Alterations in Development, Behavior, and Physiology in Drosophila Larva That Have Reduced Ecdysone Production

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Introduction

Hormones have essential roles in developmental changes in the life of Drosophila melanogaster, from the larval instars to the adult form. 20-hydroxyecdysone (20-HE), currently regarded as the active form of ecdysone, is associated with developmental changes during metamorphosis (Baehrecke 1996; Cayre et al. 2000; Farkas and Šultaková 1998, 1999; Henrich et al. 1993, 1999; Riddiford 1985; Steel and Davey 1985). This particular hormone is important in causing the behavioral and physical changes during the development stages of each molt (Truman 1996). In Drosophila, high titer levels of ecdysteroid are reached during the third instar larva, between the late feeding stage and prepupa, and in the pupal stage (Kim et al. 1999; White et al. 1997). It has been observed that steroids cause both physiological and anatomical effects on neurons (Jacobs and Weeks 1990; Levine and Weeks 1996; Thummel 1996). There is also a substantial accumulating literature documenting nongenomic effects, especially of membrane-bound steroid receptors that cause relatively rapid changes in cellular processes (Baulieu 1997; Benten and Chang 1999; Christ et al. 1999; Hanaya et al. 1997; Schmidt et al. 1998; Watson and Gametchu 1999). We have investigated the development and maintenance of motor neuron structure, function, and sensitivity to exogenous application of ecdysone using the ecdysoneless mutant strain of Drosophila, ecd1, which contains a recessive, temperature-shock-sensitive allele of a gene required for ecdysteroid production. The homozygote ecd1/ecd1 is larval lethal at 29°C, but at 25°C, it will survive to the late third instar (Henrich et al. 1993), thus allowing behavioral, anatomical, and physiological studies.

Surprisingly, there are few studies of nongenomic actions of molt-related steroid (i.e., 20-HE) compounds in crustaceans and insects (Cooper and Ruffner 1998; Cromarty and Kass-Simon 1998; Ruffner et al. 1999). Behavior of the Drosophila larvae changes immediately before pupation, but the mechanisms modulating behavior are poorly understood. On exposure of an isolated crayfish or early third instar Drosophila nerve-muscle preparation to 20-HE, there is a pronounced reduction in the size of the excitatory junction potentials (EJPs) recorded in the muscle (Cooper and Ruffner 1998; Ruffner et al. 1999). These studies also showed a quick change in the quantal release properties of synaptic transmission in response to 20-HE (Cooper and Ruffner 1998). The rapid rate of response within the presynaptic terminal and the lack of transcriptional regulation, since the neuron is anucleated, implies nongenomic action. The purpose of this study using Drosophila is to investigate both the behavior of the whole animal and the physiology and morphology of the NMJs of third instar under the conditions of reduced ecdysone production.

Segments of this work have appeared in abstract form (Cooper et al. 2000; He et al. 1998, Li et al. 1999a,b).

Methods

Husbandry

Dr. Vincent C. Henrich, University of North Carolina at Charlotte, supplied the ecd1/TM6B, Tabby (Tb) fly genotype (Lindsay and Zimm 1992) used for these experiments. Eggs from ecd1/TM6B, Tb
flies were collected for 2-h periods on apple juice-agar plates with yeast paste. The eggs were allowed to hatch and develop at 25°C until they reached the third instar larval stage.

Developmental and behavioral assays

The second instar larvae were collected as they molted and examined for the presence of nontubby morphology (indicating the ecd<sup>+/ecd<sup>1</sup> genotype) approximately 48 h after the eggs were laid. Sibling animals with the TB mutant morphology (Craymer 1980) are ecd<sup>1</sup>/TM6B and were used as controls in all experiments because they are wild type for the ecd, due to the ecd<sup>+</sup> allele carried on the TM6B chromosome. Thirty to 50 second instar larvae of ecd<sup>+/ecd<sup>1</sup></sup> and wild-type ecd<sup>1</sup>/TM6B. Tb siblings from each plate were transferred separately into vials containing standard cornmeal-dextrose-agar-yeast medium. The time and number of white pupa that formed were determined.

Feeding and locomotory behavior was assessed in early third instar larvae of both genotypes as described in Neckameyer (1996). The number of body contractile motions and mouth hook contractions were counted for 1 min. Ten animals were assayed in each of six independent experiments.

Electrophysiology

The preparations were taken from early and late third instar larvae staged as described in the preceding text. The larval dissections were performed as described in Cooper et al. (1995b). The physiological solution used is the same as previously described (Stewart et al. 1994).

Intracellular recording were made with microelectrodes filled with 3 M KCl (30–60 mΩ). The responses were recorded with a 1× LU head stage and an Axoclamp 2A amplifier to a VHS tape (Vetter, 400) as well as on-line to a PowerMac 9000 via a MacLab/4 s interface (ADIInstruments). All events were measured and calibrated with the MacLab Scope software version 3.5.4.

Anatomy

With the use of a fluorescent anti-HRP primary antibody and confocal microscopy, the Type I endings of the two major axons (Is and Ib) can be distinguished on the basis of their range of bouton size and total bouton complement (Atwood et al. 1995). With confocal microscopy, the quantitative data of bouton number, terminal length, and muscle dimensions were obtained. Fluorescent images of the nerve terminals were viewed with a Leica DM RE upright fluorescent microscope using a ×40 water-immersion objective with appropriate illumination. The composite images of Z-series were collected with a Leica TCS NT/SP confocal microscope for illustration. The number of varicosities can be counted from the images directly. The Leica software was used to measure and quantify the terminal length directly from the images.

Statistical analysis

Numerical data are represented as means ± SD. The one-way ANOVA test was used for comparison of means in the responsiveness to 20-HE, with P < 0.05 chosen as the level of significance. Two-way ANOVA test was used to examine the differences of morphological data. When the basic assumption of parametric ANOVA test was not valid, the nonparametric ANOVA rank test was used.

RESULTS

General morphology and behavior

To determine if the developmental rate is altered for the homozygous ecd<sup>+/ecd<sup>1</sup></sup> as compared with the control sibling ecd<sup>1</sup>/TM6B animals, the time it took for 50% of the population to form pupae was monitored. The genotypic identity of these animals was determined based on the dominant phenotypic marker for body size and shape, Tubby (Tb), which is present on the TM6B balancer chromosome. The relative differences shown for the late third instar stadium are prevalent throughout all the instars and pupa. To determine if the developmental rate is altered for the homozygous ecd<sup>+/ecd<sup>1</sup></sup> as compared with the control sibling ecd<sup>1</sup>/TM6B animals, the time it took for 50% of the population to form pupa was monitored. The time from the 2-h egg laying period until a pupa formed was considered the time to puation, and the time taken for all viable larva to become pupae was used as the total time taken. By plotting the commutative sum till total pupae formation occurred, a 50% index value could then be determined and compared between the genotypes (Fig. 1A). The ecd<sup>+/ecd<sup>1</sup></sup> had a substantial phase lag in developmental timing for the population to form pupae as indicated in the 50-h lag for the 50% index of mean puation time (Fig. 1B).

Since it was previously demonstrated that application of 20-HE on exposed neuromuscular preparations resulted in depression of synaptic transmission (Ruffner et al. 1999), we compared locomotive behavior between the wild-type and ecd<sup>1</sup>/ecd<sup>1</sup> larvae when endogenous 20-HE titers are expected to be different. Body-movement assays consisted of the rate in body-wall contraction and mouth-hook movements. These behavioral measures are standardized procedures to examine larval function (Neckameyer and Cooper 1998; Sewall et al. 1975). The early third instars corrected
Neuromuscular measures

The muscle m6 is innervated by both Ib and Is motor neurons (Kurdyak et al. 1994). Both Ib and Is neurons can be recruited together to produce a composite excitatory junction potential (EJP) as shown in Fig. 2A. The composite Ib and Is EJP amplitudes are significantly reduced in late third controls as compared with the ecd/ecd animals ($P < 0.01$, $n = 6$, t-test). There is no difference in the EJP amplitudes observed between the early third stage of ecd/ecd and controls (Fig. 2B). This suggests that the presence of ecdysone or a secondary effect of ecdysone results in the reduction in the EJP amplitude in late third instar of control sibling ecd/TM6B animals. To test the postulate that a lower titer of ecdysone in ecd/ecd is protecting the EJP amplitude in the late third stadium, direct application of 20-HE on exposed neuromuscular junctions was preformed. It was previously demonstrated that early third-staged larvae are susceptible to exposure to 20-HE in such a manner that the EJP amplitude would decrease rapidly within minutes (Ruffner et al. 1999). Such nongenomic actions were also demonstrated in crustaceans during intermolt (Cooper and Ruffner 1998). In both Drosophila and crayfish, the nongenomic actions of 20-HE were shown to be presynaptic, in decreasing the number of neurotransmitter containing vesicles to be released during nerve terminal depolarization. Changes in the EJP amplitudes on exposure to 20-HE were examined in the present study within late third instars of ecd/ecd and controls. Only the ecd/ecd flies showed a substantial decrease in EJP amplitude ($P < 0.05$, t-test, Fig. 3). This result suggests that these endogenous production of ecdysone in late third instar control flies already elicited the nongenomic actions and further application thus showed little effect.
there are significant differences as indicated between (Fig. 4), A 2 and ecd B 0.05, Tukey test; Fig. 5 shorter terminals than the ecd between the early and late third instar stages (controls, there were significant developmental differences between the reduced ecdysone genotype, ecd B 1, and controls in early third instars (Fig. 5). For both early and late third instars there are significant differences as indicated between ecd B 1 and control sibling ecd B/TM6B animals (Fig. 5A) although in both genotypes there is a significant difference between the early third and late third instars, thus indicating a developmental increase in the length of both the Is and Ib terminals (P < 0.05, Tukey test; Fig. 5A). The number of varicosities of the Ib versus Is terminals showed a significant difference between ecd B/ecd 1 and control sibling ecd B/TM6B animals for only the late third instar (—, P < 0.05, Tukey test; Fig. 5B). No significant differences could be found between the ecd B/ecd 1 and controls in early third instars (Fig. 5B). For ecd B/ecd 1 and controls, there were significant developmental differences between the early and late third instar stages (⋯⋯, P < 0.05, Tukey test; Fig. 5B). The developmental differences for both ecd B/ecd 1 and controls are that there are more varicosities along Is terminals as compared with Ib terminals (P < 0.05, Paired t-test).

**Nerve terminal morphology**

The two motor axons, Ib and Is, innervating muscle 6 and 7 are readily distinguishable by the size of the varicosities along the terminals (Fig. 4). The Ib axon has big terminal varicosities as compared with the Is axon. The ecd B/TM6B animals have shorter terminals than the ecd B/ecd 1 animals; this is readily seen in the overview of the terminals for ecd B/TM6B (Fig. 4A) and ecd B/ecd 1 (Fig. 4B1) as well as in the higher magnification (Fig. 4, A2 and B2) images. For both early and late third instars there are significant differences as indicated between ecd B/ecd 1 and control sibling ecd B/TM6B animals (Fig. 5A) although in both genotypes there is a significant difference between the early third and late third instars, thus indicating a developmental increase in the length of both the Is and Ib terminals (P < 0.05, Tukey test; Fig. 5A). The number of varicosities of the Ib versus Is terminals showed a significant difference between ecd B/ecd 1 and control sibling ecd B/TM6B animals for only the late third instar (—, P < 0.05, Tukey test; Fig. 5B). No significant differences could be found between the ecd B/ecd 1 and controls in early third instars (Fig. 5B). For ecd B/ecd 1 and controls, there were significant developmental differences between the early and late third instar stages (⋯⋯, P < 0.05, Tukey test; Fig. 5B). The developmental differences for both ecd B/ecd 1 and controls are that there are more varicosities along Is terminals as compared with Ib terminals (P < 0.05, Paired t-test).

**Discussion**

The ecdysoneless temperature-sensitive [l(3)ecd 1] mutant of *Drosophila* is a conditional larval lethal when raised at a restrictive temperature of 29°C (Garen et al. 1977). Ecdysteroid production in the larval ring gland is reduced as low as 10% of the control level in this mutant (Garen et al. 1977; Henrich et al. 1993), and pleiotropic effects of this mutant strain have also been examined (Redfern and Bownes 1983). It appears the brain, ring gland, and larval salivary glands are smaller in this mutant.

In this study, we investigated if behavior, physiology, sensivity to 20-HE, and nerve terminal structure showed differences between the reduced ecdysone genotype, ecd B/ecd 1, and wild-type control ecd B/TM6B animals during the early and late third instars when raised at 25°C. The ecd B mutants were able to survive through larval development and form pupae. However, the results demonstrate that the time to pupation is lengthened by about 50 h for the ecd B/ecd 1 as compared with the wild-type control siblings. There is variation in both phenotypes within the population, but with the use of the 50% to pupation index, comparisons between strains can be made. In addition to the lengthened larval cycle in the mutants, ecd B/ecd 1 animals also display behavioral differences as compared with controls. The rate of body-wall contraction and mouth-hook movements are reduced in the early third instar of ecd B/ecd 1 as compared with the wild-type ecd B/TM6B. The physiological measure of EJP amplitude for the combined Is and Ib terminals did not reveal any differences among the two genotypes during the early third instar, but the synaptic strength is reduced in the late third instars for the ecd B/TM6B. Since locomotive behavioral differences are observed between the ecd B/ecd 1 and the wild-type ecd B/TM6B in early third instars although without measurable differences in the experimentally evoked synaptic responses on m6, one is left to speculate that possible the CNS command of the motor neurons may be different during locomotion. In addition, we have not addressed the function of the other muscles associated with body contractions between these altered genotypes that may in part account for the behavioral observations. It is possible that there maybe pleiotropic effects in the ecd B mutant that result in a variety of developmental and behavioral problems, such as lower locomotor activities.

Application of exogenous 20-HE is still effective during the late third instar stadium for the ecd B/ecd 1 but not the controls. This suggests that endogenous production of ecdysone has already taken place in the wild-type but not the ecd B/ecd 1 larvae, thus the rapid nongenomic responses could still be observed in the late third ecd B/ecd 1 larvae. It has been shown that motor neurons in the hawkmoth, *Manduca sexta*, undergo various responses such as apoptosis, regression, and regrowth during various stages of development where levels of ecdysteroids are also high (Truman and Reiss 1995). In addition, mushroom bodies isolated from *Drosophila* during metamorphosis showed enhanced neurite outgrowth by direct application of 20-HE in culture (Kraft et al. 1998). However, the hormonal control of ecdysone on motor nerve terminal growth
and synaptic strength has not been fully addressed, thus our interest in the use of the ecd1/ecd1 mutant for this study.

The mechanisms that govern abrupt behavioral change associated with molting are also not well understood. In late third instar, the animals reverse from negative to positive phototactic behavior, and they slow down their locomotor functions to begin to form pupae. Reduced synaptic strength may contribute to their lower locomotor activity at this stage and could possibly be related to the increased ecdysone titers (Ruffner et al. 1999). The nongenomic action of ecdysone is not known but since fewer vesicles are released during evoked stimulation as measured from quantal analysis studies obtained at NMJs of Drosophila and crayfish there are some likely sites of action that are feasible (Cooper and Ruffner 1998; Ruffner et al. 1999). The protein-protein interactions of the SNARE proteins may possibly be effected by ecdysone, leading to fewer vesicles to be docked and released with evoked stimulation. It is possible that ecdysone could even be disrupting the protein interaction of already docked vesicles. Electron microscopy studies of the presynaptic terminals are needed to address this issue further. In addition, if steroidal action is affecting evoked calcium entry within the nerve terminal, this would result in fewer evoked vesicles. The synthetic ecdysone agonists RH-5849 is known to block a 4-aminopyridine (4-AP)-sensitive voltage-gated K⁺ channel (Ortego and Bower 1996), which could then alter the entry of calcium through voltage-gated calcium channels. Potentially with the use of calcium-sensitive indicators and confocal microscopy, this issue can be examined (Cooper et al. 1995a). At present, we are examining intact vesicle dynamics in the nerve terminals with the exposure of 20-HE with the use of the dye (FM1–43) that allows visualization of vesicle populations within nerve terminals. In addition, there are rapid effects on insect behavior when 20-HE or ecdysone is placed in their diet that have been correlated to rapid changes in the activity of sensory neurons (Tanaka et al. 1994).

Structurally the number of varicosities and the terminal length showed significant differences between ecd1/ecd1 and the wild-type ecd1/TM6B genotype in the late third instars. The relationship between terminal morphology and synaptic strength in Drosophila NMJs has been examined previously. For instance, a larger ratio of terminal size per muscle size gives rise to larger EJP amplitudes (Lennicka and Keshishian 2000). It is also suggested that the cell-adhesion molecule Fasciclin II plays a role in controlling synaptic stabilization and growth in Drosophila NMJs. In e76 mutant flies that possess a hypomorphic allele of the Fas II gene, there are fewer synapse-bearing nerve terminal varicosities (Stewart et al. 1996). Schuster et al. (1996a,b) demonstrated that the increase or decrease of axon sprouting in Drosophila NMJs depends on the expression level of Fas II. In these mentioned cases, the synaptic strength is maintained at a normal level for the muscle cell as a whole in spite of differences in the length and number of varicosities of the nerve terminals. Our results indicate that the ecd1/ecd1 late third instar larvae have longer nerve terminals with more varicosities than the ecd1/TM6B larvae. These morphological differences in the motor nerve terminals could account for the larger EJPs measured in the ecd1/ecd1 late third instars, although there are several other factors that need to be considered as well that will require further investigation to determine the true nature of why the EJPs are more pronounced in the ecd1/ecd1 late third instars. Such scenarios other than nerve terminal morphological differences are that the ecdysteroid titers are higher in the ecd1/TM6B larvae than the ecd1/ecd1 larvae and therefore a greater suppression in transmitter release is measured. In addition, the background strain ecd1/ TM6B larvae are of the Tubby (Tb) fly genotype, which is observed by the larvae being shorter yet wider than the ecd1/ecd1 strain. This difference in body morphology is also noted among the longitudinal muscles (e.g., m6), meaning that m6 is shorter and wider in ecd1/TM6B larvae than in ecd1/ecd1 larvae; this may also account for differences in total surface area.
of the muscle. This morphological difference of the muscle can lead to a difference in input resistance of the muscle fiber, thus altering the amplitude of the EJP given the same synaptic current. Studies are currently underway in our laboratories in examining such differences as synaptic physiology, nerve terminal development, and structure using these ecdysoneless strains with other background strains.

With several of the mechanisms underlying maintenance and modulation of synaptic strength during development and maturation being elucidated in various experimental systems, much still remains to be uncovered. In particular, little is known about the nongenomic actions of steroids on synaptic efficacy. One model system for studying fundamental questions about steroid action is the Drosophila neuromuscular junction. In Drosophila, the advantage of known identifiable cells with the powerful techniques of molecular genetics and the ability to perform anatomical analysis as well as electrophysiological measures allow experimental insights that are not possible, at present, in other systems. Taking advantage of mutations that result in lower ecdysone titers allows further investigations into the steroid actions of ecdysone. There are well-documented genomic effects of steroids such as estradiol, aldosterone, vitamin D3, and cortisol. Processes such as activation of the IP3-, cGMP-, and cAMP-signaling pathways and increased release of internal calcium are future avenues to be investigated (Orchink et al. 1991; Thummel 1996; Wehling 1995). Since this model system has played, and continues to play, important roles in answering questions of regulation of chemical synaptic transmission, we feel that investigations of steroid action will be fruitful in providing answers to the mechanistic actions of a variety of steroids.

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