Chapter 2
Cell Adhesion Molecules at the *Drosophila* Neuromuscular Junction

Franklin A. Carrero-Martínez and Akira Chiba

Abstract A major goal in neuroscience is the understanding of organizational principles underlying cellular communication and the ensuing molecular integrations that lead to a functional nervous system. The establishment of neuromuscular connections (junctions) is a complex process that requires enumerable cellular and molecular interactions. There are many known and well-characterized molecular events involved in every aspect of neuromuscular junction (NMJ) formation. For instance, at the presynaptic side the motoneuron must differentiate, polarize, undergo dendrogenesis and axogenesis, and extend its processes out to the muscle field. This requires axon guidance, pathfinding, and finally synaptogenesis. At the postsynaptic side, the muscle cell must differentiate and find its correct place in the embryonic body plan to receive motor axons. There are many molecules known to play essential roles during each step in these self-organizational processes. Genetic and biochemical studies have identified molecules that facilitate accurate synaptic target recognitions, as well as those responsible for pre- and postsynaptic specializations. Cell adhesion molecules (CAMs) are known to play an essential role in establishing the NMJ. In this chapter, we begin by exploring *Drosophila* and its NMJ as a model system for glutamatergic synapses in the mammalian central nervous system. We continue by discussing selected CAMs, with known roles in *Drosophila* NMJ formation. We also explore the role these CAMs play in establishing the basic cytoarchitecture that ultimately results in functional neuromuscular synapses. We then examine the role CAMs play in synapse formation and plasticity. We conclude by providing an integrative model for CAMs function during synapse formation.

Keywords *Drosophila* · Filopodia · Myopodia · Cell adhesion molecule (CAM) · Capricious (Caps) · Connectin (Con) · Down syndrome cell

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adhesion molecule (Dscam) · Fasciclin II (FasII) · Fasciclin III (FasIII) · Integrin · N-Cadherin · Neuroglian (Nrg) · Toll

2.1 Introduction

Considering the number of neurons (billions in the human brain) and the connections among them (trillions), the study of how neuronal networks emerge is a daunting task. Even with available modern tools, addressing this fundamental question is difficult and appears virtually impossible. While animals display seemingly endless variations of different developmental strategies, the underlying molecular mechanisms of assembling a functional neuromuscular network are surprisingly well conserved between chordate and arthropod species.

For this reason, the use of simpler model organisms such as the fruit fly *Drosophila melanogaster* has allowed the identification, cloning, and functional assessment of genes at the molecular, cellular, and organism levels. This model organism offers a well-characterized repertoire of genetic tools, a relatively short life span, a rapid reproduction rate, a panel of efficient molecular techniques, and a completely sequenced and mapped genome (Adams et al. 2000). In addition, due to a high degree of evolutionary conservation, the analysis of gene functions in *Drosophila* yields information that is usually relevant for and applicable to more complex organisms, such as mice and humans.

The vertebrate nervous system is divided into two main systems: central nervous system (CNS) and peripheral nervous system (PNS). The CNS is composed of the spinal cord and the brain, while the PNS is composed of sensory neurons and the neurons that connect them to the brain. In *Drosophila*, the nervous system is divided into two systems as well: CNS and PNS. The fly CNS is composed of a series of neuronal cell bodies grouped into clusters, called ganglia. These ganglia are connected to each other by parallel connectives along the ventral midline axis of the organism as well as perpendicular commissures, giving rise to the characteristic ladder-like organization of the ventral nerve cord (VNC). Motor neurons send their axons away from the VNC forming an anterior and posterior fascicle, also known as intersegmental nerve and segmental nerve, respectively. The PNS is formed by sensory input neurons (multiple dendritic neurons, external sensory organs, and chordotonal organs), which carry information to the CNS using the anterior and posterior fascicles (Hartenstein 1993).

The *Drosophila* neuromuscular network has been established as a standard genetic and cell biological model by several pioneers such as Corey Goodman, Michael Bate, Haig Keshishian, and many others. Developmental processes can be analyzed in *Drosophila* at the level of a single gene or a single cell, an ability that is essential for studying the underlying fundamental organizational principles of complex self-organizing cellular networks (Hoang and Chiba 2001).

Motor neurons in the developing CNS and their muscle cell targets are experimentally accessible during embryonic development and follow a stereotypic pattern in each segment (Landgraf et al. 1997, Schmid et al. 1999), which persists through larval development. Individual neuron lineages, axon pathways, synaptic target muscles, and the types of synaptic boutons axons develop have all been documented (Chiba 1999, Schmid et al. 1999, Landgraf et al. 2003). In each half-segment, a total of 34 neurons, including 2 which are bilaterally innervating ventral unpaired median (VUM) motoneurons, make up the motor neuron pool that innervates 30 embryonic muscle cells by the end of embryogenesis. This means that muscle and neuronal cells are each uniquely identifiable with numbers considerably smaller than in vertebrate nervous systems. This innervation ratio, together with a stereotypical spatial arrangement, means that a given neuron/muscle synaptic pair can be reproducibly accessed for analysis during well-defined embryonic developmental stages (Fig. 2.1). A diagram of the stereotypical neuronal and muscle cells localization is provided in Fig. 2.2. Table 2.1 provides a convenient conversion for the two existing muscle nomenclature systems.

The *Drosophila* NMJ is glutamatergic and thus often considered as a convenient model for studying regulatory mechanisms for mammalian central glutamatergic synapses (Johansen et al. 1989, Budnik 1996, Keshishian et al. 1996, Davis and Goodman 1998, Chiba 1999). Thus, the underlying general

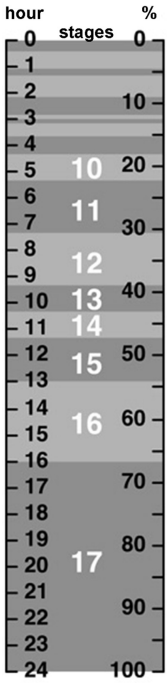


Fig. 2.1 ***Drosophila* embryonic development**. Wild-type embryonic development at 25°C has been characterized in different scales such as (*left*) hours after egg laying (AEL), (*center*) morphological and developmental events defining stages (Campos-Ortega and Hartenstein 1985), and (*right*) completed development as a percentage function

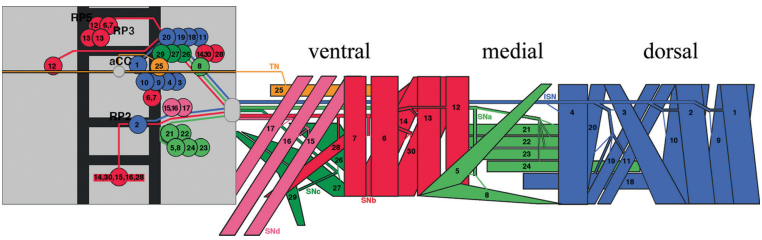


Fig. 2.2 Schematic representation of *Drosophila* neuromuscular network. Synaptic matchmaking between motoneurons (*left*) and embryonic muscles (*right*) is color coded according to the innervating nerve branch. Neuronal cell body localization is presented with the muscle number they innervate. Neurons commonly referenced throughout (RP5, RP3, aCC, RP2) are specifically named. Axons of the intersegmental nerve (ISN) and their partner muscles are shown in *blue*, while the transverse nerve (TN) is shown in *orange*. The segmental nerve (SN) branches are shown as follows: SNa (*green*), SNb (*red*), SNc (*green*), SNd (*pink*). There are two different naming conventions for *Drosophila* embryonic muscles. In this diagram we used the muscle numbering convention. Please refer to Table 2.1 for the corresponding name in the muscle location convention. For reference, the anteroposterior axis of the *Drosophila* embryo is always presented top to bottom, while the dorsolateral (ventral) axis is from right to left. That is, CNS is located to the left of the muscle field

Table 2.1 Muscle nomenclature conversion table

Number	Name	Number	Name
1	Dorsal acute 1 (DA1)	16	Ventral oblique 5 (VO5)
2	Dorsal acute 1 (DA2)	17	Ventral oblique 6 (VO6)
3	Dorsal acute 3 (DA3)	18	Dorsal transverse 1 (DT1)
4	Lateral longitudinal 1 (LA1)	19	Dorsal oblique 4 (DO4)
5	Lateral oblique 1 (LO1)	20	Dorsal oblique 5 (DO5)
6	Ventral longitudinal 3 (VL3)	21	Lateral transverse 1 (LT1)
7	Ventral longitudinal 4 (VL4)	22	Lateral transverse 2 (LT2)
8	Segmental border muscle (SMB)	23	Lateral transverse 3 (LT3)
9	Dorsal oblique 1 (DO1)	24	Lateral transverse 4 (LT4)
10	Dorsal oblique 2 (DO2)	25	Ventral transverse 1 (VT1)
11	Dorsal oblique 3 (DO3)	26	Ventral acute 1 (VA1)
12	Ventral longitudinal 1 (VL1)	27	Ventral acute 2 (VA2)
13	Ventral longitudinal 2 (VL3)	28	Ventral oblique 3 (VO3)
14	Ventral oblique 1 (VO1)	29	Ventral acute 3 (VA3)
15	Ventral oblique 4 (VO4)	30	Ventral oblique 2 (VO2)

There are two existing naming conventions for the embryonic and larval musculature. Throughout this chapter we use the muscle numbering nomenclature (Bate and Rushton 1993); however, since some references use the muscle location naming convention (Crossley 1978), we provide this table to ease cross-referencing.

principles presented here may apply to other systems. Ultimately (ignoring the specific identities of the cells discussed in this chapter), the fundamental question is (reduced to) why and how two cells decide to connect (synapse), remodel that connection (synaptic plasticity), or abnormally end their interaction (neurodegeneration).

2.2 CAMs at the NMJ

CAMs play critical roles in every single developmental stage leading up to the formation of functional NMJs. The study of CAMs has provided us with a functional explanation for the observed explicit cell motilities and required molecular integration within the emerging NMJ network. Here we provide a short list of cell-specific membrane-spanning CAMs that have been identified as target recognition molecules in the *Drosophila* neuromuscular system. Figure 2.4 provides a visual representation of the expression pattern of the molecules discussed below.

2.2.1 Capricious

Capricious (Caps) is a single-span transmembrane protein with 14 leucine-rich repeats (LRRs) in its extracellular portion. Caps is regulated by the transcription factor Krüppel and necessary for proper defasciculation of SNb axons (Abrell and Jackle 2001). Presynaptically, Caps is expressed in the anterior corner cell (aCC), RP2, U, and RP5 motoneurons. These cells innervate their Caps-positive muscle partners, muscles 1, 2, 9, 10, and 12. Caps-positive muscles innervated by Caps-negative neurons are muscles 14, 28, 15, 16, and 17 (Shishido, Takeichi and Nose 1998). Caps loss-of-function (LOF) results in muscle 12's motor axons miswiring to muscle 13. In muscles, Caps intracellular domain mediates target recognition, but not in neurons (Taniguchi et al. 2000). However, when Caps is overexpressed in all muscles, RP5 initially contacts muscle 12, before innervating muscle 13 (Shishido et al. 1998, Taniguchi et al. 2000). Taken together, these results suggest a mechanism by which upstream molecular events mediate Caps expression.

2.2.2 Connectin

Drosophila connectin (Con) is a cell surface protein with ten LRRs thought to mediate homophilic attractive adhesion (Nose et al. 1997). Starting at embryonic stage 12, Con is expressed in ventral and lateral muscles and on the intersegmental nerve (ISN) and segmental nerve (SN) axonal tracts that innervate them (Nose et al. 1992). This protein is proposed to play a dual role at NMJs, where it specifies (a) muscle pattern formation and (b) synapse formation. For instance, in muscles 18 and 21–24 an accumulation of Con protein to high levels is observed at muscle–muscle boundaries. In Con null mutants, gaps between these muscles are visible, while other Con-negative muscles develop normally (Raghavan and White 1997). Con gain-of-function (GOF) conditions, which are induced with pan-muscular promoters, do not result in major CNS, PNS, or muscle defects (Nose et al. 1992, 1997). At the presynaptic side, the protein is negatively regulated by the engrailed gene product (Siegler and Jia 1999). Con is

also expressed on the surface of glial cells PG1, PG3, and glial-like cell PG4 (Nose, Mahajan and Goodman 1992), which are thought to provide guidance cues for motoneuron axons. Given the dual roles in muscle pattern formation and synaptogenesis, we propose Con to play a general role in target selection at the NMJ.

2.2.3 Down Syndrome Cell Adhesion Molecule

Down syndrome cell adhesion molecule (Dscam, see also Chapter 9) is the *Drosophila* homologue of human Down syndrome cell adhesion molecule (DSCAM) and participates in NMJ presynaptic cell (motor neuron) pattern formation. It has been proposed that it may modulate the actin cytoskeleton through activation of the adaptor proteins Pak and Dock. The fly gene encodes a transcript that can be alternatively spliced to generate more than 38,000 predicted protein isoforms. These protein isoforms usually contain ten immunoglobulin (Ig) and six fibronectin (FN) type III extracellular domains. Dscam mutants are lethal during early larval development and exhibit a mild disorganization of the connective and commissural tracts within the ventral nerve cord (Schmucker et al. 2000). This protein is expressed in all muscles and all motor neurons; however, expression patterns of alternatively spliced isoforms are not known. This information may lead to a better understanding of adhesive regulation and activation of intracellular events.

2.2.4 Fasciclin II

Fasciclin II (FasII) is a homophilic CAM known to be important for the development, maintenance, and plasticity of the NMJ pattern. This protein contains five Ig and two FN type III domains (Grenningloh et al. 1991) and is considered as the fly ortholog of the mammalian neuronal cell adhesion molecule (NCAM). All motoneuron axon pathways and their growth cones express this protein from axonal outgrowth to synapse formation (van Vactor et al. 1993). The protein is also expressed at low levels in all muscle cells (Davis et al. 1997). Genetic increase in presynaptic FasII results in fusion of motoneuron axons, while genetic decrease leads to a complete or partial defasciculation of motor axon pathways (Lin et al. 1994, Lin and Goodman 1994). In pioneer axons such as aCC/RP2, FasII has been demonstrated to be required and sufficient to facilitate guidance of follower axons (Sanchez-Soriano and Prokop 2005). This suggests that FasII plays an essential role in the establishment of the presynaptic cell pattern. Postsynaptically, FasII is necessary for the postsynaptic accumulation of various proteins, including the scaffolding protein Discs large (Dlg), glutamate receptor subunits GluRIIA and GluRIIB, and FasII itself (Kohsaka et al. 2007). Hypomorphic mutant alleles (in which FasII levels were reduced by 50%) show a significant increase in presynaptic bouton numbers, but not in synaptic transmission (Schuster et al. 1996b, a). Furthermore, a

transient increase in FasII levels in specific muscle cells results in the formation of new ectopic functional synapses in those muscle cells (Davis et al. 1997). There is considerable evidence that FasII is able to activate intracellular signaling events through its interactions with PDZ (*P*ostsynaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and *Z*onula occludens-1 protein (zo-1)) domain-containing proteins such as Dlg (Kohsaka et al. 2007) and dX11/Mint/Lin-10 (Ashley et al. 2005). These experiments focused on embryonic and larval developmental stages and raise the possibility of a developmentally regulated choice between various PDZ scaffolding proteins as their interacting molecules, which either initiate synapse formation or modify NMJs during later developmental stages. For additional regulatory mechanisms involving FasII, refer to Packard et al. (2003). Taken together, all this evidence, together with its expression pattern (Fig. 2.4d), suggests that FasII plays essential roles in pattern formation and synapse initiation and maintenance, but not in target selection.

2.2.5 Fasciclin III

Fasciclin III (FasIII) is a single-span transmembrane immunoglobulin superfamily (IgCAM) member with three extracellular Ig domains that mediate homophilic adhesion and a PDZ-binding cytoplasmic domain that mediates interaction with postsynaptic Dlg (Woods et al. 1996). FasIII is normally expressed in muscle 6 and muscle 7 and the RP motoneuron axons, including RP3 which is part of the SNb nerve branch. LOF results in a failure of RP3 axons to innervate their normal target, while GOF experiments show that RP3 mistargets neighboring muscles misexpressing FasIII. In wild-type embryos, both aCC motoneurons, which are part of the ISN, and muscle 2 are FasIII negative. However, when FasIII is misexpressed in both aCC motoneurons and muscle 2, aCC axons misinnervate muscle 2 (Chiba et al. 1995). Furthermore, when a cytoplasmically truncated form of FasIII, which maintains its homophilic interacting domain, is misexpressed in all neurons, axon–muscle adhesion is observed. However, whether or not this leads to successful synaptogenesis is still unknown (Rose et al. unpublished). FasIII's cell-specific expression pattern may dictate its function as a positive target recognition molecule.

2.2.6 Integrins

Integrins are part of a large family of heterodimeric transmembrane proteins with five α subunits and a single β subunit in *Drosophila*. These six subunits generate five different integrin heterodimers. In addition to many other roles during embryonic development, it has been suggested that integrins play a role in linking the presynaptic partner axon with the postsynaptic muscle cell. *Drosophila* embryonic muscles express α_{PS1}/β_{PS} (PS1) and α_{PS2}/β_{PS} (PS2)

heterodimeric integrins. PS1, encoded by the gene *myospheroid*, is reported to bind to the ECM component laminin, while PS2 (encoded by the gene *inflated*) has RGD-binding activity (Gotwals et al. 1994). α_{PS1} and α_{PS2} integrin knockout mutations lead to widespread miswiring and reduced synaptogenesis (Hoang and Chiba 1998). That is, axonal fasciculation appears normal, but embryonic motoneuron axons overshoot their target muscles. Neuronal expression of an integrin transgene into the knockout greatly reduces axonal misguidance, but still fails to rescue synaptogenesis (Hoang and Chiba 1998). In embryos lacking postsynaptic α_{PS} integrins, NMJ adhesion is affected, but presynaptic synaptotagmin accumulation occurs at wild-type levels (Prokop et al. 1998). β_{PS} null mutant animals exhibit a muscle fiber twitch, even after the characteristic detached phenotype (refer to Section 2.3.2), suggesting that synaptic transmission still occurs in this altered NMJ (Prokop et al. 1998). However, at the larval NMJ synaptic arborization is greatly reduced (Beumer et al. 1999). This observation may be explained by integrins' ability to recruit essential postsynaptic components such as Dlg and FasII to the postsynaptic membrane (Beumer et al. 2002). These observations suggest that *Drosophila* integrins play multiple roles during NMJ formation and their postembryonic development.

2.2.7 N-Cadherin

N-cadherin (N-Cad) is an evolutionarily conserved classical cadherin with a large, complex extracellular domain that is composed of 15 cadherin repeats, a Fcc box (fly classic cadherin box), 2 cysteine-rich domains, and a laminin A globular segment. In addition it contains a catenin-binding cytoplasmic domain (Salinas and Price 2005, Suzuki and Takeichi 2008). Identification of 12 developmentally regulated alternative splice variants highlights a role of classical cadherins in synaptogenesis (reviewed in Halbleib and Nelson 2006). A common molecular architecture among splice variants, with differences in their extracellular and membrane-spanning domains, has been described (Yonekura et al. 2006). N-Cad regulates axonal pattern formation, presumably by regulating axonal fasciculation in the developing embryo (Iwai et al. 1997). However, a new study highlights the importance of these splice variants at the onset of synaptogenesis as they are differentially expressed in either presynaptic neuronal cells or the postsynaptic muscle cells (Hsu et al. unpublished). Identification of splice variants expression pattern is an essential step toward the understanding of how an organism fine-tunes its cellular connectivity.

2.2.8 Neuroglian

Neuroglian (Nrg) contains six Ig-like domains and five FN type III domains and participates in homophilic interactions (Hortsch 2000). Alternative splicing

generates 2 isoforms with an identical extracellular domain and 53 additional amino acid residues in the cytoplasmic domain of the neuronally expressed protein isoform (Hortsch et al. 1990). Protein expression pattern is negatively regulated by engrailed (Siegler and Jia 1999), with a shorter protein form expressed in body wall muscles, trachea, and gut and the longer form expressed in CNS and peripheral nervous system (PNS) neurons and their processes (Hall and Bieber 1997). The neuron-specific isoform is expressed in RP1, RP2, RP3, aCC, and pCC (posterior corner cell) motoneuron axonal projections and glial cells associated with them as they exit the CNS (Bieber et al. 1989). The muscle-specific isoform is expressed at high levels in muscles 7, 6, 13, 12, and 4 and at lower levels in other muscle cells and accumulates at the future site of synaptogenesis. Nrg null mutant analysis revealed motoneuron axon misprojections and stalling close to the target postsynaptic muscle cell. These mutants show complete embryonic development, but fail to hatch (Hall and Bieber 1997). The fact that Nrg accumulates at the future site of synaptogenesis raises the possibility that Nrg plays an essential at the NMJ. As proposed below, it will be interesting to investigate Nrg distribution within the myopodia and myopodial cluster.

2.2.9 Toll

Toll is a member of the LRR family of transmembrane proteins. This protein contains 15 extracellular LRR domains and is expressed in the embryonic muscles but preferentially accumulates at muscle–muscle contact. Toll displays a dynamic spatiotemporal expression pattern during axon targeting and exerts an inhibitory influence on motoneuron axons (Rose et al. 1997, Rose and Chiba 1999, Suzuki et al. 2000). Genetic misexpression of Toll in muscle 12 beyond hour 15 AEL results in RP5 motoneuron stalling just before its partner muscle. It has been proposed that Toll spatiotemporal regulation is crucial for its role in development, specifically the local inhibition of synaptogenesis of specific motoneurons (Rose et al. 1997).

In general, the CAMs reviewed here have specific expression patterns. In some cases there is a general expression pattern in both neurons and muscles, while the expression of other CAMs is restricted to a subset of neurons and/or muscle cells. Further studies addressing splice variants and their developmental regulation will lead to a better understanding of the affinity-based selection process in support of NMJ pattern formation and connectivity. The expression patterns of individual CAMs are presented in Fig. 2.4.

2.3 CAMs and Neuromuscular Network Formation

In this section we look at the essential roles that CAMs play in the establishment of the neuromuscular circuits at the stereotypical locations characteristic of the *Drosophila* NMJ (Fig. 2.2). Starting at around embryonic stage 12, myoblasts

fuse and motor axons start to navigate out of the ventral nerve chord. Muscle development occurs independently of motoneuron innervation and innervation occurs at the correct muscle partner cell even if position and/or morphology of its partner muscle are altered (Cash et al. 1992, Broadie and Bate 1993). Although both of these CAM-mediated events occur almost simultaneously we look at them separately to facilitate discussion.

2.3.1 Presynaptic Cell Pattern Formation

Drosophila neuronal network pattern formation is a critical, developmentally regulated process, in which CAMs and guidance cues help the axon to navigate the muscle field in search of its synaptic partner. CAMs play a critical role in establishing the neuronal network pattern by regulating three distinct types of adhesion: axon–extracellular matrix (ECM), axon–axon (i.e., fasciculation/defasciculation), and axon–muscle adhesion. In this section we cover axon–ECM and axon–axon adhesion events.

2.3.1.1 CAMs and Axon–ECM Adhesion

During embryonic development, motor axons navigate out to the periphery in search of their postsynaptic partners in a process known as axon pathfinding. All of these CNS axons must navigate and sort through many non-partner cells before contacting their respective synaptic targets. During this process, interactions with the ECM play a critical role for NMJ development and pattern formation (Ackley et al. 2003). At embryonic *Drosophila* stage 12 before muscle formation, mesoblasts intermingle with somatic mesoderm and start the deposition of collagen type IV (Mirre et al. 1988). Immunostaining confirmed the presence of this ECM component at this early developmental stage and showed collagen sheaths enveloping muscles, CNS, and other structures (Lunstrum et al. 1988). In general, integrin-mediated cell adhesion to the ECM provides a way for cells to adhere to a substrate in support of axon navigation toward its postsynaptic partner without engaging in a direct cell–cell interaction. This may account for the observation that integrin LOF mutants show severe patterning defects (Brown 2000). In this context, attachment of motoneuron axons to the ECM is a crucial and essential step to provide the mechanical stability that is essential for continued navigation. This principle has been demonstrated through surgical axotomy in a live, undissected embryo. When the developing aCC axon is cut, the resulting ends slowly recoil away from each other. This slow recoil suggests adhesion to the surrounding ECM (Siechen et al. unpublished). Dynamic regulation of these ECM interactions may be provided through matrix metalloproteinases (MMPs). MMPs are a large family of conserved proteases with two representatives in the *Drosophila* genome (Page-McCaw 2008). They are strongly expressed starting at embryonic stage 14 (Miller, Page-McCaw and Broihier

2008) and are able to degrade the basement membrane proteins fibronectin and type IV collagen and the ECM (Llano et al. 2000), which has led to the hypothesis that they clear ECM materials for supporting axonal growth cone pathfinding (McFarlane 2003). This type of cell adhesion may provide physical stability as the axon further explores the peripheral muscle field in search of its synaptic partner, even in the presence of a moving target (see below).

2.3.1.2 CAMs and Axon–Axon Adhesion

Axons that exit the CNS at the anterior fascicle eventually form the ISN, while those exiting the CNS at the posterior fascicle form the segmental nerves a–d (Hartenstein 1993). CAMs play an essential role in axon pattern formation (please refer to Fig. 2.2). For example, FasII is expressed on all motoneurons during embryogenesis and is necessary to maintain adhesion between axons in a process called fasciculation (van Vactor et al. 1993, Lin and Goodman 1994). When this FasII is removed, axonal growth cones do not extend properly and fail to fasciculate (Grenningloh et al. 1991). Fasciculation and defasciculation must be spatiotemporally regulated in order to allow for the formation of the highly stereotypical pattern of motor axons at the embryonic NMJ. It has recently been shown that MMPs may not be required for motoneuron axon extension, but instead promote FasII-mediated motor axon fasciculation and antagonize the semaphorin signaling pathway (Miller et al. 2008). The semaphorin pathway is essential for motor axon defasciculation. FasII or Con LOF mutations suppress Semaphorin LOF phenotypes, indicating that defasciculation of motoneuron axons occur through interference with axon–axon adhesion (Winberg et al. 1998, Yu et al. 2000). Other studies show that FasII is required to facilitate guidance of follower axons (Sanchez-Soriano and Prokop 2005), therefore playing an essential role in the establishment of the neuronal pathway. Taken together, these observations suggest that the right amount of cellular adhesion must be present or at least dynamically regulated in order for motoneuron axons to fasciculate/defasciculate at choice points en route to their synaptic targets.

2.3.1.3 CAMs and Axon–Muscle Adhesion

ECM deposition begins during early embryonic stages (Lunstrum et al. 1988, Mirre et al. 1988), even before the muscle cell pattern is established (see below). It is therefore likely that the interaction of a growth cone with the muscle surface is mediated by the ECM. Therefore, axon–muscle adhesion may not directly contribute to the establishment of the presynaptic cell pattern formation, but instead ECM interaction plays a larger role than previously considered. However, recent observations provide a novel role for axon–muscle adhesion in support of synaptogenesis. Please refer to Section 2.7.1 below for a discussion of these findings.

2.3.2 Postsynaptic Cell Pattern Formation

During embryonic development, myoblasts fuse to give rise to a multinucleated single syncytial muscle cell. In *Drosophila* this process involves neuronal CAM (NCAM/FasII), sticks and stones, and dumbfounded (also called Kirre) (Dworak and Sink 2002), all of which are members of the Ig superfamily. By the end of embryonic stage 13, the characteristic muscle cell pattern (Fig. 2.3) starts to emerge. The establishment of the embryonic muscle

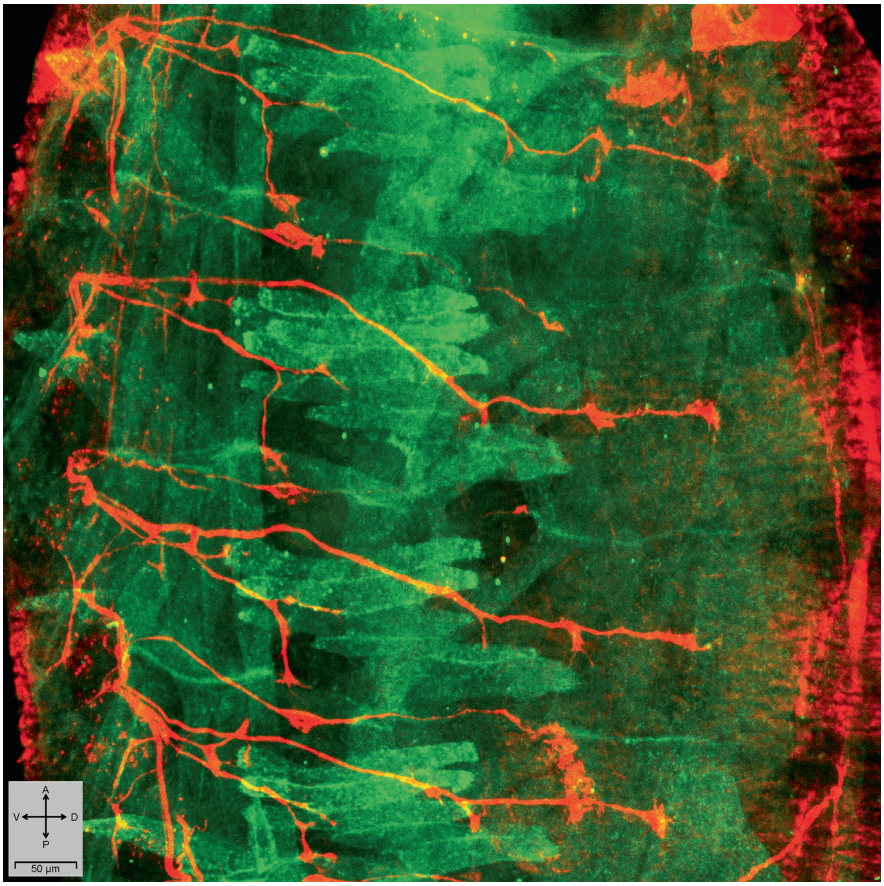


Fig. 2.3 Stereotypic embryonic neuromuscular cellular pattern. Laser scanning confocal micrograph projection reveals the highly stereotypic musculature (*green*; membrane-targeted GFP expression driven by the pan-muscle driver line Gal4-24B) and axonal (*red*; HRP antibody staining) pattern. The distinct stereotypical cellular pattern formed by each uniquely identifiable muscle and neuron, together with powerful genetic tools, makes the *Drosophila* NMJ an ideal system to dissect both cellular and molecular interactions under in vivo conditions. In this image, the anteroposterior axis runs from top to bottom and the dorsoventral axis is from right to left. This notation will be used throughout unless otherwise indicated

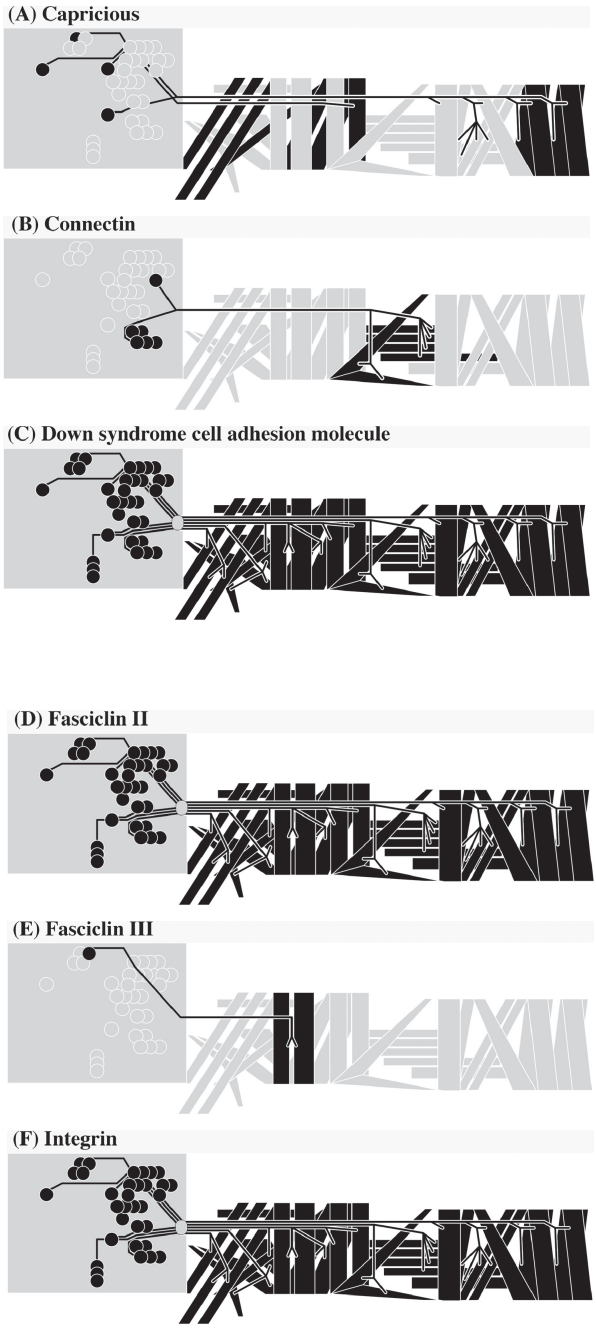
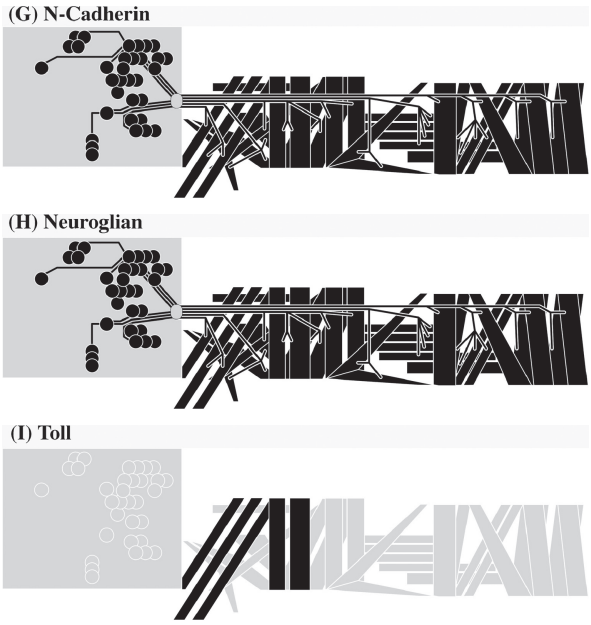


Fig. 2.4 CAM expression pattern at the embryonic NMJ. Diagrammatic representation of membrane-spanning adhesion molecules at the *Drosophila* neuromuscular junction. (**A**) capricious, (**B**) connectin, (**C**) Down syndrome cell adhesion molecule, (**D**) fasciclin II, (**E**) fasciclin III, (**F**) integrin, (**G**) N-cadherin, (**H**) neuroglian, and (**I**) Toll

Fig. 2.4 (continued)



pattern is governed in part by the direct apposition of integrins. α_{PS1}/β_{PS} (PS1) and α_{PS2}/β_{PS} (PS2) integrin complexes in both the epidermis and the muscle surface facilitate muscle insertion at apodemes of the lateral epidermis (Fessler and Fessler 1989). Removal of these molecules results in the embryonic lethal *myospheroid* phenotype. Myospheroid is characterized by muscle detachment from their respective insertion sites as soon as contractions begin, resulting in rounded muscle fibers (Wright 1960, Bokel and Brown 2002). Another essential PS2 function during myogenesis is the recruitment of non-muscle myosin II, which is also required for proper embryonic muscle pattern development (Bloor and Kiehart 2001). Integrins are also involved in the organization of the actin–myosin contractile structure into sarcomeres (Brown 2000, Bloor and Kiehart 2001). This is an important postsynaptic structure that will eventually support muscle function after NMJ formation. Taken together, these observations highlight the roles that CAMs play in establishing the muscle cellular pattern and in the maturation of the postsynaptic cell in preparation for its contractile function.

2.4 CAM-Mediated Intracellular Signaling Activation at the NMJ

The list of required proteins to successfully assemble a synapse is fairly extensive and continues to grow as research efforts are directed toward the functional characterization of CAMs at the *Drosophila* NMJ. These research efforts have

identified roles for a number of *Drosophila* CAMs in the activation of intracellular pathways on either side of the neuromuscular synapse. This activation may be mediated either directly by the CAM or indirectly through intermediary/linker proteins. Here we examine several examples in which CAMs facilitate the activation of intracellular events at the NMJ.

It has recently been shown that postsynaptic Dlg recruitment is mediated by FasII (Kohsaka et al. 2007), hinting at a CAM-mediated sequence of events at the onset of neuromuscular synapse formation. Dlg, the *Drosophila* representative of the mammalian PSD-95 family of scaffolding proteins, is essential for synaptogenesis of the embryonic NMJ (Carrero-Martínez et al. unpublished). It has been postulated that the multiple PDZ protein–protein interaction domains facilitate the molecular organization of the postsynaptic density (Thomas et al. 2000). Among the known postsynaptic molecular proteins interacting with Dlg are glutamate receptors, Shaker potassium channels, and many other essential postsynaptic proteins, including the CAMs FasIII, Nrg (Woods et al. 1996), and FasII (Thomas et al. 1997). Through these adhesive interactions, Dlg directly apposes its molecular counterparts on the presynaptic side (Thomas et al. 1997, Thomas et al. 2000), facilitating its role as a postsynaptic membrane coordinator.

Another example of CAM-mediated signaling at the NMJ involves *Drosophila* Ankyrin2 (Ank2). Ankyrins are adaptor proteins that mediate attachment of integral membrane proteins to the underlying actin cytoskeleton. *Drosophila* Ank2 is a neuron-specific ankyrin (Bouley et al. 2000), which is required for the proper organization of Nrg and FasII at the NMJ and for synaptic maintenance and functionality (Koch et al. 2008, Pielage et al. 2008). Currently, it is unknown whether Nrg is the Ank2-binding partner at the NMJ, but as Nrg is the major membrane-associated ankyrin2 ligand in *Drosophila* neurons (Bouley et al. 2000) it is an excellent candidate for interacting with Ank2 at the NMJ. Ank2 removal from the presynaptic cell results in disassembly of the larval NMJ due to the retraction of the microtubule-based cytoskeleton (Koch et al. 2008, Pielage et al. 2008). Ank2 is also associated with β-spectrin and when β-spectrin is removed from the presynaptic cell, FasII and Nrg become disorganized and eventually lose their NMJ localization. However, when spectrin is removed postsynaptically, similar defects were not observed (Pielage et al. 2005). These observations may explain why spectrin knockout flies have morphologically normal neurons at embryonic stages with reduced neurotransmission (Featherstone et al. 2001). At the larval NMJ, α- and β-spectrin RNAi knockout leads to synaptic disassembly and elimination (Pielage et al. 2005). Interestingly, the formation of the embryonic NMJ is not affected in Ank2 mutants, but as development progresses, motoneuron axons innervating posterior segments show a decreased bouton number, which manifests as posterior paralysis in the fly. Further dissection of these Ank2-mediated synaptic disintegration may prove relevant in the context of human motor neuron conditions (Koch et al. 2008).

CAMs provide the critical point-of-contact interactions that facilitate transduction of adhesive events intracellularly at both ends of the developing synapse. These CAM-mediated events are either directly or indirectly coupled to cytoplasmic proteins, which coordinate additional intracellular events, including essential interactions to the underlying cytoskeleton. The question of which protein initiates NMJ formation remains to be answered. Are CAMs the *first* molecules to set a signaling cascade in motion that leads to the activation of intracellular events? Or do intracellular events result in the clustering of CAMs at the site of synapse formation? According to the model we present below, a combination of both events may contribute to the successful genesis of the *Drosophila* neuromuscular synapse.

2.5 CAMs Mediate FORCES

By mutual and exclusive adhesion, CAMs are the ideal candidates to initiate intracellular signaling after the correct synaptic partner has been found. Recently, we have observed that muscles start to contract even before they become innervated (Siechen et al. unpublished). This is consistent with prior observations which established that muscle contractions precede the formation of the sarcomere in embryos (Volk et al. 1990, Bloor and Brown 1998). This type of movement is thought to originate either myogenically and/or due to the contraction of neighboring muscles sharing a common insertion point (Carrero-Martínez and Chiba unpublished). The timing of synaptogenesis initiation for each muscle, as assessed by axon–muscle contacts, varies slightly from muscle to muscle and is highly reproducible. Therefore, at the onset of neuromuscular synaptogenesis in vivo, the motoneuron is presented with a mechanically rich environment from which to select its correct synaptic target muscle.

While the significance of muscle contractions in the context of the developing NMJ remains to be addressed, we propose that CAMs mediate FORCES. *C*ell *A*dhesion *M*olecules mediate *F*orce-*O*rchestrated *R*etrograde *C*ommunication to *E*nhance the *S*ynapse and support its continued development. Preliminary observations support this model in which sufficient cellular adhesion is provided to withstand the muscle contractions while providing a way to transduce the axon–muscle adhesion event to both synaptic partners. While addressing this question we found that when a motoneuron axon is surgically cut at different developmental time points, different retraction/recoiling rates are observed. Prior to synaptogenesis, the resulting ends slowly recoil, but after the synapse is established, exogenously applied forces, which exceed physiological conditions, are insufficient to separate both synaptic partners (Siechen et al. unpublished). We concluded that adhesive molecules progressively accumulate as the NMJ develops into a functional structure. Taken together, these observations suggest that during axon navigation the source of adhesive stability may be provided by interactions with the surrounding ECM. As

development proceeds, adhesive forces provided by precisely matched synaptic partners increase the mechanical stability of the developing synapse. Stay tuned as we and other research groups continue to dissect this novel role for CAMs at the NMJ.

2.6 CAMs in NMJ Plasticity

During the life cycle of *Drosophila*, there are two distinct classes of NMJs: the embryonic/larval NMJs (reviewed by Keshishian et al. 1996) and the adult NMJs (reviewed by Patricia K. Rivlin 2004). A popular and versatile model for studying NMJ development and plasticity has been established in a set of motoneurons found in abdominal segments. In the context of metamorphosis, adult muscle cells develop from twist-expressing myoblasts, which are present in the larva (Currie and Bate 1991). These cells are closely associated with neural cells and continue this association throughout metamorphosis, to at least until 51 hours after puparium formation (APF) when the adult muscle pattern has been completed (Currie and Bate 1991). In specific cases, surgical denervation at the onset of metamorphosis impairs muscle development, but not its characteristic distribution pattern (Currie and Bate 1995). A genetically induced reduction of FasII affects the morphology of adult NMJs by reducing the area, but not the size of individual synaptic boutons. Conversely, a genetically encoded increase of FasII results in increased numbers of boutons and presynaptic area (Hebbar et al. 2006).

Taken together, these results suggested a role of adhesion molecules in maintaining the muscle–neuron contact even during the dramatic reorganization that takes place during metamorphosis and that this interaction is essential for the development of adult muscle morphology and thus for adult fly function and behavior. The astounding NMJ cellular reorganization that takes place during the process of metamorphosis makes this system an excellent choice to study the process of synaptic plasticity.

2.7 A Two-Step Model for CAM-Mediated NMJ Formation

The body of knowledge, which has been generated during the last 20 years in the fields of adhesion molecules and synapse formation, has provided us with a staggering amount of molecular players, cellular events, and their respective interactions, which are essential for the formation of a functional NMJ synapse. Here we propose an integrative model, which takes into consideration earlier observations and recent work describing the appearance of postsynaptic filopodia and their role in NMJ formation. This two-step model puts the role that CAMs play during the process of synaptogenesis into a new perspective. The first step involves the presentation of CAMs at the tips of myopodia to facilitate

attractive or repulsive growth cone recognition. The second step occurs locally at the postsynaptic cell surface and involves the CAM-mediated creation of a subcellular space that enhances the chance of molecular complexing, which is required for the establishment of a functional neuromuscular synapse.

2.7.1 Myopodia Brings CAMs Closer to Navigating Motor Axons

Using three- and four-dimensional analyses of undissected, live *Drosophila* embryos expressing GFP-based bioprobes, we have observed highly motile actin-based microprocesses called *myopodia* (Fig. 2.5) that extend from muscle cells before neuromuscular synaptogenesis is initiated in *Drosophila* (Ritzenthaler et al. 2000). Appearance of myopodia coincides with motoneuron outgrowths in both *Drosophila* (Ritzenthaler et al. 2000) and mouse embryos (Uhm et al. 2001, Misgeld et al. 2002). In vivo, these myopodia reach up to 30 μm in length translating to nearly the full length of a segment in a *Drosophila* embryo (Ritzenthaler et al. 2000). When considered in context with previous reports showing that neurofilopodia reach up to 15 μm in length (Johansen et al. 1989), it is conceivable that prior to synaptogenesis all synaptic partners in a given segment fall within direct reach of each other. Furthermore, the rapid protrusion/retraction rates of each individual myopodia may serve as a mechanism to break through ECM (please refer to Section 2.3.1.1 for

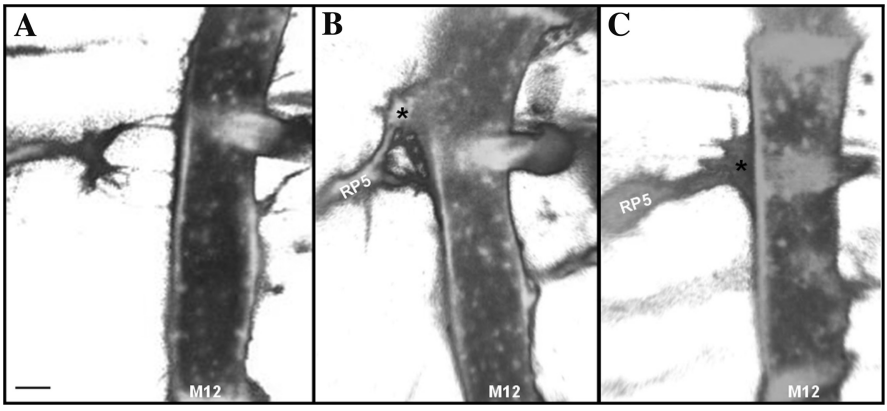


Fig. 2.5 Myopodial behavior at the onset of synaptogenesis. Myopodia are dynamic filopodia-like structures emerging from the postsynaptic muscle cell surface prior to synaptogenesis. (**A**) As development proceeds (hour 14:00 AEL), a precisely matched synaptic partner (RP5 motor neuron) starts its interaction with muscle 12's myopodia. (**B**) Within 30 min of initial contact, presynaptic and postsynaptic filopodia form a myopodial cluster (* in **B** and **C**). (**C**) This new subcellular space serves as a signaling hot spot where the postsynaptic density is organized at around hour 15:00. Live confocal imaging of membrane-targeted GFP expressed in muscle 12 and in neurons (b–d). Scale bar = 10 μm

additional details), making this an ideal structure for the presentation of 'recognition molecules', such as CAMs.

If we consider that every muscle presents its myopodia and every growth cone displays a number of filopodia, then the potential combinatorial interactions among pre- and postsynaptic filopodia are staggering. However, two developmentally regulated events greatly restrict the number of possible interactions. First, initial outgrowth of pioneer ISN axons takes place before muscle fibers have formed (Campos-Ortega and Hartenstein 1985). Second, with few exceptions myopodia have a strong tendency to aggregate toward the ventral nerve cord (Ritzenthaler and Chiba 2003). Thus, lateral and ventral muscles are not able to interact with pioneer axons which have navigated dorsally by the time these muscles are formed and start extending their myopodia. This spatiotemporal restriction is important and may explain why certain CAMs, which are simultaneously expressed by different muscles (i.e., Caps), support selective NMJ formation.

Since myopodia formation is a transient and unique embryonic event, which has only recently been described (Ritzenthaler et al. 2000, Ritzenthaler and Chiba 2003), many studies have not taken into consideration its role in CAM presentation and CAM-mediated synaptic enhancement (see FORCES above). However, in recent studies, FasII (Kohsaka et al. 2007) and Caps (Nose 2008) have been shown to be present in myopodia. Caps is expressed by both dorsal and ventral muscles (Shishido et al. 1998) and is enriched at the tip of myopodia. When mutated, the initial neurofilopodia–myopodia contact fails to stabilize (Nose 2008). These observations suggest a mechanism in which localized regions of the postsynaptic membrane guide and facilitate the formation of the NMJ by bringing appropriate CAMs and other signaling components in direct contact with their presynaptic partners, well before synaptogenesis is initiated. Thereby, precisely matched synaptic partners present CAMs through filopodial (i.e., neurofilopodia–myopodia) interactions to support axonal growth cone guidance, pathfinding, and synaptogenesis (Fig. 2.6). In addition, these CAMs may provide the necessary adhesive force to keep synaptic partners connected even in the presence of a moving target.

2.7.2 CAM-Mediated Postsynaptic Signaling Hub

We have shown that every neuromuscular synapse in live embryos is preceded by a transient stabilization/clustering of presynaptic and postsynaptic filopodia from matched partners upon contact. These interactions result in the creation of a new postsynaptic subcellular space, called *myopodial cluster* (Fig. 2.5d). In a prospero mutant background, which has severe motor neuron axon outgrowth delays from the CNS, myopodial cluster fails to form. Furthermore, filopodial interactions between non-partner cells, though extensive and essential for proper axon guidance, do not result in myopodial clustering. Myopodial cluster

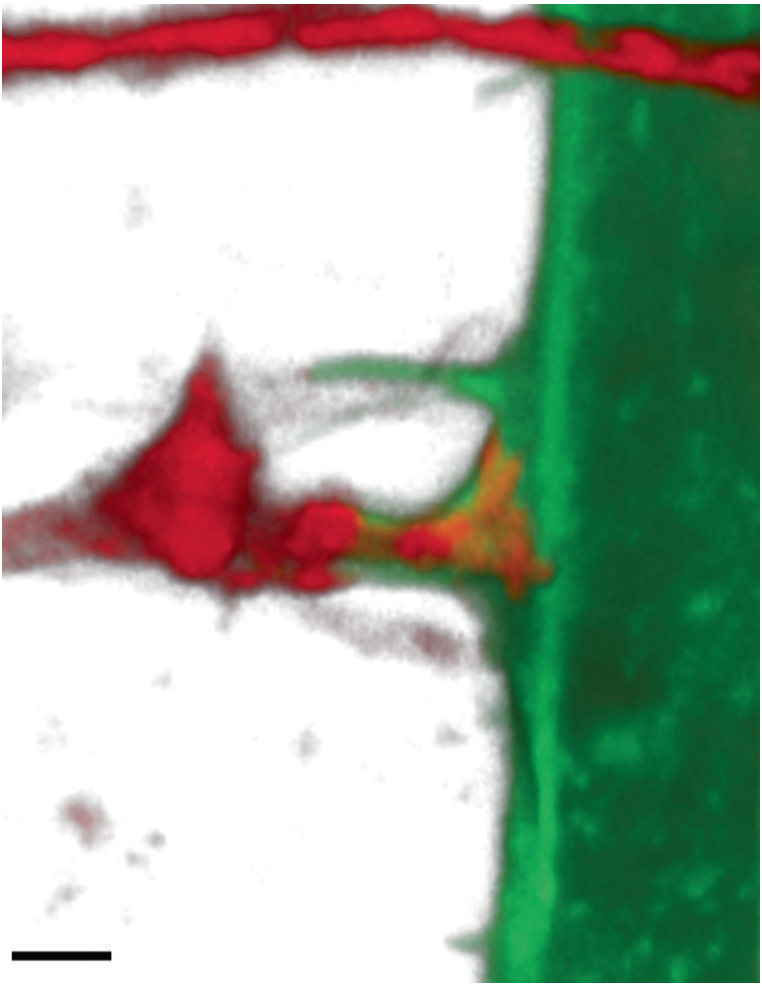


Fig. 2.6 Synaptogenesis. Muscle 12 expressing membrane-targeted GFP (*green*) and RP5 motor axon, as revealed by HRP antibody staining (*red*), in an intact (i.e., undissected) embryo fixed at hour 14:30 AEL. Scale bar = 10 μm

formation is also impaired in α_{PS2} integrin or Toll misexpression mutants, which exhibit abnormal axon targeting (Ritzenthaler et al. 2000, Ritzenthaler and Chiba 2003). These observations suggest that myopodial clustering only occurs in response to correctly matched synaptic binding partner.

Myopodial structures have not been observed at later larval developmental stages when new synaptic boutons are continuously added to the synaptic terminal. This may provide an explanation for earlier observations that inappropriate synaptic connections could be stabilized when FasII is increased in specific muscles during embryogenesis, but not when a similar increase is

induced during larval stages (Davis et al. 1997). A recent research report may provide us with a potential cellular and molecular mechanism that may underlie this effect. FasII aggregates at the postsynaptic myopodial cluster and is necessary for postsynaptic Dlg accumulation (Kohsaka et al. 2007). Functional genetic dissection of Dlg reveals that it is essential for NMJ formation, but is not required for myopodial cluster formation. Furthermore, when myopodial clustering is genetically inhibited by a dominant-negative form of ezrin, synaptogenesis still proceeds, but the resulting NMJs are morphologically altered (Carrero-Martínez et al. unpublished). These observations suggest a model in which presynaptic FasII facilitates the recruitment and clustering of postsynaptic FasII, which in turn recruits Dlg molecules and initiates the formation of a functional synapse (Fig. 2.7). Taken together, these results highlight the role for CAMs in the development of the embryonic postsynaptic subcellular space, which facilitates and perhaps even initiates intracellular signaling events that are essential for the development of the embryonic NMJ (Fig. 2.8).

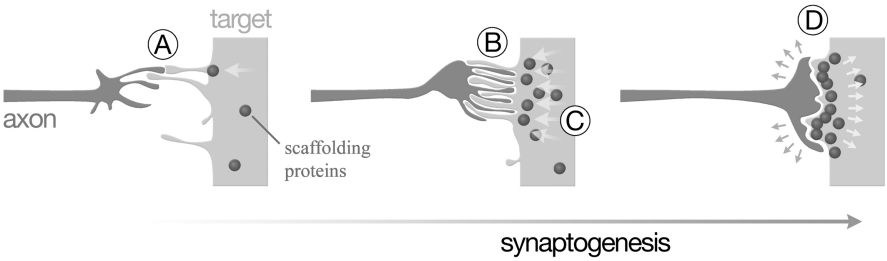


Fig. 2.7 Sequence of events leading to the successful formation of the NMJ. CAMs are presented at a distant site from the muscle surface (**A**). This interaction allows the muscle cell to start the assembly process of the postsynaptic specialization as the synaptic partner approaches. Increased and continued interaction between precisely matched synaptic partners results in the formation of a new subcellular space, called myopodial cluster (**B**). The creation of this space serves as s signaling hot spot to which several proteins such as the scaffolding protein Dlg are recruited (**C**). This space facilitates molecular interactions and the formation of signaling complexes, which are required for the formation of the neuromuscular synapse (**D**)

2.8 CAMs: The Cellular Glue that Holds Our Thoughts Together

Over the last decade, progress has been moderate in terms of identifying new CAMs that are involved in the formation of the *Drosophila* NMJ. Instead, attention in the field has shifted to more contextual issues such as filopodia. However, pioneer work in CAM and NMJ research was performed before postsynaptic filopodia had been described. Therefore, the early analyses did not take into consideration myopodia as active players for CAMs presentation

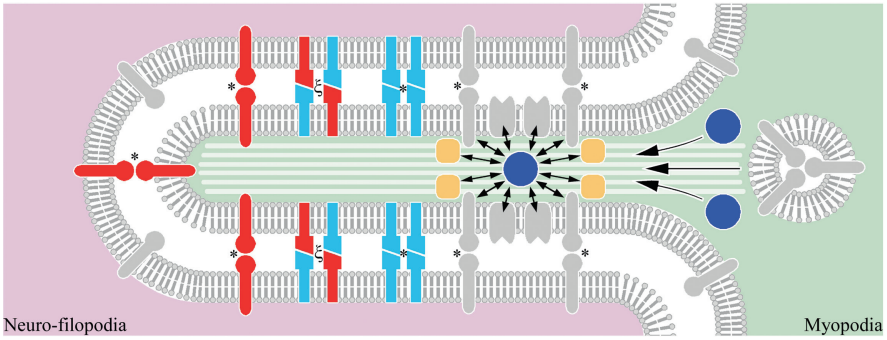


Fig. 2.8 CAMs facilitate and sustain NMJ formation. We propose that membrane-spanning proteins (such as CAMs) presented by developmentally regulated filopodia in the presynaptic (i.e., neurofilopodia; *left*) and postsynaptic (i.e., myopodia; *right*) cells may provide a mechanism that facilitates axonal pathfinding and target selection (Fig. 2.7). This is the first step toward the successful formation of the neuromuscular junction (see Section 2.7.1). As development of the NMJ progresses, myopodia aggregates to form a myopodial cluster (see Section 2.7.2). The increased surface area presented at the myopodial cluster/neurofilopodial interface locally increases the chance for trans-synaptic interaction among homophilic (*) and heterophilic (ξ) CAMs and initiate trans-synaptic signaling (*double-headed arrows*) between both synaptic partners. The narrow cytoplasmic space within each individual myopodia facilitates interaction between membrane-spanning CAMs and cytoplasmic scaffolding proteins such as Dlg, Ankyrin2, Pak, Dock, and other proteins (*circles* and *squares*). Polarization of the cytoskeleton may facilitate the recruitment of vesicles packed with other components essential for the development of the NMJ

during the process of embryonic synaptogenesis. This has led us to propose a two-step model in which CAMs facilitate the formation of the NMJ. The first step occurs during the process of growth cone extension and pathfinding. At this developmental stage CAMs are presented by both neurofilopodia and postsynaptic filopodia. This means that synaptic partner recognition may take place further away from the site of synaptogenesis than previously considered. According to our model, this ensures that both synaptic partners could form a CAM-mediated stable interaction and are able to withstand the forces generated by non-myogenic muscle contraction. At the same time, axons that are not appropriately matched with their corresponding synaptic partners will not be able to withstand the intercellular mechanical tension produced by these early muscle contractions and thus fail to activate appropriate postsynaptic signal transduction events. This activation of postsynaptic signaling events is the second step in our model. The early interactions are eventually transformed into the myopodial cluster, which serves as a signaling hot spot for the transformation of the presynaptic filopodia into synaptic boutons, a process that is concluded by the end of embryogenesis. Our model only accounts for the generation of the embryonic neuromuscular network pattern, which remains largely intact through larval stages. This is because myopodia are only transient

structures, which uniquely respond to the mutual recognition by synaptic partners at the onset of embryonic synaptogenesis.

Identification of new synaptic CAMs may provide additional insights into how the *Drosophila* NMJ is established and maintained. Furthermore, identification of CAM splice variants, their developmental regulation, and localization may provide us with additional insights into how the *Drosophila* NMJ is fine-tuned. For now, the existence and importance of these isoforms in NMJ development remain largely unknown.

Myopodia, myopodial clustering, and their interaction with presynaptic filopodia may offer an opportunity to further dissect the molecular integrations of this glutamatergic synapse in an in vivo model. If we consider that flies which are heterozygous for a specific integrin mutation have short-term memory defects (Grotewiel et al. 1998) and that FasII-mediated adhesion may be involved in long-term memory processes (Cheng et al. 2001), we might hypothesize that CAMs are the cellular glue that holds our thoughts together.

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