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Ultrastructure of Neuromuscular Junctions in *Drosophila*: Comparison of Wild Type and Mutants with Increased Excitability

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SUMMARY

The ventral longitudinal muscles of the *Drosophila* larval body wall are innervated by at least four types of synaptic terminals that can be distinguished on morphological grounds at the light microscopical level. The innervation of these muscles has been previously shown to be regulated by neuronal activity. In this report we investigate the ultrastructural basis for synaptic bouton differences by using serial sections, and examine the structure of synaptic terminals in mutants with increased excitability. We report that individual identifiable muscle fibers are innervated by terminals containing two to three types of synaptic boutons that can be distinguished in terms of synaptic vesicle population, presynaptic and postsynaptic specialization, and general shape. We propose a model to account for the bouton types observed at the light microscopical level. We find that in the hyperexcitable mutant *eag Sh*, there are dramatic ultrastructural alterations at synaptic boutons. These alterations include a partial depletion of two types of synaptic vesicles and a change in appearance of a third type, changes in number and appearance of synaptic densities, and the presence of multivesicular bodies. Our results show that an increase in neuronal excitability produces profound effects in synaptic terminal structure.

Keywords

shaker; *eag*; synaptic bouton; synapse; activity-dependent mechanisms; body wall muscles

INTRODUCTION

The fruit fly *Drosophila melanogaster* has been widely used as a model system for the study of the development of the nervous system using molecular and genetic approaches (Doe, 1992). In particular, the body wall neuromuscular junction has recently received attention as a promising system in which to study synapse function and development (reviewed in Keshishian et al., 1993). This system is composed of a relatively simple pattern of identifiable muscles which are innervated by a few motoneurons whose synapses are accessible for both physiological and developmental studies (Wu and Ganetzky, 1988; Johansen et al., 1989a,b; Budnik, Zhong, and Wu, 1990; Halpern et al., 1991; Sink and

Whittington 1991a,b,c; Cash, Chiba, and Keshishian, 1992; Chiba et al., 1992; Nose, Mahajan, and Goodman, 1992; Zhong, Budnik, and Wu, 1992; Broadie and Bate, 1993).

An ever increasing number of putative neurotransmitters and neuromodulators has been localized to axon terminals that innervate identifiable body wall muscles. The most characterized of these transmitters is glutamate, which, as in most invertebrate neuromuscular junctions, is believed to be the main excitatory transmitter (Jan and Jan, 1976b; Johansen et al., 1989a; Broadie and Bate, 1993). Immunocytochemical studies indicate that this transmitter is ubiquitously expressed at synaptic terminals that innervate all body wall muscles (Johansen, 1989b). The presence of other putative neurotransmitters or neuromodulators such as proctolin (Anderson, Halpern, and Keshishian, 1988), octopamine (Halpern et al., 1988), leucokinin I (Cantera and Nässel, 1992), and insulin (Gorczyca, Augart, and Budnik, 1993) has been demonstrated by immunocytochemical and/or biochemical techniques. However, their physiological role remains unclear. These putative transmitters /modulators are believed to be co-expressed with glutamate at subsets of synaptic terminals in a muscle-specific pattern. Synaptic terminals containing different transmitter molecules can be distinguished in terms of their characteristic shape and the muscle domain that they innervate (Johansen et al., 1989a; Gorczyca et al., 1993).

One of the mechanisms that has been related to the regulation of synaptogenesis and synapse modification in many systems is electrical activity (reviewed in Lnenicka and Murphey, 1989; Schmidt and Tieman, 1989). In vertebrates activity is believed to control both motoneuron survival and neuromuscular junction size (Brown and Ironton, 1977; Oppenheim and Nuñez, 1982). At the crustacean neuromuscular junction, motor axon terminals with different neurotransmitter output show different ultrastructural properties (reviewed in Govind and Walrond, 1989; also see Hill and Govind, 1981; Atwood and Marin, 1983). Chronic stimulation of relatively inactive motoneurons can elicit morphological transformation of terminals to resemble active terminals (Lnenicka, Atwood, and Marin, 1986; Chiang and Govind, 1986; Lnenicka et al., 1991).

In *Drosophila*, electrical activity is also thought to regulate body wall muscle innervation (Budnik et al., 1990; Zhong et al., 1992; Jarecki et al., 1992). For example, it has been shown that in the potassium channel double mutant *eag Sh*, in which neurotransmitter release and motoneuron firing is dramatically increased (Ganetzky and Wu, 1983; Zhong and Wu, 1991), there is an increase in the number of synaptic boutons and motor axon terminal branching (Budnik et al., 1990; Zhong et al., 1992). In addition, TTX application to cultured embryonic body wall muscles produces an increase in the number of processes on developing synapses and changes in growth cone morphology during neuromuscular junction formation (Jarecki et al., 1992).

The main objectives of the present study are (1) to define the ultrastructural bases for the differences of synaptic bouton morphology observed at the light microscopical level, and (2) determine whether the change in light microscopical structure of motor axon terminals observed in the hyperexcitable mutant *eag Sh* is also accompanied by changes in synaptic terminal ultrastructure.

We report that identifiable wild-type body wall muscles are innervated by two to three different classes of synaptic boutons that can be distinguished by their general shape, vesicle population, postsynaptic specialization, and position in the muscle. We also show that two *eag Sh* mutant alleles exhibit dramatic ultrastructural alterations. The most striking abnormalities include partial depletion of synaptic vesicles in two classes of synaptic boutons, an increase in the number of synaptic densities, and the presence of membranous cisternae in the boutons. Our results show that light microscopical differences in synaptic bouton morphology represent different populations of terminals that presumably release different substances, and that an increase in electrical activity can dramatically modify synaptic bouton structure. Some of this work has been previously presented in abstract form (Jia and Budnik, 1992).

MATERIALS AND METHODS

Fly Stocks

All fly stocks were reared at 25°C in standard *Drosophila* food. As a wild type, the strain Canton-Special (CS) was used. Mutant fly lines used for this study were

eag¹ Sh^{KS133} / Y / $\widehat{X}X$ yf and *eag^{4PM} Sh^{rKO120} / Y / $\widehat{X}X$ yf*. Both wild type and mutant strains were obtained from Dr. C-F. Wu.

Electron Microscopy

Samples were dissected in 0.1 mM Ca²⁺ saline (128 mM NaCl, 2 mM KCl, 4 mM MgCl₂, 35 mM sucrose, 5 mM Hepes, pH 7.2), and fixed in freshly made modified Trump's fixative (1 % glutaraldehyde (EM Science), 4% paraformaldehyde (EM Science), 0.1M cacodylate buffer (CB) pH 7.0, for 2 h at room temperature, and then at 4°C overnight. Samples were then washed three times with CB containing 264 mM sucrose (CBS) to match the osmolarity of the saline, and post-fixed in 2% OsO₄, in CBS for 30 min. After post-fixation, samples were washed three times with CBS, three times with distilled water, and stained *en bloc* in 2% uranyl acetate for 0.5 h. Samples were then dehydrated in an ethanol series and embedded in Spurr's. Transverse ultrathin sections (100 nm thick) were cut from the ventral longitudinal muscles 6, 7, 12, and 13 (identified in semithin sections; nomenclature for muscle identification is according to Crossley, 1978) at the third abdominal segment, using a diamond knife. Grids were poststained in 2% uranyl acetate (15–20 min) and 1% lead citrate (5 – 8 min), and visualized using a Jeol JEM 100S.

Data Analysis

Analysis of wild-type body wall muscles was based on three CS samples, three *eag¹ Sh^{KS133}* mutants, and one *eag^{4PM} Sh^{rKO120}*. Two of the CS samples were serially sectioned in a transversal plane, and one in a longitudinal plane. The four *eag Sh* samples were transversally sectioned. Serial sections were performed around the nerve branch point area [Fig. 2(A, arrow)] and at distal regions of the muscle. In this study we centered on the ventral longitudinal muscles 6, 7, 12 and 13 of the third abdominal segment of wandering third instar larvae. Overall, approximately 1500 sections containing synaptic boutons were photographed and analyzed for each muscle fiber in wild type and about 360 for each of the

four muscles in the mutants. Mutant samples were processed for electron microscopy in parallel with wild-type samples in three separate batches (three different experiments), so differences between samples could not be accounted for by differences in fixation, or variations between different batches.

Vesicle size and density were measured at a final magnification of 168,000x. The cross-sectional area of boutons, mitochondria, and area of the bouton occupied by vesicles was determined as follows. Images from EM negatives of serially sectioned boutons were placed on an intensity adjustable light box and entered into a Macintosh IIfx computer using a Cohu solid stage camera. The area of boutons, mitochondria, and area of the bouton occupied by vesicles were measured in each section by using the NIH program Image (version 1.44). The area of the bouton occupied by vesicles was measured by tracing a line around regions of the bouton that contained vesicles and determining the area using the Image program. Measurements were compared by using the Student's *t* test, and expressed as mean $\pm$ S.E.M. Analysis of covariance was used to compare the number of synapses/bouton in wild type and *eag Sh* mutants.

Immunocytochemistry

Body wall muscles were dissected in Ca^{2+} -free *Drosophila* saline and fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.2. Samples were then processed for anti-HRP immunocytochemistry according to Budnik et al., 1990. As a secondary antibody, a rhodamine-conjugated goat anti-rabbit antibody (Cappel) at a 1:100 dilution was used. Samples were visualized on a Bio RAD M600 confocal unit attached to a Nikon microscope. Images obtained were enhanced using the Image program, and photographed from a computer screen with Kodak T-Max 100 film.

RESULTS

The body wall muscles of *Drosophila* larvae are composed of 30 uniquely identifiable supercontracting muscle fibers arranged in a regular, segmentally repeated pattern (Crossley, 1978). Some of the most characterized muscles, both anatomically and physiologically, are the abdominal ventral longitudinal muscles 6, 7, 12, and 13 (Jan and Jan, 1976a; Wu and Ganetzky, 1988; Johansen et al., 1989a,b; Budnik et al., 1990; Sink and Whittington, 1991a,b,c). These muscles are innervated by several motoneurons, each one of which establishes synaptic contacts with the muscle fiber at multiple sites over the length of the muscle (multiterminal innervation) characteristic for each muscle fiber (Johansen et al., 1989a). Along each motor axon terminal there are periodic enlargements of the neurites, termed *synaptic boutons* or *varicosities*. These boutons are believed to be the terminal's active sites, at which neurotransmitter is released (Johansen et al., 1989a,b; Budnik et al., 1990). At least four distinct subsets of synaptic boutons can be identified (at the light microscopical level) that can be distinguished in terms of their morphology (Fig. 1), neurotransmitter immunoreactivity, or expression of cell-specific antigens (Johansen et al., 1989a; Budnik and Gorczyca, 1992; Gorczyca et al., 1993). Table 1 summarizes the different bouton types at ventral longitudinal muscles 6, 7, 12, and 13.

All bouton types have been reported to stain with both anti-glutamate and anti-HRP antibodies (Johansen et al., 1989a,b). Muscles 6 and 7 are typically innervated by terminals containing large boutons [Type I; Fig. 2(A)]. These boutons have been subdivided into Type Ib (big) and Type Is (small) by Atwood et al. (1993—this issue). Muscle 13 also contains Type Ib and Type Is boutons, but the size difference between the two is greater than that in muscles 6 and 7. Both Type Ib and Type Is boutons at muscle 13 are localized at the area of the muscle where axons emerging from the nerve first contact the muscle [branch point area; arrows in Fig. 2(A)] and possess a relatively thick axonal process between boutons. Muscle 13 also contains a third type of terminal with Type II boutons (Johansen et al., 1989a). In contrast to Type I boutons, Type II boutons are very small, extend most of the muscle length [Fig. 1 (B)], and are connected by a thin axonal process. These small boutons selectively stain with antibodies to the “Small Synaptic Bouton” antigen (SSB; Budnik and Gorczyca, 1992).

Muscle 12 is innervated by at least three types of boutons, large (Type Ib), small (Type II), and an intermediate type [Type III, Fig. 1 (C)]. Intermediate boutons are typically elliptical in shape, and express an insulin-like peptide (Gorczyca et al., 1993). Large boutons at muscle 12 are localized at the branch point area; intermediate boutons extend more distally. Small boutons extend over most of the length of muscle 12 and usually run parallel to intermediate boutons [Fig. 1(A,C)]. Branches exhibiting characteristics of Type Is boutons also appear but no quantitative analysis has been done to determine if these are indeed a separate type in this muscle.

In order to study the bases of synaptic bouton differences at the ultrastructural level, muscles 6, 7, 12, and 13 at the third abdominal segment were serially sectioned. Overall, the presence of four distinct populations of boutons that could be clearly distinguished in terms of vesicle types, postsynaptic specialization, and position at the postsynaptic muscle were observed. Muscle 6 and 7 were innervated by two types of boutons, muscle 12 by three types of boutons, and muscle 13 by three types of boutons. These bouton types are described below.

Bouton Types at Muscle 12 in the Wild Type

Boutons at muscle 12 could be divided into three groups according to the type of synaptic vesicles that they contained: boutons containing exclusively small clear vesicles (CV) [Figs. 2(B,C); 3(A)], boutons with predominantly round dense-core vesicles and relatively few small clear vesicles (DV) [Fig. 2(B,C,D); 6], and “mixed boutons” containing both small clear vesicles and ellipsoid dense-core vesicles (MV) [Fig. 2(B); 3(C)].

Clear Vesicle Boutons

CV boutons had a relatively spherical shape, and were deeply invaginated in the muscle [Fig. 2(B,C)]. All CV boutons were localized around the branch point area (about 30 μm), and were not found at muscle regions distal to the branch point. CV boutons measured 1.5–3 μm in diameter ($n = 8$ serially sectioned boutons) and were separated from the postsynaptic muscle by a cleft of 17 ± 1 nm. They were completely surrounded by an elaborate system of

membrane folds, the subsynaptic reticulum, which was localized at the postsynaptic muscle [Fig. 3(A)].

CV boutons contained large numbers of 44.0 ± 0.3 nm clear vesicles ($n = 280$) whose distribution occupied most of the bouton volume ($60.5\% \pm 6.1\%$; see Fig. 11, for distribution) at a density of about 200 vesicles/ μm^2 . Putative neurotransmitter release sites (active zones) at CV boutons appeared as T-shaped electron-dense bodies in thin sections, which were surrounded by a higher density of synaptic vesicles [Fig. 3(B); 4]. T bars were composed of an electron-dense stalk, and a bar (accessory bar) perpendicular to the stalk and parallel to the bouton membrane [Fig. 3(B)].

A more detailed analysis of T bars was performed for 17 serially sectioned dense bars. Figure 4(A,B) shows two representative examples of serial sections through T bars. A diagrammatic representation of the approximate three-dimensional structure of T bars, based on the 17 serial sections, is shown in Figure 4(C). At the presynaptic membrane underlying T bars, numerous putative exocytotic pits were observed (Fig. 4), in agreement with the suggestion that these structures might be neurotransmitter release sites (Govind, De Rosa, and Pearce, 1980; Propst and Ko, 1987).

In order to determine the distribution of putative synaptic sites within the boutons, the number of T bars, at stretches of the terminal containing CV boutons, was measured (Fig. 5). It was found that synapses were exclusively localized to synaptic boutons. In the eight boutons examined in detail, no T bars were found in the bottleneck region between boutons. We found that boutons had from three to 18 T bars and that there was a direct correlation between number of T bars and size of the bouton ($r = 0.96$; Fig. 14).

Dense-Core Vesicle Boutons

These boutons, which were elongated in shape ($0.6\text{--}1.7\ \mu\text{m}$ by $1.6\text{--}6\ \mu\text{m}$), were localized on superficial grooves of the muscle [Fig. 2(B,C,D); Fig. 6]. They were observed in the area surrounding the nerve branch point, but extended more distally than CV boutons (approximately $40\ \mu\text{m}$ from the nerve branch point in the samples analyzed). They were filled with spherical dense-core vesicles which could be divided into three discrete populations according to their degree of electron density (dark, intermediate, and light) and their size [Fig. 6(A)]. Dark dense-core vesicles were significantly smaller than the other two types ($p < 0.001$) measuring 73 ± 2 nm ($n = 58$). Intermediate and light dense-core vesicles measured 108 ± 6 nm ($n = 157$) and 97 ± 1 nm ($n = 150$), respectively. Because the diameter of these vesicles was the same as the mean section thickness (100 nm), our measurements of vesicle size probably represent an underestimate of the true vesicle diameter (Leitch, Heitler, Pitman, and Cobb, 1992).

In addition to dense-core vesicles, small translucent vesicles were observed at DV boutons [Fig. 6(A,D)]. These clear vesicles were significantly ($p < 0.001$) smaller in diameter (33.0 ± 0.5 nm; $n = 128$) than the translucent vesicles in CV boutons (44 nm in diameter at CV boutons). They were almost exclusively concentrated at restricted sites of the presynaptic membrane, surrounding putative synapses [Fig. 6(A,D)].

The putative synaptic release sites for clear vesicles in DV boutons were also very different from the electron-dense T bars typical of CV boutons. In thin sections they appeared as darker thickenings of the presynaptic membrane surrounded by translucent vesicles [Fig. 6 (A,D)]. Membrane specializations resembling septate junctions were also observed at various sites of DV boutons, especially near the branch point [Fig. 6 (B)]. In several instances putative exocytotic pits were observed at the surface of the bouton that faced the muscle [Fig. 6(C)]. These exocytotic pits did not appear to be associated with clear-vesicle synaptic areas or septate junctions. DV boutons, unlike CV boutons, were almost devoid of postsynaptic subsynaptic reticulum (see Figs. 3(A) and Fig. 6(A) for comparison).

“Mixed” Vesicle Boutons (MV)

Like DV boutons, MV boutons were localized superficially on the muscle cell and were devoid of subsynaptic reticulum [Fig. 2(B), 3(C)]. They were smaller than CV and DV boutons (less than 2 μm in diameter), and contained both clear and dense-core vesicles. These boutons extended much farther along the muscle fiber than the other two bouton types (about 180 μm from the nerve branch point). The clear vesicles were similar in appearance and size (43.5 ± 0.4 ; $n = 226$) to the clear vesicles observed in CV boutons. The dense-core vesicles differed from DV dense-core vesicles in that they displayed an ellipsoidal shape [Fig. 3(C)]. At a given section, dense-core vesicles appeared polymorphic, probably representing the ellipsoid vesicles being viewed at different planes of section. The largest of these vesicles measured 75 nm wide by 190 nm long. At the nerve branch point area, MV boutons were often found in close proximity to DV boutons [Fig. 3(C)]. No clear synaptic specializations were discerned in the MV boutons examined.

Axons Innervating Muscle 12

The number of axons that innervated muscle 12 was determined by looking at transversal [Fig. 7(B)] and longitudinal [Fig. 7(A, C)] serial sections of the nerve trunk after it branched to innervate muscle 13, and before it reached muscle 12 (Figs. 1(A); 2(A)). Four axonal profiles could be distinguished in both wild-type and *eag Sh* mutant larvae [Fig. 7(B)]. These axons appeared to branch as they reached muscle 12, since up to seven axonal profiles were found just proximal to the muscle 12 branch point area (not shown). The axons were characterized by the presence of abundant microtubules [Fig. 7(B, C)], and they were often joined by extensive septate junctions [Fig. 7(C)]. Axons giving rise to DV boutons were also found to contain relatively large numbers of dense-core vesicles with similar appearance and size to dense vesicles at DV boutons, often clustered at discrete areas of the axon [Fig. 7(A)]. On several occasions dense-core vesicles were also found in close proximity to axonal microtubules [Fig. 7(C)].

Boutons at Muscles 6, 7, and 13—Most boutons observed at muscles 6 and 7 were of the CV type (data not shown). Although a quantitative analysis of bouton size was not performed, they resembled CV boutons found at muscle 12 in that they contained 44 nm clear vesicles, had dense T bars at multiple sites of the bouton, and were enveloped by a highly elaborated subsynaptic reticulum. Another less frequently found bouton type at muscles 6 and 7 (CV₀) resembled CV terminals in that it contained 44-nm clear vesicles and was surrounded by subsynaptic reticulum [Fig. 8(A)]. However, in contrast to CV boutons,

CV_o boutons also contained a few dense-core vesicles of about 94 nm in diameter, and a few large translucent vesicles (about 112 nm in diameter). In addition, the subsynaptic reticulum was less extensive at CV_o boutons. No boutons resembling DV or MV boutons were observed at muscles 6 and 7. (For a more detailed analysis of muscles 6 and 7, see Atwood et al., 1993, this issue.)

Three types of boutons were found in muscle 13. The most common types were similar to both the CV and MV boutons described for muscle 12 (data not shown). As in muscle 12, CV boutons were localized in the muscle region surrounding the nerve branch point, whereas MV boutons extended most of the length of the muscle fiber. Another more infrequent bouton type found in muscle 13 was similar to the CV_o bouton found at muscles 6 and 7 [Fig. 8(B)]. Figure 9 summarizes all bouton types found at muscles 6, 7, 12, and 13.

Hyperexcitable Mutant *eag Sh* Shows Ultrastructural Alterations of Synaptic Boutons—Previous studies have shown that mutations in both potassium channel genes *eag* and *Sh* produce synergistic interactions that result in dramatic physiological and anatomical changes at the neuromuscular junction (Ganetzky and Wu, 1983; Wu and Ganetzky, 1988; Budnik et al., 1990; Zhong and Wu, 1991). Synaptic transmission is increased many fold, and there is an increase in endogenous motoneuron firing (Ganetzky and Wu, 1983; Budnik et al., 1990; Zhong and Wu, 1991). In addition, *eag Sh* double mutants show an increase in the number of synaptic boutons and terminal branches at some body wall muscles, as observed at the light microscopical level (Budnik et al., 1990; Zhong et al., 1992).

To determine the structural basis for these physiological and morphological changes, we examined the ultrastructure of motor axon terminals by serial thin sections at muscle fibers 6, 7, 12, and 13 at the third abdominal segment of two *eag Sh* alleles (*eag¹ Sh^{KS133}* and *eag^{4PM} Sh^{rKO120}*). We found that in both *eag Sh* allele combinations, synaptic boutons were characterized by a number of features very different from wild-type synaptic boutons.

Synaptic Vesicles at CV, MV, and DV Boutons in *eag Sh*

One of the most dramatic changes found in *eag¹ Sh^{KS133}* and *eag^{4PM} Sh^{rKO120}* mutants was that CV and MV boutons were partially depleted of synaptic vesicles [Fig. 10(A, F); 13(A)]. For example, in wild type, CV boutons contain small translucent vesicles whose distribution occupied most of the volume of the bouton [Fig. 3(A)]. In the two *eag Sh* alleles examined, vesicles at CV boutons were concentrated in the vicinity of the bouton membrane, but they were absent in the central region of the bouton. This did not seem to be a fixation artifact, because it was invariably observed in all *eag Sh*, but not in wild type preparations, even though they were processed in parallel (three sets of wild type and mutant samples processed in independent experiments). Figure 11 shows the cross-sectional area of the bouton compared to the area of the bouton which is occupied by synaptic vesicles in four wild type and four *eag¹ Sh^{KS133}* boutons, demonstrating that in the mutant there was a substantial decrease ($p < 0.01$) in the area of the bouton occupied by synaptic vesicles. At the central region of the bouton, the area of the terminal occupied by vesicles in wild type was $55\% \pm 6.8\%$, and $28\% \pm 3.3\%$ in *eag¹ Sh^{KS133}* mutants.

No apparent increase in the presence of exocytotic pits was observed at depleted mutant boutons. However, *eag Sh* boutons contained a large number of membranous structures (multivesicular bodies) very rarely observed in wild type [Fig. 10(F,G)]. Vesicular structures similar to clear vesicles in CV terminals were also observed in association with mitochondrial membranes, appearing to bud off or fuse to them (Fig. 12).

The partial depletion of synaptic vesicles in *eag Sh* was even more pronounced in MV boutons, many of which appeared completely devoid of synaptic vesicles [Fig. 13(A)]. These boutons could only be identified as MV boutons because of their position in the muscles, and because they were continuous with boutons that contained a few of the elliptic-dense vesicles that are characteristic for these boutons [Fig. 13(A)]. Numerous putative endocytotic or exocytotic pits were observed at these boutons in the mutant [Fig. 13(A)].

In contrast to CV and MV boutons, DV boutons did not appear affected by the *eag Sh* mutations in terms of bouton vesicle content. However, in *eag¹ Sh^{KS133}*, these boutons differed from wild type in the appearance of dense-core vesicles. In wild-type DV, boutons show at least three populations of boutons that vary in electron density. In *eag Sh* mutants, all vesicles appeared nearly translucent (Fig. 13). This phenomenon was not observed in the one *eag^{4PM} Sh^{KO120}* specimen. The population of small clear vesicles in DV boutons did not appear to be altered in either *eag Sh* body wall muscles.

Synapses at CV Boutons in *eag Sh*

Another change observed in both *eag Sh* alleles was that synapses were less defined and less dense than wild type [Fig. 10(B–E)]. This was consistently found in both *eag Sh* alleles. On many occasions, synaptic vesicles appeared arranged along the presynaptic membrane as they appear around wild-type synapses. However, no defined T bars were found on the serial sections [Fig. 10(B–E)]. On other occasions, dense T bars were present, but were much lighter than those in wild type. Because of this appearance, it was difficult to precisely determine the number of synapses per bouton. Taking into account only those synapses defined by the presence of a T bar, we found that there were three to 21 synaptic sites in mutant CV terminals. As in wild type there was a direct correlation between the size of the bouton and the number of synapses ($r = 0.91$; Fig. 14). We, therefore, used analysis of covariance to compare the number of synapses in CS and *eag Sh*. We found that in *eag Sh* there was a significant increase in the number of synapses/bouton ($p < 0.001$) (Fig. 14). Since other areas with some features of synaptic areas, but without clear synaptic densities, were also found in the mutant, it is possible that the number of synapses reported above represents an underestimate of the total number of synapses per bouton in the mutants.

DISCUSSION

Types of Boutons Innervating Muscle 6, 7, 12, and 13 at the Third Abdominal Segment

The present study shows that the diversity of synaptic boutons at the body wall muscles observed at the light microscopical level is accompanied by distinct ultrastructural characteristics, and that an increase in motoneuron electrical activity produced profound changes in synaptic bouton structure.

Four synaptic bouton types can be demonstrated at the *Drosophila* larval body wall muscles at the light microscopical level (Johansen et al., 1989a; Budnik and Gorczyca, 1992; Gorczyca et al., 1993; Atwood et al., 1993; this report) and are summarized in Table 1. All bouton types have been reported to contain glutamate, the main excitatory transmitter at these neuromuscular junctions (Jan and Jan, 1976b; Johansen et al., 1989a,b). Type I terminals are short and contain mostly 3–5- μ m diameter boutons. Muscles 6, 7, 12, and 13 are all innervated by motor axon terminals containing this type of bouton (Johansen et al., 1989a,b). Atwood et al. (1993) have divided the large boutons into Type Ib and Type Is based on a quantitative analysis of muscles 6 and 7. Muscle 13 contains large Type Ib and small Type Is boutons that are clearly separable on the basis of size. In muscle 12 the distinction is not as great and it is not yet known if Type Is-like boutons represent a distinct class of terminals or if they are simply smaller branches of variable-sized Type Ib boutons. Type II axon terminals contain boutons of less than 2 μ m in diameter, and can be considerably long, often extending over most of the length of the muscle fiber. Both muscles 12 and 13, but rarely 6 and 7, are innervated by Type II terminals (Johansen et al., 1989a; Budnik et al., 1990; Budnik and Gorczyca, 1992). These terminals can be immunocytochemically distinguished from the other terminals because they express the SSB antigen which stains an as yet unidentified molecule only present at these boutons (Budnik and Gorczyca, 1992). A third type of terminal (Type III) is intermediate in size, found only on muscle 12, and has been shown to contain insulin-immunoreactive material (Gorczyca et al., 1993) in addition to glutamate (Johansen et al., 1989a). Proctolin is also expressed at synaptic boutons of muscles 12 and 13, as well as in other body wall muscles (Anderson et al., 1988). In addition, octopamine immunoreactivity has been reported at muscle 12 (Halpern et al., 1988). However, the exact identity of the boutons containing proctolin and octopamine is still unclear.

Ultrastructure of Synaptic Boutons at Muscles 6, 7, 12, and 13 at the Third Abdominal Segment

Our ultrastructural analysis in wild type, based on serial-sectioned neuromuscular junctions demonstrates the presence of four distinct types of synaptic boutons: CV boutons, containing clear vesicles, CV_o boutons containing clear vesicles, large clear vesicles, and a few large dense-core vesicles, DV boutons containing three populations of large dense-core vesicles, and a smaller population of small clear vesicles, and MV boutons, containing elliptical dense vesicles and clear vesicles similar to CV vesicles (Fig. 9; Table 1). Based on their size and position at the branch point region, we propose that CV boutons correspond to Type I boutons. Additional evidence for this is that in muscles 6 and 7, which contain only Type I boutons, CV boutons are the predominant type. We also believe that some of the smaller CV boutons may belong to the Type II bouton class, because SSB antigen, which exclusively stains Type II boutons has been identified at CV boutons (Budnik and Gorczyca, 1992). Clear vesicles, such as the ones present in CV terminals, have been shown to contain glutamate-immunoreactive material (Johansen et al., 1989a).

According to our model, DV boutons correspond to Type III-intermediate boutons (Fig. 9). This is based on the fact that DV boutons are only found at muscle 12, are localized close to the branch point area, but extending further than CV boutons, and are of elongated shape.

Type III boutons have been previously shown to contain insulin-like peptide (Gorczyca et al., 1993), and may also contain proctolin and/or octopamine (Anderson et al., 1988; Halpern et al., 1988). Infrequently, insulin-like-immunoreactive boutons are also found at muscle 13 (Gorczyca et al., 1993). In many animal species dense-core vesicles have been associated with monoamine or peptide-containing vesicles (see for example, Lee and Wyse, 1991). DV terminals also contained small clear vesicles. Based on morphometric analysis, we showed that these vesicles were significantly smaller than the clear vesicles found at CV boutons. Immunoelectron microscopy will be required to determine the transmitter/modulator identity in each of these vesicle types.

We believe that MV boutons correspond to Type II boutons because of their small size, their location over most of the length of the muscle, and the fact that they are found in both muscles 12 and 13, the same muscles that are innervated by Type II terminals. Because of the similarity of the clear vesicles observed at MV terminals to those found at CV terminals, it is reasonable to suggest that they contain glutamate. However, so far it is unclear what transmitter/modulator elliptic dense-core vesicles may contain. It is interesting to note that at muscle 12, DV boutons were often found in association with MV terminals. At the light microscopical level, Type III boutons run parallel to Type II boutons (Fig. 1; and Gorczyca et al., 1993).

An apparent discrepancy of this model for different bouton types is the fact that we have previously shown that the SSB antibody also stained some dense-core vesicle boutons similar to the DV boutons described here, although the staining intensity was light (Budnik and Gorczyca, 1992). A possible explanation is that SSB is undetectable with indirect immunohistochemistry in boutons other than Type II, and that the silver intensification technique used brings this level above the level of detection. However, at the light microscopical level, we never detect SSB immunoreactivity at boutons other than Type II. Another explanation for this is the fact that our previous study was based on nonserial sections of muscles 6, 7, 12, and 13 and the segment sectioned was not identified. It is possible that those results reflect a segmental difference in the innervation of muscle 13. In fact, segmental differences in the distribution of proctolin, octopamine, and insulin-like peptide have been reported (Anderson et al., 1988; Halpern et al., 1988; Gorczyca et al., 1993).

Muscle 12 is innervated by three classes of synaptic boutons indicating that this muscle is probably innervated by at least three different motoneurons. Sections through the motor nerve prior to muscle 12 showed four axonal profiles. This “extra” profile could be another neuron whose boutons appear as smaller Type I varicosities that are often observed. Alternatively, it could be a branch of the axon of Type I boutons that diverged early on. Nerve sections very close to muscle 12 showed the presence of at least seven axonal profiles indicating that axons can undergo considerable branching before contacting with muscle 12. The identity of the motoneuron cell bodies that innervate muscle 12 are at present unknown.

Based on the bouton types observed, muscle 13 appears to be innervated by at least three motoneurons, and muscles 6 and 7 by two. Based on nerve backfills it has been suggested

that at least 30 neurons innervate each body wall hemisegment (Sink and Whittington, 1991a). Four identifiable motoneurons, RP1, RP4, RP5, and a VUM motoneurons have been shown to innervate muscle 13 (Sink and Whittington, 1991a,b; Chiba et al., 1992). In addition, two motoneurons, RP3 and 6/7b have been reported to innervate muscles 6 and 7 (reviewed in Keshishian et al., 1993).

The physiological significance of several classes of synaptic boutons innervating a single muscle fiber is unclear. However, at crustacean neuromuscular junctions it has been shown that phasic and tonic motoneurons, and motoneurons receiving inhibitory input, have a distinct terminal morphology (Hill and Govind, 1981; Atwood and Marin, 1983; Lnenicka et al., 1986, 1991; Tse et al., 1991). The presence of peptide- and monoamine-containing terminals at a subset of axon terminals (Anderson et al., 1988; Halpern et al., 1988; Gorczyca et al., 1993) indicates that, as in other species, these may have a modulatory role (Evans and O'Shea, 1977; Rane, Gerlach, and Wyse, 1984; Orchard and Lange, 1987). It is also possible that some of the boutons may have a neurosecretory role: releasing peptide hormones into the body wall cavity. These hormones may have their action not only at the body wall muscles but at other tissues in the body wall cavity. This possibility may explain the surprising diversity of molecules with putative transmitter/modulator properties at muscle 12.

The most prominent putative synaptic regions were found at CV terminals. These putative synapses were observed as dense T-shaped structures in thin sections. Similar electron-dense T bars have also been described at neuromuscular junctions of other insect species (reviewed in Osborne, 1975), and at neuromuscular junctions of adult *Drosophila* (Koenig and Ikeda, 1989). Because these T bars are easily identifiable, we analyzed their number and distribution in more detail. Several exocytotic pits were often found at the presynaptic membrane underlying T bars indicating that these structures may represent synaptic sites. T bars were exclusively localized at synaptic boutons. No T bars were found between boutons at bottleneck regions. This is in agreement with the notion that boutons represent synaptic regions at the body wall muscles. However, this is not the case in all species studied. For example, at crustacean neuromuscular junctions, synaptic sites are concentrated at boutons, but are also present in bottleneck regions of the motor axon terminal (Lnenicka et al., 1986). It was interesting to find that a single synaptic bouton may contain up to 18 putative release sites, and that the number of synapses is directly correlated with the size of the bouton.

Studies in Hyperexcitable Mutants

An important question in neurobiology is in regard to the factors that regulate the number and strength of synaptic inputs. In several systems it has been proposed that one of these factors is electrical activity (reviewed in Lnenicka and Murphey, 1989; Schmidt and Tieman, 1989). In *Drosophila* the innervation of the larval body wall muscles appears to be affected by activity. For example, mutant combinations that increase electrical activity produce an increase in the number of boutons and number of terminal branches at the body wall muscles (Budnik et al., 1990; Zhong et al., 1992). In addition, activity block by the sodium channel blocker TTX, produces an increase in projections from newly forming neuromuscular junctions (Jarecki et al., 1992). In this study we used two allele combinations

of the potassium channel mutants *eag Sh* (*eag*¹ *Sh*^{KS133} and *eag*^{4PM} *Sh*^{rKO120}) to determine whether an increase in electrical activity also affected synaptic ultrastructure.

It was found that hyperexcitable mutants showed a number of abnormalities, including partial depletion of synaptic vesicles, an increase in the number of putative synaptic sites, the presence of multivesicular cisternae, and changes in the appearance of a subset of vesicles. The fact that most of these abnormalities were found in two different allelic combinations suggests that the changes observed are due to the mutations at the *eag* and *Sh* locus, and not to an undefined locus elsewhere in the genome.

The most interesting change in mutant boutons was that in both *eag Sh* alleles there was a significant increase in the number of synapses at CV terminals. Our determination of the number of synapses in the mutants was based on counts of clearly visible T-shaped densities. However, we found that there were many instances of presynaptic sites that had a number of features characteristic of synapses, and that surrounded an area of increased electron density. Because T bars were not distinctly identified at these sites, it was difficult to determine if these areas could be putative synapses, and, therefore, we did not include these in the counts of synapse number. If these areas were actually active sites, then our estimate of synapses in *eag Sh* mutants would be an underestimate of the true number.

At *Drosophila* larval body wall muscles, the neuromuscular junction expands throughout the larval period (Gorczyca et al., 1993). This expansion occurs by both the addition of new boutons and an increase in bouton size (Gorczyca et al., 1993). In this report we have shown that there is a strong correlation between the number of synapses and bouton size. It is, therefore, likely that synapses are also continuously forming during development to keep pace with enlarging boutons. The expansion of neuromuscular junctions may be a mechanism to match the growth of muscles that occurs during the larval period (Johansen et al., 1989b; Gorczyca et al., 1993). It is possible that neuronal activity is involved in this phenomenon, because in *eag Sh* mutants there is an increase in the number of synaptic boutons (Budnik et al., 1990). Here, we show that this is also accompanied by an increase in the number of synapses.

At crustacean neuromuscular junctions, motor terminals with different release properties have characteristic ultrastructural differences in shape and size of terminals, area of synapses, and volume of the terminal occupied by mitochondria (Hill and Govind, 1981; Atwood and Marin, 1983; Lnenicka et al., 1986, 1991; Tse et al., 1991). In addition, studies show that chronic increases in levels of activity at normally inactive terminals produce a transformation of those terminals to resemble normally active terminals (Lnenicka et al., 1986; Chiang and Govind, 1986). These results, together with ours, suggest that neuromuscular junctions of invertebrate species have a significant degree of plasticity.

The increase in the number of synapses together with the increase in the number of synaptic boutons observed in *eag Sh* may explain the fact that these mutants show a considerable increase in neurotransmitter release. This increased neurotransmission has been previously attributed to a decrease in potassium currents (Ganetzky and Wu, 1983; Wu and Ganetzky,

1988). Our results indicate that an additional factor might be an increase in the number of release sites.

A puzzling observation was that T bars in the mutants were significantly less electron-dense than those in wild type. Since at present it is not known what factors confer electron density to synaptic specializations, this result is difficult to interpret. If the electron density of T bars is due to the molecular composition of these structures, then our result may indicate a structural defect at mutant T bars.

One of the most dramatic observations in *eag Sh* was that synaptic boutons were partially depleted of synaptic vesicles. This was especially obvious at the central region of CV boutons, and throughout MV boutons. A possible explanation for this is that the abnormal increase in firing and neurotransmitter release in these mutants results in a higher than normal release of synaptic vesicles. Under these conditions, the motoneuron may be unable to produce enough vesicles to maintain a vesicle reservoir in the bouton such as the one observed in wild type. Another possibility is that the partial depletion of vesicles observed in *eag Sh* mutants might be due to an increased release in the mutant due to the fixation process. However, we performed the dissections in the presence of low Ca^{2+} concentration (0.1 mM) at which little evoked release is observed in electrophysiological measurements (Wu and Ganetzky, 1982). In addition, experiments were done in parallel to wild type, and we did not observe partial depletion in wild-type samples.

Koenig and Ikeda (1989) have studied the process of vesicle formation in *Drosophila* adult flight neuromuscular junctions by using the temperature-sensitive mutant *shibire* in which membrane recycling is blocked at restrictive temperature. The synaptic boutons at these neuromuscular junctions greatly resemble the CV terminals described in this paper. These authors found that during what they believe to be the process of vesicle reformation, enlarging uncoated invaginations of the presynaptic membrane pinched off and formed large cisternae. Newly formed synaptic vesicles were observed to be associated with these cisternae. They proposed that these cisternae are the source for newly formed vesicles. It is interesting to note that in *eag Sh* boutons, in which there was a partial depletion of synaptic terminals, the presence of multivesicular bodies was often observed. However, it is not clear if these are associated with the recycling of vesicles.

We also found that in *eag¹ Sh^{KS133}*, vesicles at DV boutons contained large translucent vesicles. We believe, based on the general morphology of these boutons, that they correspond to dense-core vesicles. It is possible that due to a high rate of release, these vesicles are immature states of dense-core vesicles. However, in some wild-type axonal processes, dense-core vesicles with a similar appearance to bouton dense-core vesicles are found. This suggests that dense vesicles are transported through the axon to the bouton, and that they have their normal appearance even before they reach the bouton. Further work will be required to determine the significance of this phenomenon.

In conclusion, we have identified four types of boutons on identifiable *Drosophila* larval body wall muscle fibers. These boutons differ in vesicle type, synaptic density, localization in the muscle, and postsynaptic specialization, and may represent the diversity of bouton

types observed at the light microscopical level. Our studies in mutants with increased electrical activity show that in *Drosophila*, synaptic structure can be regulated by electrical activity.

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References

- Anderson MS, Halpern ME, Keshishian H. Identification of the neuropeptide transmitter proctolin in *Drosophila* larvae: characterization of fiber-specific neuromuscular endings. *J Neurosci.* 1988; 8:242–255. [PubMed: 2892897]
- Atwood HL, Marin L. Ultrastructure of synapses with different transmitter-releasing characteristics on motor axon terminals of a crab *Hyas areneae*. *Cell Tissue Res.* 1983; 231:103–115. [PubMed: 6133625]
- Atwood HL, Govind CK, Wu CF. Neuromuscular junction ultrastructure of ventral longitudinal abdominal muscles in *Drosophila* larvae. *J Neurobiol.* 1993; 24:1008–1024. [PubMed: 8409966]
- Broadie KS, Bate M. Development of the embryonic neuromuscular synapse of *Drosophila melanogaster*. *J Neurosci.* 1993; 13:144–166. [PubMed: 8093713]
- Brown MC, Ironton R. Motoneurone sprouting induced by prolonged tetrodotoxin block of nerve action potential. *Nature.* 1977; 265:459–461. [PubMed: 834299]
- Budnik V, Zhong Y, Wu CF. Morphological plasticity of motor axon terminals in *Drosophila* mutant with altered excitability. *J Neurosci.* 1990; 10:3754–3768. [PubMed: 1700086]
- Budnik V, Gorczyca M. SSB, an antigen that selectively labels morphologically distinct synaptic boutons at the *Drosophila* larval neuromuscular junction. *J Neurobiol.* 1992; 23:1054–1066. [PubMed: 1460464]
- Cantera R, Nässel DR. Segmental peptidergic innervation of abdominal targets in larval and adult dipteran insects revealed with an antiserum against leucokinin I. *Cell Tissue Res.* 1992; 269:459–471. [PubMed: 1423512]
- Cash S, Chiba A, Keshishian H. Alternate neuromuscular target selection following the loss of single muscle fibers in *Drosophila*. *J Neurosci.* 1992; 12:2051–2064. [PubMed: 1318955]
- Chiba A, Hing H, Cash S, Keshishian H. Growth cone choices of *Drosophila* motoneurons in response to muscle fiber mismatch. *J Neurosci.* 1992; 12:2051–2064. [PubMed: 1318955]
- Chiang RG, Govind CK. Reorganization of synaptic ultrastructure at facilitated lobster neuromuscular terminals. *J Neurocytol.* 1986; 15:63–74. [PubMed: 3012002]
- Crossley, CA. The morphology and development of the *Drosophila* muscular system. In: Ashburner, M.; Wright, TRF., editors. *The Genetics and Biology of Drosophila*. Vol. 2b. Academic Press; New York: 1978. p. 499–560.
- Doe, C. The generation of neuronal diversity in the *Drosophila* embryonic central nervous system. In: Shankland, M.; Macagno, E., editors. *Determinants of Neuronal Identity*. Academic Press; New York: 1992. p. 113–154.
- Evans PD, O'Shea M. An octopaminergic neurone modulates neuromuscular transmission in the locust. *Nature.* 1977; 270:257–259. [PubMed: 22815]
- Ganetzkzy B, Wu CF. Neurogenetic analysis of potassium currents in *Drosophila*: synergistic effects on neuromuscular transmission in double mutants. *J Neurogenet.* 1983; 1:17–28. [PubMed: 6100303]
- Gorczyca MG, Augart C, Budnik V. Insulin-like receptor and insulin-like peptide are localized at neuromuscular junctions in *Drosophila*. *J Neurosci.* 1993; 13 in press.
- Govind CK, De Rosa RA, Pearce J. Presynaptic dense bars at neuromuscular synapses of the lobster, *Homarus americanus*. *Cell Tissue Res.* 1980; 207:81–88. [PubMed: 7388913]

- Govind CK, Walrond JP. Structural plasticity at crustacean neuromuscular synapses. *J Neurobiol.* 1989; 20:409–421. [PubMed: 2664081]
- Halpern ME, Anderson MS, Johansen J, Keshishian H. Octopamine immunoreactive nerve terminals are found on a single identified muscle fiber of the *Drosophila* larval body wall. *Soc Neurosci Abstr.* 1988; 14:383.
- Halpern ME, Chiba A, Johansen J, Keshishian K. Growth cone behavior underlying the development of stereotypic synaptic connections in *Drosophila* embryos. *J Neurosci.* 1991; 11:3227–3228. [PubMed: 1658247]
- Hill RH, Govind CK. Comparison of fast and slow synaptic terminals in lobster muscle. *Cell Tissue Res.* 1981; 221:303–310. [PubMed: 7307054]
- Jarecki J, Anderson MDS, Sun YA, Keshishian H. The role of neural activity in establishing neuromuscular ending morphology in *Drosophila* embryogenesis. *Soc Neurosci Abstr.* 1992; 18:580.
- Jan LY, Jan NY. Properties of the larval neuromuscular junction in *Drosophila melanogaster*. *J Physiol (Lond).* 1976a; 262:215–236. [PubMed: 186587]
- Jan LY, Jan YN. L-Glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. *J Physiol (Lond).* 1976b; 262:215–236. [PubMed: 186587]
- Jia X, Budnik V. Ultrastructural characterization of neuromuscular junctions at *Drosophila* larval body-wall muscles. *Soc Neurosci Abstr.* 1992; 18:39.
- Johansen J, Halpern ME, Johansen KM, Keshishian H. Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *J Neurosci.* 1989a; 9:710–725. [PubMed: 2563766]
- Johansen J, Halpern ME, Keshishian H. Axonal guidance and the development of muscle fiber specific innervation in *Drosophila* embryos. *J Neurosci.* 1989b; 9:4318–4332. [PubMed: 2512376]
- Keshishian H, Chiba A, Chang TN, Halfon MS, Harkins EW, Jarecki J, Wang L, Anderson M, Cash S, Halpern ME, Johansen J. Cellular mechanisms governing synaptic development in *Drosophila melanogaster*. *J Neurobiol.* 1993; 24:757–787. [PubMed: 8251016]
- Koenig JH, Ikeda K. Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockade of membrane retrieval. *J Neurosci.* 1989; 9:3844–3860. [PubMed: 2573698]
- Lee HM, Wyse GA. Immunocytochemical localization of octopamine in the central nervous system of *Limulus polyphemus*: a light and electron microscopic study. *J Comp Neurol.* 1991; 307:683–694. [PubMed: 1869636]
- Leitch B, Heitler WJ, Pitman RM, Cobb JLS. Two classes of vesicles are present and change in relative proportion during post-embryonic development of rectifying electrical synapses in the crayfish. *J Comp Neurol.* 1992; 321:267–276. [PubMed: 1500543]
- Lnenicka GA, Atwood HL, Marin L. Morphological transformation of synaptic terminals of a phasic motoneuron by long-term tonic stimulation. *J Neurosci.* 1986; 6:2252–2258. [PubMed: 3746407]
- Lnenicka GA, Murphey RK. The refinement of invertebrate synapses during development. *J Neurobiol.* 1989; 20:339–355. [PubMed: 2664077]
- Lnenicka GA, Hong SJ, Combatti M, Le Page S. Activity-dependent development of synaptic varicosities at crayfish motor terminals. *J Neurosci.* 1991; 11:1040–1048. [PubMed: 2010803]
- Nose A, Mahajan VB, Goodman CS. Connectin: a homophilic cell adhesion molecule on a subset of muscles and motoneurons that innervate them in *Drosophila melanogaster*. *Cell.* 1992; 70:553–567. [PubMed: 1505024]
- Oppenheim RW, Nuñez R. Electrical stimulation of hindlimb increase neuronal cell death in chick embryo. *Nature.* 1982; 295:57–59. [PubMed: 7057873]
- Orchard I, Lange AB. The release of octopamine and proctolin from an insect visceral muscle effects of high-potassium saline and neural stimulation. *Brain Res.* 1987; 413:251–258. [PubMed: 3111640]
- Osborne, MP. The ultrastructure of nerve-muscle synapses. In: Usherwood, PNR., editor. *Insect Muscle*. Academic Press Inc; New York: 1975. p. 151–205.

- Propst JW, Ko CP. Correlations between active zone ultrastructure and synaptic function studied with freeze-fracture of physiologically identified neuromuscular junctions. *J Neurosci.* 1987; 7:3654–3664. [PubMed: 3500282]
- Rane SG, Gerlach PH, Wyse GA. Neuromuscular modulation in *Limulus* by both octopamine and proctolin. *J Neurobiol.* 1984; 15:207–220. [PubMed: 6145754]
- Schmidt JT, Tieman SB. Activity, growth cones and the selectivity of visual connections. *Comments Dev Neurobiol.* 1989; 1:11–28.
- Sink H, Whittington PM. Location and connectivity of abdominal motoneurons in the embryo and larva of *Drosophila melanogaster*. *J Neurobiol.* 1991a; 22:298–311. [PubMed: 1909747]
- Sink H, Whittington PM. Pathfinding in the central nervous system and periphery by identified embryonic *Drosophila* motor axons. *Development.* 1991b; 112:307–316. [PubMed: 1769336]
- Sink H, Whittington PM. Early ablation of target muscles modulates the arborization pattern of an identified embryonic *Drosophila* motor axon. *Development.* 1991c; 113:701–707. [PubMed: 1782875]
- Tse FW, Marin L, Jahromi SS, Atwood HL. Variation in terminal morphology and presynaptic inhibition at crustacean neuromuscular junctions. *J Comp Neurol.* 1991; 304:135–146. [PubMed: 2016409]
- Wu CF, Ganetzky B. *Drosophila* mutants with opposing effects on nerve excitability: genetic and spatial interactions in repetitive firing. *J Neurophysiol.* 1982; 47:501–514. [PubMed: 6279790]
- Wu, C-F.; Ganetzky, B. Genetic and pharmacological analyses of potassium channels in *Drosophila*. In: Lunt, GG., editor. *Molecular Basis of Drug and Pesticide Action*. Elsevier Science Publishers BV (Biomedical division); New York: 1988. p. 311-323.
- Zhong Y, Budnik V, Wu CF. Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of cAMP cascade. *J Neurosci.* 1992; 12:644–651. [PubMed: 1371316]
- Zhong Y, Wu CF. Alteration of four identified K⁺ currents in *Drosophila* muscle by mutations in *eag*. *Science.* 1991; 252:1562–1564. [PubMed: 2047864]

Figure 1.

Confocal images of innervation to ventral longitudinal muscles 6, 7, 12, and 13 of the third abdominal segment. (A) Overview of the four muscle fibers and terminals stained with anti-HRP. A branch of the segmental nerve makes contact between muscles 6 and 7. Another branch travels below muscle 6 and rises to innervate muscles 12 and 13. Numerous nuclei (arrowheads) and tracheoles (open arrow) can be seen within the fibers. At bottom left (asterisk) is innervation to muscles 5 and 8. Muscle 5 partially overlies the posterior region of muscles 12 and 13. N = transverse nerve. T = large trachea. (B) Enlargement of the branch point at muscle 13 showing different bouton types. Open arrow = Type I (large boutons); thin arrow = Type II (small boutons); wide arrow = Type Is. (C) Enlargement of the branch point at muscle 12. Curved arrow = Type III. Anterior is to the right. Scale bar = 40 μm in (A), 10 μm in (B, C).

Figure 2.

Synaptic boutons at muscles 12 and 13. (A) Camera lucida drawing of muscles 6, 7, 12, and 13 of a third instar larvae stained with anti-HRP immunocytochemistry (Budnik et al., 1990). Note the presence of three types of morphologically different classes of synaptic boutons at muscle 12. Arrow indicates the nerve branch point area. (B) Transverse ultrathin section through muscle 12 near the nerve branch point area, showing CV, DV, and MV boutons. Arrows at the muscle region indicate diads (junctions between *t* tubules and sarcoplasmic reticulum). (C and D) Longitudinal section through muscle 12 near the branch point area showing CV and DV (arrows) boutons. These two sections are contiguous areas of the muscle. M = muscle. m = mitochondria. Scale bar = 2.8 μ m in (B), and 3.6 μ m in (C, D).

Figure 3.

CV and MV boutons at muscle 12. (A) CV bouton showing 44-nm translucent vesicles (small arrows), T-bar synaptic densities (large arrows), and a well-developed subsynaptic reticulum (SSR). (B) High-magnification view of the three T-bar synaptic densities (arrows) shown in (A). The upper and lower T bars appear sectioned at a plane perpendicular from each other. Note that many synaptic vesicles are clustered around synaptic densities. (C) Two MV boutons at muscle 12. Note the presence of both dense ellipsoid vesicles (short arrow) and 44-nm translucent vesicles (arrowheads). MV terminals such as the one shown in this micrograph are often found in association with DV terminals. Long arrow points to the bottleneck region between the two MV boutons. M = muscle. Scale bar = 0.8 μm in (A, C), and 0.3 μm in (B).

Figure 4.

Structure of T-bar synaptic densities at CV boutons. (A, B) Serial sections through synaptic densities (arrows). Note that synaptic vesicles are highly concentrated around the densities. Small arrows in A-3 and A-4 point to putative exocytotic pits at the presynaptic membrane. Scale bar = 0.3 μm . (C) 3-dimensional model of a T-bar synaptic density based on serial sections through 17 T bars.

Figure 5.

Distribution of synapses in a representative CV bouton. Each white horizontal bar corresponds to a $0.1\text{-}\mu\text{m}$ section, and its length corresponds to the diameter of the bouton at that section. Black bars correspond to T-bar synaptic densities.

Figure 6.

DV bouton at muscle 12, (A) showing three classes of dense-core vesicles (dense = arrowhead, light = longer arrow, intermediate = shorter arrow), and a synapse surrounded by 33-nm translucent vesicles (open arrow). Note that DV terminals have an elongated shape, and are almost devoid of subsynaptic reticulum and mitochondria. (B) High-magnification view of a septate junction at a DV bouton (arrows). (C) High-magnification view of a DV bouton showing an exocytotic pit (arrow). (D) High-magnification view of a synaptic density at a DV terminal surrounded by 33-nm translucent vesicles (arrow). Note that the appearance of these synaptic densities and the translucent vesicle size is different from the ones observed at CV boutons. m = mitochondria, M = muscle. Scale bar = 0.8 μm in (A), 0.3 μm in (B, C, D).

Figure 7.

Nerve branch point area and axonal profiles in the nerve innervating muscle 12. (A) Branch point and nerve. Note the presence of a CV and DV bouton at muscle 12. Also note that dense-core vesicles can be found clustered at regions of the axon (arrows). (B) Cross-section through the nerve after it leaves muscle 13 and before it innervates muscle 12. Note the presence of four axonal profiles (asterisks), and abundant microtubules (arrowheads) inside the axons. (C) High-magnification view of a longitudinal section of the nerve showing the presence of abundant microtubules (arrowheads) and dense-core vesicles similar to the ones in DV terminals (tiny arrow). Short arrows point to regions of the axon membrane where septate junctions were observed. These are shown at high magnification in the inset. M =

muscle, N = nerve. Scale bar = 1.7 μm in (A), 0.5 μm in (B), 0.8 μm in (C), and 0.2 μm in the inset.

Figure 8.

CV_o boutons at muscles 6 (A) and 13 (B). Note the presence of small clear vesicles (arrowhead), dense-core vesicles (shorter arrow), and large translucent vesicles (longer arrow). SSR = subsynaptic reticulum, M = muscle. Scale bar = 0.6 μ m.

CV : 6-7, 13, 12

CV₀ : 6-7, 13

MV : 13, 12

DV : 12

Figure 9.

Diagrammatic representation of the four bouton types observed in this study.

Figure 10.

Partial depletion of synaptic vesicles and synapse appearance in *eag¹ Sh^{KSI33}* mutants. (A) CV terminal at muscle 12 showing a dramatic decrease in the number of synaptic vesicles in the central region of the bouton. Arrow = putative synapse at this bouton. (B, E) Serial sections through a typical *eag Sh* synaptic T density (arrow). (F) Small CV terminal showing the presence of a multivesicular body (arrow). (G) High-magnification view of another multivesicular body (arrow) at a CV bouton. SSR = subsynaptic reticulum, M = muscle, m = mitochondria. Scale bar = 0.6 μm in (A, F), 0.3 μm in (B–E), and 0.2 in (G).

Figure 11.

Area of boutons occupied by synaptic vesicles in wild type and *eag^I Sh^{KS133}*. Each of the graphs represents a different bouton at muscle 12. ○ = Cross-sectional area of the bouton at each serial section. □ = cross-sectional area of the bouton occupied by synaptic vesicles. Note the marked reduction in the area of the bouton occupied by synaptic vesicles in *eag Sh* mutants.

Figure 12.

Association of clear vesicles to mitochondria at an *eag¹ Sh^{KS133}* CV bouton. Note that membranous structures with the appearance of clear vesicles appear to be budding off or fusing to the mitochondria outer envelope (arrows). Scale bar = 0.2 μ m.

Figure 13.

DV and MV boutons in an *eag¹ Sh^{KS133}* bouton. (A) A DV and a MV terminal at muscle 12. Note that DV terminals contain large translucent vesicles and some smaller dense-core vesicles (arrowhead). Large translucent vesicles are never found in wild-type DV terminals. Also note that MV terminals are almost devoid of synaptic vesicles, and that numerous endocytotic or exocytotic pits (arrows) are observed. (B) High-magnification view of another section through the same DV bouton in (A), showing the presence of a multivesicular body (large arrow) and a synapse typical of DV boutons (small arrow). Scale bar = 0.6 μm in (A) and 0.3 μm in (B).

Figure 14.

Relationship between number of synaptic T bars and bouton diameter in wild-type (●) and *eag Sh* mutants (□).

Table 1

Bouton Types and Transmitters Found in the Ventral Longitudinal Muscles

	Muscle 6	Muscle 7	Muscle 12	Muscle 13
Bouton types	Type Ib ^{1,2}	Type Ib	Type Ib	Type Ib
Light microscope	Type Is ^{1,2}	Type Is	Type Is	Type Is
			Type II ^{1,4}	Type II
			Type III ⁵	
Bouton types	CV	CV	CV	CV
Electron microscope	CV ₀	CV ₀	MV	CV ₀
			DV	MV
Transmitter	Glutamate ^{1,3}	Glutamate	Glutamate	Glutamate
			Proctolin ⁶	Proctolin
			Insulin ⁵	
			Octopamine ⁷	

Abbreviations: As per text.

Sources:

¹ Johansen et al., 1989a;² Atwood et al., 1993;³ Jan and Jan, 1976b;⁴ Budnik and Gorczyca, 1992;⁵ Gorczyca et al., 1993 and this report;⁶ Anderson et al., 1988;⁷ Halpern et al., 1988.