The Sticky Synapse

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Editors

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Cell Adhesion Molecules and Their Role in Synapse Formation and Maintenance

Springer

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Cover illustrations: Developing Synapses - Synapses are formed at points of contact between axons and their targets. From left, Drosophila neuromuscular junctions (motor axons, red; muscles, green), mouse neuromuscular junctions (motor axons, green; neuromuscular junctions, pink), and mouse cerebellar synapses in culture (pontine axons, blue; cerebellar granule cell dendrites, pink; synapses, green).
Courtesy of Carrero-Martinez and Chiba (Drosophila) and Harris and Umemori (mouse).

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Preface

The molecular mechanisms, which are responsible for the functional differences between the various types of neuronal synapses, have become one of the central themes of modern neurobiology. It is becoming increasingly clear that a misregulation of synaptogenesis and synaptic remodeling and dysfunctional neuronal synapses are at the heart of several human diseases, both neurological disorders and psychiatric conditions. As synapses present specialized cellular junctions between neurons and their target cells, it may not come as a surprise that neural cell adhesion molecules (CAMs) are of special importance for the genesis and the maintenance of synaptic connections. Genes encoding adhesive molecules make up a significant portion of the human genome, and neural CAMs even have been postulated to be a major factor in the evolution of the human brain. These are just some of the many reasons why we thought a book on neural CAMs and their role in establishing and maintaining neuronal synapses would be highly appropriate for summarizing our current state of knowledge. Without question, over the near future, additional adhesive proteins will join the ranks of synaptic CAMs and our knowledge, and how these molecules enable neurons and their targets to communicate effectively will grow. We hope that this book will provide a comprehensive and timely synopsis of the role of CAMs at synaptic connections and will encourage other researchers to join this exciting field of neuroscience, which has the promise not only to yield new insights into the functioning of our brain but also to shed light on some devastating human diseases.

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Chapter 1
A Short History of the Synapse – Golgi Versus Ramón y Cajal

Michael Hortsch

The history of the synapse started not only as a struggle between two ideas but also as a feud between the two founding fathers of modern neuroscience, the Italian Camillo Golgi (1843–1926) and the Spaniard Santiago Ramón y Cajal (1851–1934). Preceding their groundbreaking portrayals of the nervous system structure, Robert Remak (1815–1865), Theodore Schwann (1810–1882), Otto Friedrich Karl Deiters (1834–1863), and others had published only rudimentary histological descriptions of nerves and of some other parts of the nervous system. However, the limited resolution of the microscopic analysis at that time did not allow them to elucidate the cellular details and the functional relationships between individual nervous system components. In 1872, Joseph von Gerlach (1820–1896) formulated the first theory to explain the cellular organization of the nervous system (Gerlach 1872). His model, the Reticular Theory, postulated that the nervous system consists of a continuous syncytial network or reticulum. Nerve fibers, dendrites, and neuronal cells would be directly connected to each other by cytoplasmic bridges with the neuronal cell bodies providing only nourishment support.[1] Over the following years, Joseph von Gerlach together with Camillo Golgi became the major proponents of the initially widely accepted Reticular Theory. Ironically, it was a fortuitous discovery by Camillo Golgi that ultimately led to its demise.

[1] J. Gerlach J (1872) Von dem Rückenmark. In: Stricker S (eds) Handbuch der Lehre von den Geweben des Menschen und der Thiere. Verlag von Wilhelm Engelmann, Leipzig on page 684: ". . .the finest divisions of the protoplasmic processes take part in the formation of the fine nerve fiber network, which I consider to be an essential constituent of the gray matter of the spinal cord. . . .(T)he neuronal and cytoplasmic extensions of the cells in the gray matter are therefore connected in two ways with the nerve fibers of the spinal cord. First, by means of the nerve process. . .and secondly through the finest branches of the protoplasmic processes, which become a part of the fine nerve fiber net of the gray matter."

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In 1873, Camillo Golgi reported a novel histological staining procedure, which selectively highlights a small number of neuronal cells at random while leaving most other neurons unstained (Golgi 1873). This effect is achieved by impregnating fixed neuronal tissues with potassium dichromate and silver nitrate. All stained cells are entirely filled with a brown or black precipitate of silver chromate, revealing even slender dendritic and axonal processes. In 1887, Santiago Ramón y Cajal learned about this novel histological method and developed it further to reveal even minute details of neuronal structures (Fig. 1.1). Over the following years, both Ramón y Cajal and Golgi used this staining technique for a detailed survey of many neuronal tissues. From his results, Santiago Ramón y Cajal concluded that the nervous system is not a continuous network, but rather consists of separate, discontinuous units or cells.

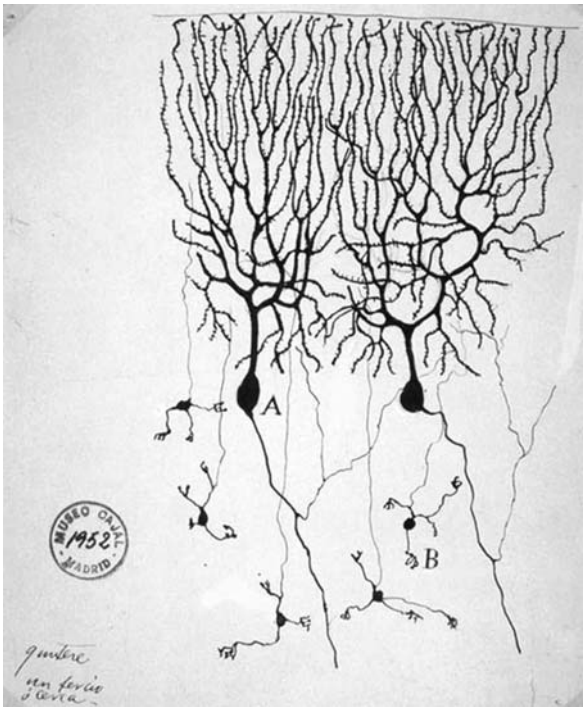

Fig. 1.1 Drawing of Purkinje (**A**) and granule cells (**B**) from an adult pigeon cerebellum by Santiago Ramón y Cajal (Golgi method), 1899. Instituto Santiago Ramón y Cajal, Madrid, Spain

Feeling scientifically isolated at his position as professor of histology and pathological anatomy in Barcelona, Ramón y Cajal traveled to the October 1889 meeting of the German Anatomical Society, which was held at the University of Berlin (Ramón y Cajal 1937). There he made the acquaintance of Rudolph Albert von Kölliker (1817–1905), Wilhelm His (1831–1904), Heinrich Wilhelm Gottfried von Waldeyer-Hartz (1836–1921), Arthur van Gehuchten (1861–1914), and other eminent histologists. After viewing Ramón y Cajal's

preparations, Albert von Kölliker in particular encouraged him to publish his findings more widely and later even confirmed and extended them with his own work.

Based on Santiago Ramón y Cajal's conclusions and the results of other researchers, Wilhelm von Waldeyer-Hartz in 1891 published a paper, in which he outlined an alternative theory, the Neuron Doctrine of the nervous system (Waldeyer-Hartz 1891), which subsequently received overwhelming support throughout the scientific community. In his publication, von Waldeyer-Hartz used for the first time the term "neuron" (Greek "νευρων" for sinew or tendon) to describe the separate cellular subunit that is common to all neuronal tissues. At that time, it had become clear that most neuronal cells consist of three different subcellular domains: the neuronal cell body or soma, fine tree-like cytoplasmic processes, and a single long fiber-like extension. Inspired by their branch-like structure and after the Greek word "δεντρο" for tree, Wilhelm His in 1889 had suggested the use of the phrase "dendrites" for the finer cytoplasmic neuronal processes (His 1889). Later in 1896, Albert von Kölliker added the term "axon" (Greek "αξον" for axle or axis) for the long, fiber-like extension (von Koelliker 1896). Over the following years, Santiago Ramón y Cajal in Spain and Arthur van Gehuchten in Belgium independently modified and extended the Neuron Doctrine by adding the Law of Dynamic Polarization, which states that neuronal signals only travel in one direction in a neuron, from dendrites and cell bodies to axons (Berlucchi 1999).

However, as the acceptance of the Neuron Doctrine grew, it raised a new problem. Neither von Waldeyer-Hartz's hypothesis nor Ramón y Cajal's morphological analysis offered an explanation of how a neuronal signal would be transferred from one neuronal cell to the next. Although specialized contact regions between neurons were soon suspected to be responsible for this process, no mechanistic explanation would be forthcoming for a considerable time. When preparing the 6th edition of his *Handbook of Human Physiology*, Sir Michael Foster (1836–1907) secured the assistance of his student Sir Charles Scott Sherrington (1857–1952) for writing the chapter on the Central Nervous System (Foster and Sherrington 1897). They both felt that a proper term for describing these special contact points between neurons was lacking and requested the help of Arthur Woolgar Verrall (1851–1912), a classical Greek scholar at the Trinity College in Cambridge (Tansey 1997). Verrall suggested the term "synapse" from the Greek "συν" (syn meaning together) and "απτειν" (haptein meaning to clasp), which was adapted by Foster and Sherrington and thereby introduced as the scientific term for describing neuronal contacts.

In 1906, the accomplishments of Camillo Golgi and Santiago Ramón y Cajal were jointly recognized with the Nobel Prize for Physiology or Medicine, the first of many to honor discoveries in the field of neuroscience (Table 1.1). The committee awarded the prize to both scientists "in recognition of their work on the structure of the nervous system" (Grant 2007). In his acceptance speech, given December 12, 1906, in Stockholm, Santiago Ramón y Cajal summarized his extensive histological work and that of other scientists, which argued against

Table 1.1 Nobel Prizes for Physiology or Medicine, which have been awarded for basic neuroscience discoveries

1906	Camillo Golgi and Santiago Ramón y Cajal "in recognition of their work on the structure of the nervous system"
1932	Sir Charles Sherrington and Lord Edgar Douglas Adrian "for their discoveries regarding the functions of neurons"
1936	Sir Henry Halett Dale and Otto Loewi "for their discoveries relating to chemical transmission of nerve impulses"
1944	Joseph Erlanger and Herbert Spencer Gasser "for their discoveries relating to the highly differentiated functions of single nerve fibers"
1957	Daniel Bovet "for his discoveries relating to synthetic compounds that inhibit the action of certain body substances, and especially their action on the vascular system and the skeletal muscles"
1961	Georg von Békésy "for his discoveries of the physical mechanism of stimulation within the cochlea"
1963	Sir John Eccles, Alan Lloyd Hodgkin, and Andrew Fielding Huxley "for their discoveries concerning the ionic mechanisms involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane"
1967	Ragnit Granit, Haldan Keffer Hartline, and George Wald "for their discoveries concerning the primary physiological and chemical visual processes in the eye"
1970	Sir Bernard Katz, Ulf von Euler, and Julius Axelrod "for their discoveries concerning the humoral transmittors in the nerve terminals and the mechanism for their storage, release, and inactivation"
1977	Roger Guillemin and Andrew Viktor Schally "for their discoveries concerning the peptide hormone production of the brain" and Rosalyn Yalow for "for the development of radioimmunoassays of peptide hormones"
1981	Roger W. Sperry "for his discoveries concerning the functional specialization of the cerebral hemispheres" and David H. Hubel and Torsten N. Wiesel "for their discoveries concerning information processing in the visual system"
1986	Stanley Cohen and Rita Levi-Montalcini "for their discoveries of growth factors"
1991	Erwin Neher and Bert Sakmann "for their discoveries concerning the function of single ion channels in cells"
1997	Stanley B. Prusiner "for his discovery of Prions – a new biological principle of infection"
2000	Arvid Carlsson, Paul Greengard, and Eric R. Kandel "for their discoveries concerning signal transduction in the nervous system"
2004	Richard Axel and Linda B. Buck "for their discoveries of odorant receptors and the organization of the olfactory system"

the Reticular Theory and in support of the Neuron Doctrine[2] (Ramón y Cajal 1967). He acknowledged that in the future, novel techniques might reveal new structures and mechanisms and how neuronal cells are connected. However,

[2] S. Ramón y Cajal, Nobel Prize Lecture (1967): "From the whole of these facts, the neuronal doctrine of His and of Forel, accepted by many neurologists and physiologists, is derived as an inevitable postulate... The irresistible suggestion of the reticular conception, of which I have spoken to you has led several physiologists and zoologists to object to the doctrine of the propagation of nerve currents by contact or at a distance. All their allegations are based on the findings by incomplete methods showing far less than those which have served to build the imposing edifice of the neuronal conception."

from the data, which were available to him, he rejected a continuous neuronal network and therefore the Reticular Theory. Much to his chagrin, Camillo Golgi in his Nobel lecture, which he had delivered the previous day, presented a diametric opposite view and a scathing rejection of the Neuron Doctrine[3] (Golgi 1967). In his autobiography, Santiago Ramón y Cajal describes Camillo Golgi's Nobel lecture as self-serving and his attitude as arrogant (Ramón y Cajal 1937). He accuses him of ignoring the experimental results of other researchers and of "worship of his own ego."[4] Certainly no love was lost between these two pioneers of neuroscience. Until his death in 1926, Camillo Golgi remained an ardent supporter of the Reticular Theory.

First insights into the mechanism and the chemical nature of synaptic signals came at the beginning of the 20th century, mainly from the laboratory of John Newport Langley (1852–1925) at Cambridge University in England. In 1904, his student Thomas Renton Elliott (1877–1961) discovered that adrenaline from the adrenal gland mimics the effect of sympathetic nerve innervation on various muscles and glands (Elliott 1905). Adrenaline had previously been recognized as a small bioactive molecule derived from the adrenal medulla; its structure had been determined and it had just been chemically synthesized. Although he mistakenly assumed that adrenaline, rather than noradrenaline, might be released from the peripheral sympathetic nerve endings, Thomas Elliott laid the conceptual foundation for the activity of neurotransmitters as small chemical molecules that bridge the synaptic gap between nerve endings and their targets (Elliott 1904). The identification of the first genuine neurotransmitter can be credited to another former student of Langley, Sir Henry Halett Dale (1865–1968) (Tansey 2006). Together with his colleague Arthur James Ewins (1882–1957) at the Wellcome Physiological Research Laboratories he identified and isolated acetylcholine from a bacterial contamination of the cereal fungus ergot and characterized its physiological activity (Dale 1914, Ewins 1914). However, the final proof of its physiological significance fell to his friend and 1936 fellow Nobel laureate (Table 1.1), the physiologist Otto Loewi (1873–1961). Otto Loewi's experiments on explanted frog hearts established that signaling across most synapses is mediated by small chemical compounds, now referred to as neurotransmitters (Loewi 1921). Nevertheless, it took a considerable time until it was generally accepted that synaptic signal transduction usually is based on a chemical and not on a bioelectrical mechanism. Even in 1937, Sir John Eccles (1903–1997), one of the 1963 Nobel laureates

[3] C. Golgi, Nobel Prize Lecture (1967): "I shall ... confine myself to saying that, while I admire the brilliancy of the (neuron) doctrine, which is a worthy product of the high intellect of my illustrious Spanish colleague, I cannot agree with him on some points of an anatomical nature."

[4] S. Ramón y Cajal, Recollections of My Life (1937): "Contrary to what we all expected, instead of pointing out the valuable facts, which he (Golgi) had discovered, he attempted in it to refloat his almost forgotten theory of interstitial nerve nets. Likewise he considered it unnecessary to correct any of his old theoretical errors, or of his lapses of observation."

for his work on the ionic mechanisms of nerve cell excitation and inhibition (Table 1.1), still favored an electrical transmission model (Eccles 1937). Only later he converted to Henry Dale's view of a chemical-centered signal transmission at synapses. Over the next decades, a number of additional neurotransmitters were identified. For example, a student of Henry Dale, Ulf Svante von Euler (1905–1983), demonstrated in 1946 that noradrenalin is the major neurotransmitter of the sympathetic nervous system (von Euler 1946). Also the first mechanistic details about the process of synaptic transmission began to emerge. At the beginning of the 1950s, Sir Bernard Katz (1911–2003) and his coworkers showed that neurotransmitter molecules were released from the presynaptic termini in discrete quantal amounts (Fatt and Katz 1952, Del Castillo and Katz 1954), and Julius Axelrod (1912–2004) and his research group demonstrated that secreted neurotransmitters were not just rapidly degraded by enzymes, but also taken up and recycled by the surrounding cells (Whitby et al. 1961). In 1961, their contributions to the understanding of synaptic processes were also recognized by the Nobel Prize committee (Table 1.1).

Although physiological and biochemical experiments settled the chemical nature of synaptic signal transmission, a new microscopic technique was needed to elucidate the fine structure of synaptic organization and to demonstrate how transmitters are released into the synaptic cleft. In 1933, Ernst August Friedrich Ruska (1906–1988) had developed the first electron microscope, and at the beginning of the 1950s, this technology was used to investigate the subcellular organization of many biological tissues including neuronal cells. These initial studies by Eduardo de Robertis (1913–1988), J. David Robertson (1922–1995), Fritiof S. Sjöstrand (born 1912), and others provided the final morphological proof for the central hypothesis of the Neuron Doctrine, the existence of a discontinuity or gap between the pre- and the postsynaptic cell (Robertson 1953, Estable, Reissig and De Robertis 1954, Sjöstrand 1958). The superior magnification and resolution of the electron microscope also revealed additional structural details, which had not been seen using other techniques. One such revelation was the presence of small secretory vesicles in the presynaptic terminus (De Robertis and Bennett 1955, Palay 1956). These membrane vesicles were soon postulated to contain neurotransmitters and thus provided an explanation for the quantal release of neurotransmitters, which had been observed by Sir Bernard Katz and his group. Early electron microscopic analyses also reported an electron-dense region at the membrane of the postsynaptic neuron, now referred to as the postsynaptic density (Akert et al. 1969). Despite this wealth of new structural information about the general subcellular organization of synaptic connections, electron microscopic studies alone were unable to identify the molecular components and proteins that form them.

Over the last 40 years, genetic, biochemical, molecular biological and genomic approaches have finally revealed a plethora of protein components, which constitute the synaptic apparatus. Among these synaptic proteins are components of the secretory pathway, which are responsible for vesicle transport, polypeptides involved in membrane vesicle docking and fusion, neurotransmitter receptors

and ion channels, enzymes responsible for neurotransmitter processing, inactivation and uptake, cytoskeletal elements and scaffolding proteins, extracellular matrix components, cellular signaling proteins, and also cell adhesion molecules (CAMs). As synapses are special contact points between neurons and their targets it may not be surprising that CAMs are important components of synaptic connections. However, it was somewhat unexpected that many CAMs, which have been found at synapses, also have important non-synaptic functions in neuronal cell and in tissues outside the nervous system, such as during neuronal differentiation, axonal pathfinding, cell migration, or epithelial stability. Only relatively few adhesive molecules appear to have an exclusive synaptic function. Several general characteristics of CAMs appear to be of special relevance for their functional role at synapses. Synaptic contacts contain not only homophilic CAMs but also heterophilic CAMs, which interact with a heterologous binding partner on the pre- or postsynaptic cell surface. Such heterophilic pairs of adhesive molecules or pre- versus postsynaptic differences in the expression of CAM-interacting proteins might play a role in the differential organization of pre- and postsynaptic membranes. Besides their extracellular adhesive specificities, many CAMs also exhibit evolutionarily well-conserved, cytoplasmic binding activities to different cytoskeletal elements. These interactions appear to be of special importance in integrating different structural and functional aspects of the synaptic apparatus. More recently, it has become increasingly clear that many adhesive proteins directly or indirectly influence various cellular signaling processes. This is relevant not only during synapse formation but also during synaptic functioning and remodeling. In turn, cellular signaling processes, especially those involving protein phosphorylation and proteolysis as well as interactions with the cytoskeleton are known to regulate the adhesive ability of many CAMs. For synaptic CAMs, this may be important for facilitating synaptic plasticity, when existing synaptic connections are weakened or severed. Therefore, synaptic CAMs may be directly involved in processes like long-term potentiation and depression and synaptic remodeling. Almost all of the major CAM families have one or more representatives that are expressed at synaptic contacts, and different classes of synapses appear to have specific subsets of adhesive proteins. Although all chemical synapses share some general characteristics, this variety of CAMs is certainly part of the structural and functional diversity between different types of synaptic contacts. While our knowledge of how different CAM families contribute to synapse formation and functioning is still incomplete, the available data support some general themes, which are summarized above and in the following chapters. In the coming years, our understanding of the crucial role of CAMs at synapses will certainly deepen and possibly new adhesive molecules will join the list of known synaptic CAMs that are discussed in this book.

Today the term synapse is used in connection with three different types of cellular junctions (Yamada and Nelson 2007). It describes contact points not only between neuronal cells but also between immune cells and epithelial cells. An immunological synapse is the interface between antigen-presenting cells

(e.g., macrophages, dendritic or activated B cells) and lymphocytes (Grakoui et al. 1999). Adhesion complexes, such as tight and adherent junctions, between epithelial cells are sometimes referred to as epithelial synapses (Yamada and Nelson 2007). However, usually the term synapse alludes to neuronal synapses. The majority of neuronal synapses are chemical based, as presumed in the preceding part of this chapter. More recently, evidence for an alternative type of neuronal synapse has emerged, which uses an electrical mode of signal transduction. These electrical synapses are formed by connexin/pannexin-containing gap junctions, which allow the direct propagation of the action potential from one neuronal cell to the next without the need for a chemical transmitter intermediate (Connors and Long 2004). As gap junctions form small cytoplasmic connections between neighboring cells, the existence of electrical synapses might be viewed as a partial exoneration of Camillo Golgi's old idea that neuronal cells are directly linked to each other. The relative importance of electrical versus chemical synapses currently remains unclear. Obviously, the structural and functional interactions between neuronal cells and their targets have grown increasingly intricate and multifaceted. As Santiago Ramón y Cajal pointed out in 1906 "Unfortunately, nature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity. Besides, we believe that we have no reason for scepticism. While awaiting the work of the future, let us be calm and confident in the future of our work" (Ramón y Cajal 1967).

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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

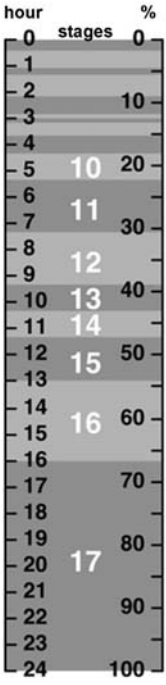

Fig. 2.1 ***Drosophila* embryonic development**. Wild-type embryonic development at 25°C has been characterized in different scales such as (*left*) hours after egg laying (AEL), (*center*) morphological and developmental events defining stages (Campos-Ortega and Hartenstein 1985), and (*right*) completed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

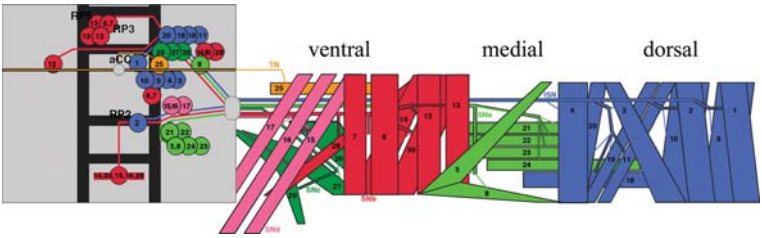

Fig. 2.2 Schematic representation of *Drosophila* neuromuscular network. Synaptic matchmaking between motoneurons (*left*) and embryonic muscles (*right*) is color coded according to the innervating nerve branch. Neuronal cell body localization is presented with the muscle number they innervate. Neurons 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.