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Role of Extracellular Matrix Proteins and Their Receptors in the Development of the Vertebrate Neuromuscular Junction

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Abstract

The vertebrate neuromuscular junction remains the best-studied model for understanding the mechanisms involved in synaptogenesis, due to its relatively large size, its simplicity of patterning and its unparalleled experimental accessibility. During neuromuscular development, each skeletal myofiber secretes and deposits around its extracellular surface an assemblage of extracellular matrix (ECM) proteins that ultimately form a basal lamina. This is also the case at the neuromuscular junction, where the motor nerve contributes additional factors. Before most of the current molecular components were known, it was clear that the synaptic ECM of adult skeletal muscles was unique in composition and contained factors sufficient to induce the differentiation of both pre- and postsynaptic membranes. Biochemical, genetic and microscopy studies have confirmed that agrin, laminin (221, 421, and 521), collagen IV (α3-α6), collagen XIII, perlecan and the ColQ-bound form of acetylcholinesterase are all synaptic ECM proteins with important roles in neuromuscular development. The roles of their many potential receptors and/or binding proteins has been more difficult to assess at the genetic level due to the complexity of membrane interactions with these large proteins, but roles for MuSK-LRP4 in agrin signaling and for integrins, dystroglycan, and voltage-gated calcium channels in laminin-dependent phenotypes have been identified. Synaptic extracellular matrix proteins and their receptors are involved in almost all aspects of synaptic development, including synaptic initiation, topography, ultrastructure, maturation, stability and transmission.

INTRODUCTION

Our understanding of the roles for extracellular matrix (ECM) proteins in the formation of the neuromuscular synapse began, in large part, with the approach of assessing motor axon or muscle regeneration after injury in the adult animal. While the presence of what we now know to be the basement membrane surrounding skeletal myofibers was first proposed by Bowman in 1840 and while the issue of topographic specificity of reinnervation at the neuromuscular junction (NMJ) was first noted by Cajal in 1928 (see (Sanes and Lichtman, 2001; Sanes, 2003), it was experiments by McMahan and colleagues carried out in the late 1970s that showed that ECM proteins at neuromuscular junction (NMJ), present in the form of a synaptic basal lamina (BL), remained after injury of either the pre- or postsynaptic cell and helped to dictate the topography and presence of synapses on regenerated cell membranes (Letinsky et al., 1976; Marshall et al., 1977; McMahan et al., 1978; Sanes et al., 1978; Burden et al., 1979). Denervation of motor axons, coupled with destruction of the muscle, led to reinnervation of original synaptic sites on the basal lamina, while postsynaptic differentiation of regenerated muscle occurred at the original synaptic sites on the basal lamina even in the absence of the nerve. Such differentiation can be maintained for many months. Thus, the synaptic BL not only marks the location of the NMJ for regenerating

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cells, it provides sufficient trophic support to allow prolonged survival in absence of a synaptic partner. These studies clearly implicated the synaptic ECM as containing essentially indelible markers of synaptic localization and differentiation, which, in turn, has led a number of investigators to search for and identify individual synaptic ECM proteins and their receptors. Due to the space considerations, we apologize in advance for our failure to cite studies, of which there are many, that are not included here. There are a number of previous reviews where one can find additional citations (see (Sanes et al., 1998; Sanes and Lichtman, 2001; Hughes et al., 2006) for general review, (Sanes, 2003) for muscle ECM, (Kummer et al., 2006; Wu et al., 2010) for agrin, (Yurchenco and Patton, 2009) for laminin, (Kadler et al., 2007) for collagen, (Massoulie and Millard, 2009) for acetylcholinesterase, (Engel et al., 1999; Voermans et al., 2008; Farrugia and Vincent, 2010) for neuromuscular disorders, (Martin, 2003; Ervasti and Sonnemann, 2008) for dystrophin-associated complex, (Korkut and Budnik, 2009) for WNTs and (Burden, 1993; Sunesen and Changeux, 2003) for neuregulin).

The neuromuscular synapse has been a favorite of developmental neurobiologists for three reasons: 1. Its large size, about 1000 times that of a typical interneuronal synapse, 2. Its simplicity of patterning, almost all mammalian synapses are ultimately innervated by only one nerve terminal and 3. Its experimental accessibility, which allows imaging and cell manipulation in the living animal. Of course, being an essential synapse that controls voluntary movement and breathing, the NMJ is also very important in its own right. Neuromuscular pathology and dysfunction are involved in a number of human diseases, for example the congenital and autoimmune myasthenias (Engel et al., 2010; Farrugia and Vincent, 2010).

In mammals, including rodents and humans, the NMJ utilizes cholinergic neurotransmission to control muscle movement. Acetylcholine made in motor neurons is packaged into secretory vesicles that reside within the nerve terminal. When the motor nerve, whose cell body resides within the ventral column of the spinal cord or the hindbrain, is excited, an action potential is generated, leading to quantal release of acetylcholine at the NMJ. Nicotinic acetylcholine receptors (AChRs) concentrated in the postsynaptic membrane are activated upon binding acetylcholine to depolarize the muscle membrane, with elevated intracellular calcium levels ultimately causing muscle contraction. Unlike many central nervous system synapses, which must integrate synaptic signals from numerous presynaptic partners, the molecular and cellular architecture of the neuromuscular synapse is designed to insure that neurotransmission occurs with high fidelity each time the motor nerve is excited.

To bring such a robust synaptic structure about, a large number of developmental events must take place. Even so, it still is a bit astounding to think that neuromuscular development in the rodent requires almost a month to be fully completed (Sanes and Lichtman, 2001). Without a doubt, the most striking aspect of NMJ development is the intense concentration of postsynaptic acetylcholine receptors, or AChRs, in the muscle membrane underlying the nerve terminal. During embryogenesis, as myoblasts fuse to form myotubes, synaptic genes, including the subunits of the AChR, are activated to produce high concentrations of AChRs (about 1000/μm^2). Through a combination of mechanisms (lateral protein migration to synapses, increased mRNA biosynthesis by subsynaptic muscle nuclei, increased protein stability in synaptic regions, increased protein degradation and reduced mRNA expression in non-synaptic regions), AChRs are reorganized and concentrated such that AChR density is 10,000–20,000/μm^2 at the adult NMJ while in extrasynaptic regions AChR density is less than 10/μm^2 (Fambrough, 1979; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). In addition, AChR subunit composition is altered, with $\alpha_2\beta\gamma\delta$ pentameric AChR channels present in the embryo and $\alpha_2\beta\delta\varepsilon$ channels present in the adult (Sunesen and Changeux, 2003). Changed AChR subunit composition alters AChR calcium permeability and channel

open time. This, coupled with the dramatic concentration of AChRs, allows the nerve to excite the skeletal myofiber to membrane potentials well beyond those required to elicit contraction, providing a safety factor for a synapse that must work with high efficiency for the organism to survive.

As the NMJ matures, its shape changes on several levels (Sanes and Lichtman, 2001): 1. The synaptic membrane transforms from a contiguous immature oval plaque to a disjointed pattern of pretzel-like regions. 2. The topography of the pre- and postsynaptic membrane is altered, such that infoldings of the postsynaptic membrane, called secondary folds, align with concentrations of secretory vesicles docked in specialized presynaptic membrane regions called active zones. 3. The extracellular matrix surrounding each skeletal myofiber and within the synaptic cleft forms and matures, both in terms of its density and its molecular constituents, 4. Motor axons that have multiply innervated single myofibers in the embryo are eliminated postnatally such that most myofibers are monoinnervated in the adult, and 5. A synaptic non-myelinating Schwann cell covers the nerve terminal in areas outside the synaptic cleft, capping the region of contact between the nerve terminal and the muscle membrane. These changes coincide with developmental changes that are further removed from the NMJ (sarcomeric patterning and determination of muscle fiber type, muscle growth, adjoining of myofibers to tendons at myotendinous junctions, myelination of motor axons by Schwann cells and development of motor neuron subtypes and pools). Within this complex soup of cellular processes, the extracellular matrix is formed and contributes in important ways to synaptic development.

DEVELOPMENT OF THE SYNAPTIC EXTRACELLULAR MATRIX

The ECM proteins that comprise the synaptic cleft at the NMJ are not fully present when the motor axon first arrives at the skeletal muscle. Shortly thereafter, the muscle begins to synthesize, secrete, and deposit ECM proteins that ultimately comprise a basal lamina that surrounds each and every skeletal myofiber (Figure 1) (Chiu and Sanes, 1984). A basal lamina is an ordered extracellular matrix structure that contains laminin and collagen IV as its main protein constituents, along with heparan sulfate glycosaminoglycans, which in muscle include agrin and perlecan, and other ECM proteins, including tenascin, fibronectin, perlecan, and nidogen (Yurchenco and O'Rear, 1994; Yurchenco et al., 2004). At the NMJ, the deposition of ECM proteins into the synaptic cleft to make the synaptic BL has the effect of appearing to push the nerve away from the skeletal myofiber such that a synaptic cleft of roughly 50nm develops between the cells. Given that many of the proteins present in this region can exceed 50nm, at least along one axis, as individual molecules (for example, laminin, agrin, and collagen IV), the ordering of the synaptic BL could be such that individual ECM proteins contact both the pre- and postsynaptic membrane.

Under the electron microscope, the synaptic BL appears contiguous with the BL surrounding the remainder of the myofiber membrane, but at the molecular level it is quite distinct (Chiu and Sanes, 1984; Engvall et al., 1990; Sanes et al., 1990; Patton et al., 1997). Both major BL proteins, laminin and collagen IV, have different synaptic and extrasynaptic isoforms (Figure 1). In addition, perlecan, agrin, and nidogen are abundantly expressed ECM glycoproteins that have synaptic forms or variants (Ruegg et al., 1992; Jenniskens et al., 2000; Fox et al., 2008). Because members of the laminin and collagen IV families and agrin have been extensively studied in mice at the genetic level, an approach that has led to clearly defined synaptic functions, we will focus primarily on these ECM proteins in this review. This is not an attempt to overlook other important ECM proteins (for example, fibronectin, tenascin, thrombospondin) or the proteins that regulate ECM expression (for example, matrix metalloproteinases and their inhibitors), some of which may have biological roles in neuromuscular biology (Arber and Caroni, 1995; Cifuentes-Diaz et al., 1998; Moscoso et al.,

1998; Voermans et al., 2011), but to focus on aspects of synaptic development for which ECM protein roles have been most clearly defined by human and mouse genetics. Perhaps the best studied of these are the laminins.

Laminins are trimeric proteins composed of three different subunits, with an α, β and γ chain. A new nomenclature has been agreed upon to indicate the subunit composition of individual laminin trimers (for example, laminin α4β2γ1 would be laminin 421 and so on (Aumailley et al., 2005). Myoblasts begin to fuse to form myotubes on or about embryonic day E10 in the rat intercostal muscle. Shortly thereafter, by E11.5, laminin 111, laminin 211 and laminin 511 begin to be synthesized and secreted into the basal lamina surrounding skeletal myofibers in a patchy discontinous pattern (Patton et al., 1997). Laminin 111 is only expressed for several days during embryogensis and is by and large only concentrated at the ends of myofibers and myotendinous junctions, while laminin 211 is expressed throughout adulthood, becoming the predominant muscle laminin. The formation of primary myofibers is followed by the formation of secondary myofibers, which coincides with synaptogenesis beginning on about day E13–14. In addition to laminin 211, laminin 411 and laminin 511 become prevalent by E15, at which time a basal lamina sheath surrounds most myofibers. Beginning at E15, the three laminin α chains (α2, α4, and α5) also form trimers with laminin β2 and laminin γ1 to make laminin 221, 421, and 521 (Chiu and Sanes, 1984; Patton et al., 1997). These laminins are expressed only at the NMJ due to the synaptic exclusivity of laminin β2, first identified by Sanes and colleagues as S- (or synaptic) laminin (Hunter et al., 1989). By E18, the β1 chain of laminin is lost from the NMJ (Chiu and Sanes, 1984; Patton et al., 1997) making laminin 211, 411 and 511 exclusively extrasynaptic. Later, in the first two weeks after birth, laminin α4 and laminin α5 expression is lost in the extrasynaptic BL, leaving only laminin 211 to surround the extrasynaptic muscle membrane (Chiu and Sanes, 1984; Patton et al., 1997).

Most other ECM proteins, and their receptors, follow one of the developmental expression patterns described for the laminins (Figure 1). For example, muscle-derived agrin and muscle-derived neuregulin follow the same pattern of expression as laminin α4 and α5. Both of these proteins have extrasynaptic expression at birth that becomes highly concentrated at the NMJ by P14-P21(Hoch et al., 1993; Moscoso et al., 1995). Ruegg and colleagues have reported that loss of extrasynaptic agrin varies between muscle groups, with some maintaining low levels of extrasynaptic expression into adulthood (Eusebio et al., 2003). Agrin and neuregulin deposited by the motor nerve, by contrast, are confined to the NMJ. MuSK, the tyrosine kinase that responds to neuronal agrin, LRP4, its essential cofactor in agrin signaling, and erbB 2 and 3 kinases, receptors for neuregulin, all show a synaptic expression pattern in the adult animal (Moscoso et al., 1995; Apel et al., 1997; Zhang et al., 2008).

The two principal receptors for both laminins in skeletal muscle are α7β1 integrin and dystroglycan (Figure 2). There are three splice forms of integrin α7 in skeletal muscle (A, B, and C) that differ due to alternative splicing within the intracellular domain (there is splicing within the extracellular domain as well) (Ziober et al., 1993). Integrin α7A-C all form heterodimers with integrin β1 chains, which in adult skeletal muscle is the β1D splice form (van der Flier et al., 1997). Integrin α7B shows an expression pattern equivalent to laminin α4 and α5, with high extrasynaptic expression at birth and synaptic localization by P14 (Martin et al., 1996). Integrin α7A, like laminin β2, shows an exclusively synaptic expression pattern, but one that becomes evident only beginning at postnatal day 4. Integrin α7C is expressed in a pattern like laminin α2, remaining extrasynaptic into adulthood. Integrin α3β1 is concentrated at the NMJ in active zones within the presynaptic membrane (Cohen et al., 2000), and integrin α1β1 and αvβ1 are also concentrated at the NMJ (Martin et al., 1996).

Dystroglycan is expressed in both synaptic and extrasynaptic regions of adult myofibers (Ervasti and Campbell, 1991; Matsumura et al., 1992). Unlike integrins, splicing is not an issue for dystroglycan, as the dystroglycan gene has only two exons (Ibraghimov-Beskrovnaya et al., 1993). The complexity in dystroglycan biology comes from posttranslational modifications, in particular glycosylation (Martin, 2003). A large number of genes contribute to glycosylation of α dystroglycan with O-linked mannose chains that are required for laminin (and also agrin and perlecan) binding (Michele et al., 2002; Martin, 2006). The presence of the CT (Cytotoxic T cell) carbohydrate, a glycan structure present on the muscle membrane at the neuromuscular junction (Martin et al., 1999), on α dystroglycan increases binding of both synaptic and extrasynaptic isoforms of laminin (Yoon et al., 2009). Galgt2, the enzyme that synthesizes the synaptic CT carbohydrate on α dystroglycan (Yoon et al., 2009), and the CT glycan itself, are expressed in extrasynaptic regions at birth and become confined to the NMJ by P14 (Martin et al., 1999; Hoyte et al., 2002). Some heparan sulfate glycosaminoglycan (GAG) structures show a similar developmental expression pattern to the CT carbohydrate, suggesting that glycosaminoglycans on synaptic proteoglycans (e.g. agrin and perlecan) are different than the glycosaminoglycans on their extrasynaptic forms (Jenniskens et al., 2000).

Like laminin α2, Collagen IV $(\alpha 1)_2(\alpha 2)$ trimers are present along the entirety of the muscle BL from an early embryonic stage (Sanes et al., 1990). By contrast to laminins, collagen IV (α3, α4, α5), collagen IV $(\alpha 5)_2(\alpha 6)$, both also secreted basal lamina components, and collagen XIII, a transmembrane collagen localized at the plasma membrane, are not expressed until after birth and are only found at the NMJ, with clear expression being evident for all at P21 (Miner and Sanes, 1994; Fox et al., 2007; Latvanlehto et al., 2010). Collagen VI, an extracellular beaded filament-forming collagen (Kadler et al., 2007), is present in the reticular lamina that surrounds the extrasynaptic BL in adult muscle (Lampe and Bushby, 2005). As the reticular lamina is not present at the NMJ, collagen VI is not present at the synapse. Another collagen-like molecule is ColQ, the non-enzymatic synaptic subunit of acetylcholinesterase (AChE) (Massoulie and Millard, 2009). ColQ is exclusively localized at the synapse, becoming present shortly after the arrival of the motor axon (Chiu and Sanes, 1984; Rotundo, 2003).

THE THREE “A”s IN THE SYNAPTIC AChR TRIUMVIRATE - AGRIN, ACTIVITY AND ARIA

Most early studies of NMJ development focused on the dramatic concentration of postsynaptic acetylcholine receptors (AChRs) in the muscle membrane. This search was greatly facilitated by access to α bungarotoxin, a 74 amino acid snake protein isolated from *Bungaris multicinctus*, that bound with specific and high, almost irreversible, affinity to muscle AChRs (Lee et al., 1967). Early studies pointed to three components important for the concentration of AChRs-Agrin, Activity and ARIA. Agrin is the synaptic ECM protein best understood to play an essential role in synaptic development, but consensus on its mechanism of action has shifted in the years since its original purification and cloning. Agrin was originally purified from the marine ray, *Torpedo californica*, as a factor that stimulated the clustering of acetylcholine receptors (AChRs) in the membrane of cultured skeletal myofibers (Godfrey et al., 1984; Wallace et al., 1985; Nitkin et al., 1987). A single agrin gene was cloned and identified as an approximately 400kDa heparan sulfate glycosaminoglycan, with the glycosaminoglycan chains representing about half the molecular weight of the molecule (Rupp et al., 1991; Tsim et al., 1992; Tsen et al., 1995) (Figure 2). Still other glycosylation domains showed the presence of N-linked glycosylation and mucin-like regions with high densities of O-linked sugars. Agrin is a roughly linear 95nm rod-like protein (Denzer et al., 1998). At the N-terminus, agrin contains a unique splice site allowing for a transmembrane domain in neurons as well as a splice form that can

be transported down the motor axon and secreted at the motor nerve terminal (Magill-Solc and McMahan, 1990; Magill-Solc and McMahan, 1990; Denzer et al., 1998; Burgess et al., 1999; Burgess et al., 2000). The N-terminal half of the molecule also contains a binding domain for laminin and a nine follistatin-like repeats (Rupp et al., 1991; Tsim et al., 1992; Denzer et al., 1998).

The AChR aggregating activity of agrin was shown to be present in the C-terminal half of the molecule, which contains four EGF-like repeats and three laminin-like G domains (LG1-3, Figure 2) (Rupp et al., 1991; Ruegg et al., 1992; Tsim et al., 1992). LG3 is the domain that is essential for the *in vitro* AChR aggregation activity of agrin (Gesemann et al., 1995; Campanelli et al., 1996; Gesemann et al., 1996). Moreover, one of two neuron-specific exons must be spliced into the z splice site in LG3, controlled by the neuron-specific splicing factors Nova 1 and 2 (Ruggiu et al., 2009), for agrin to have high AChR clustering activity. Mutagenesis of the z splice exons has further defined the essential amino acids required (Scotton et al., 2006). This LG3 domain is also required for agrin binding to the α3 subunit of the sodium-potassium ATPase (Hilgenberg et al., 2006). The LG2 domain binds α dystroglycan (Bowe et al., 1994; Campanelli et al., 1996; Gesemann et al., 1996; Gesemann et al., 1998). LG2 confers higher affinity agrin binding to muscle cells, which alters its potency in inducing AChR clustering (Campanelli et al., 1996; Gesemann et al., 1996). The presence of LG1 increases further agrin binding to α dystroglycan and agrin potency in inducing AChR clustering on cultured muscle cells(Gesemann et al., 1996). This may be mediated by one of several integrins (e.g. αvβ1) that bind to this domain (Martin and Sanes, 1997; Burgess et al., 2002). At least one EGF repeat (EGF4) is also a site for O-fucosylation by Pofut1 (Protein O-fucosyltransferase 1) that can alter the muscle form of agrin's bioactivity (Kim et al., 2008). Ectopic expression of agrin can induce postsynaptic specializations *in vivo* (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1998; Meier et al., 1998; Rimer et al., 1998) and deletion of agrin, even deletion of only the z splice exons, leads to aberrant neuromuscular development, including increased motor nerve branching, decreased apposition of pre- and postsynaptic specializations, dramatically fewer AChR clusters in the postsynaptic membrane and paralysis (Gautam et al., 1996; Burgess et al., 1999).

These experiments all pointed to agrin being an important inducer of AChR clustering in the postsynaptic membrane, but more recent work has defined agrin as a stabilizer of the postsynaptic membrane rather than as an inducer of it. Classic studies from Burden and colleagues and Lee and colleagues showed that mice lacking the motor nerve (the phrenic nerve, via deletion of DNA topoisomerase II β or the transcription factor HB9) or mice lacking the neurotransmitter acetylcholine (via deletion of choline acetyltransferase) maintain ectopic expression of postsynaptic specializations along the majority of the myofiber membrane(Yang et al., 2000; Lin et al., 2001; Yang et al., 2001; Brandon et al., 2003). Such specializations, while present in the middle region of the muscle during embryogenesis, normally dissipate by birth, leading to a concentrated endplate band of postsynaptic AChRs. Thus, the muscle pre-patterns AChR-rich postsynaptic membranes rather than the nerve instructing the muscle to do so. While mice lacking agrin showed ectopic AChR aggregates before birth, these were lost more quickly than in normal muscles, suggesting that agrin counteracted the pre-patterning of AChR-rich postsynaptic domains within the muscle membrane. Mice deficient in choline acetyltransferase (ChAT) rescued the agrin null phenotypes, with double mutant mice having normal postsynaptic AChR density and relatively normal innervation(Lin et al., 2005; Misgeld et al., 2005). This supports a model wherein agrin serves to stabilize and offset the dissipation of postsynaptic membrane formation normally driven by muscle activity (the second A). This AChR dispersing activity of acetylcholine is mediated in part by activation of serine/threonine cyclin-dependent kinase 5 (Cdk5), calpain and calcium/calmodulin protein kinase II (Tang

et al., 2004; Fu et al., 2005; Lin et al., 2005; Chen et al., 2007). Cdk5-null mice phenocopy ChAT null animals in their formation of ectopic pre-patterned AChR aggregates in muscle, and Cdk5 inhibitors can block the effects of acetylcholine addition to muscle cells (Fu et al., 2005; Lin et al., 2005). Thus, while *in vitro* studies of agrin activity in inducing AChR clustering are legion (and true), such clusters form without agrin *in vivo,* and agrin instead serves to locally counteract the negative dispersal of postsynaptic AChRs by activity.

Studies by Yancopoulos and colleagues implicated MuSK, a muscle transmembrane tyrosine kinase (Jennings et al., 1993), as the receptor for agrin (DeChiara et al., 1996). Mice lacking MuSK have no postsynaptic membranes, even during early development and even in the absence of acetylcholine. Importantly, MuSK is also essential for the pre-patterning of AChRs in skeletal muscle (Lin et al., 2001) and overexpression of MuSK in muscle can lead to ectopic synapse formation, suggesting that such pre-patterning is instructive for defining the ultimate sites of innervation (Kim and Burden, 2008). This also appears to be true in zebrafish, where pre-patterned, aneural, postsynaptic AChR aggregates ultimately become innervated by motor neurons (Flanagan-Steet et al., 2005; Panzer et al., 2005).

Addition of agrin to myofibers stimulates the phosphorylation of tyrosine residues on MuSK in cultured cells, demonstrating MuSK's ability to respond to agrin, but agrin does not bind directly to MuSK (Glass et al., 1996). Rather, agrin binds Lrp4 and activates MuSK via an agrin-Lrp4-MuSK signaling complex (Kim et al., 2008; Zhang et al., 2008). Lrp4 is a member of the low-density lipoprotein receptor family that is concentrated at the NMJ and contains, like other Lrps, an extracellular protein motif that is very densely glycosylated (May et al., 2007). Mice lacking Lrp4, like mice lacking MuSK, fail to make proper NMJs (Weatherbee et al., 2006), and expression of Lrp4 in non-muscle cells can recover MuSK activation by agrin (Kim et al., 2008; Zhang et al., 2008).

MuSK interacts with a number of important cytoplasmic proteins to carry out its signaling functions (Figure 3). These include adaptor protein Dok7 (downstream of tyrosine kinase 7) (Okada et al., 2006; Inoue et al., 2009; Bergamin et al., 2010), dishevelled 1(Dvl1) and p21-activated kinase (Pak1) (Luo et al., 2002), and tumourous imaginal discs protein (Tid1) (Linnoila et al., 2008). Likewise, rapsyn (Sobel et al., 1977; Sealock et al., 1984; Frail et al., 1988), a myristylated muscle protein that binds in a one-to-one stoichiometry to the AChR (LaRochelle and Froehner, 1986), interacts with MuSK (Apel et al., 1997) and is essential for AChR aggregation (Gautam et al., 1995; Borges et al., 2008). Rapsyn interactions can be altered by calpain, a muscle protease (Chen et al., 2007). The small G proteins Rac and Rho and geranylgeranyltransferase I, an enzyme that can modify and activate G proteins via prenylation, also play important roles in this process(Luo et al., 2003; Weston et al., 2003), as does APC (adenomatous polyposis coli), an actin-binding protein (Wang et al., 2003).

Agrin binding to Lrp4-MuSK stimulates MuSK autophosphorylation on tyrosine 553 near the transmembrane region, beginning the process of MuSK signaling (Watty et al., 2000). This recruits Dok7 dimers, which cross-link and dimerize MuSK subunits along with Crk and Crk-L (Herbst and Burden, 2000; Herbst et al., 2002; Till et al., 2002; Inoue et al., 2009; Bergamin et al., 2010; Hallock et al., 2010). MuSK then autophosphorylates tyrosines 750, 754 and 755. This is thought to release the auto-inhibitory domain within the cytoplasmic tail, allowing substrate binding (Watty et al., 2000; Till et al., 2002). Casein kinase 2 (CK2) phosphorylates serines on MuSK (Cheusova et al., 2006), enhancing AChR clustering, and the tyrosine kinases Src and Fyn affect AChR β –subunit phosphorylation in a MuSK-dependent manner (Smith et al., 2001; Mittaud et al., 2004; Sadasivam et al., 2005). Abelson kinase (Abl), a tyrosine kinase, and Shp2 (Src homology 2-containing protein tyrosine phosphatase 2), a tyrosine phosphatase, have been suggested to provide positive and

negative feedback loops, respectively, for MuSK phosphorylation (Finn et al., 2003; Mittaud et al., 2004; Madhavan et al., 2005; Zhao et al., 2007).

Overexpression of MuSK is sufficient to induce ectopic AChR aggregates, even in the absence of agrin, and MuSK overexpression expands the topographic zone of innervation in the myofiber membrane (Kim and Burden, 2008). This may be the result of spontaneous MuSK autoactivation through increased MuSK-MuSK associations. In such muscles, ectopic AChR aggregates can become innervated, even when far removed from the normal sites of innervation, suggesting that MuSK expression, and thereby increased MuSK activity, pre-patterns sites within the postsynaptic membrane for innervation by nerve terminals. How is this pre-patterning of MuSK expression normally achieved? Expression of MuSK, and thereby its degree of autoactivation, is most likely controlled at the transcriptional and posttranslational levels. As for transcription, the MuSK gene contains an E box DNA binding element for the transcription factor myogenin, which suppresses transcription of genes in extrasynaptic nuclei of adult myofibers (Tang et al., 2006), and an N box DNA binding element (Lacazette et al., 2003), which induces transcription of genes in synaptic nuclei via Ets factors, which are, in part, activated by neuregulin (Burden, 1993). The late onset expression of myogenin in embryogenesis may help to dictate the suppression of MuSK, thereby tying the topography of innervation to muscle differentiation. Myogenin expression, in turn, can be controlled by histone diacetylase 4 (HDAC4), which, when deleted, increases extrasynaptic AChR clustering and expression of synaptic genes (Tang et al., 2009). As for posttranslational regulation, MuSK contains a carboxyterminal motif that can associate with an E3 ubiquitin ligase, PDZRN3 (Lu et al., 2007) and putative ariadne-like ubiquitin ligase (PAUL) (Bromann et al., 2004), both of which may also control protein levels, as may association with N-ethylmaleimide sensitive factor (NSF) (Zhu et al., 2008) and calpain (Hezel et al., 2010). Mutation of the N-linked glycosylation sites on MuSK can also alter protein stability (Watty and Burden, 2002). While agrin is a very large ECM protein, a wealth of biochemical and genetic studies point to the clear importance of the Agrin-Lrp4-MuSK signaling pathway in stabilizing the postsynaptic membrane under the nerve terminal. These are supported by the findings of human mutations in agrin, MuSK and MuSK-binding proteins that cause congenital myasthenias (Huze et al., 2009; Engel et al., 2010; Farrugia and Vincent, 2010).

The third A in the AChR triumvirate is ARIA-coined Acetylcholine Receptor Inducing Activity for its ability to stimulate AChR mRNA levels in cultured muscle cells (Falls et al., 1993). ARIA is more commonly called neuregulin (NRG) (though it goes by other names as well, including glial growth factor (GGF), neu differentiation factor (NDF), and heregulin (HRG) (Lemke and Brockes, 1984; Holmes et al., 1992; Marchionni et al., 1993). Neuregulin activity is associated with its extracellular EGF domain, which activates dimeric forms of the erbB2-erbB3 or erbB2-erbB4 transmembrane tyrosine kinase (Gassmann and Lemke, 1997). The erbB kinases are present in skeletal muscles and/or in Schwann cells (Altiok et al., 1995; Chu et al., 1995; Goodearl et al., 1995; Jo et al., 1995; Moscoso et al., 1995; Sandrock et al., 1995; Zhu et al., 1995; Rimer et al., 1998; Lemke, 2006). Neuregulin has many biological activities. Perhaps most importantly here, it is essential for nerve-dependent survival and function of Schwann cells (Trachtenberg and Thompson, 1996; Lemke, 2006).

Three groups of studies originally pointed to neuregulin as also being important for the increased concentration of AChR mRNAs in subsynaptic muscle nuclei: 1. Motor neurons synthesize, transport, and secrete neuregulin protein into the synaptic ECM at the NMJ (Han and Fischbach, 1999), 2. Addition of neuregulin to cultured myotubes increases transcription of AChR mRNAs (Schaeffer et al., 2001) and 3. Mice heterozygous for deletion of neuregulin-1, mice deleted for neuregulin receptors (erbB kinases 2 or 3), where cardiac

defects are transgenically rescued and mice overexpressing dominant activated forms of neuregulin receptors in skeletal muscles all have altered AChR transcription, altered neuromuscular development, or are myasthenic (Sandrock et al., 1997; Woldeyesus et al., 1999; Lin et al., 2000; Ponomareva et al., 2006). Other experiments subsequently pointed to the fact that neuronally derived neuregulin, indeed the whole nerve, is indispensible for concentration of AChR mRNAs and the transcription of synaptic genes (Lin et al., 2001; Yang et al., 2001). In addition, motor neuron- or muscle-specific knockouts of neuregulin, or skeletal muscle-specific knockouts of its receptors, erbB2 and/or erbB4, cause, at best, only a slight deficit in synaptic AChR transcription (Leu et al., 2003; Escher et al., 2005; Jaworski and Burden, 2006). These mice have relatively normal NMJ development and neurotransmission, though other findings, for example defects in muscle spindle formation, can be present (Leu et al., 2003). Thus, neuregulin-deficient phenotypes, when found, most likely result from its effects on Schwann cell development and survival rather than from effects of its secretion from the nerve and signaling to skeletal muscle.

SYNAPTIC LAMININS – DEFINING SYNAPTIC ULTRASTRUCTURE

Laminin 211, the predominant extrasynaptic muscle laminin, and laminin 211-associated membrane proteins, are essential for the maintenance of the integrity of the skeletal myofiber membrane (Mayer et al., 1997; Hayashi et al., 1998; Blake et al., 2002; Durbeej and Campbell, 2002; Michele and Campbell, 2003; Barresi and Campbell, 2006; Martin, 2006; Ervasti and Sonnemann, 2008; Chandrasekharan and Martin, 2010; Hara et al., 2011). Of this there can really be no doubt. Mice and humans deficient in laminin α2 (the 2 in laminin 211) have congenital muscular dystrophy, mice and humans lacking enzymes that make glycans on α dystroglycan that are necessary for laminin 211 binding have muscular dystrophy, mice lacking dystroglycan in skeletal muscles have muscular dystrophy (as can humans with a particular missense mutation in dystroglycan), and mice and humans lacking cytoplasmic dystroglycan binding proteins (e.g. dystrophin, Plectin 1) have muscular dystrophy. Mice and humans lacking the principal laminin binding integrin in skeletal muscle, integrin α7β1, also have muscular dystrophy.

Overexpression of certain ECM proteins (laminin 111 (Gawlik et al., 2004; Rooney et al., 2009; Rooney et al., 2009) or agrin (Moll et al., 2001; Bentzinger et al., 2005), or overexpression of synaptic laminin-associated proteins or their modifiers (Galgt2, a synaptic enzyme that glycosylates α dystroglycan (Nguyen et al., 2002; Xu et al., 2007; Xu et al., 2009), integrin α7B1, a synaptic integrin (Burkin et al., 2001; Burkin et al., 2005), or utrophin, a synaptic orthologue of dystrophin (Deconinck et al., 1997; Rafael et al., 1998; Tinsley et al., 1998) all can functionally compensate for the loss of members of the extrasynaptic laminin 211 complex in skeletal muscles and inhibit muscle disease. These experiments suggest that synaptic ECM proteins serve structural roles in maintaining the synaptic membrane akin to what their extrasynaptic counterparts do in the rest of the muscle cell. As the synaptic basal lamina comprises only about 0.1% of the myofiber membrane, loss of synaptic laminin chains does not lead to muscle damage, but such loss does lead to profound changes in synaptic ultrastructure.

At the NMJ, laminin 221, 421 and 521 are defined as uniquely synaptic by either the expression of a synaptic α chain (laminin α4 or laminin α5), which occurs postnatally, or by synaptic expression of laminin β2, which occurs embryonically (Figure 1) (Patton et al., 1997). As laminin β2 is present in all three synaptic laminin trimers, it is perhaps not surprising that mice lacking laminin β2 show aberrant NMJ development (Noakes et al., 1995). In laminin β2-deficient mice, the three cells of the NMJ fail to develop their proper alignment. Instead, the synaptic Schwann cell sends processes between the nerve terminal and muscle to invade the synaptic cleft, removing about half the extent of normal nerve-

muscle interaction. This may be due to loss of laminin 521. Schwann cells show little ability to migrate on laminin 521 (Cho et al., 1998; Patton et al., 1998), therefore its absence may in effect remove the synaptic break on Schwann cell migration. In addition, synaptic ultrastructure fails to form properly in laminin β2-deficient mice (Noakes et al., 1995). Secondary folds in the postsynaptic membrane are reduced, as are active zones in the presynaptic membrane. Moreover, both structures, when present, are not precisely aligned with one another. Consistent with ultrastructural changes, laminin β2-deficient mice have deficits in endplate potentials consistent with their altered synaptic architecture (Noakes et al., 1995). Almost all developmental phenotypes become significant shortly after birth (by P7) (Fox et al., 2007). To some extent, laminin β2-dependent phenotypes may overlap with FGF-dependent phenotypes, as FGFR2/Lam β2 double mutants show more pronounced changes (Fox et al., 2007).

Laminin β2 is confined to the synapse via its secretion from skeletal myofibers through a combination of mechanisms, which include concentration of laminin β2 mRNAs in subsynaptic nuclei and the synaptic targeting of laminin β2-containing proteins (Martin et al., 1995; Moscoso et al., 1995; Miner et al., 2006). Skeletal myofibers cultured in the absence of nerves make postsynaptic membranes where laminin β2 becomes exclusively localized, even when the protein is overexpressed by all muscle cell nuclei (Martin et al., 1995). Likewise, transgenic mice made to overexpress laminin β2 in all skeletal myofiber nuclei nevertheless concentrate laminin β2 exclusively at the NMJ (Miner et al., 2006). Thus, the laminin β2 protein is capable of driving its own synaptic localization. Synaptic localization requires a region near the C-terminus of laminin β2 within the coiled-coil domain that interacts with the other laminin chains (Martin et al., 1995).

Nishimune, Sanes, and Carlson identified a presynaptic receptor for laminin β2, the voltage-gated calcium channel (Nishimune et al., 2004). N- and P/Q-type voltage-gated calcium channels, the subtypes present in the motor nerve terminal during early and late NMJ development, respectively, bind laminin β2 via their eleventh extracellular loop (in $Ca_V2.2$ or $Ca_V2.1$) (Nishimune et al., 2004). This loop binds the region of laminin β2 containing the LRE tripeptide originally shown to be the adhesion site for motor neurons (Hunter et al., 1989). Mice deficient in N-type calcium channels copy the loss of active zones seen in laminin β2-deficient mice (Noakes et al., 1995; Nishimune et al., 2004). This phenotype could also be induced by injection of a peptide from the 11^{th} extracellular loop of the N-type channel into wild type NMJs (Nishimune et al., 2004). Similar findings have been found for sensory innervation of the skin in *Xenopus laevis* (Sann et al., 2008). Mice deficient in both N- and P/Q- calcium channels show a further reduction in active zones proteins, including Bassoon, Piccolo, and CAST/Erc2, supporting the presence of a direct laminin β2-calcium channel-cytoskeletal active zone scaffold (Chen et al., 2011). The loss of active zones in laminin β2 and calcium channel mutant mice is akin to what occurs in Eaton-Lambert myasthenic syndrome, where patients develop autoantibodies to voltage-gated calcium channels (Farrugia and Vincent, 2010). It is possible that some such autoantibodies disrupt laminin β2-calcium channel interactions. Humans with mutations in laminin β2 (LAMB2) can also develop congenital myasthenia. This disease has ultrastructural changes at the NMJ similar to those found in laminin β2 deficient mice (Maselli et al., 2009).

Receptors for laminin β2 on the skeletal muscle membrane are not well understood, though laminins containing the β2 chain bind postsynaptic membranes even in the absence of nerves (Martin et al., 1995). While the β chains of laminin do not have the laminin LG domains required for binding to α dystroglycan and many integrins, it would not be surprising if laminin β2 helped to define such interactions through its interactions with laminin α chains. There is at least one report of a β2-containing laminin (laminin 421) binding α3 integrin (Carlson et al., 2010), which is also concentrated within active zones

(Cohen et al., 2000). Another issue with phenotype interpretation is that laminin β1, which is normally excluded from the synaptic ECM in the adult (Chiu and Sanes, 1984), is present at the NMJ in laminin β2-deficient animals (Martin et al., 1996). Thus, aberrant expression of extrasynaptic laminins (211 and 411) at the NMJ in laminin β2 deficient mice may contribute to the phenotypes seen.

Work by Patton, Sanes and colleagues on laminin α4-deficient mice has shown that the laminin α4 protein is essential for the proper alignment of presynaptic active zones with the crests of AChR-rich secondary folds in the postsynaptic membrane (Patton et al., 2001). Laminin α4, as laminin 421, is restricted to small domains within the synaptic BL at the crests of the secondary folds, where such alignments take place. Mice lacking laminin α4, unlike mice lacking laminin β2, show normal numbers of pre- and postsynaptic specializations with regard to active zones and secondary folds, but these structures are no longer precisely aligned with one another (Patton et al., 2001). The misalignment of active zones and secondary folds ultimately is associated with alterations in synaptic geometry more commonly found in aged NMJs (Balice-Gordon and Lichtman, 1990; Valdez et al., 2010). For example, laminin α4-deficient nerve terminal branches appear to atrophy, showing thinner varicosities even at young ages (Patton et al., 2001). The receptors responsible for laminin α4-dependent phenotypes are not well understood. The direct binding of voltage-gated calcium channels to laminin β2 (Nishimune et al., 2004) and its role in active zone development (Noakes et al., 1995; Nishimune et al., 2004; Chen et al., 2011) suggests that laminin α4-deficient mice could merely be laminin β2 hypomorphs. This could result from reduced, but not absent, expression of laminin β2 in laminin α4-deficient mice, as laminin 521 and 221 would still be present (Noakes et al., 1995; Patton et al., 1997; Patton et al., 2001). Laminin α4 also plays a role in controlling Schwann cell proliferation and myelination, which could impact NMJ development (Yang et al., 2005).

Analysis of the role of laminin α5 has been complicated by additional phenotypes (exencephaly, syndactyly, and dysmorphogenesis of the placenta) that cause neonatal death when this laminin is deleted in the whole mouse (Miner et al., 1998). A conditional muscle-specific deletion was made to more easily assess the role of laminin α5 at the NMJ (Nishimune et al., 2008). The topological maturation of AChR clusters (from plaque to pretzel) was delayed in the absence of muscle laminin α5, and this was accentuated significantly in mice lacking both muscle laminin α5 and laminin α4 (Nishimune et al., 2008). Interestingly, expression of a chimeric laminin α1/α5 transgenic protein, where the LG3-LG5 domains of laminin α1 replaced the respective regions in laminin α5, failed to rescue laminin α5 deficient phenotypes (Nishimune et al., 2008). While the topological maturation defects observed in muscle-specific laminin α5-deficient mice were more subtle than those in mice lacking laminin β2 (Noakes et al., 1995), they add support to the notion that laminin α chains may, either directly or by proxy, impact postsynaptic development as well as presynaptic development. Laminin 511 can also interact with SV2 (Son et al., 2000), a synaptic vesicle transmembrane protein, and laminin 521 can associate with and prevent the migration of Schwann cells and motor axons (Cho et al., 1998; Patton et al., 1998). Thus, laminin α5 may also have presynaptic roles, though the impact of those roles on synaptic ultrastructure appears to be muted.

Trimeric laminins have an intrinsic ability to polymerize into an ECM, but certain proteins can both facilitate polymerization or cross-link laminin, once present, with other ECM proteins (Yurchenco and Patton, 2009). Nidogen, also known as entactin, provides such a cross-link between laminins and collagen IV (Fox et al., 1991; Aumailley et al., 1993). Nidogen 1 and 2 are 150kDa proteins comprised mostly of three globular domains that make a protein of about 40nm in length. The G2 domain binds collagen IV while the G3 domain binds laminin γ1 (Reinhardt et al., 1993; Poschl et al., 1996). There is good evidence that

nidogen 2 is synaptically expressed at the adult NMJ (Chiu and Ko, 1994; Fox et al., 2008). Nidogen 2 is present in the entire extrasynaptic BL at birth and becomes confined to the NMJ by the third postnatal week (Figure 1). The absence of nidogen 2, however, does not alter expression of any other synaptic ECM proteins; nidogen 2 is present in mice lacking synaptic laminins (β2, α4, or α5) or synaptic collagen IVs (α3-α6), and all of these proteins are present in mice lacking nidogen 2 (Fox et al., 2008). Nevertheless, mice deficient in nidogen 2 show subtle changes in the postsynaptic structuring of AChRs at the NMJ, making NMJs seem more immature in appearance (Fox et al., 2008). This phenotype is variable between muscle groups. As some muscles (e.g. extensor digitorum longus) have almost no abnormal synapses, this is clearly not an essential protein for synaptogenesis.

Almost all laminin α chains also bind α dystroglycan and members of the β1 family of integrins (Figure 2) (Yurchenco et al., 2004). Integrin α7β1 is the predominant muscle integrin, including at the NMJ (Song et al., 1992; Ziober et al., 1993; Martin et al., 1996). Several early studies suggested that integrin α7 altered agrin-induced AChR clustering *in vitro* (Burkin et al., 1998; Burkin et al., 2000). Mice lacking integrin α7, however, show no significant neuromuscular abnormalities, though they do develop an unusual form of muscular dystrophy (Mayer et al., 1997). Expression of some synaptic integrin α7 splice forms, however, is altered in laminin β2 deficient mice, suggesting a more direct synaptic integrin-laminin connection (Martin et al., 1996). Mice lacking other synaptic integrin α chains also do not appear to have NMJ phenotypes. Such lack of effects may be due to molecular compensation between different integrin α chains. Tissue-specific deletion of integrin β1, which is required for expression of almost all muscle integrin α chains (as integrins are αβ heterodimers), has been a means of getting beyond such problems (Schwander et al., 2003; Schwander et al., 2004). Interestingly, early loss of β1 integrin in muscle impaired myoblast fusion and yielded aberrant myotubes with altered costameric patterning (Schwander et al., 2003). Those myofibers that were formed, however, did not show altered expression of muscle ECM proteins or receptors (e.g. laminin α2, collagen IV, nidogen, perlecan, dystroglycan) (Schwander et al., 2003). Mice with muscles lacking integrin β1 fail to be innervated, despite pre-patterning of AChRs in muscle cells, and die perinatally (Schwander et al., 2004). This experiment strongly suggests that muscle β1 integrins have important roles in NMJ development. Specific deletion of β1 integrin in motor neurons, by contrast, appears to have no impact on NMJ development (Schwander et al., 2004). Thus, the role of integrins in the presynaptic membrane remains unclear.

Like β1 integrin, deletion of dystroglycan results in embryonic lethality when this gene is removed from the whole animal (Williamson et al., 1997). Myotube-specific deletion of dystroglycan results in muscular dystrophy (Cohn et al., 2002). Dystroglycan deletion in specific myofibers caused neuromuscular abnormalities that suggested a role for dystroglycan in postsynaptic development (Cote et al., 1999). Interestingly, muscle cells lacking laminin α4 and laminin α5 also have reduced expression of synaptic dystroglycan at AChR-rich regions, while muscle cells lacking dystroglycan have altered AChR patterning similar to cells lacking laminin α4 and α5 (Nishimune et al., 2008). Unfortunately, laminin α4 and α5 are often upregulated in dystrophic skeletal muscles (Patton et al., 1999), making it difficult to assess their role in dystroglycan deficiency *in vivo*. Laminin LG domains are usually removed from laminin to study their interaction with receptors such as α dystroglycan, as native laminin is a spontaneously forming polymer. Such studies show that recombinant laminin LG α chains bind α dystroglycan and that differential glycosylation of α dystroglycan can alter such binding. For example, laminin LG1-5 domains from laminin α2 (LG1-5(α2)), the predominant integrin/dystroglycan-binding region on the predominant extrasynaptic laminin, binds the extrasynaptic glycoform of α dystroglycan one hundred times better than laminin LG1-5 (α4), a synaptic laminin (Talts et al., 1999; Talts et al., 2000). By contrast, binding of synaptic LG1-5(α4) and LG1-5(α5) laminin are increased

significantly more than binding of LG1-5(α2) when α dystroglycan is glycosylated with the synaptic CT carbohydrate (Yoon et al., 2009). Moreover, transgenic overexpresson of Galgt2, the enzyme that creates the synaptic CT carbohydrate, in skeletal myofibers stimulates CT glycosylation of α dystroglycan along the extrasynaptic membrane and induces ectopic overexpression of laminin α4 and laminin α5 (Xia et al., 2002), both normally synaptic dystroglycan-binding proteins. This upregulation likely involves a combination of increased laminin transcription and increased laminin membrane affinity or ECM stability. As a group, the ectopic overexpression of this complex of synaptic laminins, synaptically glycosylated dystroglycan, and utrophin (and other synaptic dystrophin surrogates), driven by Galgt2, can functionally compensate for loss of laminin α2 or dystrophin in the extrasynaptic muscle membrane (Nguyen et al., 2002; Xu et al., 2007; Martin et al., 2009; Xu et al., 2009). Loss of these extrasynaptic muscle proteins, for example in laminin α2 deficient dy^W mice or in dystrophin-deficient mdx mice, normally leads to muscular dystrophy, and expression of the Galgt2 transgene inhibits muscle damage from occurring in these disease models.

Laminin signaling via dystroglycan and laminin/collagen signaling via integrins are markedly complex processes, well beyond the scope of this review, however, we will discuss here some of the protein components in these pathways proven relevant to changes in synaptic development and structure (Figure 3). In particular, deletion of cytoplasmic dystroglycan-binding proteins has suggested a role for intracellular dystroglycan-binding proteins in the NMJ ultrastructure and development. In the extrasynaptic membrane, laminin 211 binds α dystroglycan on the outside of the myofiber membrane and dystrophin binds β dystroglycan on the intracellular side. Dystrophin, the 400kDa protein whose absence give rise to Duchenne muscular dystrophy (Hoffman et al., 1987; Koenig et al., 1987), links dystroglycan and associated proteins in the membrane to the F-actin cytoskeleton via a variety of molecular interactions, including binding to other cytoskeleton binding proteins (plectin 1 and ankyrin), cytoplasmic DAG complex proteins (syntrophins and dystrobrevins), and signaling molecules (neuronal nitric oxide synthase, nNOS) (Blake et al., 2002). While dystrophin is also present at the NMJ, a dystrophin orthologue called utrophin is uniquely present at the synapse (Ohlendieck et al., 1991). Utrophin is very similar in protein domain structure to dystrophin (Love et al., 1989) and binds a similar cohort of molecules, but some of these, for example, β2 syntrophin and α1 dystrobrevin (Peters et al., 1997; Peters et al., 1998), like utrophin, are either uniquely present or are highly concentrated at the NMJ. Mouse genetics has shown that deletion of utrophin, syntrophins, or dystrobrevins all give rise to changes in NMJ structure akin to those observed in dystroglycan-deficient muscles, suggesting that these synaptic proteins translate structural information and/or signals from laminins in the synaptic BL through the postsynaptic membrane (Figure 3) (Deconinck et al., 1997; Grady et al., 1997; Cote et al., 1999; Adams et al., 2000; Grady et al., 2000). There are a variety of other proteins that can associate with dystroglycan (for example, Grb2, Rapsyn, Plectin, Ezrin) or that can signal from laminin via dystroglycan (for example, Src, MAP kinase, Akt) that could be involved in NMJ development. Similarly, there are a host of cytoplasmic and membrane integrin-associated proteins (for example, Talin, Vinculin, Paxillin, Focal adhesion kinase (FAK), Alpha actinin, CD47, etc.) (Figure 3) and related signaling molecules (for example Grb2, Cib2, Crk, Src, Fgr, Ras, Rho, PI-3 Kinase, MAP Kinase) that may be involved in laminin/collagen signaling at the NMJ. To our knowledge, there has been no definition of specific intracellular integrin protein binding interactions in mammals that define specific synaptic laminin- or collagen IV-integrin NMJ functions, though these may certainly exist. Because deletion of all β1 integrins in muscle has been very complicated to analyze due to changes in muscle development and lack of innervation (Schwander et al., 2003; Schwander et al., 2004), it is perhaps not surprising that these integrin-associated proteins have not been more intensively explored regarding NMJ development.

THE SYNAPTIC COLLAGENS – MATURATION OF THE SYNAPSE

In addition to laminin, the other major class of muscle BL proteins are the non-fibrillar collagens of the collagen IV family (Yurchenco and Patton, 2009). Collagen IV proteins are approximately 400nm long threadlike coils comprised of three very homologous polypeptide chains (Yurchenco and O'Rear, 1994). Each collagen IV trimer contains an N-terminal cysteine-rich domain, a long collagenous triple helical domain, and a carboxy-terminal NC1 domain (Figure 2). The N- and C-terminal regions serve to connect neighboring trimers. Like laminins, collagen IVs spontaneously aggregate into an extracellular matrix. There are six (α1-α6) collagen IV chains, and these are arranged in head-to-head pairs, each using a common bifunctional promoter (α1-α2,α3-α4, and α5-α6). In skeletal muscle, collagen IV$(\alpha1)_2(\alpha2)$ is the major form of collagen IV in the extrasynaptic BL, while collagen IV (α3, α4, α5) and collagen IV$(\alpha5)_2(\alpha6)$ are exclusively present at the NMJ (Sanes et al., 1990; Miner and Sanes, 1994; Fox et al., 2007) (Figure 1). Collagen IVs (α3- α6) become expressed at the NMJ very late, on or after the third postnatal week, after the NMJ has taken on its adult form (Fox et al., 2007). There are human diseases associated with defective expression of (or autoimmune responses to) collagen IV(α3, α4, and α5), including Goodpasture syndrome and Alport syndrome. These disorders result in kidney disease due to altered collagen IV expression in the renal glomerular basement membrane, but these diseases show no obvious neuromuscular phenotype (Miner, 2011).

Mice lacking collagen IV (α5) lack all synaptic collagen IVs, as collagen IV(α5) is required in both collagen IV trimers found at the NMJ (Fox et al., 2007). Collagen IV(α5)-deficient mice show no NMJ phenotype 3 weeks after birth, as expected since collagen IVs are not expressed until this age. By two months, however, these mice show fragmented pre- and postsynaptic specializations (Fox et al., 2007). In addition, they have NMJs with partially retracted or distended nerve terminals, sometimes leaving portions of postsynaptic AChR unopposed by the nerve, and have ring-like structures of neurofilament in some nerve terminals (Fox et al., 2007). These phenotypes are not apparent in mice lacking collagen IV (α3), which still make collagen IV $(\alpha5)_2(\alpha6)$, or in mice lacking collagen IV (α6), which still make collagen IV (α3, α4, α5) (Fox et al., 2007). Thus, there likely is some molecular compensation between different collagen IV trimers. Additional support for collagen IVs having roles in synaptic development comes from the finding that proteolyzed NC1 domains of certain collagen IVs induce presynaptic differentiation of cultured motor neurons (Fox et al., 2007). The NC1 domains of collagen IV (α2, α3, and α6) have such activity while the NC1 domains of collagen IV (α1, α4, and α5) do not. While others have certainly gone down blind alleys studying proteolytic fragments of collagens, these findings supplement the *in vivo* evidence showing that collagen IVs have roles in the maturation and the maintenance of NMJ.

Collagen XIII, a transmembrane collagenous trimer flanked by NC sequences (Hagg et al., 1998; Snellman et al., 2000), is expressed by skeletal muscle and may be cleaved and deposited in the synaptic ECM (Latvanlehto et al., 2010). At the NMJ, Collagen XIII is detectable at very low levels in the early postnatal period, with expression more apparent after two weeks of age (Latvanlehto et al., 2010). While collagen XIII is expressed in many tissues (Hagg et al., 2001; Sund et al., 2001), muscle-specific deletion leads to incomplete adhesion of the pre-and postsynaptic cell to the synaptic BL at the NMJ (Latvanlehto et al., 2010). Like laminin β2 mutants (Noakes et al., 1995), collagen XIII-deficient mutants also show invasion of the synaptic cleft by the capping synaptic Schwann cell. Functionally, collagen XIII-deficient mice show slightly but significantly reduced miniature endplate potential (mEPP) amplitude, but they have dramatic reductions in mEPP frequency (Latvanlehto et al., 2010). These mice also have a deficit in the readily releasable pool,

indicating a presynaptic functional defect (Latvanlehto et al., 2010). These phenotypes become apparent in the adult animals, and animals develop tremors as they age.

The final collagen at the neuromuscular junction is ColQ, the collagenous subunit of acetylcholinesterase (AChE) (Figure 2) (Rotundo, 2003; Massoulie and Millard, 2009). AChE within the synaptic cleft degrades acetylcholine secreted by the nerve terminal after nerve excitation. AChE forms tetrameric, octameric, and dodecameric complexes of catalytic subunits in muscle cells. These bind to ColQ at the NMJ to make “asymmetric” AChE forms (Hall, 1973; Sanes and Hall, 1979; Krejci et al., 1997). Mice lacking ColQ fail to localize AChE at the NMJ (Feng et al., 1999; Bernard et al., 2011). NMJs still form, however, albeit with slightly aberrant shape, and animals are born and survive for at least several weeks, with 10–20% surviving for up to three months (Feng et al., 1999). This is a pretty remarkable result, as inhibition of AChE should be fatal (as certain insecticide and military nerve gas AChE inhibitors attest to). Compensatory mechanisms, perhaps mediated by the capping synaptic Schwann cell, appear to make up for lost ACh clearance in the ColQ mutant animals, allowing the mice to live for some period of time. Legay and colleagues have argued that ColQ also interacts with MuSK and that ColQ-deficient myotubes have altered postsynaptic organization via this interaction (Cartaud et al., 2004; Sigoillot et al., 2010). ColQ also interacts with perlecan. Mice lacking perlecan, like mice lacking ColQ, fail to express AChE at the neuromuscular junction, but these mice, unlike ColQ mutants, die at birth from respiratory failure (Arikawa-Hirasawa et al., 2002). Heparin, which mimics the heparin sulfate glycosaminoglycans on perlecan, can remove AChE from the NMJ (Torres and Inestrosa, 1983; Brandan and Inestrosa, 1984). These studies also support a role for perlecan in localizing AChE to the synapse. Perlecan is an abundant muscle ECM protein that can also interact with laminin, nidogen, fibronectin, and collagen IV in the ECM and dystroglycan and other receptors in the muscle membrane (Figure 2) (Sanes et al., 1986; Talts et al., 1999). By virtue of its heparan sulfate glycosaminoglycans, perlecan may also interact with growth factors, including FGFs and HB-GAM (Peng et al., 1998). Thus, perlecan, along with agrin, may serve to localize many trophic factors to the NMJ. It is conceivable that loss of synaptic AChE in ColQ- and perlecan-deficient mice are related to the development of a coordinated synaptic protein complex. Alternatively, interactions of ColQ-AChE with MuSK and other synaptic proteins may localize AChE, while interactions of ColQ-AChE with perlecan may stabilize ECM expression, or vice versa.

CONCLUDING REMARKS

Extracellular matrix proteins that reside within the synaptic cleft at the neuromuscular junction play essential roles in almost all aspects of synaptic development, including synaptic initiation, topography, ultrastructure, maturation, stability and transmission. While the NMJ serves as a model to understand the formation of other types of synapses, one compelling difference is that interneuronal synapses in the CNS, as well as the NMJs of invertebrates (see Broadie et al., Wlodarcyk et al, this issue), do not possess a synaptic basal lamina. Protein interacting motifs present at the NMJ, however, are present nonetheless at such synapses. Agrin can exist in a transmembrane form in neurons that can localize to CNS synapses (Burgess et al., 2000), laminin LG domains are present on CNS synaptic transmembrane proteins like neurexins (Ushkaryov et al., 1992), which also interact with dystroglycan (Sugita et al., 2001). Integrins and dystroglycan are localized to certain classes of CNS synapses (Montanaro et al., 1995; Gerrow and El-Husseini, 2006). Neuroligins, synaptic neurexin-binding proteins, contain non-catalytic domains of acetylcholinesterase (Ichtchenko et al., 1995), and the list goes on. Indeed, laminins containing laminin β2 can be present at certain CNS synapses, albeit in a non-BL configuration(Egles et al., 2007). Thus, while the ECM may not be present in the same form as is found at the neuromuscular junction, it is likely that many of the same types of protein-protein or protein-glycan

interactions found at the NMJ will be present at synapses elsewhere in the nervous system. The difference primarily may lie in the context for such adhesive interactions and signaling, with cell-cell adhesion and signaling mechanisms driving CNS synapse formation and cell-ECM mechanisms predominating at the vertebrate NMJ.

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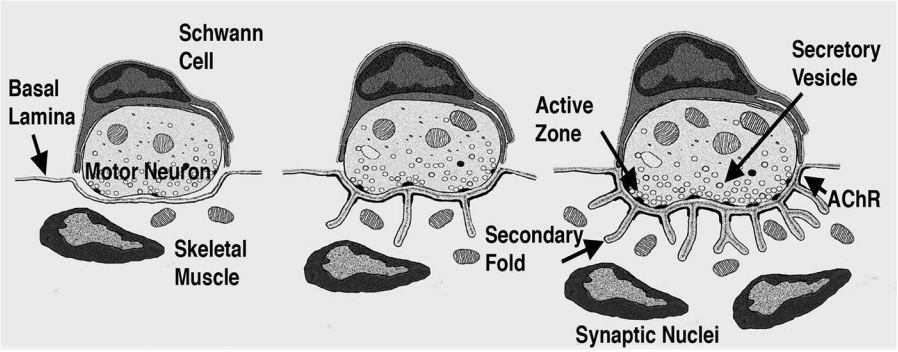

	E15		P0		P21	
Ubiquitously Expressed	ESYN	SYN	ESYN	SYN	ESYN	SYN
LN 211	+	+	+	+	+	+
Col IV (α1)$_2$(α2)	+	+	+	+	+	+
Fibronectin	+	+	+	+	+	+
Nidogen 1	+	+	+	+	+	+
Perlecan	+	+	+	+	+	+
Uniquely Synaptic						
LN 221	-	+	-	+	-	+
LN 421	-	+	-	+	-	+
LN 521	-	+	-	+	-	+
Agrin (z8)	-	+	-	+	-	+
ColQ	-	+	-	+	-	+
AChE	-	+	-	+	-	+
Col IV (α3)(α4)(α5)	-	-	-	-	-	+
Col IV (α5)$_2$(α6)	-	-	-	-	-	+
Col XIII	-	-	-	-	-	+
Ubiquitous Then Synaptic						
Agrin (z0)	+	+	+	+	-	+
Neuregulin	+	+	+	+	-	+
Nidogen 2	+	+	+	+	-	+
Ubiquitous Then Extrasynaptic						
LN 411	+	+	+	-	+	-
LN 511	+	+	+	-	+	-
Other						
LN 111	+	-	-	-	-	-

Figure 1. Developmental expression of synaptic extracellular matrix (ECM) proteins at the rodent neuromuscular junction

By embryonic (E) day 15, skeletal myofibers have formed and are innervated the nerve terminals of motor neurons at the neuromuscular junction. The nerve terminal is capped by a non-myelinating Schwann cell. Skeletal myofibers express a number of ECM proteins, including laminin (LN), collagen IV, fibronectin, perlecan, ColQ and nidogen, while motor nerves express ECM proteins including agrin (z8, 11, or 19) and neuregulin. Some ECM proteins are localized within the synaptic (SYN) basal lamina, which exists only at the neuromuscular junction, while other ECM proteins are present within the extrasynaptic (ESYN) basal lamina that surrounds the remainder of the skeletal myofiber membrane.

ESYN and SYN composition shifts during neuromuscular development as synaptic ultrastructure develops, such that unique laminin and collagen chains become expressed exclusively in the synaptic basal lamina in the postnatal (P) animal.

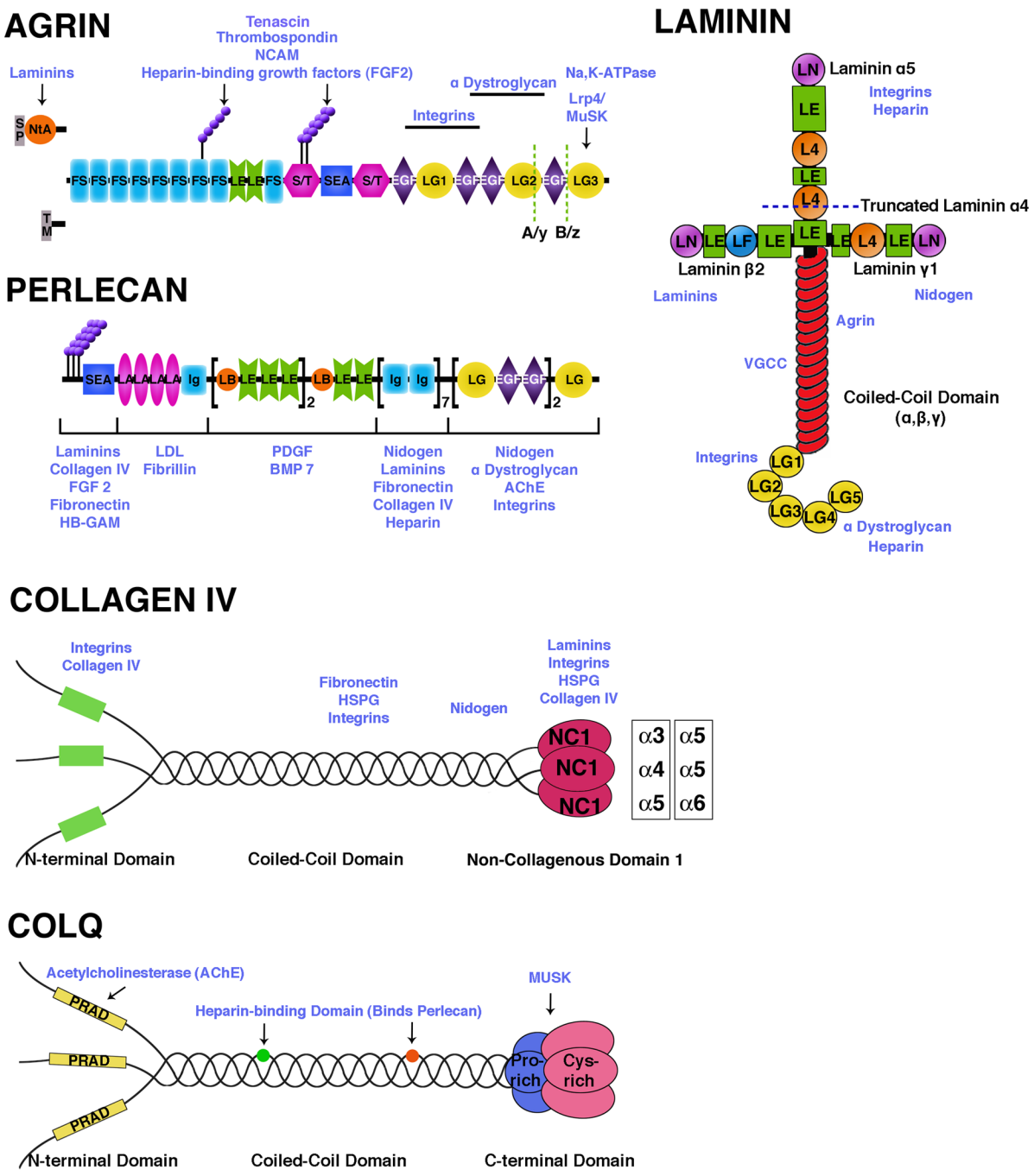

Figure 2. Synaptic extracellular matrix (ECM) proteins and their receptors at the neuromuscular junction

ECM proteins within the synaptic basal lamina at the neuromuscular junction include agrin, laminins, perlecan, collagen IVs, and ColQ. Potential ECM-receptor and ECM-ECM interactions are indicated in blue. Agrin contains splice sites in the N-terminus to allow for secreted and transmembrane forms and A/y and B/z splice sites in the C-terminus that allow heparin binding and AChR clustering, respectively. Laminins are heterotrimeric proteins comprised of an α, β, and γ chain. At the NMJ, the uniquely synaptic laminin chains are laminin β2, laminin α4, and laminin α5. Collagen IVs are heterotrimeric proteins of

different three alpha chains. The NMJ contains two synaptic collagen IV trimers ((α3, α4, α5) and $(\alpha5)_2(\alpha6)$).

Legend: EGF, Epidermal growth factor-like repeat; FS, Follistatin-like domain; Ig, Immunoglobulin domain; LF, Laminin four domain (of the β chain); LB, Laminin B domain; L4, Laminin domain IV (of the γ or α chain); LE, Laminin EGF-like domain; LG, Laminin-like globular domain; LN, Laminin N-terminal domain; LA, LDL receptor domain class A; NtA, N-terminal agrin-laminin binding domain; PRAD, Proline-rich attachment domain; S/T, Serine/threonine-rich mucin-like domain; SP, Signal peptide; SEA, Sea urchin sperm protein, enterokinase, and agrin domain; TM, Transmembrane domain; AChE, Acetylcholinesterase; NCAM, Neural cell adhesion molecule; FGF, Fibroblast growth factor, HB-GAM, Heparin-binding growth-associated molecule; BMP, Bone morphogenetic protein; Lrp4, Low-density lipoprotein receptor-related protein 4; MuSK, Muscle-specific kinase; PDGF, Platelet-derived growth factor, LDL, Low density lipoprotein; HSPG, Heparan sulfate proteoglycan; VGCC, Voltage-gated calcium channel.

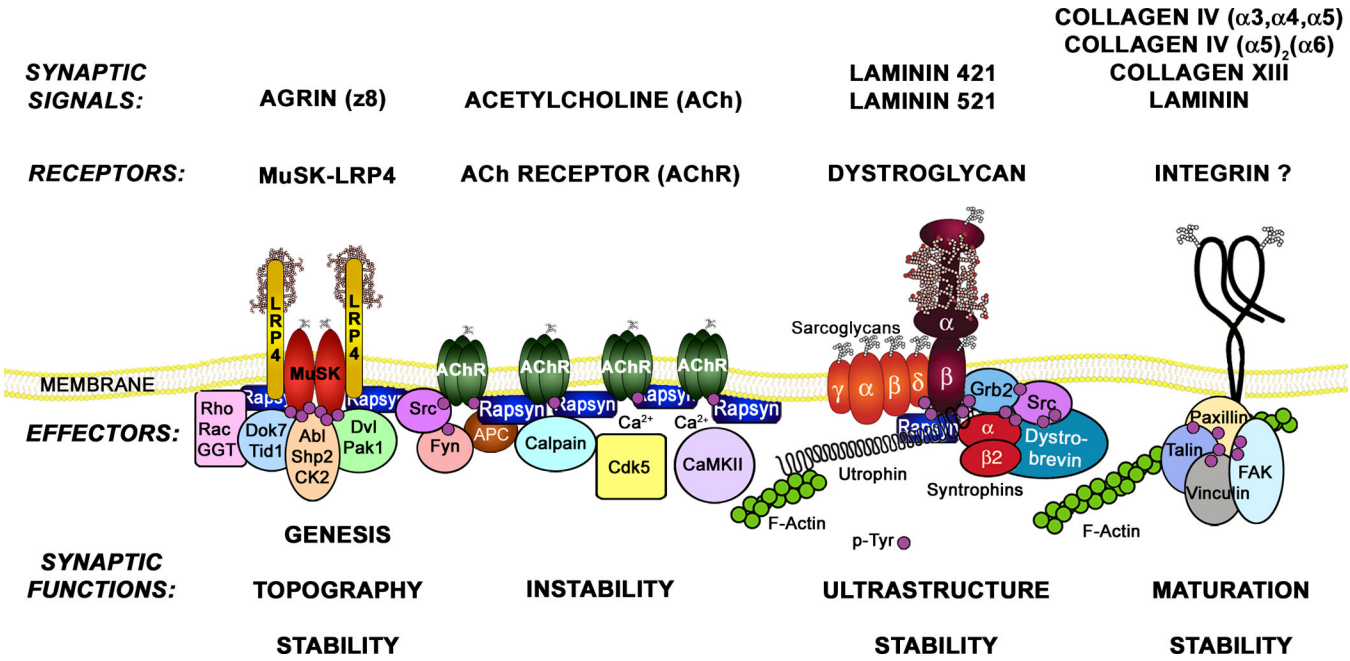

Figure 3. Synaptic ECM Signaling in Neuromuscular Development

Genetic evidence in mice and humans points to three central ECM-mediated groups of signals that are essential for proper neuromuscular development in muscle, those involving synaptic agrin, synaptic laminins and synaptic collagens. Signals from neuron-specific splice forms of agrin (for example z8) in the synaptic basal lamina (BL) work via the MuSK-LRP4 complex in the membrane to induce downstream tyrosine phosphorylation signals that drive synaptogenesis, which is agrin-independent, and to organize synaptic topography and induce synaptic stabilization, which are agrin-dependent. Down-stream signals are mediated or modulated by a variety of cytoplasmic effectors, including structural and adaptor/signaling proteins (Dok7, Dvl, Pak1, Tid1, APC, Rapsyn), proteases (Calpain), tyrosine kinases (Abl, Src, Fyn), tyrosine phosphatases (Shp2), serine/threonine kinases (Cdk5, CaMKII and CK2), small G proteins (Rho, Rac) and a geranylgeranyltransferase (GGT). Calcium entry stimulated by acetylcholine works, in part via Calpain, Cdk5 and CamKII to destabilize synaptic membranes. Laminins, particularly Laminin 421 and 521, in the synaptic BL signal via dystroglycan and possibly integrins in muscle to induce changes in synaptic ultrastructure and to maintain synaptic stability. Ultrastructural changes at the synapse are mediated, in part, by cytoplasmic dystroglycan-binding proteins including utrophin, syntrophins and dystrobrevins. These proteins help to link the ECM outside the muscle membrane to filamentous (F-) actin in the cytoskeleton. Laminin 421 and 521 also bind to the voltage-gated calcium channels in the presynaptic membrane to organize active zones. Collagens IV in the synaptic BL and collagen XIII in the postsynaptic membrane may signal via integrins to induce synaptic maturation and stability. Integrins may bind and signal through a variety of intracellular proteins (for example Focal adhesion kinase (FAK), vinculin, paxillin,talin).