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EFFECTS OF THE ECDYSONELESS MUTANT ON SYNAPTIC EFFICACY AND STRUCTURE AT THE NEUROMUSCULAR JUNCTION IN *DROSOPHILA* LARVAE DURING NORMAL AND PROLONGED DEVELOPMENT

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Abstract—Hormonal regulation in development and maintenance of synaptic transmission involves examination of both the presynaptic and postsynaptic components and a system in which the hormones can be controlled. We used the ecdysoneless heat-sensitive mutation ($l(3)ecd^1/l(3)ecd^1$) of *Drosophila* to provide the ability to regulate endogenous ecdysone production at various larval stages. In conjunction, we used the neuromuscular junctions of *Drosophila* since they offer the advantage of assessable preparations for both morphological and physiological measures. The growth in the Ib and Is motor nerve terminals and the corresponding muscle 6 in segment 4 of the larval *Drosophila* throughout the third instar stage in the presence of normal and a much reduced endogenous ecdysone level was investigated. Muscle 6 and the motor nerve terminals parallel in growth throughout the third instar. The nerve terminals increase in length and varicosity number, thus providing an increase in the number of synaptic release sites. The ecdysoneless larvae also show an increase in muscle size, however the Is and Ib motor nerve terminals do not mature to the extent of the wild-type ecdysone producing flies. The motor nerve terminal length is shorter with fewer numbers of varicosities per terminal. In spite of a shorter nerve terminal and fewer varicosities, with an increasing muscle fiber, the compound excitatory junctional potentials of Ib and Is in the ecdysoneless flies are larger, which is suggestive of synaptic structural modification.

This study demonstrates ecdysone's role in modifying nerve terminal development and neuromuscular junction function.

Key words: neurotransmission, neuromuscular junction, synapse, ecdysone, hormone, insect.

Synaptic communication, which is conducted as graded electrical potentials within a postsynaptic cell, is greatly influenced by size of the postsynaptic cell. As the postsynaptic cell increases in size, either more transmitter must be released or alterations in receptivity to the presynaptic signals will have to occur in order to maintain a consistent postsynaptic potential. Neuromuscular junctions (NMJs) of *Drosophila* offer a unique opportunity to investigate synaptic efficacy and maintenance during development since they contain identified motor neurons and muscle fibers. In addition, the larval temporal development can be tightly monitored for each instar. It has been shown that 20-hydroxyecdysone (20-HE), the active form of ecdysone, is associated with developmental changes during metamorphosis in insects (Riddford, 1985; Steel and Davey, 1985; von Richter et al., 1989; Farkas and Sutakova, 1998; Henrich et al., 1993, 1999). This hormone is important in causing the behavioral and physical changes during the developmental stages of each molt (Truman, 1996). It is also well established that ecdysone has effects in altering morphology of cultured neurons of *Manduca* (Truman, 1996), crickets (Cayre et al., 2000) and *Drosophila* mushroom bodies (Kraft et al., 1998). The majority of currently described actions of ecdysteroids are genome-based (Segraves, 1994; Levine and Weeks, 1996; Thummel, 1996). The non-genomic effects of 20-HE in reducing synaptic efficacy at NMJs have also been demonstrated previously in cockroach (von Richter, 1979), *Drosophila* (Ruffner et al., 1999) and crayfish (Cooper and Ruffner, 1998).

Taking advantage of genetic manipulation in *Drosophila*, we used a mutation that has altered levels of ecdysone referred to as the ecdysoneless mutant ecd^1/ecd^1 (Garen et al., 1977). This mutation allows investigation into the role of the steroid hormone in the process of neuromuscular development and function within the intact animal, thus avoiding problems associated with culturing neurons and muscles. The ecdysoneless temperature-sensitive [$l(3)ecd^1/l(3)ecd^1$] 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

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Abbreviations: EcR, ecdysone receptor; EJP, excitatory junctional potential; 20-HE, 20-hydroxyecdysone; HRP, horseradish peroxidase; MANOVA, multivariate analysis of variance; NMJ, neuromuscular junction.

and Bownes, 1983; Farkas and Suakova, 1999). It appears that the brain, ring gland and larval salivary glands are smaller in this mutant, as compared to background strains. However, the hormonal control of NMJ development has not been fully studied. We have shown earlier that exogenous application of 20-HE during the late third instar to the ecd^1/ecd^1 mutant will result in a decrease in the excitatory junctional potential (EJP) amplitude, whereas the background strains with normal ecdysone levels will not (Li et al., 2001).

Thus, the purpose of this study was to determine the developmental pattern in motor nerve terminal morphology (i.e. length and numbers of varicosities) for the low- and high-output motor neurons in the presence of normal and suppressed levels of ecdysteroids. In addition, the growth of the muscle and measures of synaptic responses were correlated to motor nerve terminal morphological characteristics. Preliminary results of this study have appeared previously (Li et al., 1999a,b; Cooper et al., 2000).

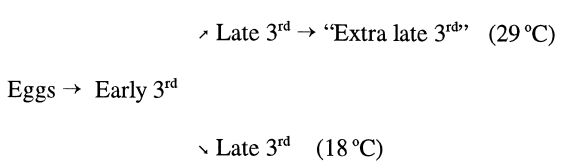
EXPERIMENTAL PROCEDURES

Strains

The Canton S (CS, wild-type) and *l(3)ecd¹/TM6* strains of *Drosophila melanogaster* (Garen et al., 1977; Henrich et al., 1993) were cultured at 18°C on a standard cornmeal–dextrose–agar–yeast medium. A 1-h prepulse of egg laying at 25°C was performed to clear the females of stored eggs in the ovipositor. Subsequently, eggs from both strains were collected separately at 25°C for 2-h periods on apple juice-agar plates with yeast paste. The eggs were allowed to hatch and develop at 18°C. About 100 early third instar larvae of CS or $l(3)ecd^1/l(3)ecd^1$ strains were collected approximately 4–6 h after they molted, and half of them were transferred to 29°C. Remaining larvae were kept at 18°C to allow further development as illustrated in Scheme 1. The genotype $l(3)ecd^1/l(3)ecd^1$ were selected from the progeny larvae of the $l(3)ecd^1/TM6$ strain. The wild-type CS flies served as controls. When third instars are transferred to 29°C, the CS strain will form pupae in 2 days, whereas the $l(3)ecd^1/l(3)ecd^1$ strain will remain as larvae for at least 3 weeks.

Anatomy

Preparations were taken from each stage for all strains as described above. A fluorescent anti-horseradish peroxidase (HRP) primary antibody (ICN Pharmaceuticals, CA, USA) was used to aid in identifying the motor nerve terminals on muscle 6 (m6) (Johansen et al., 1989b). The terminals are primarily type I endings from the two major axons (Is and Ib) (Atwood et al., 1993). 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 confocal images were quantified for varicosity number, terminal length, and muscle dimensions with the Leica confocal software.



Scheme 1.

Electrophysiology

The larval dissections were performed as described in Cooper et al. (1995b). In brief, a longitudinal mid-dorsal incision was made and the edges pinned so that the preparation was spread out on a glass slide with magnetic tape adhered to one side in the preparation dish, as originally described for studies in the leech (Muller et al., 1981). Internal organs were carefully removed to expose the body wall muscles, particularly the ventral longitudinal muscles of segments 3 and 4. The recordings were obtained from the prominent longitudinal m6.

The physiological solution used is the same as previously described (Stewart et al., 1994). In brief, the physiological saline contains (in mM): 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 5 BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid). All experiments were performed at room temperature (19–21°C).

Intracellular recordings 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 online to a PowerMac 9000 via a MacLab/4s interface (ADInstruments, Mountain View, CA, USA). All events were measured and calibrated with the MacLab Scope software version 3.5.4.

Supplement of 20-HE in the diet

Since the ecdysoneless (ecd^1/ecd^1) third instars, while maintained at 29°C, will not form pupae for 2–3 weeks we chose to examine if supplementing the diet with 20-HE would accelerate pupal formation. The ecd^1/ecd^1 flies were divided into two groups: control and 20-HE fed (Sigma, St. Louis, MO, USA; 1 mg/ml, with the use of 5% ethanol, in a yeast-paste diet of 0.5 g yeast/ml). This feeding paradigm is similar to that successfully used by Garen et al. (1977). The early third instars were moved at 18–29°C for 1 day prior to the start of the experimental feeding paradigm. Forty animals in each group were used. The percentage of animals over time that pupated were determined. Every 4 h, the number of larvae forming pupae outside the yeast paste was counted. The percent of larvae forming pupae over time was used as an index to assess the dietary supplemental effects of 20-HE.

Statistical analysis

Numerical data were represented as mean ± S.E.M. Since correlations among the variables of interests can be expected, multivariate analysis of variance (MANOVA), followed by Tukey test (between-group comparison) or planned comparisons (within-group comparison) (Statistica, Statsoft, Tulsa, OK, USA), was used to examine the differences of morphological and physiological data, with $P < 0.05$ chosen as the level of significance. The sample size $n = 70$ for morphological data and $n = 40$ for physiological experiments (10 animals for each experimental condition). When the basic assumption of parametric MANOVA tests was not valid, the non-parametric MANOVA rank tests were used.

RESULTS

The development of the nerve terminals, Is and Ib, and m6 were quantified for the early and late third instars in both CS and ecd^1/ecd^1 strains at 18°C and 29°C to examine the effects of normal ecdysone expression (18°C) and a much reduced level (29°C). The identification of Is and Ib terminals is readily obtained with the use of the fluorescent-tagged anti-HRP antibody and composites of optical sections of the NMJ with confocal microscopy. To illustrate the developmental changes and the effects of ecdysone on nerve terminals, representative images are

shown in Fig. 1. The nerve terminals of the ecd^1/ecd^1 (Fig. 1A1, B1) and CS (Fig. 1A2, B2) strains continue to develop from the early to late third instars at 18°C. When early third instars of both ecd^1/ecd^1 and CS are transferred from 18°C to the restrictive temperature of 29°C for the heat-sensitive allele of the ecd^1/ecd^1 strain, a continued development is observed in both strains. The CS strain will pupate within 48 h at 29°C, whereas the ecd^1/ecd^1 flies will not. The ecdysoneless larvae will continue to remain as third instars for up to 3 weeks when they are maintained at 29°C, at which time many will attempt to pupate, but they die as pupae. The ecdysoneless larvae after 1 week appear to be in good health as assayed by locomotive behavior and robust amplitudes of evoked EJPs measured in m6. In addition, the resting membrane potentials of the muscle appear normal. For these reasons, we chose to use the 1-week staged ecd^1/ecd^1 larvae for further analysis of terminal morphology and physiological responses. The morphology of the Ib and Is motor nerve terminals from late ecd^1/ecd^1 (Fig. 1C1) and CS (Fig. 1C2), raised at 29°C, are still able to be differentiated. The extra-late (1 week) ecd^1/ecd^1 animals (29°C) show a significant variation in terminal morphology as compared to the 48-h ecd^1/ecd^1 (29°C) flies (Fig. 1D). The varicosities of the terminals are in clusters instead of being spread out over the muscle surface as seen in the earlier developmental periods. With high magnification of the terminals, they are able to be identified as Is or Ib for quantification purposes.

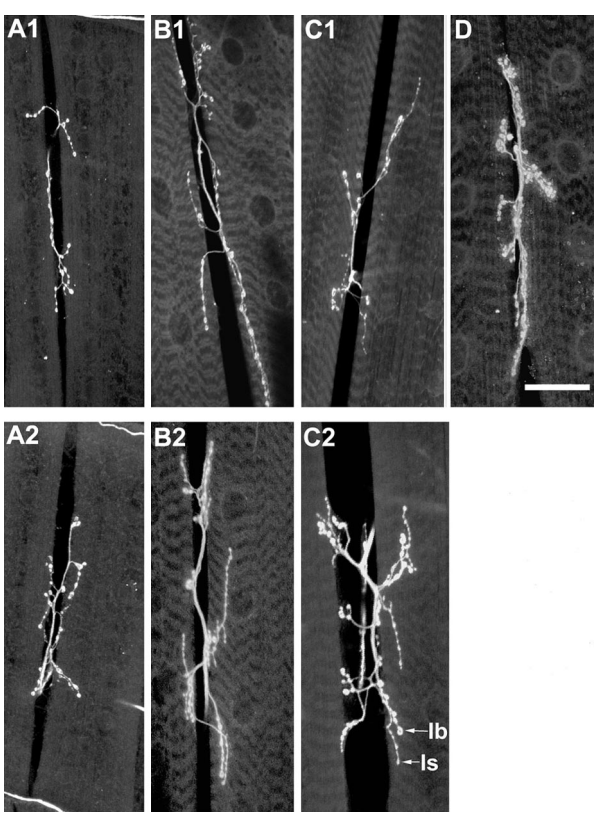


Fig. 1. NMJs on m6 and m7 in segment 4 in the ecd^1 mutant (upper panel) and in the wild-type CS strain (lower panel). Motor terminals were labeled with a fluorescein tagged anti-HRP primary antibody and subsequently imaged with a confocal microscopy. Early third instars (A1, A2) and late third instar larvae (B1, B2) were raised continuously at 18°C. Early third instars transferred from 18°C to 29°C showed differences in the motor nerve terminal morphology (C1, C2). The ecd^1/ecd^1 larva, if maintained at 29°C, will prolong the third instar stage and the nerve terminals will under an alteration in structure (D). Note the identification of the Ib and Is terminals in C2 for descriptive purposes. Scale bar = 45 μm.

There are quite distinctive differences in the morphological characteristics between the Ib and Is terminals and the associated m6 throughout development from the early third to the late third instar (Fig. 2). Larvae maintained at 18°C from an embryonic stage in both the ecd^1/ecd^1 and CS strains show a significant increase in muscle size (MANOVA followed by Tukey test, $P < 0.001$ for both strains). The length and width of the muscle fiber increases, thus resulting in an overall increase in the dorsal surface area of the fiber (Fig. 2A). When the early third instars of CS and ecd^1/ecd^1 flies are transferred to 29°C, the muscle surface area of the CS strain does not increase as much, as compared to ecd^1/ecd^1 larvae (MANOVA followed by Tukey test, $P < 0.001$). The muscle in the ecd^1/ecd^1 strain appears to remain the same size from the late third instar to the extra-late stage (i.e. 1 week) when maintained at 29°C (Fig. 2A, extra late).

The Is and Ib terminals also show developmental growth from early to late third instars when the larvae are maintained at 18°C. The length of the terminals (MANOVA followed by Tukey test, $P < 0.002$ for both Ib and Is axons from each strain) (Fig. 2B) and the number of varicosities along the terminals (MANOVA followed by Tukey test, $P < 0.01$ for both Ib and Is axons from each strain) (Fig. 2C) are increased significantly from the early to late third instar. The Is terminals are longer than Ib terminals in late third stage for both CS (MANOVA followed by planned comparisons, $P < 0.001$) and ecd^1/ecd^1 strains (MANOVA followed by planned comparison, $P < 0.02$). In addition, the Is terminal has more varicosities compared to the Ib terminal at both early and late third stages for both strains (MANOVA followed by planned comparisons, $P < 0.01$ in all cases). This difference is present in both the ecd^1/ecd^1 and CS strains without any difference between the strains (18°C). However, when the strains are transferred to 29°C at the early third stage, there are significant differences between the strains for both the terminal length (MANOVA followed by Tukey test, $P < 0.05$ for both Ib and Is axons) (Fig. 2B) and number of varicosities (MANOVA followed by Tukey test, $P < 0.001$ for both Ib and Is axons) (Fig. 2C). The CS flies at 29°C maintained a significant difference between the Is and Ib terminals, with the Is having a greater length (MANOVA followed by planned comparisons, $P < 0.01$) and more varicosities (MANOVA followed by planned comparisons, $P < 0.005$). The ecd^1/ecd^1 strain at 29°C does not continue to show a difference in terminal length between the Is and Ib terminals (Fig. 2B), but there is still a significantly larger number of varicosities on the Is terminal compared to the Ib terminal (MANOVA fol-

lowed by planned comparisons, $P < 0.05$) (Fig. 2C). When the ecd^1/ecd^1 strain is maintained at 29°C for 1 week (extra late), the same morphological characteristics are observed as those measured after 48 h (late), except no differences are found in the number of varicosities between Ib and Is terminals (Fig. 2B, C).

To compare the relative development of the nerve terminals along with the muscle, ratios in the numbers of varicosities along the Ib and Is terminals (Fig. 2C) to the dorsal surface area of the muscle (Fig. 2A) were determined (Fig. 3A). Since the majority of the synapses within the terminal are contained in the varicose regions (Atwood et al., 1993), it would appear to be more relevant to compare the number of varicosities than the terminal length, with the surface area of the muscle between strains and temperatures. The large increase in muscle area from early third to late third (18°C) is followed by as large of an increase in the number of varicosities for Ib axon, thus the ratio does not change (Fig. 3A). In addition, the continued growth of the muscle for the ecd^1/ecd^1 (maintained at 29°C) occurs without the concomitant increase in number of varicosities, as compared to the CS (29°C), resulting in a reduced ratio in the number of varicosities per surface area of the muscle for both late (MANOVA followed by Tukey test, $P < 0.001$ for Ib and $P < 0.01$ for Is) and extra-late stages (MANOVA followed by Tukey test, $P < 0.001$ for Ib and $P < 0.002$ for Is) (Fig. 3A).

The number of varicosities per length of nerve terminal, for Is and Ib terminals, is not different between the ecd^1/ecd^1 and CS strains at either 18°C or 29°C. However, there is a significant difference between Is and Ib terminals for all experimental conditions (MANOVA followed by planned comparisons, $P < 0.01$ in all cases) except for the early third ecd^1/ecd^1 mutants at 18°C ($P = 0.113$) and the late CS strain at 29°C ($P = 0.114$) (compare top and bottom halves in Fig. 3B).

One might expect that the EJP amplitudes for either Ib or Is terminals would be different for late third instars between the CS and ecd^1/ecd^1 mutants raised at 29°C, since the ratios of the varicosities per muscle surface

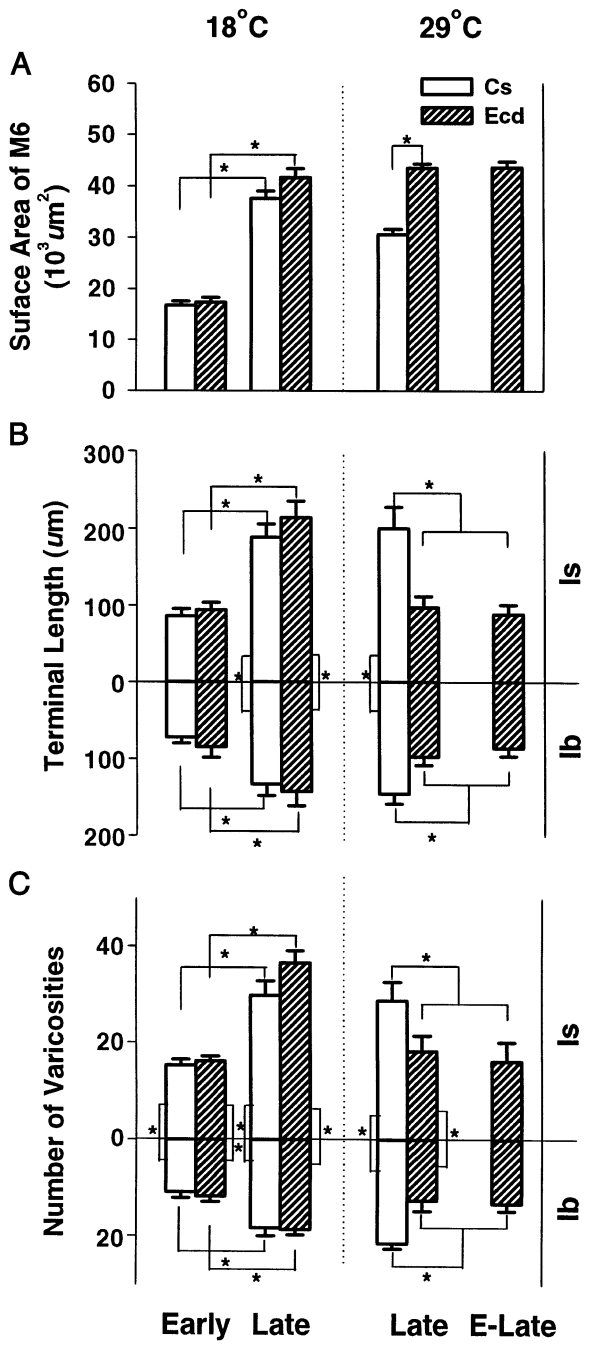


Fig. 2. Morphological characteristics of motor nerve terminals Ib and Is and m6 within segment 4 for CS and ecd^1/ecd^1 of the early and late third instar raised either at 18°C or 29°C. (A) The dorsal surface area of m6 increases during development from the early to late third stage when raised at 18°C without differences between CS and ecd^1/ecd^1 (Ecd). Although, at 29°C the ecd^1/ecd^1 strain has a significantly larger surface area compared to CS ($P < 0.001$) and the size of the muscle is maintained in this state even for up to a week. (B) The terminal lengths of Ib (upper half) and Is (lower half) continued to increase from the early to late third stage for both CS and *ecd1/ecd1* strains ($P < 0.002$ for both strains). When the early third instars are placed at 29°C, only the CS exhibits the normal terminal development. In comparison, the ecd^1/ecd^1 flies showed a substantial lack of growth to the late third instar (48 h), as well as a week later (extra late). The lengths for both Ib and Is terminals are significantly greater in CS strain than the late ($P < 0.05$ for both Ib and Is) and extra-late ecd^1/ecd^1 strain ($P < 0.05$ and $P < 0.02$, respectively). The terminal lengths are also greater for Is than for Ib axons in both CS and ecd^1/ecd^1 strains raised at 18°C ($P < 0.001$ and and $P < 0.02$, respectively) and CS strain at 29°C ($P < 0.001$). (C) The number of varicosities on the Is and Ib terminals increase with larval development from the early to the late third instar in both the CS ($P < 0.01$ and $P < 0.001$, respectively) and the ecd^1/ecd^1 strain ($P < 0.001$ for both Ib and Is) (18°C). When the strains are maintained at 29°C, only the CS shows normal development. The number of varicosities of both Ib and Is terminals are significantly larger in the CS strain than the late ($P < 0.001$ for both Ib and Is) and extra-late ecd^1/ecd^1 strain ($P < 0.001$ for both Ib and Is). The number of varicosities are also significantly larger for Is than Ib for all stages ($P < 0.01$ in all cases) except for extra-late mutant strain. In this and Figs. 3 and 4, horizontal comparison bars together with stars indicate the significant difference of measurements between experimental conditions (MANOVA followed by Tukey test), whereas the vertical comparison bars together with stars indicate the difference of measurements within each experimental condition (MANOVA followed by planned comparisons). E-Late represents the extra-late stage in this and Figs. 3 and 4.

are different (Fig. 3A, lower half for 18°C), but they are not (Fig. 4, Ib+Is). The only significant differences in the EJP responses are for the ecd^1/ecd^1 mutants when both the Ib and Is terminals were recruited together (MANOVA followed by Tukey test, $P < 0.05$) (Fig. 4, Ib+Is). However, the EJP amplitude produced by the Is neuron is significantly larger than those from the Ib axon within each preparation (MANOVA followed by planned comparisons, $P < 0.001$ in all cases) (Fig. 4, Ib+Is).

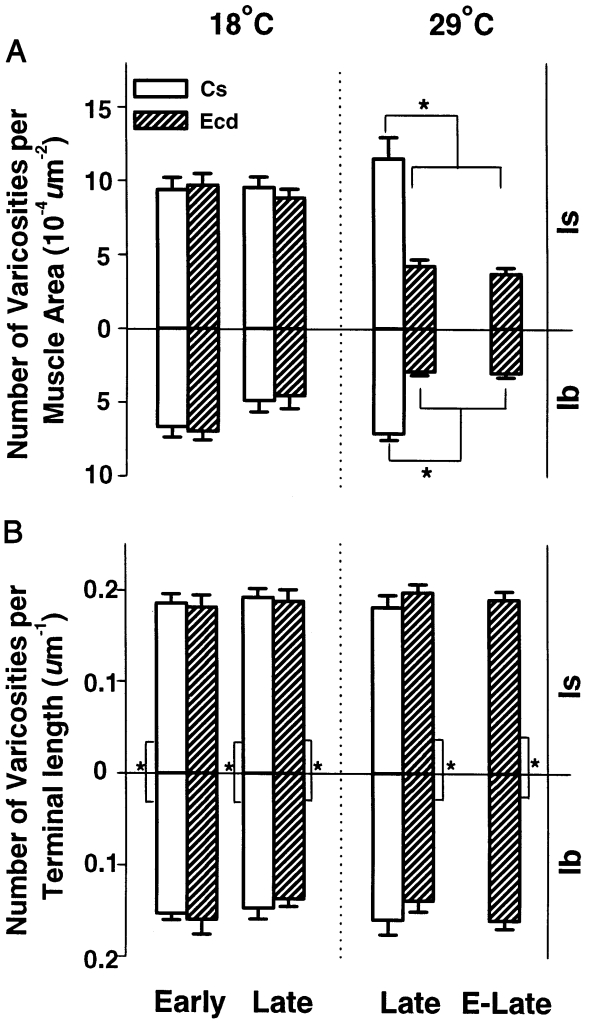


Fig. 3. The number of varicosities along the Ib and Is terminals in relation to growth of m6 and the nerve terminals. (A) The number of varicosities for terminals Is (upper half) and for Ib (lower half) per surface area of the muscle are not significantly different for the Ib terminals of both CS and ecd^1/ecd^1 (Ecd) during development at 18°C from the early to late third stage. Early third instars placed at 29°C showed a significant increase for only Is terminals in the CS strain ($P < 0.05$), but a decrease for the ecd^1/ecd^1 for both the Is ($P < 0.001$) and Ib ($P < 0.01$) terminals. The smaller values for the ecd^1/ecd^1 were maintained even after 1 week while the animals were maintained at 29°C ($P < 0.001$ and $P < 0.002$, respectively). Since there is a significantly larger number of Is varicosities as compared to the Ib, this is also reflected in the ratio in the numbers of varicosities per muscle surface area. (B) The number of varicosities per terminal length show no differences between CS and ecd^1/ecd^1 for early or late stages at 18°C or for the 29°C, but there are significant differences between the Is and the Ib terminals for all the stages ($P < 0.01$ for all cases) except for early third ecd^1/ecd^1 mutant at 18°C ($P = 0.113$) and late CS strain transferred to 29°C ($P = 0.114$) (compare the upper and lower halves).

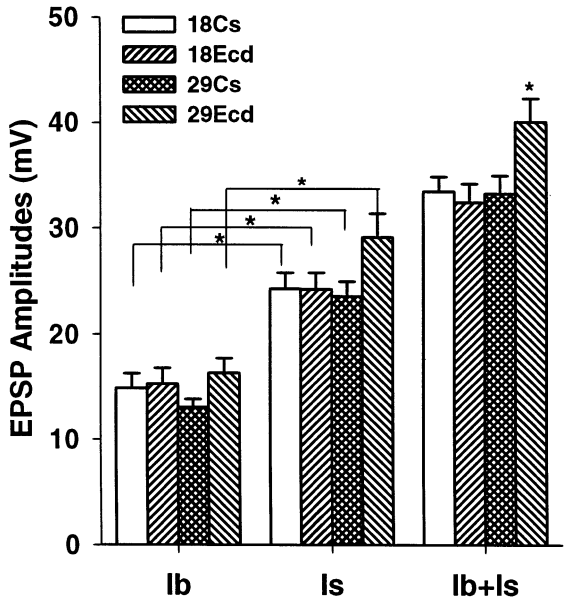


Fig. 4. Amplitudes of the excitatory postsynaptic potentials EPSP in m6 produced by Ib and Is motor nerve terminals in late third CS and ecd^1/ecd^1 (Ecd) are shown. There is no significant difference for either the Ib or Is when stimulated at 1 Hz in all experiments. However, the combined EJP measured from the mutant strain raised at 29°C is significantly higher than from other experimental conditions (*, MANOVA followed by Tukey test, $P < 0.05$). For each experiment the EJP amplitude for the Is axon was larger than for the Ib axon (MANOVA followed by planned comparisons, $P < 0.001$ in all cases).

Effects of 20-HE in the diet

Hormone replacement experiments for the third instar ecdysoneless larvae (ecd^1/ecd^1) revealed that the mutation can be rescued with a dietary 20-HE supplement, since time to pupation was drastically speeded up from 2 or 3 weeks to only 36 h when the animals were maintained at 29°C and fed 20-HE. Control groups were also examined to determine how accelerated their rates were to forming pupae. The controls were also examined at 29°C. Forty individual larvae were used in the control and 20-HE-fed groups. Ninety percent of the fed animals formed pupae within 36 h after being placed in food containing 20-HE. The results were quite clear, as compared to the 2–3 weeks for the non-fed 20-HE ecd^1/ecd^1 group. Long-term studies on the NMJ are not possible to compare to the non-fed ecd^1/ecd^1 group since they pupated so rapidly after consuming 20-HE. We did notice that the 20-HE-fed group did not appear to wander as much over the dish or through the yeast paste as compared to the control group. This is fitting with observations for other insects fed ecdysone (Tanaka et al., 1994). Possibly, more refined studies will be possible in the future, in which the amount of 20-HE added to the diet can be titrated as well as if a continuous ingestion or periodic feeding can result in a delay to the time of pupation.

DISCUSSION

In this study, we have characterized the growth in the Ib and Is motor nerve terminals and the corresponding m6 in segment 4 of the larval *Drosophila* throughout the third instar stage in the presence of a normal and a much reduced endogenous level of ecdysone. In *Drosophila* with normal ecdysone production, m6 increases in size throughout the third instar and the motor nerve terminals parallel the growth by increasing their length with a concomitant increase in the number of varicosities along the axon terminals to provide an overall enhancement in the number of synaptic release sites. The ecdysoneless larvae also show an increase in muscle size, although the Is and Ib motor nerve terminals do not mature to the extent as in the wild-type ecdysone-producing flies. The motor nerve terminal length is shorter with fewer numbers of varicosities per terminal. Although one might expect that the EJP amplitudes would be smaller in the late third instar ecdysoneless flies, this was not the case. Instead, a larger EJP amplitude was measured from the ecdysoneless mutant when both the Is and Ib terminals were recruited together.

Developmental differences of the motor nerve terminals in the ecdysoneless flies were expected, given that it is known that exogenously applied ecdysone can alter the growth of the mushroom bodies and axon outgrowth in crickets (Cayre et al., 2000) and in *Drosophila* (Kraft et al., 1998). It was also noted that the ecdysoneless flies, as used in this study, form enlarged type III synaptic varicosities (Morales et al., 1999). It would be of interest to know if the lengths and numbers of varicosities of those terminals are also altered. Some effects of ecdysone appear to be neuronal-specific in their genomic response since growth is promoted in particular neurons, while other neurons retract their process or die with exposure, as was demonstrated for insect neurons in culture (Truman, 1996; Jiang et al., 1997; Angevin et al., 2000). The mechanism of ecdysone's action to either increase or decrease neuronal growth is likely through multiple selective genomic actions, since a variety of cellular processes are needed to mediate such actions over a period of days. Because there are diverse actions of ecdysone *in vivo* and *in vitro* concerning neuronal development, from promoting growth to death, there may indeed be various isoforms of the ecdysone receptor (EcR) expressed in different neurons, which results in the varied responses, or possibly the EcRs are the same, but translational differences occur. These issues have not yet been resolved in the selective actions of ecdysone on the motor neurons in *Drosophila*. Although it is conceivable that at different times in insect development when titers of ecdysone may vary substantially, there could be selective actions due to receptor levels and various physiological states of the neurons (Cayre et al., 2000), as well as competitive actions of other hormones, such as juvenile hormone, which also varies in titer throughout development (Cayre et al., 1994). As far as we are aware, no one has measured juvenile hormone levels in the ecd^1/ecd^1 larvae when ecdysone titers are low, which may result in altered juvenile hormone production. It is known that different EcR isoforms can be induced by 20-HE during development in *Bombyx mori* (Kamimura et al., 1999) and that the expression of the EcR does vary in the nervous system of *Manduca sexta* throughout development (Fahrbach, 1992).

There are non-genomic actions of ecdysone, as well as for the ecdysone agonist, RH-5849. Application of 20-HE on the NMJ of the crayfish (Cooper and Ruffner, 1998) and *Drosophila* (Ruffner et al., 1999) results in a decrease in the probability of evoked vesicular release from the presynaptic nerve terminal. In the cockroach, application of ecdysone results in a rapid selective decrease in motor neuron firing that corresponds to the endogenous fluctuations in ecdysone titer, as well as motor neuron activity throughout nymph development (von Richter, 1979). This study by Richter is likely the first report of non-genomic actions on invertebrate neurons by ecdysone. The application of the ecdysone analog RH-5849 (used as an insecticide) causes an increased activity and results in a loss of motor coordination, followed by paralysis and death (Ortego and Bowers, 1996). One site of action for RH-5849 was shown to be a 4-AP-sensitive K^+ voltage-gated channel (Ortego and Bowers, 1996). It has not been established yet if 20-HE has a direct action on these same type of K^+ channels. Also, when armyworms *Spodoptera littoralis* were fed RH-5849, they displayed a rapid decrease in wandering behavior (Pszczółłkowski and Kuszczak, 1996). Such behavioral changes have been observed when 20-HE was directly injected into the silkworm (Tanaka et al., 1994), suggesting again a rapid non-genomic action possibly acting at the NMJ.

The non-genomic actions of 20-HE may also be playing a role in the development and maintenance of terminal morphology, along with the genomic actions. Since it is well established in mammals that a reduction in synaptic release within motor nerve terminal by application of botulinum, toxins will cause the terminals to sprout and make new connections to maintain effective synaptic transmission (Bonner et al., 1994; Lagueny and Burbaud, 1996). Since the non-genomic actions of 20-HE are to depress transmission, the nerve may compensate by enhancing synaptic strength over a longer period of time. Even though both the Ib and Is nerve terminals are shorter and have fewer varicosities in the late third instar ecdysoneless strain, the amplitudes of the EJPs are normal or even larger in amplitude than the controls with normal levels of ecdysone. This suggests a compensatory mechanism at the synaptic level for an enhancement of vesicular release, as demonstrated for the FasII *Drosophila* mutant, which has shorter Ib and Is nerve terminals with fewer numbers of varicosities on m6 (Stewart et al., 1996). In this FasII mutant there is an increase in the number of active zones per synapse which is likely the mechanism for the compensation. This type of structural complexity in the number of active zones per synapse has been shown to be directly correlated to synaptic efficacy in low- and high-output varicosities for crayfish NMJs (Cooper et al., 1995a,b; King et al., 1996; Msghina et al., 1998) and with computational analysis related to calcium dynamics within the synapse (Cooper

et al., 1995b). In addition, postsynaptic compensatory mechanisms are possible. Additional glutamate receptors could be recruited to provide for a greater receptor density and thus increased overall responsiveness, or possibly the receptors themselves have altered sensitivity. These scenarios have also been postulated to account for the alterations observed in the FasII mutant (Davis and Goodman, 1998). Bi-directional communication of the motor nerve terminal and the muscle is implied in a number of studies involving arthropods (Lnenicka and Mellon, 1983; Atwood and Cooper, 1996a,b; Lnenicka and Keshishian, 2000), but the mechanism by which this occurs has not been resolved.

Since developmental abnormalities such as ring gland, brain and salivary glands are noticed in the larval and for the adult, follicle epithelial cells, there is a potential that these were not directly effected by the low levels of ecdysteroids, but from indirect effects of the mutation (Redfern and Bownes, 1983). It could also be likely that there are some pleiotropic effects in the ecd^1 mutation which result in a wide variety of developmental problems, even the shorter nerve terminals that we have observed. In considering that feeding 20-HE to ecd^1/ecd^1 resulted in them forming pupae suggests that the lack of ecdysone in this mutation is the primary deficit for development. It would be of interest to know at what amount of 20-HE in the diet is required to trigger pupation, as well as if continuous ingestion or periodic feeding would result in differences in pupation, as has been studied in lobsters with their molting process (Gilgan and Zinck, 1975).

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