DEVELOPMENT OF DROSOPHILA LARVAL NEUROMUSCULAR JUNCTIONS: MAINTAINING SYNAPTIC STRENGTH

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Abstract—In spite of the available information about the development of Drosophila neuromuscular junctions, the correlation between nerve terminal morphology and maintenance of synaptic strength has still not been systematically addressed throughout larval development. We characterized the growth of the abdominal longitudinal muscle 6 (m6) and the motor terminals Ib and Is that innervate it within segment 4. In addition, we measured the evoked excitatory junction potential (EJP) amplitudes while the Ib and Is axons were selectively recruited. Regression analysis with natural log transformation of response variables indicated that the developmental curves for m6 and the motor axons Ib and Is were best fitted as second order polynomial regressions during larval development. Initially Is terminals are longer and possess more synaptic varicosities at the first instar stage. The Is terminals also grow faster in subsequent developmental stages. The growth of nerve terminals and their target m6 are not proportional although tightly correlated. This results in a larger average muscle area innervated by a single varicosity as the animal develops. The amplitudes of the EJPs of Ib and Is neurons show no developmental difference in their amplitudes from the first to the late third larval instar. The Is axon consistently produced larger EJPs than the Ib axon at each developmental stage. The time constants for both rising and decay phases of EJPs increase exponentially throughout larval development.

The results presented not only help in quantifying the normal development of Drosophila neuromuscular junctions, but also provide a framework for future investigations to properly interpret developmental abnormalities that may occur in various mutants.

Key words: neurotransmission, neuromuscular junction, synapse.

During development of neuromuscular junctions (NMJs) in mammals and arthropods, the morphology of both pre- and postsynaptic components changes dramatically. As the postsynaptic muscle 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. Thus, the morphology of nerve terminals and their target in developing animals is unique in its usefulness in investigating how the synaptic strength of two distinct motor nerve terminals is regulated in relation to the changes of the same target during development.

Several lines of evidence suggest that terminal morphology is correlated with synaptic transmission in Drosophila larva NMJs (Schuster et al., 1996a,b; Stewart et al., 1996; Lnenicka and Keshishian, 1990). The most commonly studied Drosophila NMJs are the ventral longitudinal abdominal muscle fibers m6 and m7 (Crossley, 1978), which are both innervated by only two motor neurons (type Ib and Is terminals) (Atwood et al., 1993). Type Ib (big) terminals have large varicosities and produce smaller excitatory junction potentials (EJPs) in comparison to the type Is (small) terminals (Kurdyak et al., 1994). The outgrowth of the two axons innervating m6 and m7 proceeds at different rates at an early embryonic stage (Broadie and Bate, 1993). Thus, this model system is unique in its usefulness in investigating how the synaptic strength of two distinct motor nerve terminals is regulated in relation to the changes of the same target during development.

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It is known in vertebrates that synaptic transmission is correlated with synaptic size and the size of the synapse is correlated with the size of the postsynaptic muscle fiber (Kuno et al., 1971; Nudell and Grinnell, 1983; Ogata and Yamasaki, 1985; Balice-Gordon et al., 1990; Herrera et al., 1991; Wilkinson et al., 1992; Wilkinson and Lumin, 1994). Several lines of evidence suggest that terminal morphology is correlated with synaptic transmission in Drosophila larva NMJs (Schuster et al., 1996a,b; Stewart et al., 1996; Lnenicka and Keshishian, 2000; Li and Cooper, 2001; Li et al., 2001).
Matching of synaptic transmission and muscle fiber size has been shown in a FasII mutant by compensatory changes in synaptic ultrastructure with reduced number of varicosities on m6 (Stewart et al., 1996). However, in the mutation highwire, motor nerve terminals are longer and have more varicosities but synaptic transmission is reduced (Wan et al., 2000). In addition, normal or even larger EJPs are observed in an ecdysoneless mutant even though both the Ib and Is nerve terminals are shorter and have fewer varicosities in the late third instar larvae in comparison to the wild type strain (Li et al., 2001).

In spite of the available information about the development of Drosophila NMJs, the correlation between nerve terminal morphology and synaptic strength has not been systematically addressed throughout the larval development from the first to late third instar.

**EXPERIMENTAL PROCEDURES**

**Fly stock and staging of larvae**

The wild type fruit fly, Drosophila melanogaster, Canton-S, was used in this study. The methods used to stage the fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985). The animals were housed at 18°C on a cornmeal-agar-dextrose-yeast medium.

A 1-h prepulse of egg laying at 18°C was performed to clear the females of stored eggs in the ovipositor. After the eggs were laid, 2 days elapsed before completion of embryonic development, 2 more days for each of the first and second instars, and about 4 days to complete the third instar stage. Five developmental larval stages were used in these studies (Fig. 1): in the first instar stage both the 1- and 24-h larvae; in the second instar stage the early stage (L1) 8 h after first molt and a late stage (L2) 10 h before second molt; and, within the third instar an early stage (E3) 8 h after second molt (still burrowing in their food) and a late stage (L3) 10 h before the last molt.

**Anatomy**

Preparations were taken from each stage as described above. A fluorescent anti-horseradish peroxidase (HRP) primary antibody was used to identify the motor nerve terminals on muscle m6 (Johansen et al., 1989). The terminals are 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 the Z-series were collected with a Leica TCS NT/SP confocal microscope. The confocal images were quantified for varicosity number, terminal length, and muscle dimensions with the Leica confocal software.

**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 in the preparation dish as originally described for studies of the leech nervous system (Müller et al., 1981). Internal organs were carefully removed to expose the body wall muscles, particularly the ventral longitudinal muscles of segment 4. The electrical recordings were obtained from the prominent longitudinal m6 muscle.

The physiological solution used was the same as previously described (Stewart et al., 1994). In brief, the physiological saline contains (in mM): 1.0 CaCl2·2H2O, 70 NaCl, 5 KCl, 10 NaHCO3, 5 trehalose, 115 sucrose, 5 N,N,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 X 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). All events were mea-
sured and calibrated with the MacLab Scope software version 3.5.4.

Selective stimulation of the Ib or the Is axons was obtained by varying the stimulation intensity, polarity, and duration until all three possible responses could be observed (i.e., Ib, Is, and Ib+Is).

**Statistical analysis**

Ten larvae were randomly chosen from each developmental stage as described earlier for either morphological (n = 50) or physiological (n = 50) studies. The data were initially plotted and analyzed descriptively, with means and standard deviations computed. Regression analysis or analysis of variance (ANOVA) was then applied to analyze the relationship between variables. When regression models were fitted on some variables such as muscle area, number of varicosities, and terminal length, with developmental time as the explanatory variable, non-constant error variance (heteroscedasticity) was detected by plots of residuals. A standard statistical method used to analyze heteroscedasticity is transformation of the response variable (Weisberg, 1985; Montgomery, 1992). We first employed the natural log transformation of the response variable, and then first and/or second order regression models were fitted to the transformed data. The modified model fit the data quite well as judged with the $F$ statistic, the correlation coefficient, and the residual plot. The characteristics of the EJP were analyzed by two-way ANOVA, and post-hoc multiple comparison were performed where appropriate. All data analyses were implemented with SAS Software Version 8.0.

**RESULTS**

**Muscle and terminal growth during development**

The development of m6 and the motor nerve terminals (both Ib and Is) on segment 4 was quantified from the first instar to the late third instar stages in Canton-S larvae raised at 18°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 taken by confocal microscopy. To illustrate the developmental changes, representative images are shown in Fig. 1 (first five panels). Larval m6 receives innervation from Ib and Is axon (Atwood et al., 1993). The measurement of m6 width and length was done at this magnification and the dorsal surface area of m6 was obtained. Under higher magnification (far right panel), the Ib and Is terminals are readily identifiable for quantification of terminal length and number of varicosities.

Using the dorsal surface area as an index, the developmental growth curve of m6 was best fitted by a second order polynomial regression ($r^2 = 0.96$, $P < 0.0001$) as shown in Fig. 2. Since the variances associated with the size parameters (such as length, weight, area) usually change along with development, the common least square regression analysis cannot be utilized without transformation of the data, thus producing unstable residuals (inset A). Therefore, regression analysis with natural log transformation of response variable (m6 area) was applied, which stabilized the residuals (inset B). This method was also used in the following studies when regression analysis applied. In addition, m6 grew symmetrically (Fig. 2C), and the increase of muscle width is
linearly proportional to its length ($r^2 = 0.92, P < 0.0001$) during larval development.

The Ib and Is terminals showed developmental growth from the first instar to the late third instar larvae (Fig. 3). Both the natural log transformed numbers of varicosities ($r^2 = 0.83, P < 0.0001$ for Ib and $r^2 = 0.85, P < 0.0001$ for Is terminal) (Fig. 3A) and terminal lengths ($r^2 = 0.78, P < 0.0001$ for Ib and $r^2 = 0.85, P < 0.0001$ for Is terminal) (Fig. 3B) best fit second order regressions during development. The Is terminal is longer (paired $t$-test: $t = 6.10, P < 0.001$) and possesses more varicosities than the Ib terminal at the first stage ($t = 3.02, P = 0.007$). The parallel regression curves with log transform of response variables indicated that the number of varicosities and terminal length for Is axon also increase more rapidly than for the Ib terminal during the subsequent developmental stages. Open circles and dashed lines represent the Is terminal and dark circles and solid lines the Ib terminal in this and subsequent figures.

As axons grow during development, new varicosities and branches are added either de novo or by budding from existing varicosities (Zito et al., 1999). The spacing between synaptic varicosities (for both Ib and Is) along the terminals was quantified. The Ib varicosities are farther apart than Is varicosities at each developmental stage (two-way ANOVA followed by multiple comparison, $P < 0.05$) and the average distances between neighboring varicosities for both Ib and Is terminals are not significantly different throughout larval development (two-way ANOVA, $P = 0.59$ and $P = 0.64$ respectively). This indicates that both Ib and Is terminals grow in a unitary fashion although at different rates.

**Dynamic nerve–muscle matching during development**

The results indicate that both muscle size and the nerve terminal length increase during larval development.
We further examined the morphological relationship between the muscle and terminals by comparing varicosity number (for both Ib and Is) and dorsal surface area in segment 4 (Fig. 4A). The number of varicosities (for both Ib and Is) is tightly correlated with muscle size, and their correlations were best described by linear regressions with natural log transformation of both response and explanatory variables ($r^2 = 0.84$, $P < 0.0001$ for both Ib and Is terminals). In addition, the number of varicosities of the Is terminal increases faster than for the Ib terminal during growth of the postsynaptic muscle fiber ($t = 2.73$, $P = 0.009$, slope).

To further examine the dynamic correlation between muscle size and Ib and Is terminal sizes during larval development, the ratios of muscle area to number of varicosities (for both Ib and Is) for the developmental time were plotted (Fig. 4B). The ratio represents the average muscle area innervated by a single varicosity (either Ib or Is). The linear increase of the ratio indicates that each varicosity (for both Ib and Is) innervates more postsynaptic muscle area during development ($r^2 = 0.82$, $P < 0.0001$, and $r^2 = 0.71$, $P < 0.0001$, respectively).

**Physiological measurements during development**

The Ib axon can usually be recruited at a lower threshold of stimulation and it produces smaller EJPs than the Is axon. Representative traces are shown in Fig. 5A. EJPs were recorded at different developmental stages while either the Ib or Is was selectively stimulated. The amplitudes of the EJPs did not show any significant difference for either the Ib or Is axons over the developmental periods examined. However, the durations of membrane depolarization increase as the muscle grows. To quantify the time courses of EJPs, time constants of rising ($t_1$) and decay ($t_2$) phases were measured as shown in A. The $t_1$ indicates the time for the membrane potential to rise to 63% of its maximum amplitude and $t_2$ represents the decay rate from the peak to 37% of its amplitude. The changes in the time constants were closely correlated with their developmental stage. (B) The developmental curves for $t_1$ best fit linear regressions with natural log transformation of response variables. The same trends were observed for $t_2$. As implied above, the two best fit curves for $t_1$ of both Ib and Is axons are not significantly different ($F = 1.72$, $P = 0.19$), which is also the case for the $t_2$ parameter ($F = 1.89$, $P = 0.16$). However, $t_2$ increases more rapidly than $t_1$ throughout larval development ($F = 81.6$, $P < 0.0001$).
DISCUSSION

In this study the growth of the Ib and Is motor nerve terminals and the corresponding m6 in segment 4 of the larval Drosophila from first instar to late third instar stages was characterized. The growth curves for both m6 and its motor axons Ib and Is were characterized by second order regressions after the data were transformed with a natural log. The number of varicosities and terminal length of Is axon increase more rapidly than for the Ib axon throughout development. Although the two axon terminals grow at different rates, they both grow unitarily. As shown in other systems (Kuno et al., 1971; Nudell and Grinnell, 1983; Ogata and Yamaski, 1985; Balice-Gordon et al., 1990; Herrera et al., 1991; Wilkinson et al., 1992; Wilkinson and Lunin, 1994), the growth of motor nerve terminals and their targets is highly correlated. We have shown that in Drosophila NMJs, the number of varicosities and the dorsal surface area of m6 are highly correlated. Developmentally, the average muscle area innervated by a single varicosity increased, which indicated that the muscle area increased faster than the growth of the nerve terminal that innervates that fiber. In spite of the dramatic size changes in both the nerve terminals and the postsynaptic muscle fiber, the EJP amplitudes are maintained during larval development. Therefore, either more transmitter must be released from each synaptic varicosity or the receptivity of the muscle fiber must increase.

An additional observation that was made in this study was that we recorded occasionally small amplitude EJPs (about 5 mV) in addition to the Ib and Is EJPs, which were in each developmental stage. This third type of EJP on m6 and m7 has been noted previously (Jareki and Keshishian, 1995; Lnenicka and Keshishian, 2000) and has been explained as a result of ectopic innervation.

The regression analysis we provided of the developmental growth pattern of Drosophila NMJ and characterization of the basic physiology during larval development will allow one to examine whether the developmental pattern of NMJs is altered under different experimental conditions. For example, it is known that environmental factors, such as temperature, greatly affect insect development. Also, the recent genetic approaches which use a variety of mutants have greatly helped in understanding the mechanisms underlying the regulation of synaptic strength (Schuster et al., 1996a; Stewart et al., 1996; Davis and Goodman, 1998; DiAntonio et al., 1999; Wan et al., 2000; Li and Cooper, 2001; Li et al., 2001).

Variances associated with growth size parameters (i.e., length, weight, area) usually increase as development progresses. Intrinsic variation and competition among individuals for limiting resources contribute to the increased variation during growth (Kirkwood and Mace, 1996; Woods, 1999; Gille et al., 2000). If competition factors dominate, skewed distributions of those size parameters are usually produced, especially at later developmental stages. To avoid the effects of individual competition on animal development, few larvae were housed together. The distribution plot of the data measured at each developmental stage also helped to examine this possibility.

In statistical analysis, the common least square method dealing with the heteroscedasticity of the data is quite misleading. In this study, we applied a natural log transformation to stabilize the variance. Other techniques, such as box-cox transformation and the weighted least square method, can also be applied to stabilize the variance. We have used the weighted least square method and have found no significant differences between these two approaches to characterize differences in growth between the Ib and Is terminals.

The basic anatomy and physiology of NMJs on m6 and m7 have been intensively examined over the past few years (Johansen et al., 1989; Budnik and Gorczyca, 1992; Gorczyca et al., 1993; Atwood et al., 1993; Kurdyak et al., 1994; Schuster et al., 1996a; Stewart et al., 1996; Davis and Goodman, 1998; DiAntonio et al., 1999; Lnenicka and Keshishian, 2000). However, most of the studies were focused on preparations at a single stage and only a few cross-stage analyses have been accomplished (Keshishian et al., 1993; Schuster et al., 1996a). Our quantification of the terminal morphology at third instar larvae was consistent with the previous work of both Kurdyak et al. (1994) and Lnenicka and Keshishian (2000), however largely dis-
creptant with Schuster et al. (1996a). The relationship between the number of varicosities and the surface area of muscle 6 and 7 was examined in their study (Schuster et al., 1996a), and a second order polynomial regression was fitted to the developmental curve. The data clearly indicated that the non-constant error variance existed, but the statistical methods of dealing with the heteroscedasticity was not described in that study. Therefore, a comparison to our results is not appropriate. Muscle m6 and m7 were usually considered as a whole in many of the past studies on developmental measures; thus, the potential differences of morphology and physiology of NMJs on m6 or m7 across segments have not been fully provided. These past analyses with pooled data either from m6 and m7, or from different segments may be misleading considering the heterogeneity of the data.

It has been reported that the muscle surface area increases 100-fold from the first instar to the wandering third instar larvae (Keshishian et al., 1993), and that the growth of m6 is a linear function with time (Wan et al., 2000). However, as shown in our study, the increase of m6 is rather a second order exponential function. The dorsal surface area increases about 64 times in size during larval development. In addition, the length and width of m6 grow proportionally, which indicates an isometric-like muscle growth. This type of growth tends to increase the rise time of the EJP and decrease the attenuation of cell response. Our data indicated that the EJP amplitudes did not change during larval development. However, the decay time constant did increase, as expected. Similar results have been reported in other systems, such as the frog (Hodgkin and Nakajima, 1972) and crayfish (Lnenicka and Mellon, 1983), over development. The decay time constant is a function of both membrane resistance and membrane capacitance. It has been reported that the membrane resistance of Drosophila body wall muscles drops ‘slightly’ during larval development (Keshishian et al., 1993), however this was not quantified. The muscle membrane capacitance has not been determined throughout the larval developmental stages. Since the membrane capacitance is related to the surface area, the exponential increase of the muscle area may lead to the exponential increase in the decay time constant of the EJPs.

The dorsal surface area has been used as a common index to study the development of Drosophila muscle, but this is by no means indicative of the total surface area. The membrane invaginations in the regions around the varicosities are substantial. These unique convoluted enfoldings are termed the subsynaptic reticulum and have been documented for m6 and m7 around the Ib and Is motor terminals (Atwood et al., 1993). To obtain an accurate assessment of the surface area, one would need serial cross-sections, and three-dimensional reconstruction from electron micrographs to account for the massive amount of surface area of the subsynaptic reticulum. This type of investigation is a forbiddingly laborious task and will probably not be undertaken soon, but it would be very beneficial in conjunction with the results we have presented for the development of the motor nerve terminals.

As for NMJs in mammals (Lichtman et al., 1987), the Drosophila NMJs are dynamic. New varicosities and branches appear throughout development. In the neuromuscular system investigated, the number of varicosities and the terminal length of the Is axon increase faster in comparison with the Ib terminal. In an edcsynoneless mutant raised at 29°C, both Ib and Is axons are shorter and have fewer varicosities, whereas the size of m6 is normal as compared with the wild type control (Li et al., 2001). This earlier report suggested an intrinsic component of neuronal growth, which has also been shown in other systems (Davis, 1989; Davis and Goodman, 1998; Chen et al., 1995). The intrinsic property of neuronal growth may be due to the expression of a distinct group of genes during development (Skene, 1989), which appear to provide specific internal states for extrinsic signals from the local environment that initiate production and control of neuronal elongation and sprouting (Caroni et al., 1997). Such local regulation of nerve terminal morphology and function is postulated to account for the regional differences of a single motor neuron in crustacean walking legs (Cooper et al., 1995a).

How homeostatic control of synaptic efficacy is maintained throughout development is not fully understood. Recent studies of different presynaptic or postsynaptic parameters selectively have begun to define how this dynamic process is achieved during development. Two good examples have made use of genetic manipulations to selectively reduce the quantal size at the Drosophila NMJ, by either reducing density (Petersen et al., 1997) or altering composition (Davis and Goodman, 1998) of the postsynaptic glutamate receptors. In both cases, a compensating increase in the number of quanta released by the presynaptic terminals was observed, which was not accompanied by an increase in terminal size. Therefore, either the density of transmitter release sites (active zones) increased or the probability that a quantum was released from each site must have increased. A similar homeostatic regulation of transmitter release has been described for the mouse null mutation of the neuregulin gene (Sandrock et al., 1997). Neuregulin is a substance which regulates the amount of the postsynaptic acetylcholine receptors at the NMJ. As in Drosophila, the receptor density was reduced in the mutant, and there was a compensatory increase of quanta released without enlargement of the terminal size. No compensatory decrease was observed in transmitter release when there was an increase in receptor density (Petersen et al., 1997) or efficacy (Davis and Goodman, 1998) of postsynaptic receptors by genetic manipulation. The muscle fibers were thus hyperactive in these flies. One possible explanation for these results is that a genetically induced reduction in quantal size mimics what would occur during normal development. As the muscle fiber grows, the depolarization by a quantum declines, reducing the quantal size. This would be detected by a yet undefined mechanism, and the retrograde signals generated could increase transmitter release. In contrast, increasing quantal size does not happen during normal development. Thus, there does not appear to be a homeostatic mechanism to reduce the amount of transmitter released when...
the postsynaptic response is too high. By genetically altering the levels of adhesion molecules FasII on the muscle fibers, it is possible to induce more or fewer synaptic varicosities along the motor nerve terminals (Schuster et al., 1996a,b; Stewart et al., 1996). In both of the cases, the synapses compensated by altering the number of quanta released per varicosity, which was inversely proportional to the number of varicosities. A similar observation was also observed in null NCAM transgenic mice (Rafuse et al., 2000). The NMJ was smaller in NCAM-deficient mice. Functionally, immature end plate potential size, end plate potential size, and quantal content did not differ from that of wild type with either normal or low release conditions. The paired-pulse facilitation, however, was impaired. A recent study by Wan et al. (2000) in the Drosophila mutant highwire, the neuromuscular synapses grew exuberantly and were greatly expanded in both the number of varicosities and the extent and length of terminal branches. These synapses appear normal ultrastructurally but have reduced quantal content physiologically. hiv encodes a large protein found at presynaptic terminals. These results along with those of the Fasciclin II hiw mutants suggested that NCAM and HIW are involved in regulating synaptic transmission, either directly or indirectly but have reduced quantal content physiologically.

Other compensatory mechanisms, including alteration of transmitter release parameters and postsynaptic receptivity, can be brought into play when mismatches occur (Grinnell, 1995). Thus, there may be a hierarchy in the types of compensatory mechanisms, with some occurring only when others are no longer possible, perhaps due to developmental constraints (Davis et al., 1997; Landmesser, 1998). In some cases, compensatory signals may be overridden, such as in Drosophila hyper-excitable mutants (Davis et al., 1997; Zhong et al., 1992).

In the Drosophila FasII mutant, it is known that the synapses are more complex in nature in that they contain more active zones. This would structurally account for a higher synaptic efficacy per varicosity, thus compensating for the shorter nerve terminals while maintaining the same sized EJP measured in the muscle fibers (Stewart et al., 1996). Such differences in the complexity of synaptic structure (i.e., the number of active zones and spacing between them) appears, in part, to explain why the varicosities on the Is motor nerve terminal have a greater synaptic efficacy than the Ib varicosities (Atwood et al., 1993; Kurdyak et al., 1994). Parallels in the synaptic structure and efficacy also hold for other arthropods, the crustaceans (Atwood and Cooper, 1995, 1996a,b; Cooper et al., 1995a, 1996a,b; Govind and Chiang, 1979) in particular, between phasic and tonic motor nerve terminals of the crayfish (King et al., 1996).

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Development of Drosophila neuromuscular junctions


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