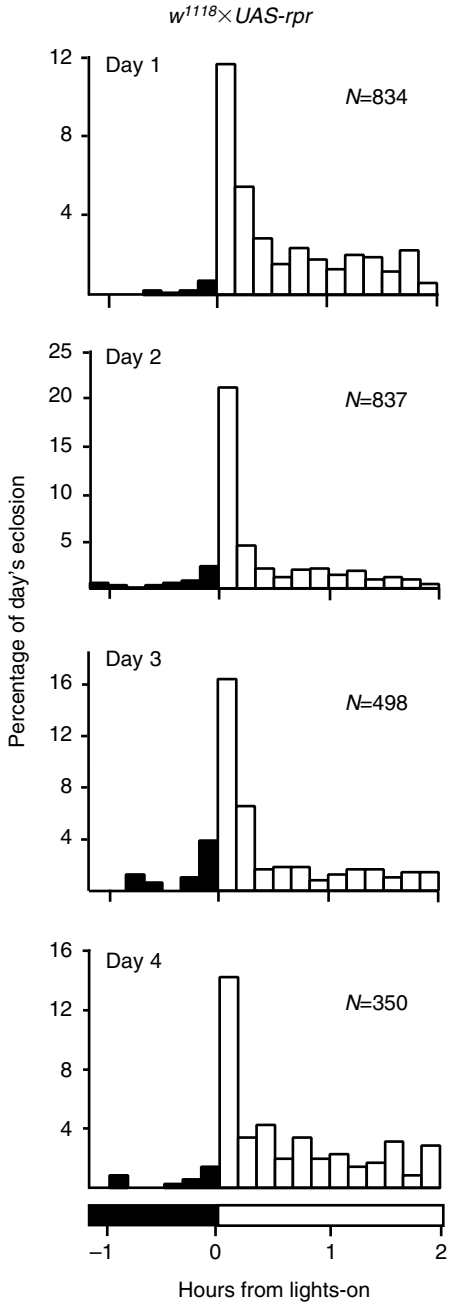


Fig. 1. Effects of developmental age on the rapid eclosion response to light. Representative data for a population of $w^{1118}\times$*UAS-rpr* flies that resulted from a 1 day egg collection, monitored every morning for 4 days. Emerging flies were collected every 10 min between –1 h and +2 h relative to lights-on (LOn, 0 h). The amount of eclosion is normalized to the day's total eclosion. The horizontal bar below the day 4 panel represents the time relative to lights-on; black for the dark, white for the light. *N*, the total number of flies collected each day.

Effects of varying the time of the LOn signal on control and EH cell knockout flies

Experiments were performed to determine the effects of early or late LOn signals on eclosion in $w^{1118}\times$*UAS-rpr* controls and in the eclosion hormone (EH) cell knockout strain. Entrained adults were

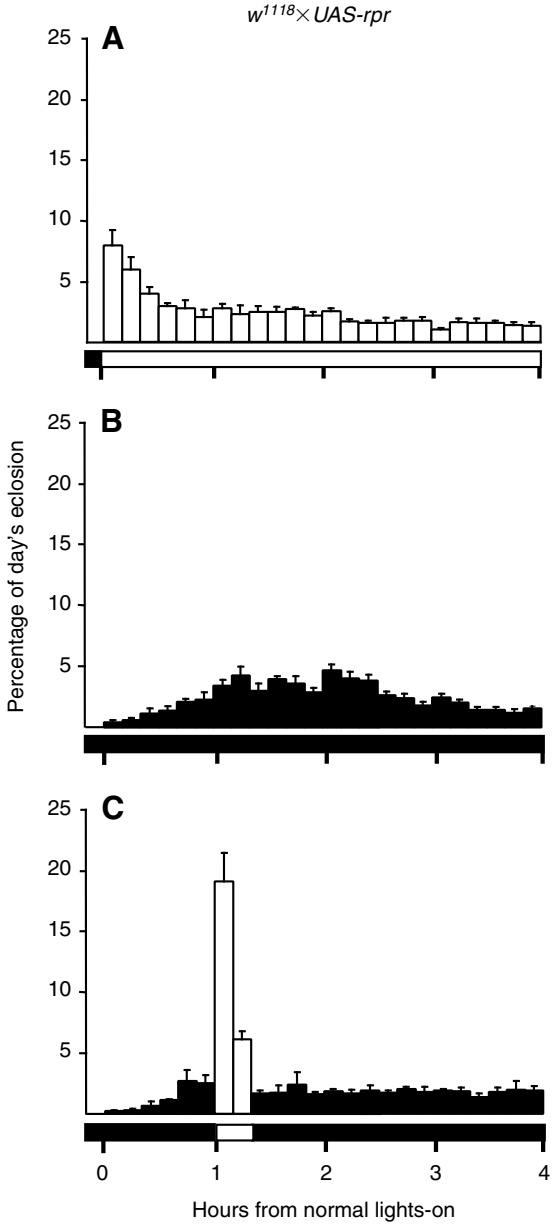


Fig. 2. Effects of a light pulse on the rapid eclosion response. Flies of the $w^{1118}\times$*UAS-rpr* strain (A) received light continuously from the time of normal LOn, (B) were held in the dark or (C) received a 20 min pulse of light beginning at +1 h from normal LOn. Axes and coloring are as described for Fig. 1. Data bars give the means ± s.e.m. for four trials (N=993–1316 per test condition per trial).

subjected to normal lighting or to a LOn signal that was either advanced (–1 h or –2 h) or delayed (+2 h) relative to the expected LOn signal. Representative results are shown in Fig. 3. For the $w^{1118}\times$*UAS-rpr* flies, the normal LOn signal resulted in a robust burst of eclosion, with 29% of the day's emergence occurring during the first 20 min (Fig. 3A). Delaying the light signal by 2 h resulted in a corresponding delay in the abrupt eclosion burst, with about 14% of the flies emerging during the 20 min following LOn (Fig. 3B). This delayed LOn peak was smaller than that observed at normal LOn because the majority of flies that would eclose during this gate had already emerged. However, this peak represented about 41% of the flies that had not yet eclosed at the time of the shifted LOn signal. This percentage is similar to that seen in Fig. 3A, where 36% of the flies emerged within 20 min of the LOn signal. When the LOn signal was advanced by 1 h there was no immediate burst of eclosion (Fig. 3C), but a substantial increase in eclosion was observed during the next hour as compared to flies that were still in the dark (compare with Fig. 3B). Similar results were obtained when the LOn signal was advanced by 2 h (Fig. 3D).

The LOn response observed for the $w^{1118}\times$*UAS-rpr* controls was absent in the EH cell knockout (KO) flies (Fig. 3E–H). The EH cell KOs did not show an eclosion burst in response to any of the conditions tested. Advancing the LOn signal failed to swell the leading edge of the eclosion distribution (compare Fig. 3G,H with dark region of Fig. 3F). Overall, the phase of eclosion relative to the L:D cycle is the same in controls and the EH cell KOs (McNabb et al., 1997). Hence, entrainment of the circadian clock for eclosion is normal. It is only the masking of the LOn signal that is missing upon removal of the EH neurons.

The role of the compound eyes and ocelli in the LOn response

A previous report showed that flies that lack both compound eyes and ocelli due to a mutant allele of the *sine oculis* (*so*) locus, so^{1}, fail to show a LOn response (Engelmann and Honegger, 1966). To determine if the LOn response requires only one or both of the sets of optic photoreceptors, we tested mutant strains that lack either the compound eyes or the ocelli. The strains that had no ocelli were mutant for the oc^{1} allele of *ocelliless* (Flybase, 1999; Lindsley and Zimm, 1992) and the so^{+2} (Heitzler et al., 1993) allele of *sine oculis*. The strains that lacked compound eyes were mutant for alleles of *eyes absent*, eya^{2} ($cli^{eya\text{-}2}$; Bonini et al., 1993) and eya^{1} ($cli^{eya\text{-}1}$; Eissenberg and Ryerse, 1991; Sved, 1986). The Canton-S (CS) wild-type strain was used as a control. Preliminary results obtained with oc^{1} and so^{+2} were essentially identical. Similarly, results obtained with eya^{2} and eya^{1} were essentially identical to each other. It was difficult to obtain large cultures of each of these mutant strains because of their reduced viability, so we focused on oc^{1} and eya^{2} for the detailed studies described below.

The eclosion patterns of the CS and $w^{1118}\times$*UAS-rpr* strains differed in a few aspects. First, for CS, the normal entrained eclosion distribution started about an hour earlier and a substantial number emerged prior to LOn. Secondly, the CS LOn response was not as robust (Fig. 4A–D). In addition, the phase of the CS eclosion gate was much broader; by 3 h after normal LOn, only 32% of the flies had eclosed *vs* 69% of $w^{1118}\times$*UAS-rpr* flies (χ^2-test, P<0.0001). The observation that the CS strain showed a definite LOn response when the LOn signal was advanced to –1 h and –2 h suggests that the CS strain is competent to respond to light earlier in the day than is the $w^{1118}\times$*UAS-rpr* strain. We assume that this difference is due to the phase of the eclosion gate being earlier for the CS than the $w^{1118}\times$*UAS-rpr* strain (compare Fig. 3B with Fig. 4B).

Fly strains that lacked either compound eyes or ocelli lacked the LOn response (Fig. 4E–L) despite normal circadian locomotor rhythms (Vosshall and Young, 1995) and geotaxis. For both the oc^{1} and the eya^{2} strains, no significant increases in eclosion rate were observed regardless of when the flies were exposed to a LOn signal (χ^2-test, P<0.0001). The apparent increase seen in Fig. 4F was not seen upon repetition of this test and thus appears to be due to random fluctuation, much like the variation observed throughout the day. In both mutant strains, particularly oc^{1}, the eclosion rate was variable and substantial eclosion preceded the LOn signal. However, overall oc^{1} eclosion appeared circadian. Few flies emerged during the night (7% for oc^{1} *vs* 8% for CS) and the proportion of the day's eclosion

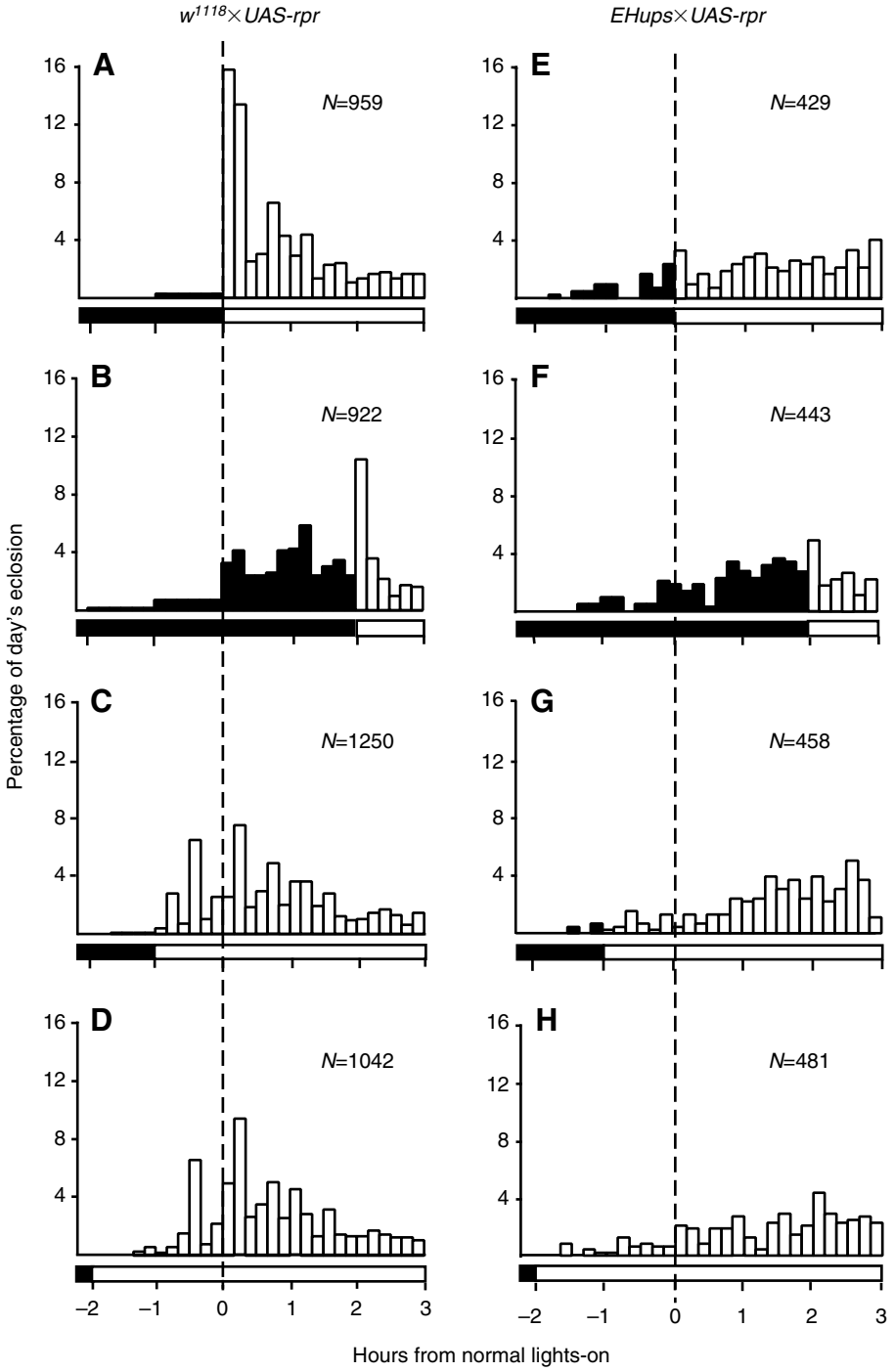


Fig. 3. Effects of varying the time of the LOn signal and of loss of the eclosion hormone (EH) neurons on the LOn response. Eclosion of (A–D) the *w*^{1118}×*UAS-rpr* control flies and (E–H) *EHups*×*UAS-rpr* flies that lack EH neurons. Flies received one of four treatments: (A,E) light beginning at normal LOn, (B,F) held in the dark until +2 h, (C,G) light beginning at –1 h or (D,H) light beginning at –2 h, as represented by the horizontal bars at the base of each panel. Legends are as described for Fig. 1. Data from an individual representative experiment is shown.

that took place within the first 3 h of LOn (28%) was similar to the CS strain (32%).

Effects of targeted expression of tetanus toxin light chain in the EH neurons

Targeted ablation of the EH cells eliminated the LOn response (above) but did not distinguish whether the loss of the LOn response was due to the lack of eclosion hormone itself or to some other function of the EH cells. One potential way to leave the EH neurons intact but block EH release was by targeting expression of an intracellular form of tetanus toxin light chain (TeTxLC) in the EH neurons. To produce these flies, we crossed the *EHups-Gal4* strain to flies that carried a *UAS-TNT-L* transgene (Sweeney et al., 1995). The control flies were progeny of the *w*^{1118}×*UAS-TNT-L* cross. The LOn shift paradigm was used to determine if the flies showed a LOn response.

The *w*^{1118}×*UAS-TNT-L* strain demonstrated a strong LOn response when the LOn signal occurred at its normal time (Fig. 5A,B), similar to that observed for the *w*^{1118}×*UAS-rpr* strain. However, moderate increases in eclosion were also detected when the LOn signal was advanced to –1 and –2 h (Fig. 5C,D), similar to the CS strain. *EHups*×*UAS-TNT-L* flies lacked the LOn response (Fig. 5E–H) irrespective of the phase of the LOn signal. In a replicate experiment, the small increase in eclosion rate at the onset of light shown in Fig. 5F was not seen.

The effectiveness of TeTxLC in suppressing EH release was assessed using immunocytochemistry. EH is normally released just prior to each ecdysis, resulting in a rapid and dramatic intracellular depletion of EH from the EH cell axons (Baker et al., 1999; Horodyski et al., 1993). We harvested the CNS of control *w*^{1118}×*UAS-TNT-L* and *EHups*×*UAS-TNT-L* flies the night before eclosion or within 1 min post-eclosion and immunostained them for EH. The CNS of *w*^{1118}×*UAS-TNT-L* flies prior to eclosion showed strong immunolabeling of the cell bodies and processes of the EH neurons (*N*=4; Fig. 5I). By contrast, after eclosion these neurons had little or no EH in descending axons in the ventral ganglion and reduced levels in the cell bodies and processes in the brain (*N*=12; Fig. 5J). In the *EHups*×*UAS-TNT-L* flies, the EH neurons also showed strong immunostaining prior to eclosion (*N*=5; Fig. 5K), but after eclosion the neurons still showed abundant EH (*N*=14; Fig. 5L). The level of EH in the CNSs from post-eclosion *EHups*×*UAS-TNT-L* flies was substantially higher than in CNSs from either control or pre-eclosion *EHups*×*UAS-TNT-L* flies. This suggests that the peptide continues to accumulate in the EH cells during the final 8–11 h before ecdysis and that TeTxLC suppresses its release.

As an additional means of assessing whether the effects of expressing TeTxLC in the EH cells was equivalent to ablating them, we compared other ecdysial and post-ecdysial effects in

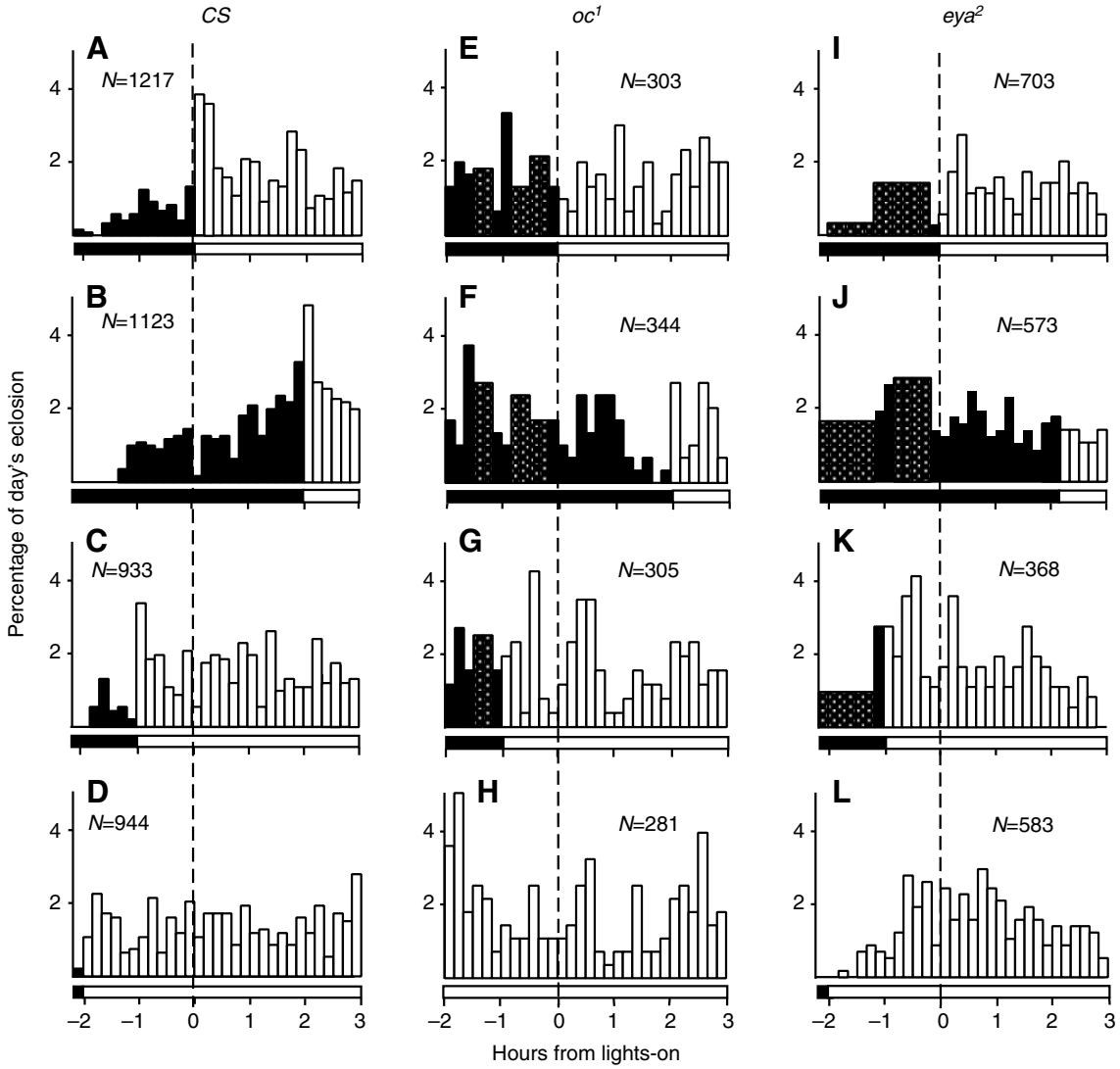


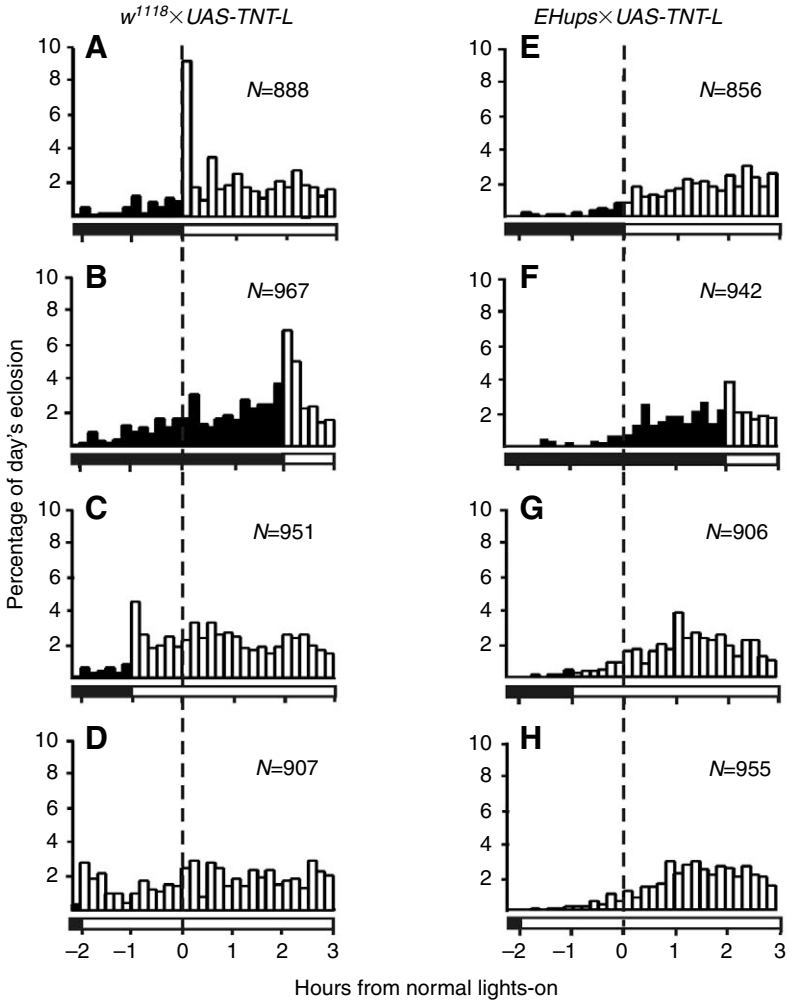
Fig. 4. Effects of lack of optic photoreceptors on the LOn response. The LOn shift paradigm was used to test the CS (Canton-S, control; A–D), the *oc*^{1} ocelliless (E–H) and the *cli*$^{eya\text{-}2}$ eyeless strains (I–L). Legends are as described for Fig. 3 except that, for *oc*^{1} and *cli*$^{eya\text{-}2}$, some collection bins were greater than 10 min intervals but were averaged in 10 min intervals as indicated by stippling.

EHups×*UAS-TNT-L* and *EHups*×*UAS-rpr* (EH cell knockout) flies. The EH cell knockout flies showed extensive larval mortality due to incomplete shedding and inflation of the trachea (McNabb et al., 1997). By contrast, the *EHups*×*UAS-TNT-L* strain exhibited no larval mortality. The principal post-ecdysial defect of the EH cell knockout is the failure to spread the wings, a phenotype observed in a large proportion (76%) of adult flies (McNabb et al., 1997). Expression of TeTxLC in the EH cells had a less severe effect on wing spreading; only 4±1% of all *EHups*×*UAS-TNT-L* adults failed to inflate their wings (three experiments). Although the magnitude of this effect is much less than that seen for the *EHups*×*UAS-rpr* strain, it is significantly elevated (two-tailed *t*-test, P=0.01) above the levels seen for w^{1118}×*UAS-TNT-L* controls (0.3±0.2%, two experiments).

Effects of light on wing spreading latency

Wing expansion follows eclosion and is the last behavioral component of the adult eclosion sequence (Fraenkel, 1935). It is regulated by Bursiconα (Dewey et al., 2004) and EH (McNabb et al., 1997; Truman and Riddiford, 1974). To determine if the LOn signal influences wing spreading latency (WSL), we compared the interval between eclosion and the completion of wing spreading for flies that eclosed in response to the LOn signal to flies that eclosed in the dark. To focus on flies that eclosed in response to the LOn signal, we monitored only those that eclosed within 20 min of LOn. We compared rates of wing spreading between strains that exhibited the LOn response and their counterparts that did not.

For the w^{1118}×*UAS-rpr* strain, the WSL was dramatically different for flies that eclosed in response to the LOn signal *vs* flies that eclosed in the dark (Fig. 6). The flies that eclosed in response to LOn took an average of 86±6 min to spread their wings (Fig. 6A). Flies that eclosed in the dark spread their wings in less than half the time, with an average of 41±4 min. For flies that eclosed in the dark, 41% spread their wings within the first 10 min after eclosion, strongly skewing the distribution to the left (Fig. 6B). By comparison, only 17% of flies that eclosed at LOn spread their wings within the first 10 min.



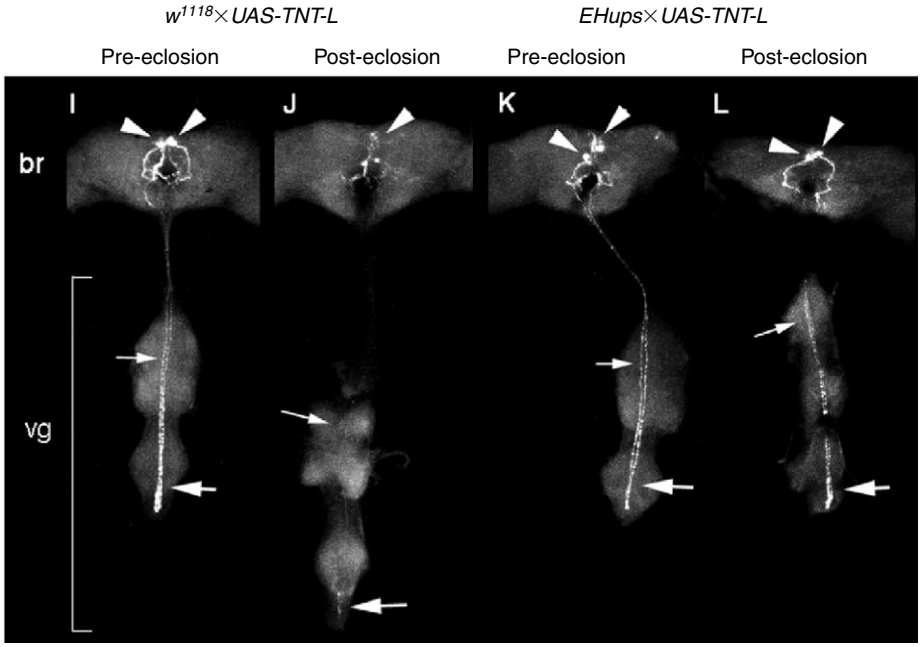


Fig. 5. Effects of expressing TeTxLC in the EH neurons on the LOn response and EH release. Representative results are shown for the LOn shift assay for $w^{1118}\times$*UAS-TNT-L* (A–D) and *EHups*×*UAS-TNT-L* (E–H) strains. Data are presented as described for Fig. 3. (I–L) Immunostaining for EH in CNSs from $w^{1118}\times$*UAS-TNT-L* flies (I) 8–11 h prior to eclosion and (J) within 1 min after eclosion, and from *EHups*×*UAS-TNT-L* flies (K) 8–11 h prior to eclosion and (L) within 1 min after eclosion. These are representative images of projected *z*-series. Arrowheads indicate the EH cell bodies located in the brain (br); large arrows, the posterior portion of the EH axon that extends down the ventral ganglion (vg); small arrows, the thoracic portion of the EH axon.

Results obtained with the *EHups*×*UAS-rpr* strain were more difficult to assess (Fig. 6B) because the vast majority (71%) of these flies failed to spread their wings, as previously seen (McNabb et al., 1997). In current experiments, only four of the 45 that eclosed within 20 min of LOn expanded their wings. Although both *EHups*×*UAS-rpr* and *EHups*×*UAS-TNT-L* flies lacked the LOn response, the latter were better for assessing the relationship of the LOn signal to wing spreading behavior because more of them spread their wings. The $w^{1118}\times$*UAS-TNT-L* control strain showed a similar WSL to that of the $w^{1118}\times$*UAS-rpr* strain (Fig. 6C) – flies that eclosed at LOn took much longer to spread their wings than those that eclosed in the dark (χ^2-test, P=0.0001). By contrast, the *EHups*×*UAS-TNT-L* flies that eclosed at LOn showed a very similar distribution to those that eclosed in the dark (Fig. 6D). The proportion of flies that spread their wings within the first 10 min after eclosing was also similar under the two conditions (χ^2-test, P>0.1). These results suggest that for strains that exhibit the LOn effect, such as the $w^{1118}\times$*UAS-rpr* and $w^{1118}\times$*UAS-TNT-L* strains, the WSLs differ substantially for flies that eclose in response to the LOn signal *vs* those that eclose in the dark. In the absence of a LOn effect, as in the case of the *EHups*×*UAS-TNT-L* strain, there is little difference under the two conditions.

The correlation between the LOn response and the WSL was further tested using the ocelliless oc^1 strain, the eyeless (eya^2) strain and a CS control (Fig. 6E–G). The differences in WSL for CS flies that eclosed in response to a LOn signal and those that eclosed in the dark were modest when compared with $w^{1118}\times$*UAS-TNT-L* and $w^{1118}\times$*UAS-rpr* strains but followed the same trends. For CS flies, the distribution of WSLs seen for flies that eclosed in the dark is slightly more skewed to the left than for flies that emerged after LOn (Fig. 6E). On average, CS flies that eclosed just after LOn completed wing spreading in 33±4.7 min, compared to 23±3.0 min for those that emerged in the dark, a

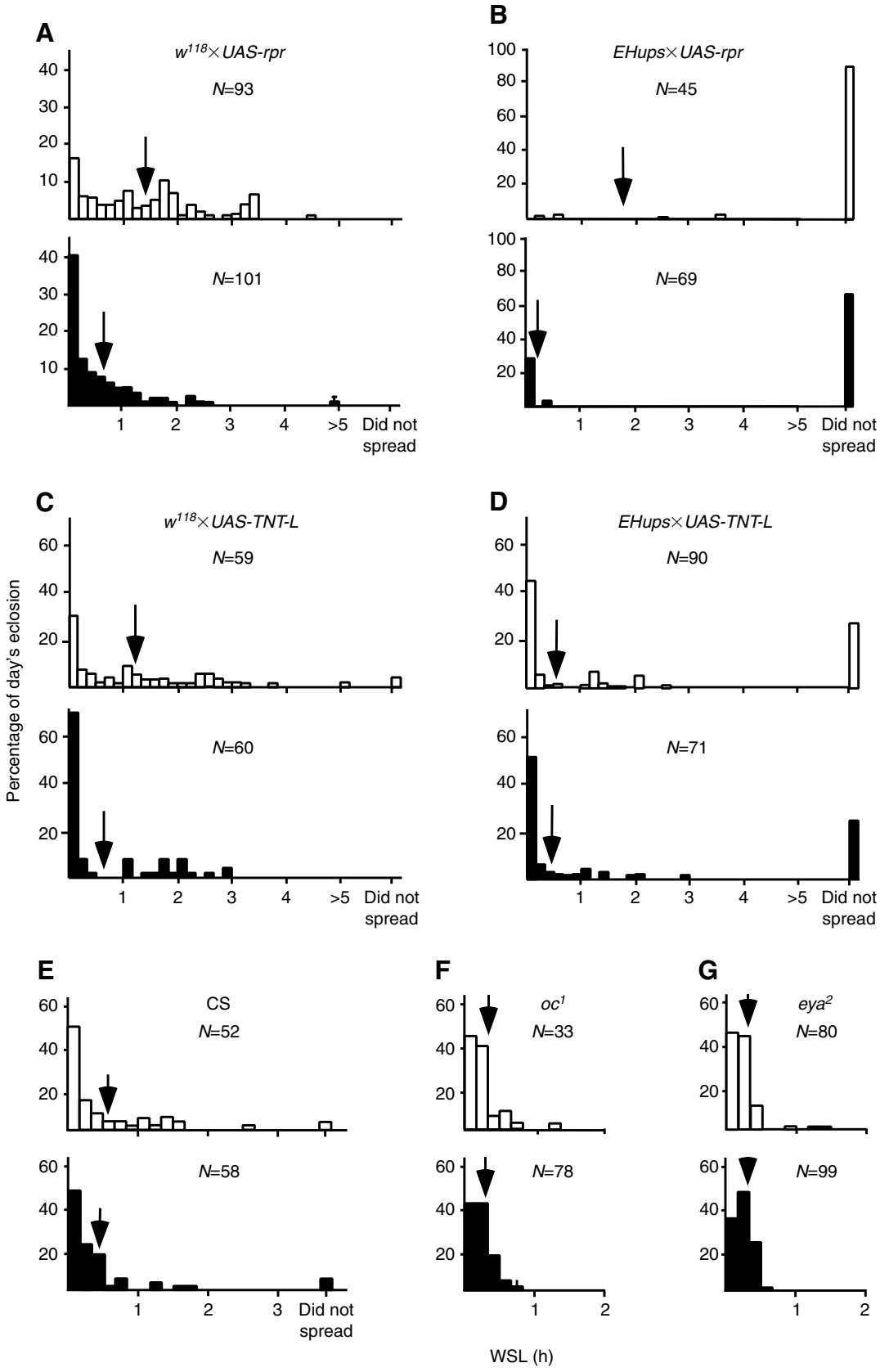


Fig. 6. Effects of the LOn signal on wing spreading latency (WSL) is shown for flies that eclosed in response to LOn (upper panels, white bars) and flies that eclosed in the dark (lower panels, black bars) for (A) w^{1118}×*UAS-rpr*, (B) *EHups*×*UAS-rpr*, (C) w^{1118}×*UAS-TNT-L*, (D) *EHups*×*UAS-TNT-L*, (E) Canton-S, (F) oc^{1} and (G) and $cli^{eya\text{-}2}$ strains. The WSL is expressed per 10 min bin. The average WSL is indicated by an arrow.

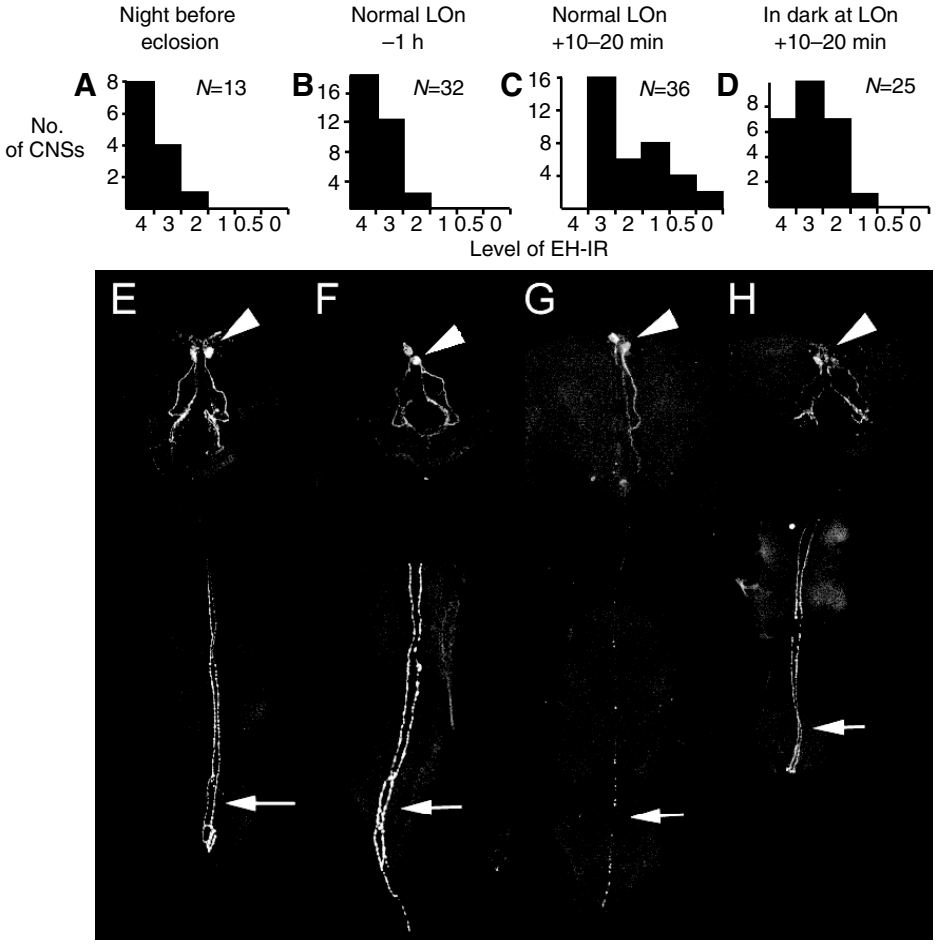


Fig. 7. Effects of light on EH release in $w^{1118}\times$*UAS-rpr* flies. Pharate adults that were not destined to eclose at the time of normal LOn were collected the night prior to eclosion and their CNSs were harvested (A,E) the night prior to eclosion, (B,F) 1 h before normal LOn, (C,G) 10–20 min after LOn or (D,H) held in the dark until 10–20 min after normal LOn. (A–D) CNSs immunostained for EH were scored for level of staining. (E–H) Representative *Z* projections.

statistically significant difference (χ^2-test, $P<0.01$). In both cases, almost half of the CS flies spread their wings within the first 10 min of eclosion. For both the oc^1 and eya^2 strains (Fig. 6F,G), flies that eclosed at LOn and those that eclosed in the dark showed similar WSLs and proportions of flies that spread their wings within 10 min of eclosion. These results are consistent with those of the two previous sets of experiments. Strains such as CS that show a LOn response complete wing expansion more rapidly when they emerge in the dark than after a LOn signal. By contrast, those that lack a LOn response, oc^1 and eya^2, show no difference under the two conditions.

Effects of light on EH release

Experiments described above suggest that a LOn signal can markedly shorten the normal 40–60 min interval between EH release and eclosion (Baker et al., 1999). In addition to suppressing descending inhibition, another mechanism by which the LOn signal could cause a shift in emergence time is by directly stimulating EH release. To determine if the LOn signal could induce EH release, we examined levels of EH immunostaining in CNSs from pharate adult $w^{1118}\times$*UAS-rpr* flies exposed to different light treatments. These pharate adults were selected carefully to ensure that they were not destined to eclose within an hour after the time of normal LOn (see Materials and methods) and a large number of CNSs (see below) were tested for each condition to ensure that developmental stage did not influence the differences observed. The night before eclosion, the EH cell bodies and their projections showed strong EH immunoreactivity, scoring 3.7 out of 4 (see Methods) (Fig. 7A,E). At –1 h, EH levels were approximately as high as they were the night before, demonstrating that the flies had not yet released EH in preparation to eclose (score 3.5; Fig. 7B,F). Some of the pharate adults were subjected to the normal LOn signal, while others were maintained in darkness. Nervous systems from flies that were collected within the first 20 min of the LOn signal showed a dramatic decrease in EH levels (score 0.5; Fig. 7C,G), with 14 of 36 nervous systems showing a score of 1 or lower. By contrast, CNSs from flies that had been maintained in the dark and then sacrificed at the same time as the above flies continued to show strong EH staining (score 2.9; Fig. 7D,H), with only one of 25 showing a score of 1 or below. The observation that the CNSs from flies maintained in the dark scored lower than the groups from before the LOn signal is consistent with the notion that some of the flies held in the dark are within the eclosion gate and have initiated EH release.

DISCUSSION

The lights-on (LOn) response is a rapid eclosion response to light

The daily distribution of adult eclosion in *Drosophila* is regulated by the interaction of two light-regulated timing pathways. One involves the circadian clock, which determines the temporal phase of the gate that restricts eclosion to a particular window during the day (Pittendrigh and Skopik, 1970). The second pathway involves the masking effect of the LOn signal that causes a pronounced skewing of the eclosion peak within this gate. We have shown that the eclosion response to the LOn signal is rapid and strong, typically occurring within 10 min and resulting in up to 22% of the day's

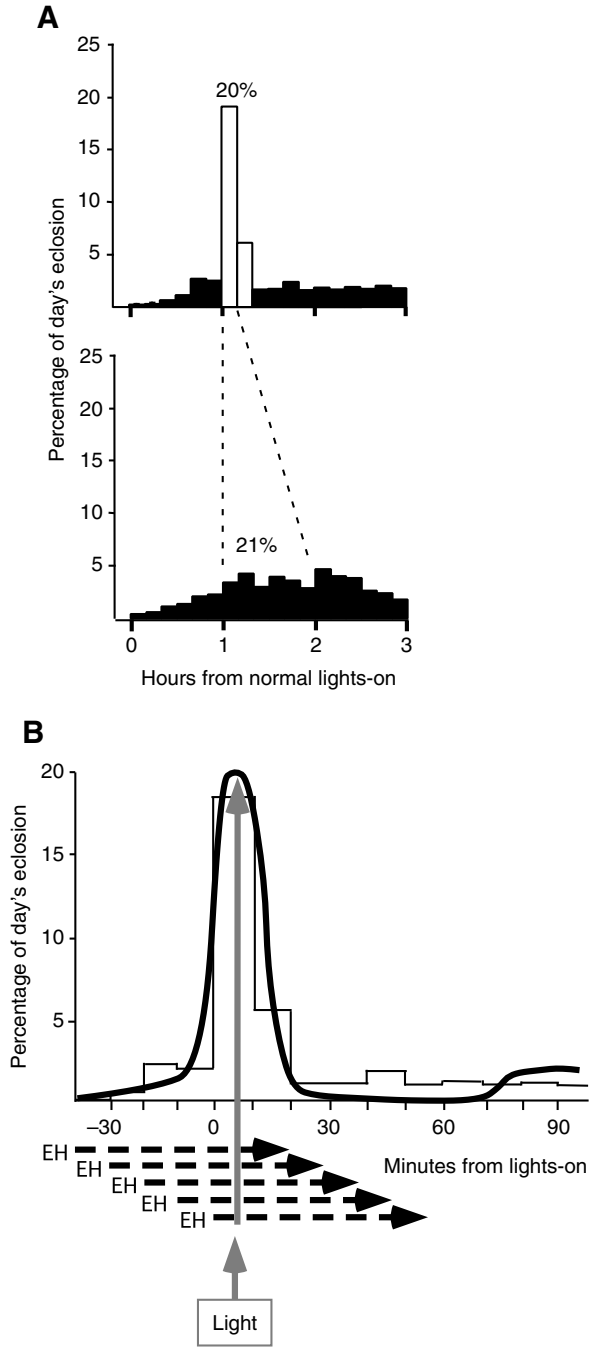


Fig. 8. Light stimulates rapid eclosion in competent flies, but also appears to have a second, delayed, effect. (A) Data from the $w^{1118}\times$*UAS-rpr* pulse experiment (Fig. 2B,C) show that the proportion of flies that eclosed during the first 10 min of the 20 min light pulse (white bars) was roughly equivalent to the proportion that eclosed over the corresponding 60 min interval in the dark (bracketed by dashed lines). (B) Summary of the effects of light on eclosion, adapted from the $w^{1118}\times$*UAS-rpr* pulse experiment (Fig. 2C). Dashed arrows indicate the intervals over which flies that have released EH are recruited to eclose by a LOn signal. Pharate adults that had released EH and were competent to eclose within approximately 60 min were recruited to eclose in the first 10 min after light exposure (gray arrow). If the pool of competent flies all eclosed at or shortly after LOn, the amount of eclosion after the light pulse would be expected to diminish to 0 (thick black line). Instead, the proportion of flies eclosing went down to only approximately 2% (thin black line) possibly as a result of light stimulating EH release in a group that was developmentally mature and ready to eclose.

eclosion within that 10 min interval. The LOn response can occur either earlier or later in the eclosion gate but it can only take place after the normal gate has opened. The response is exhibited in the absence of a normal circadian rhythm in *per* and *tim* mutants (S.L.M., unpublished data). A temperature transition may also exert a masking effect on eclosion *via* the EH neurons (Jackson et al., 2005).

The role of the EH neurons in the LOn response

The EH neurons are essential for the LOn response as shown by the results of specifically expressing either cell death genes to ablate them (Fig. 3; McNabb et al., 1997) or tetanus toxin light chain to suppress synaptic release (Fig. 5). How do the EH neurons mediate the LOn response? As seen in Fig. 7, one effect of the LOn signal appears to be the stimulation of EH release. Our immunocytochemical studies show that the LOn signal caused a rapid depletion of EH in pharate adult flies that were expected to release EH later in the day.

A second effect of the LOn signal appears to be in decreasing the latency of eclosion relative to EH action. In *Drosophila*, the normal latency from EH release to eclosion is 40–60 min (Baker et al., 1999) but the LOn response occurs within 10 min (e.g. Fig. 1). Experiments in both *Manduca* and *Drosophila* suggest that the delay between EH release and ecdysis is due to a descending inhibition that is set up as a consequence of EH release. EH release normally takes place well in advance of ecdysis. In *Manduca*, the delay period is about 20–25 min for larval ecdysis (Ewer et al., 1994), and 2–3 h for adult eclosion (Ewer and Truman, 1996). Decapitation of *Manduca* pharate adults during the delay period leads to the rapid onset of eclosion behavior (Ewer et al., 1997; Reynolds, 1980), suggesting that the delay is due to descending inhibition from the head. Transection of the CNS at different levels shows that neurons from the subesophageal and thoracic ganglia contribute to this descending inhibition (Fuse and Truman, 2002; Zitnan and Adams, 2000). Similarly in *Drosophila*, decapitating pharate adults that have released EH results in their rapid eclosion (Baker et al., 1999). This rapid behavioral response to decapitation is not seen in EH cell knockout flies (Baker et al., 1999), suggesting that EH release is required for the inhibition to be set in place.

A LOn signal appears to be another way to elicit early eclosion in flies that have released EH. The simplest interpretation is that the LOn signal acts by suppressing the descending inhibition. This mechanism is favored by the rapid nature of the LOn response and by evidence from the pulse experiment with the $w^{1118}\times$*UAS-rpr* strain. If the LOn response results from the removal of the descending inhibition, flies that eclose during the LOn peak should include all of the pharate adults that have released EH and are in the delay period at the time of the pulse. This can be seen by comparing the results of the pulse and dark experiments (Fig. 8A). In the dark-maintained group, about 21% of the flies that eclosed during the day emerged between 1 and 2 h after the time of normal LOn. Given a latency of 40–60 min between EH release and ecdysis (Baker et al., 1999), most of these flies would have been expected to have released EH by the time the LOn pulse was given at 1 h. In this example, in the group that received the pulse, the LOn signal was followed by 20% of the flies emerging within the first 10 min, similar to the 21% predicted to be in the waiting period. This is consistent with the interpretation that flies that demonstrate the LOn response have already released EH, are competent to respond to the LOn signal, and are rapidly released from the inhibition that causes the normal delay (Fig. 8B). If the LOn signal acted only to suppress the descending inhibition and hence reduce the eclosion latency in

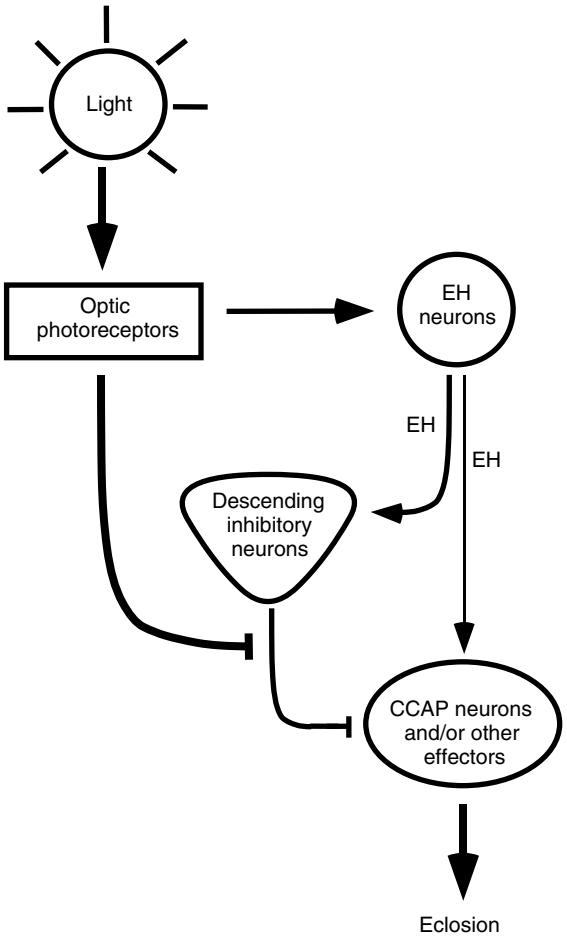


Fig. 9. A model of effects of light on eclosion. EH activates both an eclosion activation pathway and a set of inhibitory neurons that repress eclosion behavior. The activation pathway may include the CCAP neurons and additional EH-downstream neurons. We postulate that the release of CCAP and other eclosion activators is inhibited by EH action. Light suppresses the inhibitory pathway to allow the release of CCAP and other factors and permit subsequent eclosion. In addition, light acts on the EH cells to stimulate EH release, probably *via* the retinal photoreceptors. Although eclosion can be accelerated by light, wing spreading cannot, suggesting that EH stimulates these behaviors *via* distinct pathways. Arrow and line thickness indicate the strengths of the different responses.

pharate adults that had released EH, we would expect eclosion to drop down to zero for 40–60 min after the LOn peak. The fact that we do not observe this severe depression is most likely explained by our observation that the LOn signal can induce premature EH release in addition to reducing the eclosion latency.

Photoreceptors and the LOn response

Unlike the circadian entrainment of eclosion, the LOn response requires a signal transduced from the eyes. Previous research demonstrated that the LOn response is mediated by retinal photoreceptors (Engelmann and Honegger, 1966). Using mutations that eliminate either the compound eyes or the ocelli, we have shown that both sets of photoreceptors are required for the response under our assay conditions. The reason that both sets of photoreceptors are required is unknown, but one possibility is that they act, either in series or in parallel, to amplify the response. We also cannot rule out the possibility that the mutants we tested had defects in other components of the ecdysis pathway that may mediate the LOn response. For example, *eyes absent* mutations affect the development of additional regions of the brain (Bonini et al., 1998; Boyle et al., 1997), and *ocelliless* and *sine oculis* also affect other developing tissues (Finkelstein et al., 1990; Serikaku and O'Tousa, 1994).

We postulate a mechanism in which a signal from the photoreceptors acts on the eclosion inhibitory neurons to suppress the descending inhibition (Fig. 9). Before the cellular pathway and the mechanism of the release of the inhibition can be elucidated, the source of the inhibition must be identified. Based on the results of head ligations described above, it must reside in the head. In *Manduca*, neurons of the cell 27/704 group are EH targets (Ewer et al., 1994; Ewer et al., 1997) and some that are located in the subesophagheal ganglion are candidate sources of the inhibition (Fuse and Truman, 2002; Zitnan and Adams, 2000). The identity of the *Drosophila* inhibitory neurons is less clear. Efforts to identify the *Drosophila* homologs of the cell 27/704 group as EH targets have been unsuccessful because they do not show a cGMP response following EH release (Ewer and Truman, 1996). Currently, the function of the *Drosophila* CCAP-expressing neurons in eclosion is uncertain, as their ablation does not prevent eclosion (Park et al., 2003). However, several other sets of neurons demonstrate calcium transients in response to ETH, suggesting candidate neuropeptide-expressing neurons as downstream activators (Kim et al., 2006). The pathway by which the optic photoreceptors signal the ecdysis inhibitory neurons also remains to be identified.

Differences in magnitude of the LOn response were observed between the control strains. The $w^{1118}\times$*UAS-rpr* flies have pale apricot eyes and are highly responsive to the LOn signal. The Canton-S and $w^{1118}\times$*UAS-TNT-L* flies have red eyes and show a weaker LOn response. The magnitude of the response may be a function of light sensitivity and thus be higher in strains with less pigmentation since screening pigments are associated with decreased sensitivity of the eyes to light (Ostroy and Pak, 1974; Zimmerman and Ives, 1971).

Expression of tetanus toxin light chain in the EH neurons suppresses the LOn response and EH release

We have demonstrated three different ways of eliminating the LOn response: (1) removal of the EH neurons by targeted expression of the cell death gene *rpr* (i.e. making an EH cell knockout), (2) removal of either ocelli or compound eyes by the use of previously identified mutants, and (3) alteration of EH cell function by targeting expression of an intracellular form of tetanus toxin light chain (TeTxLC) to the EH cells. The ability of EH neuron-targeted TeTxLC to block the LOn response shows that the elimination of this response in the EH cell knockouts is not due to death of EH cells, but rather to loss of EH cell function. Since TeTxLC, which acts by inhibiting synaptobrevin-mediated docking (reviewed by Humeau et al., 2000), inhibits the release of EH (Fig. 5K,L), synaptobrevin appears to play a role in the release of neuropeptides. Flies that express TeTxLC in their EH neurons also show a partial failure to spread their wings, a post-ecdysial effect that is characteristic of the EH cell knockouts (McNabb et al., 1997). We do not yet understand the difference in penetrance of this phenotype between these two strains. It may reflect a low level of EH release by the flies that express targeted TeTxLC, interstrain variation (as seen previously in McNabb et al., 1997) or a combination of these factors.

Wing spreading, a post-ecdysial behavior that is regulated by EH, is not accelerated by the LOn signal

For strains that exhibited a strong LOn response, flies that eclosed in response to LOn took substantially longer to spread their wings than flies that eclosed in the dark. For $w^{1118}\times$*UAS-rpr* flies, it took

an average of 47 min longer; for $w^{1118}\times UAS\text{-}TNT\text{-}L$ flies, about 40 min longer. This time difference is within the 40–60 min latency range between EH release and eclosion (Baker et al., 1999). Our findings are consistent with EH playing roles in initiating both eclosion and wing spreading, and with these two behaviors having separate downstream pathways with distinct latencies. The LOn signal interacts with the ecdysis delay pathway to result in early ecdysis, but does not affect the wing spreading circuit. Hence, the LOn signal causes early ecdysis and a corresponding increase in latency between eclosion and wing expansion.

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