

Presynaptic Development at the *Drosophila* Neuromuscular Junction: Assembly and Localization of Presynaptic Active Zones

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Summary

We describe the extent to which presynaptic structures at the embryonic neuromuscular junction of *Drosophila* can form in mutants where development of postsynaptic somatic muscles is affected. Although *twist* mutant embryos lack mesoderm, motor axons still grow out of the CNS and form morphologically normal presynaptic active zones, independent of their target cells. In *myoblast city* mutant embryos, myoblasts do not fuse but form fully differentiated mononucleate muscles, which make functional neuromuscular synapses with correctly localized presynaptic active zones. Myoblasts also fail to fuse but still attract appropriate innervation in *mef2* mutant embryos. However, these myoblasts fail to differentiate into muscles and presynaptic active zones fail to localize at neuromuscular contacts. Thus, the process of synapse formation can be genetically separated from the process of target recognition, revealing that localization of presynaptic active zones requires *mef2*-dependent muscle differentiation.

Introduction

During the formation of synapses, specialized regions of pre- and postsynaptic cells associate to form a single functional transmission unit (Hall and Sanes, 1993; Burns and Augustine, 1995). This process can be divided into two main steps: first, during target recognition, growth cones of presynaptic neurons interact specifically with their postsynaptic targets to establish the contacts that underlie functional circuitry; second, during synapse assembly, a number of different morphological and molecular features required for transmission develop or become localized at sites of contact. On the presynaptic side, these include Ca^{2+} -channels, vesicle pools, transmitter pumps, and the machinery of vesicle release and recycling; on the postsynaptic side, transmitter receptors and signaling and second messenger systems. Alignment of pre- and postsynaptic features is ensured by exchange of information between synaptic partners. For example, at the cholinergic neuromuscular junction (NMJ) of vertebrates, postsynaptic transmitter receptors are induced to cluster in response to presynaptic signals (Hall and Sanes, 1993; Bowe and Fallon, 1995). Likewise, alignment of presynaptic active zones

and vesicle clusters at sites of synaptic contact requires signals that are localized in the synaptic basement membrane, and at least one of these signals, s-Laminin, is derived from the postsynaptic muscle (Sanes et al., 1978; Noakes et al., 1995; Gautam et al., 1996). Whereas a great deal of attention has been given to presynaptic signals inducing postsynaptic maturation (Hall and Sanes, 1993; Bowe and Fallon, 1995; Broadie and Bate, 1993b), the influence of postsynaptic cells on the development of presynaptic structures has not been so intensively studied.

We have used the glutamatergic NMJ of *Drosophila* to investigate mechanisms of synaptogenesis. In *Drosophila*, embryonic myogenesis generates a stereotyped set of muscles, reproducibly contacted by a consistent set of motoneurons. The formation of the muscle pattern is initiated by the segregation of a special class of myoblasts, muscle founder cells, each of which seeds formation of a specific muscle (Bate, 1990). Founder cells can be identified by the expression of position-specific marker genes before they fuse with neighboring myoblasts to form myotubes (Dohrmann et al., 1990; Bourguoin et al., 1992; Bate et al., 1993). Specificity of the founder cell appears to condition specificity of the whole muscle, so that it can establish neuromuscular contacts with appropriate presynaptic partners during target recognition (Rushton et al., 1995). During the subsequent step of synapse differentiation, NMJs acquire common features, including T-shaped active zones with clustered vesicles presynaptically and a characteristically organized synaptic cleft (Broadie et al., 1995; Keshishian et al., 1996). Functional synapses differentiate even when motoneurons are misrouted to contact wrong muscles (Cash et al., 1992; Chiba et al., 1993), and therefore we assume that correct differentiation of NMJs requires presynaptic properties that are common to all motoneurons and postsynaptic properties that are common to all muscles.

We tested this assumption with the help of mutations in genes required for different steps in the sequence of muscle development. Because the NMJ develops in the embryo, we can work with lethal mutations in genes essential for synaptic development (Broadie, 1994). *twist* is required for gastrulation and its loss results in complete absence of mesoderm and, therefore, muscles (Simpson, 1983; Nüsslein-Volhard et al., 1984). *myoblast city* (*mbc*) is required for myoblast fusion to establish syncytial muscles and its loss results in the formation of mononucleate muscles within a field of unfused myoblasts (Rushton et al., 1995). *mef2* is required for muscle differentiation and in its absence myoblasts fail to develop into mature muscle (Bour et al., 1995; Ranganayakulu et al., 1995). We used mutations in these genes to block postsynaptic development specifically at different stages. We show that NMJ differentiation is an event that can be genetically separated from the preceding process of target recognition. Furthermore, we show that differentiation of the NMJ comprises two key aspects: first, the formation of presynaptic active zones in motoneurons, which is independent of the postsynaptic

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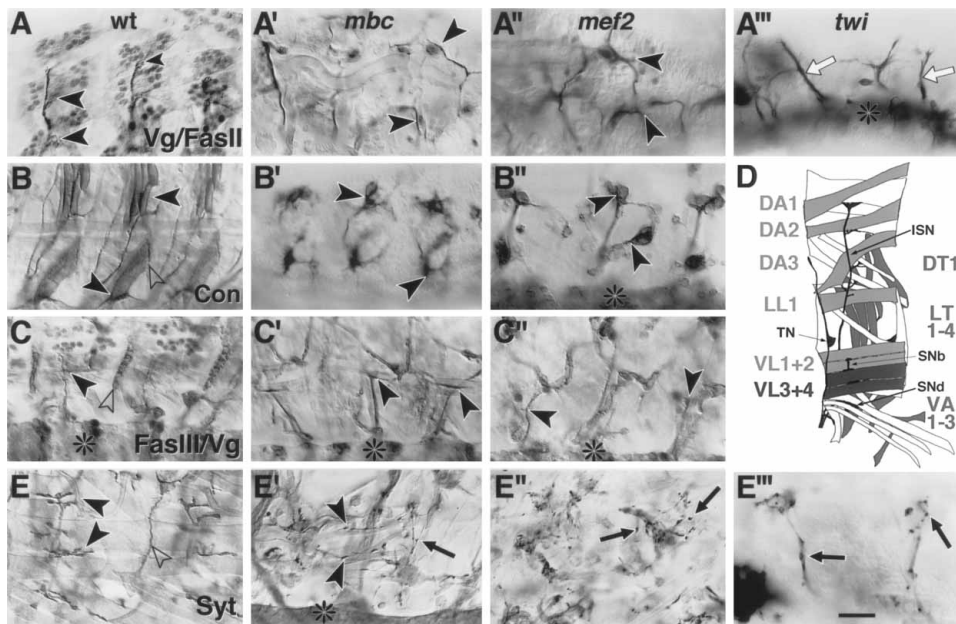


Figure 1. Connectivity and Synaptogenesis in Wild-Type and Mutant Embryos

In all panels, dorsal is uppermost and anterior to the left. Columns show wild-type (A–E), *mbc* (A'–E'), *mef2* (A''–E''), and *twist* (A'''–E''') embryos, stained with antibodies against Vestigial (Vg) and FasII (A–A'''), Connectin (Con) (B–B'''), Vg and FasIII (C–C'''), and Synaptotagmin (Syt) (E–E'''). (D) diagrams the layout of nerves and muscles in an abdominal hemisegment and shows distribution of marker expression within muscles: Vestigial (medium grey; Bate et al., 1993); Connectin (heavier grey; Nose et al., 1992; Meadows et al., 1994); FasIII (dark grey; Halpern et al., 1991). TN, ISN, SN: transverse, intersegmental, and segmental nerves, respectively (according to Goodman and Doe, 1993). Upper three rows show nerve connections at stage 16 (arrowheads) formed with the dorsal muscles/muscle-like cells DA2 and DA3 (A–A'''), the lateral muscles/muscle-like cells LT1–4 and VL2 (B–B'''), and the ventral muscles/muscle-like cells VL3 and VL4 (C–C'''). Muscles/muscle-like cells are correctly innervated according to the following criteria: DA1–3 and LL1 innervated from IS; DT1 by Connectin-expressing axon from IS; LT1–4 by Connectin-positive axons from SNa; VL1 by FasII-positive axon from SNb; VL2–4 by FasIII-positive axons from SNb; VA1–3 by Connectin-positive axons from SNC. (A''') shows FasIII-stained peripheral nerves (arrowed) in *twist* mutant embryo. (E–E''') show synaptic structures at late stage 17 (arrowheads); dots of Synaptotagmin staining are visible along nerves (arrowed) in all three mutants. Asterisks, where shown, indicate the CNS, open arrowheads, muscle attachments. Scale bar, 15 μ m.

target cell; second, the differentiation of properties within postsynaptic muscle that are required for formation of synapses and localization of active zones at sites of synaptic contact.

Results

The Neuromuscular System in the *Drosophila* Embryo

Motor axons in *Drosophila* embryos can be detected by antibodies against the cell adhesion protein FasII (VanVactor et al., 1993), which reveal a pattern of two main nerve branches, the segmental and the intersegmental nerve, from which motor axons defasciculate to innervate their target muscles (Figure 1). At late stage 17 (Campos-Ortega and Hartenstein, 1985), these sites of neuromuscular contact can be labeled with an antibody against the vesicle-associated protein Synaptotagmin (Littleton et al., 1993), revealing synaptic varicosities, called boutons, which are arranged in characteristic numbers and patterns on individual muscles (Broadie and Bate, 1993c; Figure 1). Ultrastructurally, presynaptic boutons are covered by basement membrane on one side and closely attached to the muscle on the other, and in the area of close apposition between

muscle and neuron, the junction is identical in size and appearance to larval synapses in *Drosophila* (Figure 2A) (Seecof et al., 1972; Atwood et al., 1993; Jia et al., 1993; Broadie et al., 1995). At these synapses, pre- and postsynaptic membranes appear electron dense over a stretch of several hundred nm and are separated by a very regular cleft of 15 nm. The cleft contains electron-opaque material, which is strikingly periodic in structure (Figure 2A) and may be a postsynaptic component, since it is closely associated with the postsynaptic membrane. Presynaptic densities (T-bars) can be seen, which are composed of a stem, about 50 nm in height, separated from a bar-like roof by a very narrow gap. Clear synaptic vesicles (40–50 nm in diameter) are concentrated and docked at T-bars, and these sites are believed to be comparable with active zones of vertebrate NMJs (Broadie et al., 1995; Stewart et al., 1996).

Active zones with T-bars and clustered and docked vesicles not only form at the neuron–muscle interface, but can also be found at other points on axons of motoneurons (data not shown). They can appear either in the absence of any postsynaptic cell, separated from the hemolymph only by a basement membrane (referred to as neurohemal active zones). Comparable structures have been found in the locust neurohemal organ (Binnington and Lane, 1982). Or, alternatively, active zones

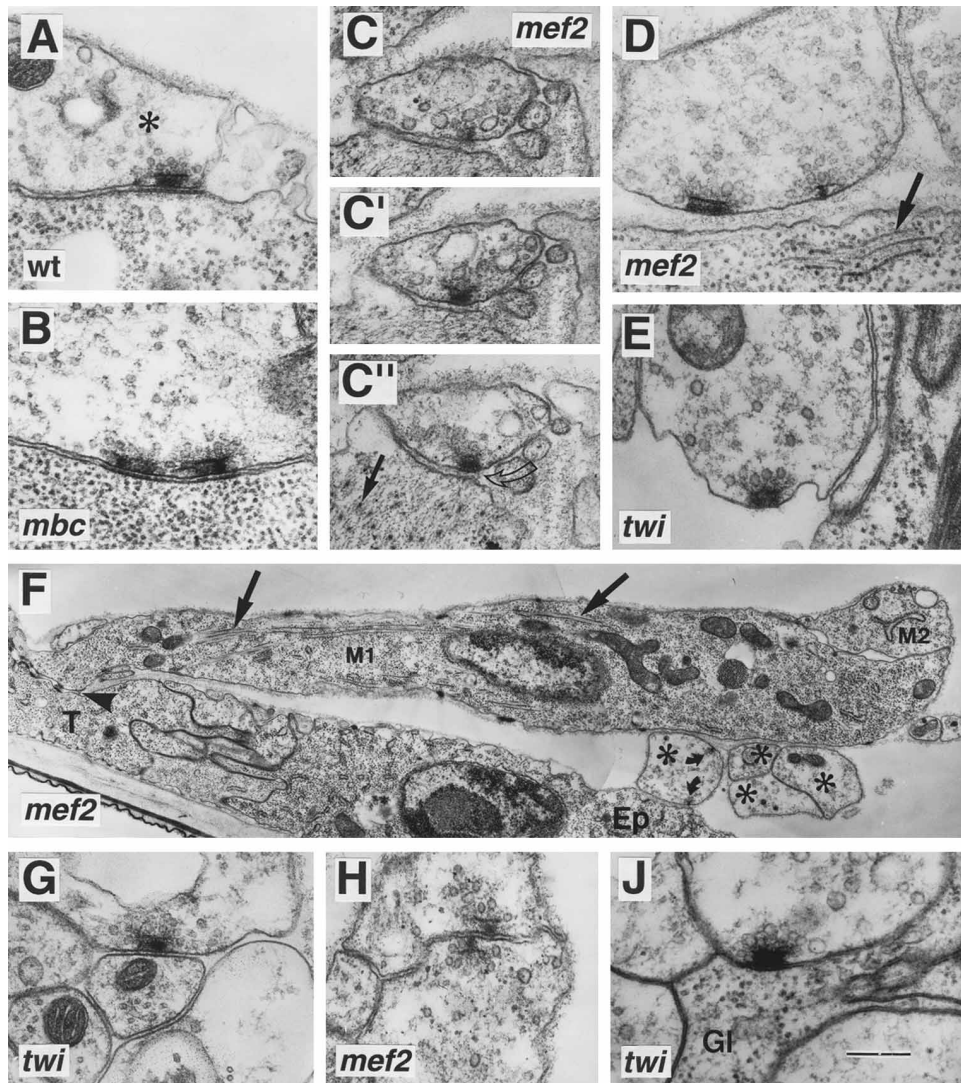


Figure 2. Synaptic Structure in Wild-Type and Mutant Embryos

(A) Wild type. Motor axons form swellings (bouton: asterisk) at sites of close contact with muscles. Active zones are characterized by a T-shaped presynaptic density (T-bar) with clustered vesicles. The T-bar is in a region of closely aligned electron-dense pre- and postsynaptic membranes separated by a 15 nm synaptic cleft, within which is a single layer of extracellular material closely associated with the postsynaptic membrane.

(B) *mbc* mutant embryo. Structure of the active zone is indistinguishable from wild type. Double T-bars, as here, are occasionally seen in wild type and in *mbc*.

(C–C'') Serial sections through an active zone in *mef2* mutant embryo. Although associated with a muscle-like cell (myofilaments arrowed), there is no close apposition of membranes (open arrow in [C'']).

(D) *mef2* mutant active zone showing separation from muscle-like cell by double basement membrane.

(E) Neurohemal active zone in *twist* mutant embryo.

(F) *mef2* mutant embryo: motor axon swellings (asterisks) associated with a muscle-like cell (epidermal attachment site, arrowhead; myofilaments arrowed). Serial sections reveal nine active zones in the motor axons (two indicated by curved arrows), none of which are neuromuscular.

(G) and (H) Neuroneuronal active zones in *twist* and *mef2* mutant embryos, respectively.

(J) Neuroglial active zone in a *twist* mutant embryo; the glial cell (Gl) ensheathes the axon bundle. Scale bar, 1 μm in (F); 250 nm in all other pictures.

can be found at close contacts with other neurons (neuroneuronal active zones). However, comprehensive analyses of serial-sectioned wild-type embryos (see Experimental Procedures) reveal that these neuroneuronal and neurohemal active zones represent only 11% of all active zones in the wild type, and the majority of active zones are located at NMJs (Figure 3).

Formation of Presynaptic Active Zones Does Not Require Target Muscles

Previously, we showed that postsynaptic clustering of glutamate receptors at the embryonic NMJ of *Drosophila* is dependent on innervation by the presynaptic motoneuron (Broadie and Bate, 1993b). Here, we test the extent to which presynaptic differentiation, for example,

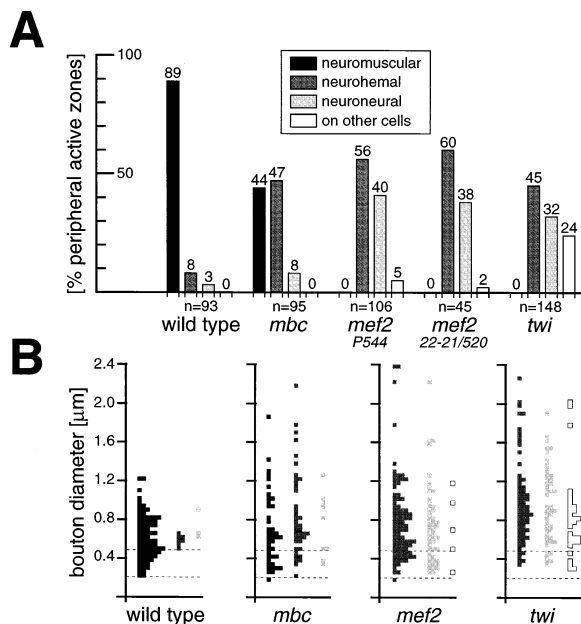


Figure 3. Distribution of Active Zones According to Category from an Analysis of Serial Sections

(A) Relative distribution of active zones that are neuromuscular, neurohemal, or neuroneural, or where the postsynaptic cell is glial or undefined. Genotypes as shown. Approximate active zone density (number of sites divided by number of sections) is comparable for all genotypes: wt, 0.51; *mbc*, 0.39; *mef2*, 0.48; *twi*, 0.45. See Experimental Procedures for further details of the analysis procedure.

(B) Axon diameters (perpendicular to the active zone membrane) for each active zone in the analysis. Note that these measurements do not represent the true volume of boutons. Dashed line shows typical diameter of axons in glial-wrapped nerve without active zones.

the formation of neuromuscular active zones, depends on the presence of a fully differentiated postsynaptic partner. First, we analyzed the structure of motoneurons in *twist* mutant embryos in which mesoderm, and hence the muscles, are never formed (Simpson, 1983; Nüsslein-Volhard et al., 1984).

Anti-FasII staining reveals that in *twist* mutant embryos motor axons grow out of the CNS, even though it is drastically malformed (see Figure 1). In each hemisegment, the nerves form a major dorsally projecting branch (probably representing the intersegmental nerve), and, in 39% of cases, a minor laterally projecting branch (probably the equivalent of the segmental nerve). As in wild type (Nose et al., 1992), both branches contain subsets of Connectin-positive axons (data not shown). In most cases, the axons stay close together until the branch opens up at its dorsalmost tip, where there is some short range defasciculation (see Figure 1).

Interestingly, spots of anti-Synaptotagmin staining are detected all along the nerves or in clusters close to the main nerve trunk (see Figure 1). Closer inspection of these peripheral axons at the ultrastructural level reveals that they are fully capable of forming apparently normal active zones (see Figures 2 and 3). In a rough calculation, the density of peripheral active zones per section is 0.45 in *twi* mutant embryos, comparable with 0.51 in wild type. These active zones are neurohemal, neuroneural, or at sites of contact with other cells (some glial, i.e.,

sheathing nerves, and some of unknown identity). Interestingly, all active zones away from neuromuscular contacts, mutant and wild type, have a clear tendency to lie in axonal swellings with diameters resembling or exceeding those of neuromuscular boutons in wild type (Figure 3). It may be that motoneurons form boutons completely independently of their target muscles and that these boutons are fundamental synaptic units within which active zones can form (see Discussion).

Thus, postsynaptic target muscles are not required for the outgrowth of presynaptic motor axons into the periphery, nor are they required to induce the expression of genes coding for the components of active zones or for the assembly of active zones themselves. Synthesis of the active zone appears to be an independent function of the presynaptic motoneuron, which is integrated into the development of the neuromuscular synapse.

Functional Synapses Can Form in the Absence of Myoblast Fusion

Although presynaptic development in motoneurons appears to be largely independent of the target muscle, we wished to investigate the possibility that establishment of active zones at neuromuscular sites might be a targeted process requiring differentiation of the postsynaptic cell. To do this, we analyzed mutant embryos in which particular aspects of muscle development are affected and investigated the extent to which NMJs can form under these conditions.

We first considered embryos homozygous for mutations in *mbc*, a gene required for myoblast fusion. In *mbc* mutant embryos, fusion of myoblasts to form myotubes is virtually absent, but muscle founder cells are formed and these cells later elongate, make epidermal contacts, and express products typical of differentiated muscles such as Myosin and the α PS2 Integrin (see Figure 1; unpublished data; Rushton et al., 1995). In mature *mbc* mutant embryos (late stage 17), elongated muscle cells are clearly visible, and ultrastructurally these cells contain normally arranged myofilaments and form contacts with the epidermis that are indistinguishable from the muscle attachment sites in wild type. Thus, general muscle characteristics develop normally in *mbc* mutant embryos and, despite the lack of fusion, founder cells appear to develop into differentiated mononucleate muscles (Rushton et al., 1995).

In normal embryos, subsets of muscles and their founder cells express nuclear proteins such as Vestigial and S59 (Dohrmann et al., 1990; Bate et al., 1993). In *mbc* mutant embryos, unfused founder cells also express these proteins and do so at appropriate positions in the overall pattern (Rushton et al., 1995). In addition, the founders express cell-surface proteins such as Connectin and FasIII, and are contacted by the terminals of appropriate motoneurons (see Figure 1) (our unpublished data; Rushton et al., 1995). Thus, while development of syncytial muscles is blocked, mononucleate muscles differentiate with the specific characteristics of individual muscles in the normal pattern, and these cells are targeted by axons of their proper presynaptic motoneurons.

We used this situation to ask whether a fully differentiated NMJ could develop in the absence of myoblast

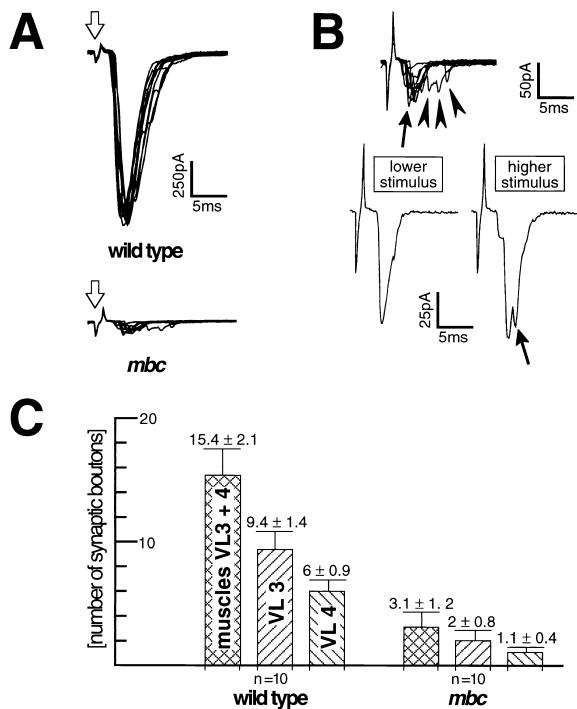


Figure 4. Electrophysiology and Bouton Counts at the Mature NMJ (22–24 hr after Egg Lay)

(A) Recordings of EJCs from muscles VL3 or 4 in wild type and from comparably positioned mononucleate fibers in *mbc* mutant embryos. Voltage-clamp; whole-cell configuration; motor nerve stimulated with suction electrode (open arrow). Wild-type muscle responds with a reliable EJC of mean amplitude 1.75 nA, whereas amplitudes one-tenth of this are recorded from the equivalent mononucleate fiber in *mbc*.

(B) EJCs from *mbc* mutant embryos. Arrowheads in upper panel indicate possible EJCs-vesicle fusions (miniature EJCs; Kidokoro and Koh-ichi, 1994; Broadie et al., 1995). The EJC itself is approximately twice as large as the miniatures. Lower panel indicates recruitment of second peak (arrow) at higher stimulus amplitudes in *mbc* mutant embryos. This is typical of wild-type junctions at VL3/4 and probably represents the double innervation of these fibers.

(C) Numbers of boutons in wild-type and *mbc* mutant embryos revealed by staining with anti-Synaptotagmin or anti-Cysteine string protein. Bouton number in *mbc* is reduced to about 20% of wild type and might be even lower, if we take into account that neurohemal active zones often lie in close proximity to muscles (see Figure 2) and might be misinterpreted as neuromuscular.

fusion. Our analyses show that functional synapses develop on the mononucleate muscles of *mbc* mutant embryos. Ultrastructurally, the neuromuscular contacts in these embryos form synapses with vesicle clusters, T-bars, a stretch of smooth electron dense pre- and postsynaptic membranes, and a synaptic cleft, 15 nm wide, containing electron-dense extracellular matrix (see Figure 2). Thus, in all respects, the ultrastructure of the neuromuscular synapses, which form in the absence of *mbc* function, is identical to the wild type.

To test the properties of these synapses electrophysiologically, we made patch-clamp recordings from muscles VL3 and 4 in wild type and from the longitudinal mononucleate muscles at the equivalent positions in *mbc* mutant embryos (Figure 4). Excitatory junctional currents (EJCs) were measured in response to suction-electrode stimulation of the motor nerve. Mononucleate

muscles in *mbc* mutant embryos have reliable EJCs, although the amplitude of these EJCs is reduced to a tenth of that typical of a wild-type muscle (Figure 4). In some cases, the EJCs have a second peak, which may represent the double innervation of the mononucleate muscles, which is characteristic of the muscles VL3 and 4 in larvae and late wild-type embryos (Figure 4) (Jan and Jan, 1976; Broadie and Bate, 1993a). A further indication of functional NMJs in *mbc* mutant embryos is the fact that the mature embryos clearly twitch when they are mechanically removed from the egg case at the normal time of hatching.

In the light microscope, Synaptotagmin-labeled boutons are clearly detectable on mononucleate muscles in *mbc* mutant embryos. However, there are far fewer than on comparable muscles in wild-type embryos, suggesting that the much smaller EJCs we observed are a result of the much smaller size of the NMJ (see Figure 1; Figure 5) and reflect a correlation between synaptic size and synaptic strength (Bailey and Kandel, 1993; Lisman and Harris, 1993) rather than any impaired transmission at the synapse. Even though the muscle cells of *mbc* embryos are much smaller than in the wild type, most of the muscle surface, as in the wild type, is still not covered by synaptic contacts. However, in *mbc* mutant embryos, spots of Synaptotagmin staining appear in areas of nerve branches that are not in contact with muscles (see Figure 1). We counted the different types of active zones ultrastructurally and found that in *mbc* mutant embryos, 55% of all active zones are of the neurohemal or neuroneural type, which form away from the NMJ (see Figure 3).

Taken together, these data indicate that unfused muscle founder cells in *mbc* are capable of differentiating into mononucleate muscle cells with specific characteristics that attract the axons of appropriate motoneurons, and the NMJs that differentiate on these fibers have morphological and physiological properties closely resembling those of the wild type. In *mbc* mutant embryos, as in wild type, only a fraction of the muscle surface is devoted to the NMJ. This fraction in *mbc* represents a far smaller surface for the location of active zones than the surface encountered by equivalent motoneurons on a wild-type muscle, suggesting that muscles are capable of supporting a limited number of boutons, which is related to their size. Clearly, motoneurons innervating the smaller mononucleate muscles in *mbc* mutant embryos fail to regulate the number of active zones to match the available muscle surface, so that there is a large number of nonattached sites, which appear as neurohemal active zones adjacent to the muscle. This situation remains unchanged as the embryos age. Thus, 24 hr after the normal hatching time, the motoneurons still have a preponderance of neurohemal active zones over a much smaller number of motoneuronal active zones (data not shown).

Localization of Active Zones Requires Differentiated Muscle

Having found that NMJs can form on founder cells in the absence of fusion, we analyzed the NMJ in *mef2* mutant embryos, where failure to fuse is part of a more profound phenotype affecting muscle differentiation. As

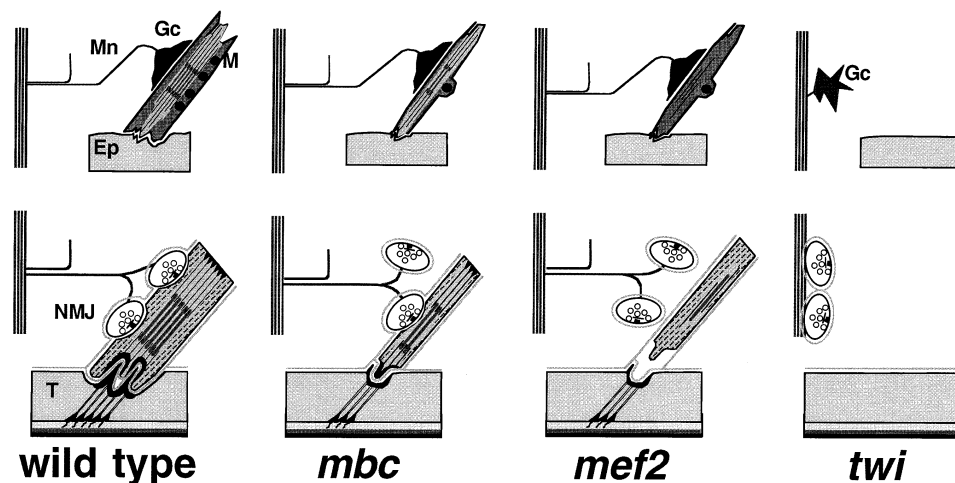


Figure 5. Summary of the Neuromuscular Phenotypes for Each of the Genotypes Analyzed

Upper panels: stage 16. Motor axons (Mn) contact muscles (M) via growth cones (Gc). The muscles are multinucleate or mononucleate and in contact with the epidermis (Ep). Lower panels: late stage 17. In wild type, muscles, tendon cells (T), and NMJs are fully differentiated, and a basement membrane (grey line) has formed. In *mbc* mutant embryos, NMJs differentiate normally, but half the active zones are in axonal swellings not attached to muscle. In the absence of *mef2* function, general muscle properties represented by myofilaments and myotendinous junctions are poorly differentiated and NMJs are not formed. In *twist* mutant embryos, motor axons defasciculate poorly and active zones form in axonal swellings within nerve bundles.

in *mbc* mutant embryos, the pattern of muscle founder cells is largely normal in the absence of *mef2* function (Bour et al., 1995; Ranganayakulu et al., 1995). However, these cells never differentiate to form mononucleate muscles as they do in *mbc* mutant embryos. Therefore, we set out to test the requirement for *mef2*-dependent muscle differentiation in presynaptic development of the NMJ. First, we repeated the observations previously made for *mbc*, to find out whether in *mef2* mutant embryos founder cells are contacted by appropriate presynaptic motoneurons.

In *mef2* mutant embryos, the Connectin-positive founder cells are usually present (see Figure 1), and the only reproducible change in the Connectin expression pattern is a doubling of the most dorsal Connectin-positive cell in each hemisegment. The proper number of Vestigial-positive (two of them also FasIII-positive) founder cells is not always detectable (present: 20% VL1-4, 50% LL1, 16% DA3, 90% DA2, and 40% DA1; n = at least 80 hemisegments). However, each of these cells can form, allowing their innervation to be investigated (see Figure 1). Analyses of the Connectin-, Vestigial-, or FasIII-expressing founder cells show that target recognition takes place in the absence of *mef2* function, and motoneurons that establish contact are always attracted by the correct founder cells, as judged by the branch pattern of innervation and the expression of the axonal markers FasIII and Connectin (see Figure 1; establishment of neuromuscular contact: 100% of LL1, DA2, and all Connectin-positive founder cells; 85% of VL1-4; 54% of DA3; but only 20% of DA1; n = at least 80 hemisegments). There is no evidence of motor axons contacting any myoblasts other than those that become founders. These observations show that *mef2*-dependent muscle differentiation is not required for target recognition by motoneurons.

Although muscles are founded in the absence of *mef2*

function and attract the correct motor axons, general aspects of muscle differentiation, such as myoblast fusion, β 3-Tubulin, and Myosin expression, are severely affected (Bour et al., 1995; Ranganayakulu et al., 1995). At stage 16, only a dorsal row of cells (and occasional ventral ones) shows some signs of differentiation. These Myosin-positive stretched cells can still be seen in mature embryos (data not shown); however, ultrastructural analyses of such cells at late stage 17 show that differentiation of myofilaments and muscle attachments is scarce and incomplete. Thus, general features of differentiated muscle are deranged or missing in *mef2* mutant embryos. Having established that founder cells are contacted by motor axons in *mef2* mutant embryos, we could now ask whether, as in *mbc*, normal neuromuscular synapses can form, or whether there are *mef2*-dependent properties missing from these embryos that are required for the assembly of a synapse.

Antibody staining reveals Synaptotagmin-positive varicosities in nerve bundles or on axons throughout the segment in *mef2* mutant embryos (see Figure 1E), and ultrastructural analysis indicates that active zones are present at frequencies comparable with wild type (*mef2* 0.48 and wild-type 0.51 active zones per section). Of 151 active zones found in *mef2* mutant embryos, 30 were within a range of 2 μ m of cells with myofilaments; however, all of them were neurohemal, neuroneural, or onto glial cells (see Figures 2 and 3). Even in the two cases where active zones were located at a neuromuscular contact that was free of basement membrane, the pre- and postsynaptic membranes failed to form the tight apposition that is typical of synapses, and in no case did we see evidence of the regularly periodic cleft material (see Figure 2). Thus, normal neuromuscular synapses are wholly absent in *mef2* mutant embryos, and the distribution of active zones in *mef2* resembles that of *twist* mutant embryos.

In summary, connections between motoneurons and muscles are formed in the absence of *mef2* function, but further differentiation of these contacts into mature NMJs fails. The development of normal synaptic contacts with localized active zones appears to depend on properties of differentiating muscles that are absent in *mef2* mutant embryos.

Discussion

Mutations Affecting Muscle Development Uncover Mechanisms Needed for Presynaptic Differentiation

The formation of the NMJ depends on an interplay between the specific properties of pre- and postsynaptic cells, as well as on general characteristics of neural and muscle lineages. Thus, in *Drosophila*, highly specific contacts are formed between individual motoneurons and their target muscles (Goodman and Doe, 1993; Bate and Broadie, 1995). Each neuron, however, makes the elements of the machinery for releasing the neurotransmitter, glutamate, just as each muscle synthesizes a field of postsynaptic glutamate receptors (Keshishian et al., 1996).

As in vertebrates, the initial assembly of the transmitter receptors is largely autonomous to the muscle, but concentration of these receptors to the synaptic site depends on an interaction with the presynaptic motoneuron (Broadie and Bate, 1993b; Hall and Sanes, 1993; Currie et al., 1995). Here, we have extended our analysis of synaptogenesis to investigate the synthesis and localization of the presynaptic machinery of neurotransmitter release. Our investigations take advantage of the fact that pre- and postsynaptic partners at the NMJ originate from different tissues, the development of which requires different sets of genes (Bate, 1993; Goodman and Doe, 1993). Thus, we can use mutations specifically affecting muscle development to investigate the role of postsynaptic cells in regulating the differentiation of their presynaptic partners. An advantage of this approach is that differentiating motoneurons are faced with a uniformly defective muscle field within which to form synaptic contacts. We have been able to show that while formation of presynaptic active zones is autonomous to motoneurons, proper localization of these zones absolutely requires the presence of a differentiated muscle fiber.

Presynaptic Active Zones Form in the Absence of Muscles

The starting point for this investigation is the demonstration that ultrastructurally normal active zones are formed by the axons of motoneurons in *twist* mutant embryos. This demonstrates that induction and assembly of active zones is independent of target cells. This apparent autonomy in the development of active zones is reminiscent of the development of the postsynaptic site in *Drosophila*, where functional glutamate receptors are expressed independently of innervation (Broadie and Bate, 1993a, 1993b; Currie et al., 1995), or in vertebrates, where myotubes can act autonomously to assemble a structure that resembles the postsynaptic apparatus (for

review, see Hall and Sanes, 1993). Presynaptic motoneurons in vertebrates are capable of releasing transmitter in the absence of target cells, but whether this represents the presence of fully differentiated active zones in these axons is unclear (Hume et al., 1983; Young and Poo, 1983).

The finding that presynaptic active zones can form independently of target cell contact has implications for the investigation and interpretation of NMJ formation in *Drosophila*. It suggests the existence of unknown muscle-independent mechanisms that trigger initiation of active zone formation. Presynaptic transmitter release onto motoneurons is unlikely to be this trigger, as previous studies have shown that proper active zones can form even in *syntaxin* mutant embryos, where transmitter release onto motoneurons is apparently blocked (Broadie et al., 1995). It could be that other cell interactions with motoneurons are required for formation of active zones, or that motoneurons simply follow a cell-autonomous program.

In addition, our data suggest the existence of muscle-independent mechanisms that allow the assembly of individual components into an ultrastructurally normal active zone. Ectopic active zones have normal T-bars, clustered vesicles, and often vesicles that appear docked (data not shown; see Broadie et al., 1995). Cysteine-string protein (Zinsmaier et al., 1990; data not shown) and Synaptotagmin (Figure 1) are also localized at ectopic active zones, and it could well be that these active zones are capable of transmitter release. This muscle-independent formation of active zones might either be analogous to a process of self-assembly, independent of any signal, or it could be elicited by a regulatory signal provided by the motoneuron. During normal synapse formation, the target muscle is required to localize either the assembly signal or the already formed or forming active zone at sites of synaptic contact.

Interestingly, active zones tend to form in axonal swellings (Figure 3). Either the formation of active zones induces such swellings or their assembly takes place in axonal areas that swell owing to independent mechanisms. In *mbc* and, to a slightly lesser extent, in *mef2* mutant embryos, such active zone-bearing varicosities tend to lie in defasciculated areas of the axon, suggesting that swellings may form preferentially at the tip of the axon, where growth cones would normally differentiate to form the presynaptic structures of the NMJs (Hall and Sanes, 1993; Broadie and Bate, 1993a). Thus, even when no proper neuromuscular contact is established, it might be that the growth cones can still differentiate into synaptic branches and swellings, bearing active zones. More detailed analyses of the architecture of unattached motor axons in mutant embryos will give further insights into the development of presynaptic terminals.

NMJ Formation Requires Differentiation of Target Muscles

By using mutations that specifically interfere with different aspects of muscle differentiation, we can show that there are muscle properties that are absolutely required for the normal development of the presynaptic site at the NMJ. For this, we used the different characteristics

of the *mbc* and *mef2* mutations that reflect the separate requirements for these two genes and their products in the myogenic lineage. Both genes are clearly required for myoblast fusion, but not for the acquisition of muscle-specific characteristics such as appropriate innervation. However, *mef2* is also required for the further differentiation of the myogenic lineage. Clearly, neither *mbc* nor *mef2* is required for initiating the muscle pattern (a normal arrangement of founder cells forms in both); both are required for fusion of myoblasts, but only *mef2* is required for the development of general muscle properties. It is this distinction that enables us to show that there are general characteristics of muscle cells that are required for the normal differentiation of the presynaptic site at the NMJ.

The *mbc* mutant phenotype reveals that myoblast fusion is not required for the formation of a neuromuscular synapse. The *mef2* mutant phenotype shows us that further muscle properties depending on *mef2* function are essential if a neuromuscular synapse is to form. Thus, *mef2* mutant embryos allow target recognition, as an early process of NMJ formation, to be separated genetically from the later process of NMJ differentiation. *mef2* is not expressed in presynaptic motoneurons (there is no expression in the ventral nerve cord, although there is some expression in the brain; Schulz et al., 1996), but is strongly expressed in the postsynaptic cells, the muscles (Taylor, 1995). This suggests that the requirement for *mef2* in the differentiation of presynaptic terminals is an indirect one mediated by the normal development of *mef2*-dependent characteristics in the muscles. The competence of motoneurons themselves is reflected by the fact that in *mef2* mutant embryos, apparently normal synapses can form between peripheral axons, characterized by tight synaptic clefts, filled with extracellular material, reminiscent of the structure of neuroneural synapses seen in the wild-type CNS (Shaw and Meinertzhagen, 1986; Burrows et al., 1989).

The assembly of the NMJ is one of a spectrum of properties of differentiating muscle that are under the control of *mef2*, including the expression of β 3-Tubulin, muscle Myosin, and α PS2 Integrin as well as the interaction of muscles with epidermal cells to form normal muscle attachment sites (Bour et al., 1995; Ranganayakulu et al., 1995). Like the formation of muscle attachment sites, assembly of the NMJ is an interactive process between two cell types, involving the coordinate expression and localization of a number of different components, including elements of the cytoskeleton, together with membrane-bound or membrane-associated proteins (Hall and Sanes, 1993). Our electron micrographs show that in *mef2* mutant embryos, there is a general failure to form a close association between motoneuron and muscle, a failure to localize the presynaptic active zone, and a failure in the assembly of at least one characteristic and possibly postsynaptic structure, the typically periodic electron-opaque material found in the synaptic cleft of the normal NMJ. Using the markers for muscles and nerves that are available, we can show that the majority of founder cells are contacted by appropriate axons in such embryos. Since we never see properly differentiated NMJs in *mef2* mutant embryos, we conclude that this phenotype is not simply caused by motoneurons making contact with inappropriate targets.

We have not yet identified the particular *mef2*-dependent feature(s) of the postsynaptic cell that are required for normal differentiation of the presynaptic terminal. It could be that the muscle secretes signals that influence the behavior of its presynaptic partner, or that there is a mechanical requirement for factors that prevent the ingrowth of basement membrane into the synaptic cleft or for transmembrane or extracellular components that promote and maintain neuromuscular adhesion. It is known that factors required for the alignment of presynaptic active zones are located in the basement membrane at the cholinergic NMJ in vertebrates (Sanes et al., 1978), and one of these components, s-Laminin, has been identified and shown to be muscle derived (Noakes et al., 1995).

Although the postsynaptic cell regulates the localization of presynaptic active zones, our experiments suggest that it does not regulate their number. Thus, the number of active zones in wild type, *twist*, *mbc*, and *mef2* is always comparable (see legend for Figure 3). The fact that occasional neuroneural and neurohemal release sites are found in wild-type as well as mutant embryos suggests that the muscle surface that is available for the localization of active zones within presynaptic boutons is limited and that these ectopic sites may simply be a consequence of the normal process of matching the relatively independent capacity of the motoneuron to assemble active zones with the capacity of the muscle to accommodate them. This view is reinforced by the fact that in *mbc* mutant embryos, the overall number of active zones formed by the motoneurons is unaffected, although the capacity of the mononucleate muscles to synthesize whatever properties are required for accommodating presynaptic active zones is greatly reduced in comparison with their multinucleate counterparts in wild-type embryos. This reduction presumably reflects the fact that each fusing myoblast contributes proportionately to the total muscle protein.

Thus, the differentiation of the presynaptic terminal at the NMJ in *Drosophila* depends on a process that integrates the development of muscles and motoneurons by targeting active zones formed by the motoneurons to the muscle surface. We can identify this as a *mef2*-dependent muscle-mediated process because of its characteristic ultrastructural and immunocytochemical phenotype. However, it is likely that many, perhaps all, of the features that are specific to the postsynaptic side of the junction also fail to develop in these embryos. The fact that these too are *mef2*-dependent properties should facilitate their identification and analysis in the future.

Experimental Procedures

Mutant Stocks

mbc^{C1} and *mbc*^{C2} appear to be null alleles of the *myoblast city* locus (Rushton et al., 1995). We used mutant alleles of the *mef2* gene, which were reported to be protein nulls: in *Df(2R)P544*, part of the *mef2* gene is deleted (courtesy of R. A. Schulz; Lilly et al., 1995; Ranganayakulu et al., 1995); in *Df(2R)P520*, most, if not all, of the coding sequence is deleted, and in the point mutation *mef2*²²⁻²¹, the sixth codon is converted into a nonsense codon (both courtesy of H. T. Nguyen; Bour et al., 1995). We used the genotypic constellations *Df(2R)P544* and *Df(2R)P520/mef2*²²⁻²¹ and could not find obvious phenotypic differences between them. The *twi*¹⁰⁹⁶ allele is a protein null (Nüsslein-Volhard et al., 1984).

Immunohistochemical Methods

Immunocytochemical staining of embryos was carried out following standard techniques for whole mounts (e.g., Rushton et al., 1995) or for dissected preparations (e.g., Broadie and Bate, 1993a). For Synaptotagmin staining of *twist* mutant embryos, specimens were injected with 4% paraformaldehyde in phosphate-buffered saline and postfixed in that solution for about 2 hr after their tips had been cut off with a razor blade splinter; then, specimens were cut in half and processed for whole-mount antibody staining. We used the following: first, anti-Myosin (rabbit; 1:1000; courtesy of D. Kiehart; Kiehart and Feghali, 1986); second, anti-S59 (rabbit; 1:5000; Baylies et al., 1995); third, anti-Vestigial (rabbit; 1:300; provided by S. Carroll; Williams et al., 1991); fourth, anti-Connectin (mouse; 1:10; provided by R. White; Meadows et al., 1994); fifth, anti- α PS2 Integrin (rat; 1:4; provided by N. Brown; Bogaert et al., 1987); sixth, anti-FasII (mouse; 1:10; provided by C. S. Goodman; VanVactor et al., 1993); seventh, anti-FasIII (mouse; 1:4; provided by C. S. Goodman; Patel et al., 1987); eighth, anti-Synaptotagmin (rabbit; 1:1000; provided by H. Bellen; Littleton et al., 1993); and ninth, anti-Cystein string protein (mouse; 1:10; provided by K. Zinsmaier; Zinsmaier et al., 1990). For the double stains, anti-Vestigial was preceded by anti-FasII or anti-FasIII. Both antibodies were developed together in the presence (flat preparations) or absence (whole mounts) of nickel chloride.

All specimens were embedded in Araldite. Flat preparations of late stage 17 embryos were postfixed in 2.5% glutaraldehyde in phosphate-buffered saline (this postfixation requires that the antibody stain was carried out without nickel chloride), cut off the glass with a razor blade splinter, and embedded. For documentation, negatives were scanned into a computer with a Nikon Scan LS1000. To achieve a more realistic impression, different focal planes were combined into one picture using Photoshop software.

Electron Microscopy

For the analysis of late stage 17, hourly egg lays were kept at 25°C until some larvae started hatching. Unhatched embryos were dechorionated with bleach and mutants selected by the following criteria: *mbc* mutant embryos fail to fill their tracheae with air, *mef2* mutant embryos have an unstricted gut (Bour et al., 1995; Lilly et al., 1995), and *twist* mutant embryos have a twisted appearance (Simpson, 1983; Nüsslein-Volhard et al., 1984). Embryos were injected with 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7–7.2) (for details, see Prokop and Technau, 1993). The tips of injected specimens were cut off with a razor blade splinter, followed by 1 hr postfixation in 2.5% glutaraldehyde in 0.05 M phosphate buffer.

Preparations were briefly washed in 0.05 M phosphate buffer, fixed for 1 hr in 1% osmium, in dH₂O, washed in dH₂O for 5 min, treated *en bloc* with an aqueous 2% solution of uranyl acetate for 30 min, dehydrated, and transferred to Araldite. Serial sections of 30–50 nm (silverygrey) thickness were obtained on a Reichert–Jung Ultracut, transferred to carbonated formvar-coated slot grids, following the method of Galey and Nilsson (1966), poststained with lead citrate for 5–10 min, and analyzed on a Jeol 200CX.

Frontal serial thin sections were taken from abdominal segments in a region about 10–15 μ m behind the anterior border of the denticle belts, which can be visualized in semithin sections with the light microscope. All active zones outside the CNS that were detectable in at least two consecutive sections were photographed and analyzed. The numbers of serial sections analyzed for active zone counts were 180 taken from two wild type, 240 from four *mbc* mutant, 226 from three *Df(2R)P544* mutant, 84 from one *mef2²²⁻²¹/Df(2R)P520* mutant, and 334 from three *twist* mutant embryos. Individuals with the same genotype always showed comparable distributions of active zone types with the exception of *twi* mutant embryos, where certain active zone types appeared in hot spots, therefore dominating the counts within these individuals.

Electrophysiology

Whole-cell recordings of muscles VL3/4 in wild type (22–24 hr; second and third abdominal segment; muscle nomenclature according to Bate, 1993) or of mononucleate muscles in equivalent position of *mbc* mutant embryos were made using standard patch-clamp techniques (Broadie and Bate, 1993a). Muscles were voltage-clamped at –60 mV. Signals were amplified using an Axopatch-1D

(Axon Instruments), filtered at 2 KHz, and analyzed using pCLAMP 5.51 software (Axon Instruments).

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