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

HAO LI,[1] DOUG HARRISON,[1] GRACE JONES,[1] DAVY JONES,[2] AND ROBIN L. COOPER[1]
[1]*School of Biological Sciences, University of Kentucky, Lexington 40506-0225; and* [2]*Graduate Center for Toxicology, Chandler Medical Center, University of Kentucky, Lexington, Kentucky 40506-0305*

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Li, Hao, Doug Harrison, Grace Jones, Davy Jones, and Robin L. Cooper. Alterations in development, behavior, and physiology in *Drosophila* larva that have reduced ecdysone production. *J Neurophysiol* 85: 98–104, 2001. We investigated behavior, physiology, sensitivity to exogenous application of ecdysone, and nerve terminal structure for differences between the reduced ecdysone genotype, ecd^1/ecd^1, and wild-type control $ecd^1/TM6B$ animals during the early and late third instars when raised at 25°C. The ecd^1 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^1/ecd^1 as compared with the wild-type control siblings. In addition to the lengthened larval cycle in the mutant, ecd^1/ecd^1 animals, they 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^1/ecd^1 as compared with controls. The physiological measure of excitatory junction potential 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 controls. Application of exogenous ecdysone is still effective during the late third instar for the ecd^1/ecd^1 but not the controls. This suggests that endogenous production of ecdysone have already taken place in the wild-type but not the ecd^1/ecd^1 larvae, thus the rapid nongenomic responses could still be observed in the late third ecd^1/ecd^1 larvae. Structurally the number of varicosities and the terminal length showed significant differences between ecd^1/ecd^1 and the wild-type $ecd^1/TM6B$ genotype in the late third instars.

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; Farkaš and Šutáková 1998, 1999; Henrich et al. 1993, 1999; Riddford 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). The majority of currently described actions of ecdysteroids are genome based (Levine and Weeks 1996; Segraves 1994; 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 1999; Chang 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*, ecd^1, which contains a recessive, temperature-shock-sensitive allele of a gene required for ecdysteroid production. The homozygote ecd^1/ecd^1 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 $ecd^1/TM6B$, *Tubby* (*Tb*) fly genotype (Lindsley and Zimm 1992) used for these experiments. Eggs from $ecd^1/TM6B$, Tb

Address for reprint requests: R. L. Cooper, 101 T.H. Morgan School of Biological Sciences, University of Kentucky, Lexington, KY 40506-0225 (E-mail: RLCOOP1@pop.uky.edu).

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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^1/ecd^1 genotype) approximately 48 h after the eggs were laid. Sibling animals with the TB mutant morphology (Craymer 1980) are $ecd^1/TM6B$ and were used as controls in all experiments because they are wild type for the *ecd*, due to the ecd^+ allele carried on the *TM6B* chromosome. Thirty to 50 second instar larvae of ecd^1/ecd^1 and wild-type $ecd^1/TM6B$, *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 (ADInstruments). 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. 1993). 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^1/ecd^1 as compared with the control sibling $ecd^1/TM6B$ 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^1/ecd^1 as compared with the control sibling $ecd^1/TM6B$ 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 pupation, 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. 1*A*). The ecd^1/ecd^1 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 pupation time (Fig. 1*B*).

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^1/ecd^1 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

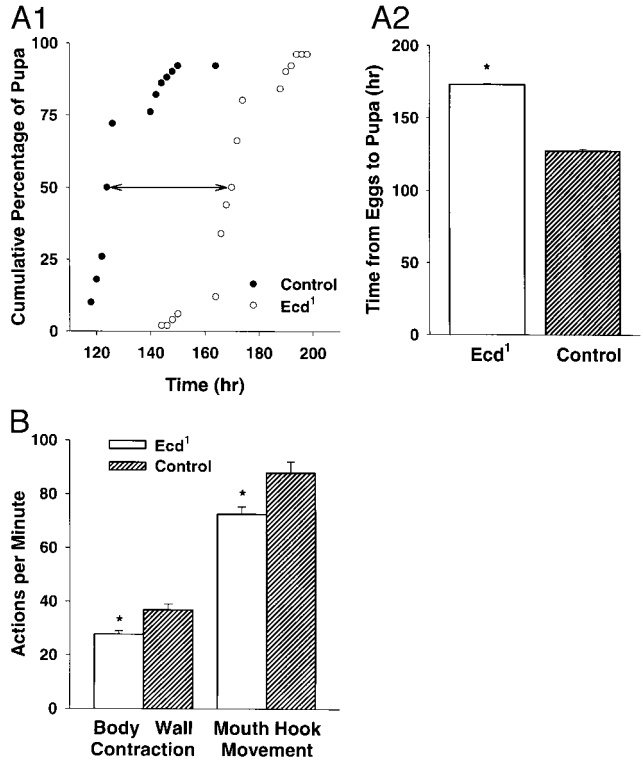

FIG. 1. *A1*: the time courses of larvae development. *A2*: the durations are compared from egg laying to 50% of them forming white pupae for both genotypes. *B*: locomotion and feeding assays of ecd^1/ecd^1 and wild-type $ecd^1/TM6B$ larvae. The body wall contractions (locomotion) and mouthhook movements (feeding) were counted within a 1-min period. The number of animals tested in each genotype was 20. The error bars represent the SE.

for the developmental phase lag were used in the behavioral tests. The ecd^1/ecd^1 had a slower mean rate in body wall contraction and mouth hook movement than control animals ($P < 0.05$, *t*-test; Fig. 1*C*).

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. 2*A*. The composite Ib and Is EJP amplitudes are significantly reduced in late third controls as compared with the ecd^1/ecd^1 animals ($P < 0.01$, $n = 6$, *t*-test). There is no difference in the EJP amplitudes observed between the early third stage of ecd^1/ecd^1 and controls (Fig. 2*B*). 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

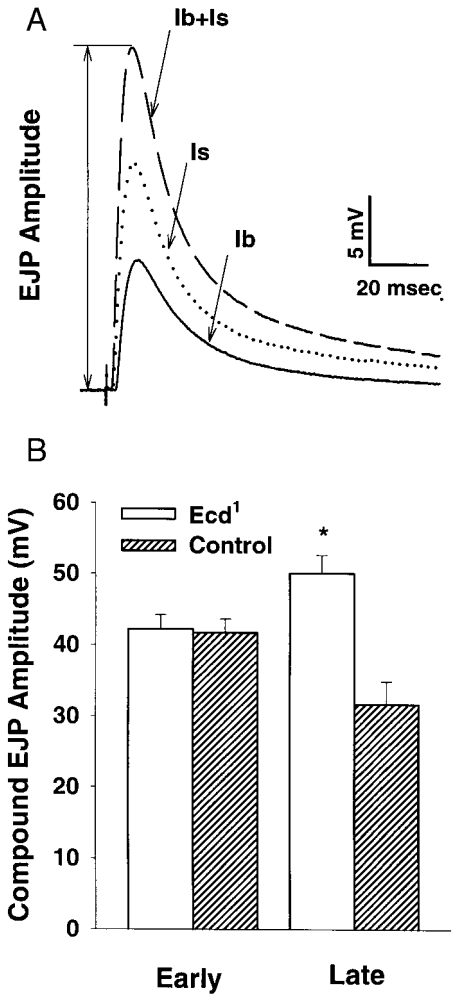

FIG. 2. The ventral abdominal muscle, m6 is innervated by 2 excitatory axons, Ib and Is. The Ib has large varicosities but gives rise to small excitatory junction potentials (EJPs) where as the Is has small varicosities and produces large EJPs. *A*: a compound EJP when Ib and Is terminals are both recruited. *B*: comparison of compound EJP amplitudes recorded from m6 of early and late 3rd instar larvae of both genotypes. The EJP amplitudes are significantly reduced in late 3rd $ecd^1/TM6$ larvae, $P < 0.01$; 6 animals were tested for each of the stages and strains. The stimulation frequency was 1 Hz, and average of 100 compound EJP amplitudes recorded from each preparation was used in the analysis.

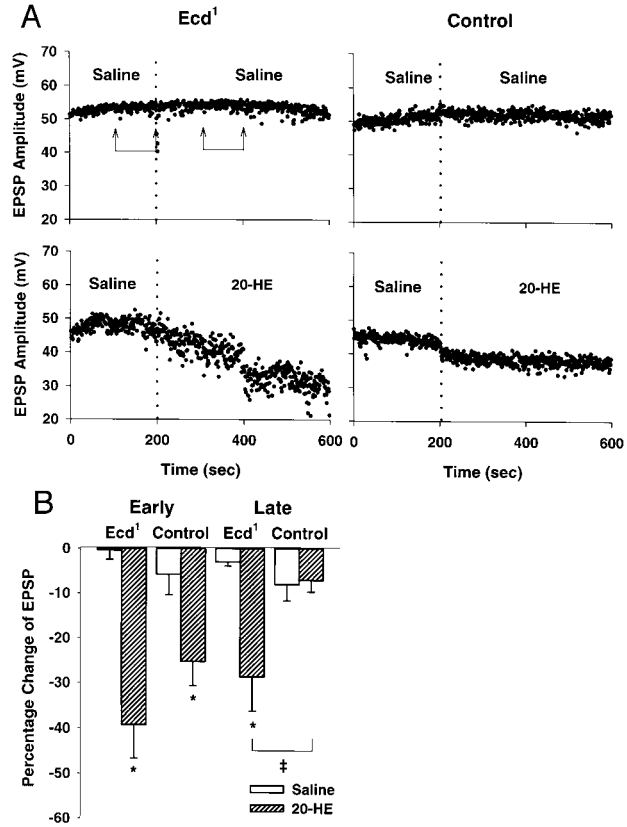

FIG. 3. The effects of 20-hydroxyecdysone (20-HE, 10 μM) on the compound EJPs. Segmental nerve 4 was stimulated while the EJPs were recorded with an intracellular electrode in muscle 6 of the stimulated segment. *A*: isolated responses recorded from ecd^1/ecd^1 and wild-type $ecd^1/TM6B$ late 3rd instar larvae are shown while both Ib and Is motor neurons recruited. The preparations were bathed in saline, and then the saline was changed with either saline or 10 μM 20-HE while recording. One hundred EJPs recorded before and starting 100 s after the saline was changed from each preparation were averaged for subsequent analysis as illustrated by the line between the arrows in the *top left panel*. $\cdots$, the time in which the solution was exchanged. *B*: the percentage change ($\pm$SE) of EJPs is shown for the 6 animals used in each experimental condition.

sibling $ecd^1/TM6B$ animals. To test the postulate that a lower titer of ecdysone in ecd^1/ecd^1 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^1/ecd^1 and controls. Only the ecd^1/ecd^1 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.

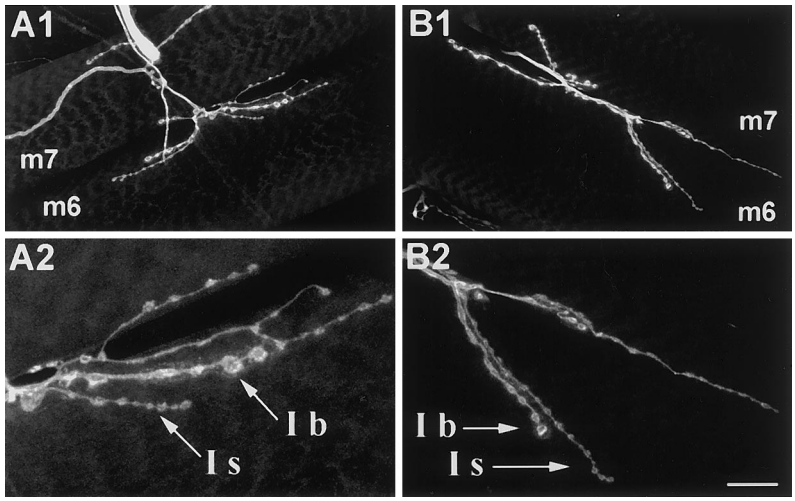

FIG. 4. Terminals of Ib and Is motor neurons on muscle 6 and 7 of segment 4. Imaged by confocal microscopy after treatment with fluorescently tagged anti-HRP antibody. Full view of the entire extent of innervation by the 2 terminals on m6 for representative $ecd^1/TM6B$ (*A1*) and ecd^1/ecd^1 (*B1*) strains. Higher magnification of the terminals to illustrate the Is and Ib anatomical differences in $ecd^1/TM6B$ (*A2*) and ecd^1/ecd^1 (*B2*). Quantification of terminal length and numbers of varicosities are readily made at this magnification. Scale bar: *A1*, 24; *B1*, 28; *A2*, 7.7; and *B2*, 14 μm.

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 $ecd^1/TM6B$ animals have shorter terminals than the ecd^1/ecd^1 animals; this is readily seen in the overview of the terminals for $ecd^1/TM6B$ (Fig. 4*A1*) and ecd^1/ecd^1 (Fig. 4*B1*) 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^1/ecd^1 and control sibling $ecd^1/TM6B$ animals (Fig. 5*A*) 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. 5*A*). The number of varicosities of the Ib versus Is terminals showed a significant difference between ecd^1/ecd^1 and control sibling $ecd^1/TM6B$ animals for only the late third instar (—, $P < 0.05$, Tukey test; Fig. 5*B*). No significant differences could be found between the ecd^1/ecd^1 and controls in early third instars (Fig. 5*B*). For ecd^1/ecd^1 and controls, there were significant developmental differences between the early and late third instar stages ($\cdots$, $P < 0.05$, Tukey test; Fig. 5*B*). The developmental differences for both ecd^1/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, sensitivity to 20-HE, and nerve terminal structure showed differences between the reduced ecdysone genotype, ecd^1/ecd^1, and wild-type control $ecd^1/TM6B$ animals during the early and late third instars when raised at 25°C. The ecd^1 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^1/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^1/ecd^1 animals also display behavioral differences as compared with controls. The rate of body-wall contraction and mouthhook movements are reduced in the early third instar of ecd^1/ecd^1 as compared with the wild-type $ecd^1/TM6B$. 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 $ecd^1/TM6$. Since locomotive behavioral differences are observed between the ecd^1/ecd^1 and the wild-type $ecd^1/TM6B$ 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^1 mutant that result in a variety of developmental and behavioral problems, such as lower locomotive activities.

Application of exogenous 20-HE is still effective during the late third instar stadium for the ecd^1/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^1/ecd^1 larvae, thus the rapid nongenomic responses could still be observed in the late third ecd^1/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

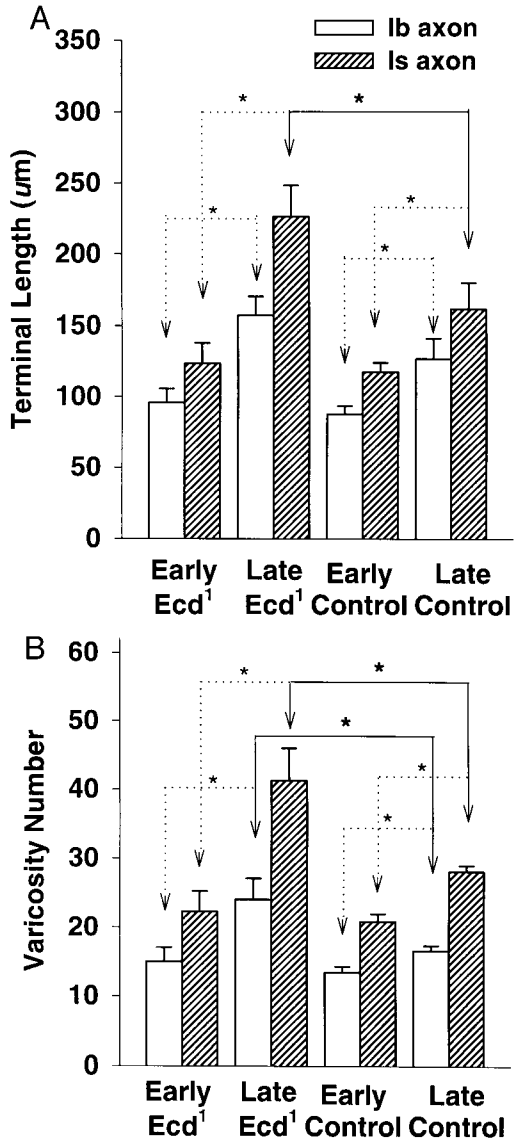

FIG. 5. Quantification of neuromuscular morphology. *A*: the total length of Ib and Is terminals are compared for early and late 3rd instars for both genotypes. The Is terminals are significantly longer than the Ib at each developmental stage in both genotypes. Both Ib and Is terminals are significantly longer at late 3rd stadium than at early 3rd one for both strains (*A*, ···). The only significant difference between genotypes of the same developmental stage is that the Is terminals are longer in the late ecd^1/ecd^1 as compared with the wild-type $ecd^1/TM6B$ (—). *B*: the number of varicosities is greater for the Is terminals than the Ib terminals at each developmental stage. There are differences between developmental stages (···) and the genotypes in the late 3rd stage for the number of varicosities of Ib and Is terminals (—). Nine animals of each genotype and each instar were examined.

and synaptic strength has not been fully addressed, thus our interest in the use of the ecd^1/ecd^1 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 possible 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 ecd^1/ecd^1 and the wild-type $ecd^1/TM6B$ 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 (Lnenicka 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 ecd^1/ecd^1 late third instar larvae have longer nerve terminals with more varicosities than the $ecd^1/TM6B$ larvae. These morphological differences in the motor nerve terminals could account for the larger EJPs measured in the ecd^1/ecd^1 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 ecd^1/ecd^1 late third instars. Such scenarios other than nerve terminal morphological differences are that the ecdysteroid titers are higher in the $ecd^1/TM6B$ larvae than the ecd^1/ecd^1 larvae and therefore a greater suppression in transmitter release is measured. In addition, the background strain $ecd^1/TM6B$ larvae are of the *Tubby* (*Tb*) fly genotype, which is observed by the larvae being shorter yet wider than the ecd^1/ecd^1 strain. This difference in body morphology is also noted among the longitudinal muscles (e.g., m6), meaning that m6 is shorter and wider in $ecd^1/TM6B$ larvae than in ecd^1/ecd^1 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 know 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 results 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 IP_3-, 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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REFERENCES

ATWOOD HL, GOVIND CK, AND WU C-F. Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. *J Neurobiol* 24: 1008–1024, 1993.

BAEHRECKE EH. Ecdysone signaling cascade and regulation of Drosophila metamorphosis. *Arch Insect Biochem Physiol* 33: 231–244, 1996.

BAULIEU EE. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Recent Prog Horm Res* 52: 1–32, 1997.

BENTEN WP, LIEBERHERR M, STAMM O, WREHLKE C, GUO Z, AND WUNDERLICH F. Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. *Mol Biol Cell* 10: 3113–3123, 1999.

CAYRE M, STRAMBI C, STRAMBI A, CHARPIN P, AND TERNAUX JP. Dual effect of ecdysone on adult cricket mushroom bodies. *Eur J Neurosci* 12: 633–642, 2000.

CHANG AS AND CHANG SM. Nongenomic steroidal modulation of high-affinity serotonin transport. *Biochim Biophys Acta* 1417: 157–166, 1999.

CHRIST M, HASEROTH K, FALKENSTEIN E, AND WEHLING M. Nongenomic steroid actions: fact or fantasy? *Vitam Horm* 57: 325–373, 1999

COOPER RL, LI H, AND SOUTHARD RC. The non-genomic actions of 20-HE in *Drosophila* and crustaceans. *Symposium "Ecdysone 2000," Rapperswil, Switzerland,* 2000.

COOPER RL, MARIN L, AND ATWOOD HL. Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. *J Neurosci* 15: 4209–4222, 1995a.

COOPER RL AND NECKAMEYER WS. Dopaminergic neuromodulation of motor neuron activity and neuromuscular function in *Drosophila melanogaster*. *Comp Biochem Physiol B Biochem Mol Biol* 122: 199–210, 1999.

COOPER RL AND RUFFNER ME. Depression of synaptic efficacy at intermolt in crayfish neuromuscular junctions by 20-hydroxyecdysone, a molting hormone. *J Neurophysiol* 79: 1931–1941, 1998.

COOPER RL, STEWART BA, WOJTOWICZ JM, WANG S, AND ATWOOD HL. Quantal measurement and analysis methods compared for crayfish and *Drosophila* neuromuscular junctions and rat hippocampus. *J Neurosci Methods* 61: 67–78, 1995b.

CRAYMER L. New mutants report. *Drosophila Information Service* 55: 197–200, 1980.

CROMARTY SI AND KASS-SIMON G. Differential effects of a molting hormone, 20-hydroxyecdysone, on the neuromuscular junctions of the claw opener and abdominal flexor muscles of the American lobster. *Comp Biochem Physiol* 120: 289–300, 1998.

FARKAŠ R AND ŠUTÁKOVÁ G. Ultrastructural changes of *Drosophila* larval and prepupal salivary glands cultured in vitro with ecdysone. *In Vitro Cell Dev Biol Anim* 34: 813–823, 1998.

FARKAŠ R AND ŠUTÁKOVÁ G. Developmental regulation of granule size and numbers in larval salivary glands of *Drosophila* by steroid hormone ecdysone. *Cell Biol Int* 23: 671–676, 1999.

GAREN A, KAUVAR L, AND LEPESANT J-A. Roles of ecdysone in *Drosophlia* development. *Proc Natl Acad Sci USA* 74: 5099–5103, 1977.

HANAYA R, SASA M, ISHIHARA K, AKIMITSU T, IIDA K, AMANO T, SERIKAWA T, ARITA K, AND KURISU K. Antiepileptic effects of 20-hydroxyecdysone on convulsive seizures in spontaneously epileptic rats. *Jpn J Pharmacol* 74: 331–335, 1997.

HE P, LI H, BONNER PH, HARRISON D, JONES G, JONES D, AND COOPER RL. Non-genomic physiological responses of ecdysone (20-HE) at the neuromuscular junctions of larval *Drosophila* that lack the ability to produce ecdysone. *Soc Neurosci Abstr* 327: 12, 1998.

HENRICH VC, LIVINGSTON L, AND GILBERT LI. Developmental requirements for the ecdysoneless (*ecd*) locus in *Drosophila melanogaster*. *Dev Genet* 14: 369–377, 1993.

HENRICH VC, RYBCZYNSKI R, AND GILBERT LI. Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. *Vitam Horm* 55: 73–125, 1999.

JACOBS GA AND WEEKS JC. Postsynaptic changes at a sensory-to-motorneuron synapse contribute to the developmental loss of a reflex behavior during insect metamorphosis. *J Neurosci* 10: 1341–1356, 1990.

KIM SJ, PARK JG, AND LEE CC. Transcript titers of ecdysteroid receptor components vary between tissues and stages during Drosophila development. *Mol Cells* 9: 61–66, 1999.

KRAFT R, LEVINE RB, AND RESTIFO LL. The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. *J Neurosci* 18: 8886–8899, 1998.

KURDYAK P, ATWOOD HL, STEWART BA, AND WU C-F. Differential physiology and morphology of motor axons to ventral longitudinal muscle in larval *Drosophila*. *J Comp Neurol* 350: 463–472, 1994.

LEVINE RB AND WEEKS JC. Cell culture approaches to understanding the actions of steroid hormones on the insect nervous system. *Dev Neurosci* 18: 73–86, 1996.

LI H, HARRISON DA, AND COOPER RL. Development of *Drosophila* larvae neuromuscular junction: maintaining synaptic strength. *Am Zool* 39: 249A, 1999a.

LI H, HARRISON DA, AND COOPER RL. Development of *Drosophila* larvae neuromuscular junction: maintaining synaptic strength. *Soc Neurosci Abstr* 25: 792.9, 1999b.

LINDSLEY DL AND ZIMM GG. *The Genome of Drosophila melanogaster*. New York: Academic, 1992.

LNENICKA GA AND KESHISHIAN H. Identified motor terminals in *Drosophila* larvae show distinct differences in morphology and physiology. *J Neurobiol* 43: 186–197, 2000.

NECKAMEYER W. Multiple roles for dopamine in *Drosophila* development. *Dev Biol* 176: 209–219, 1996.

NECKAMEYER WS AND COOPER RL. GABA transporters in *Drosophila melanogaster*: developmental expression, behavior, and physiology. *Invert Neurosci* 3: 279–294, 1998.

ORCHINIK M, MURRAY TF, AND MOORE FL. A corticosteroid receptor in neuronal membranes. *Science* 252: 1848–1851, 1991.

ORTEGO F AND BOWERS WS. Induction of autotomy in the American bird grasshopper *Schistocerca americana* (Drury) by the ecdysone agonist RH-5849 and investigation of its mode of action. *Experientia* 52: 42–50, 1996.

REDFERN CPF AND BOWNES M. Pleiotropic effects of the "*ecdysoneless-1*" mutation of *Drosophlia melanogaster*. *Mol Gen Genet* 189: 432–440, 1983.

RIDDFORD LM. Hormone action at the cellular level. In: *Comprehensive Insect Physiology Biochemistry and Pharmacology*, edited by Kerkut GA and Gilbert LI. New York: Pergamon, 1985, vol. 8, p. 37–84.

RUFFNER ME, CROMARTY SI, AND COOPER RL. Depression of synaptic efficacy in high- and low-output *Drosophila* neuromuscular junctions by the molting hormone (20-hydroxyecdysone) during intermolt. *J Neurophysiol* 81: 788–794, 1999.

SCHMIDT BM, CHRIST M, FALKENSTEIN E, AND WEHLING M. Nongenomic steroid actions: completing the puzzle. Aldosterone as an example. *Exp Clin Endocrinol Diabetes* 106: 441–445, 1998.

SCHUSTER CM, DAVIS GW, FETTER RD, AND GOODMAN CS. Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. *Neuron* 17: 641–654, 1996a.

SCHUSTER CM, DAVIS GW, FETTER RD, AND GOODMAN CS. Genetic dissection of structural and functional components of synaptic plasticity. II. Fasciclin II controls presynaptic structural plasticity. *Neuron* 17: 655–667, 1996b.

SEGRAVES WA. Steroid receptors and other transcription factors in ecdysone response. *Recent Prog Hormone Res* 49: 167–195, 1994.

SEWELL D, BURNET B, AND CONNOLLY K. Genetic analysis of larval feeding behavior in *Drosophlia melanogaster*. *Genet Res Camb* 24: 163–173, 1975.

STEEL CGH AND DAVEY KG. Integration in the insect endocrine system. In: *Comprehensive Insect Physiology Biochemistry and Pharmacology*, edited by Kerkut GA and Gilbert LI. New York: Pergamon, 1985, vol. 8, p. 1–36.

STEWART BA, ATWOOD HL, RENGER JJ, WANG J, AND WU C-F. Improved stability of *Drosophila* larval neuromuscular preparation in haemolymph-like physiological solutions. *J Comp Physiol [A]* 175: 179–191, 1994.

STEWART BA, SCHUSTER CM, GOODMAN CS, AND ATWOOD HL. Homeostasis of synaptic transmission in Drosophila with genetically altered nerve terminal morphology. *J Neurosci* 16: 3877–3886, 1996.

TANAKA Y, ASAOKA K, AND TAKEDA S. Different feeding and gustatory responses to ecdysone and 20-hydroxyecdysone by larvae of the silkworm, *Bombyx mori*. *J Chem Ecol* 20: 125–133, 1994.

THUMMEL CS. Flies on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet* 12: 306–310, 1996.

TRUMAN JW. Steroid receptors and nervous system metamorphosis in insects. *Dev Neurosci* 18: 87–101, 1996.

TRUMAN JW AND REISS SW. Neuromuscular metamorphosis in the moth Manduca sexta: hormonal regulation of synapse loss and remodeling. *J Neurosci* 15: 4815–4826, 1995.

WATSON CS AND GAMETCHU B. Membrane-initiated steroid actions and the proteins that mediate them. *Proc Soc Exp Biol Med* 220: 9–19, 1999.

WEHLING M. Looking beyond the dogma of genomic steroid action: insights and facts of the 1990s. *J Mol Med* 73: 439–447, 1995.

WHITE KP, HURBAN P, WATANABE T, AND HOGNESS DS. Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* 276: 114–117, 1997.