The Ecdysoneless (ecd\textsuperscript{1ts}) Mutation Disrupts Ecdysteroid Synthesis Autonomously in the Ring Gland of 
Drosophila melanogaster

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Abstract:
Ring glands dissected from homozygous l(3)ecd\textsuperscript{1ts} wandering larvae and upshifted in vitro to the restrictive temperature, 29°C, synthesize abnormally low quantities of ecdysteroid. Nevertheless, ecd\textsuperscript{1} ring glands retain the ability to respond at 29°C to an extract prepared from wild-type larval neural tissues that presumably contain prothoracicotropic hormone (PTTH), although both basal and stimulated levels of synthesis are lower than those in wild-type ring glands. Extracts prepared from ecd\textsuperscript{1} neural tissue exhibit an unusually high level of PTTH activity. Mutant ring glands downshifted in vitro to the permissive temperature after removal from larvae maintained at 29°C regain the ability to produce normal basal and stimulated ecdysteroid levels. Collectively, these experiments demonstrate that the ecd\textsuperscript{1} mutation disrupts the physiology of the ring gland at 29°C autonomously and may also interfere with PTTH release.

MATERIALS AND METHODS

Stock maintenance. All larvae utilized in these experiments were reared on standard agar food medium under a constant light, 18°C regime until the time of experimentation, unless otherwise noted. Eggs were collected at 4-hr periods from individuals homozygous for ecd\textsuperscript{1} st red e ca. These adults were the selected progeny of mass crosses between individuals of a conditional balanced lethal stock (TM6/ecd\textsuperscript{1} st red e ca) at the permissive temperature, 18°C. Homozygous individuals can be distinguished by the orange eye color that results from the
interaction of the recessive eye color marker mutations and wild-type halteres (TM6 carries $Ubx^{67b}$) and $e$). Constant selection for the parents of tested progeny was necessary because genetic modifiers accumulate within a few generations in a homozygous population, even at 18°C. Similar egg collections were made from members of a wild-type Canton-S strain.

**Dissection and incubation methods.** For the experiments to measure the response of an *in vitro* temperature upshift, individual ring glands or brain-ventral ganglion-ring gland complexes were dissected from early wandering larvae at 18°C and placed in 15-µl drops of Grace's medium preincubated at 18 or 29°C in accordance with a previously developed protocol for another dipteran (Roberts et al., 1984). Larvae were taken as they wandered off the food medium and those with swollen salivary glands were discarded. After either 2 or 4 hr of incubation in vitro, a 10-µl sample was removed from the culture medium and the ecdysteroid content quantified by radioimmunoassay (RIA) (Warren et al., 1984). The data are expressed in ecdysone equivalents. It should be noted that the general term ecdysteroid is used here although it appears that ecdysone is the major product of the *Sarcophaga* ring glands (Bollenbacher et al., 1976) and *Manduca* prothoracic glands (King et al., 1974). In the case of *Drosophila* ring glands, however, the *in vitro* incubation products include ecdysone, 20-deoxymakisterone, and an unidentified ecdysteroid (Redfern, 1984; Warren, Henrich, and Gilbert, unpublished data).

Another group of early wandering *ecd1* larvae were transferred to 29°C for 1 or 4 hr prior to dissection of brain-ventral ganglion-ring gland complexes and these complexes were incubated for 2 hr at 29°C. The *in situ* portion of this experiment could not be performed with a Canton-S control because wild-type larvae at this developmental stage pupariate within a few hours of an upshift from 18 to 29°C. Brain-ventral ganglion-ring gland complexes from *ecd1* larvae upshifted 24 hr prior to dissection and treated according to the procedures described below also were included in the *in situ* temperature upshift experiment. It is important to note that this experiment involves subjects that are not developmentally equivalent.

For the tests of ring gland responsiveness to neural (PTTH) extracts and *in vitro* reversibility, cultures of homozygous *ecd1 st red e ca* larvae were upshifted from 18 to 29°C in the third instar 9 days after egg collection. Ring glands were removed 24 ± 2 hr later as described above and incubated for 2 hr either in Grace’s medium alone or in Grace's medium containing PTTH extract. These media had been temperature equilibrated at either 18 or 29°C. In addition to the experimental groups, a culture of homozygous *ecd1* larvae and wild-type Canton-S larvae was maintained at 18°C and the ring glands from early wandering larvae at 18°C were tested for a response to PTTH extract. Another Canton-S group was upshifted as early third instar larvae to 29°C and their ring glands were dissected 24 ± 2 hr later to test for a response to PTTH extract. This group served as a control for the manipulation of the *ecd1* culture. Finally, the rest of the *ecd1* culture used for these experiments was kept at 29°C to ensure that the temperature upshift caused the lethality expected because of the expression of the mutant gene.

**Preparation of PTTH extracts.** Brain-ventral ganglion complexes were collected from wandering third instar Canton-S larvae at 25°C and stored at —70°C until extracted. After homogenization in Grace’s medium at 4°C, the mixture was heated to 100°C for 3 to 5 min (Bollenbacher et al., 1979; Roberts et al., 1984) and centrifuged, and the supernatant designated as PTTH extract. The concentration of the extract was adjusted to 8 brain-ventral ganglion eq/10 µl of final supernatant. All extracts were tested for the presence of ecdysteroids by RIA and appropriate adjustments were made in calculating the extent of ring gland activation. For control groups, Grace's medium was subjected to the same regimen and this had no measurable effect upon the *in vitro* performance of the ring glands.

The *ecd1* PTTH extract was prepared from brain-ventral ganglion complexes removed from *ecd1* larvae upshifted to 29°C as early third instar larvae and dissected at the same temperature. The concentration was adjusted to 8 brain-ventral ganglion eq/10 µl, as above.

**Statistical methods.** An unpaired Student *t*- or *T*-test was used for all comparisons between two groups. To test
for significant differences among the four "in situ upshift" groups (Fig. 2) and the six "basal synthesis" groups (Fig. 3), a one-way analysis of variance was employed (Sokal and Rohlf, 1969). A statistically significant difference (P < 0.05, F-test) led to the use of Duncan's multiple range test to identify those groups that differed from the others. The six "stimulated synthesis" groups (Fig. 3) were treated in identical fashion.

RESULTS

**In vitro analysis of brain-ventral ganglion-ring gland complexes and ring glands from ecd1 and Canton-S larvae.** If the ecd1 mutation disrupts the physiology of the neuroendocrine axis of Drosophila, then the in vitro transfer of dissected brain-ventral ganglion-ring gland complexes to a restrictive temperature could reveal a failure of ecdysteroid synthesis. This approach eliminates the possibility that observed physiological abnormalities arise as an indirect consequence of a developmental alteration expressed peripheral to the neuroendocrine axis. At 18°C, ecd1 complexes placed in vitro produce about the same levels of ecdysteroid as Canton-S complexes (Fig. 1a). The observed pattern of synthesis by Canton-S complexes from early wandering larvae resembled previously reported results for an Oregon-R strain (Redfern, 1983) although the levels of synthesis at 18°C were somewhat higher than those reported for 25°C. When transferred to 29°C immediately after dissection, ecd1 and wild-type brain-ventral ganglion-ring gland complexes displayed differences in ecdysteroid synthesis that were apparent after 2 and 4 hr of incubation. Wild-type complexes produced about 50% more ecdysteroid in 4 hr than they did at 18°C (P < 0.01, t-test). By contrast, ecd1 complexes produced about 40% less ecdysteroid at 29°C than at 18°C (P < 0.01, t-test).

This experiment does not allow a distinction between direct effects of the mutation on the ring gland and the effects of the brain that may modulate ring gland function by neural or neurohormonal means. However, wild-
type and mutant ring glands generated patterns of ecdysteroid synthesis (Fig. 1b) that resemble those obtained from complexes, indicating that the observed differences involve the ring gland.

The decrease in ecdysteroids seen at 29°C could result from a failure to synthesize ecdysone or a failure of the prothoracic gland to secrete ecdysone after it is synthesized. The latter possibility was tested by measuring the amount of ecdysteroid in Canton-S and ecd1 ring gland extracts after a 4-hr incubation at 29°C. In both groups, the amount of ecdysteroid that remained in the ring gland after incubation in vitro was below reliable levels of detection (<15 pg/ring gland) and therefore could not account for the differences seen in the incubation medium (data not shown). This is consistent with the finding that the prothoracic gland appears to release ecdysone as soon as it is synthesized (Bollenbacher et al., 1979).

**Effect of in situ upshifts followed by in vitro incubation of ecd1 brain-ventral ganglion-ring gland complexes.**

Neural and neurohumoral cues may modulate the activity of the ring gland in situ, even at 29°C, and such ring glands could continue to respond to stimuli that are completely absent in vitro. This possibility is supported by the observation that the ecd1 ecdysteroid titer does not drop immediately after transfer of the larvae to 29°C (Berreur et al., 1984; Redfern and Bownes, 1983).

Brain-ventral ganglion-ring gland complexes from ecd1 larvae were dissected 0, 1, 4, and 24 hr after a whole-animal temperature upshift and then in vitro ecdysteroid synthesis was examined (Fig. 2). Complexes from larvae placed at 29°C 1 hr prior to dissection produced more ecdysteroid than complexes upshifted immediately after dissection and placement in the incubation medium (P < 0.01, F-test). Brain-ventral ganglion-ring gland complexes from larvae dissected 4 hr after a temperature upshift produced considerably less ecdysteroid in vitro (P < 0.01; F-test) than those upshifted just 1 hr prior to dissection, while the complexes from larvae upshifted
Response of wild-type and ecd1 ring glands to PTTH extract. When early wandering ecd1 larvae are up-shifted to 29°C, they pupariate later than similarly treated wild-type larvae and die as pharate adults. A reasonable explanation for this observation based on the in vitro experiments described above is that the ecd1 mutation disrupts the normal function of the prothoracic gland cells, although the relatively high levels of ecdysteroid synthesis observed in ring glands from larvae unshifted in vivo 1 hr before dissection and then incubated at 29°C suggest that mutant glands respond to internal stimuli, presumably PTTH. In other insects, PTTH is released into the hemolymph and stimulates higher levels of ec dysone synthesis in the prothoracic (ring) glands (Bollenbacher et al., 1979; Roberts et al., 1984). Although PTTH has not been characterized in any Drosophila species, the studies described below are based on the assumption that the Drosophila neuroendocrine system is analogous to those of Manduca and Sarcophaga.

At both 18 and 29°C, ring glands from wild-type larvae synthesized significantly larger quantities of ecdysteroids in the presence of PTTH extract (Fig. 3; P < 0.01, t-test). Likewise, ecd1 ring glands attained similar stimulated levels of ecdysteroid production at 18°C. By contrast, ecd1 ring glands produced abnormally low basal levels at 29°C (P < 0.01; F test) although they could be induced to synthesize significantly higher levels of ecdysteroid in the presence of PTTH extract (P < 0.01; t test). While these stimulated levels of synthesis in ecd1 ring glands at 29°C were lower than those of other groups (P < 0.01; F test), the ratio between stimulated and basal levels was comparable (Fig. 3). PTTH extract prepared from ecd1 larval tissue actually evoked an un-usually high level of activity from wild-type ring glands incubated in vitro at 29°C, perhaps indicating an accumulation of PTTH in the nervous system. This suggests that the delay in pupariation noted for ecd1 larvae up-shifted in the early third instar arises from the mutation's interference with the precise temporal release of PTTH.

Reversibility of ecd1 effects. If ecd1 interferes with PTTH release and/or ecdysteroid synthesis, then normal function might be restored autonomously by an in vitro transfer of the ring gland to 18°C after dissection from mutant larvae held at 29°C. As shown in Fig. 3, almost normal basal and stimulated rates of ecdysteroid synthesis could be restored by performing such a transfer, demonstrating that the ecd1 lesion acts autonomously upon the physiology of the ring gland at the restrictive temperature.

DISCUSSION

These results indicate that at least one focus of activity for the ecd1 gene resides within the ring gland, resulting in the synthesis of lower than normal quantities of ecdysteroids. This dysfunction can be detected in vitro before the phenotype becomes detectable in situ. At 29°C, the mutation lowers both the basal level of ecdysteroid synthesis and the stimulated level elicited by PTTH extract. Normal basal and stimulated rates of synthesis can be restored by the in vitro transfer of ring glands to 18°C. This reversibility demonstrates that the ecd1 lesion acts autonomously upon the physiology of the ring gland.

The ecd1 mutation apparently does not affect any aspect of PTTH synthesis or function since extracts of ecd1 brains elicit a response from wild-type glands at 29°C, indicating that the extract contains a functional neuropeptide. However, the apparently greater quantity of PTTH in the mutant brain suggests that the mutation interferes with PTTH release. In fact, the situation described here resembles a phenomenon found in diapausing pupae of Manduca where development is arrested because the neurohemal organ does not release PTTH (Bowen et al., 1984). The constant ratio between basal and stimulated levels of ecdysteroid synthesis in ecd1 ring glands at the permissive and restrictive temperatures suggests that the components of the ring gland involved in the response to neurohormonal stimuli (e.g., PTTH receptors) remain intact. The ability to respond to PTTH extract also eliminates the possibility that the mutation imposes a rate-limiting restriction upon ecdysteroid biosynthesis at the restrictive temperature. The reversibility of ecd1 both in vivo and in vitro, further indicates that the mutation does not permanently incapacitate larvae subjected to the restrictive temperature. In fact, the elevation of stimulated levels of ecdysteroid synthesis observed when the ring glands are returned to 18°C after a prolonged exposure to 29°C suggests that prothoracic gland cells do not undergo cytolysis. This
premise is supported by electron microscopic examination of ecd1 prothoracic gland cells held at 29°C for several days (Hanton, Henrich, and Gilbert unpublished data). Therefore, the developmental defects associated with ecd1 larvae maintained at 29°C probably arise as a long-term secondary consequence of physiological defects such as those observed during the in vitro analysis. The mutation's autonomous action upon the imaginal discs (Sliter, 1986) indicates that the ecd1 locus does not code for an enzyme in the ecdysteroid biosynthetic pathway.

In summary, while ecd1 clearly exerts pleiotropic effects (Redfern and Bownes, 1983; Berreur et al., 1984) the mutation directly and measurably disrupts ecdysteroid synthesis in the neuroendocrine axis of Drosophila. The enigmatic action of this mutation, therefore, must be viewed as a reflection of the largely unknown regulatory mechanisms that govern endocrine function during the larval-pupal metamorphosis of Drosophila.

REFERENCES


