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THE ROLE OF LAMININS IN THE ORGANIZATION AND FUNCTION OF NEUROMUSCULAR JUNCTIONS

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Abstract

The synapse between motor neurons and skeletal muscle is known as the neuromuscular junction (NMJ). Proper alignment of presynaptic and post-synaptic structures of motor neurons and muscle fibers, respectively, is essential for efficient motor control of skeletal muscles. The synaptic cleft between these two cells is filled with basal lamina. Laminins are heterotrimer extracellular matrix molecules that are key members of the basal lamina. Laminin α4, α5, and β2 chains specifically localize to NMJs, and these laminin isoforms play a critical role in maintenance of NMJs and organization of synaptic vesicle release sites known as active zones. These individual laminin chains exert their role in organizing NMJs by binding to their receptors including integrins, dystroglycan, and voltage-gated calcium channels (VGCCs). Disruption of these laminins or the laminin-receptor interaction occurs in neuromuscular diseases including Pierson syndrome and Lambert-Eaton myasthenic syndrome (LEMS). Interventions to maintain proper level of laminins and their receptor interactions may be insightful in treating neuromuscular diseases and aging related degeneration of NMJs.

Keywords

aged; Bassoon; neuromuscular junction; laminin; voltage-gated calcium channels

Introduction

Neuromuscular junctions (NMJs) are chemical synapses located between nerve terminals and specialized sites on the post-synaptic skeletal muscle fiber plasma membrane (i.e., motor endplates). Innervation of muscle fibers by motor neurons establishes proper control of skeletal muscle contraction by the nervous system, and this innervation can become disrupted during disease and aging. Depolarization of motor neurons results in subsequent

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depolarization of the skeletal muscle fiber plasma membrane or sarcolemma. On the presynaptic side, depolarization of motor neurons results in synaptic vesicle fusion with the plasma membrane and exocytosis of a chemical neurotransmitter, acetylcholine (ACh), into the synaptic cleft. Synaptic vesicle release sites of the nerve terminal are well-organized structures known as active zones (see Box 1 for expanded description of active zones) [1–5]. At the endplate, the sarcolemma uniquely folds to form junctional folds, and acetylcholine receptors (AChRs) accumulate at the crest of these junctional folds to rapidly and efficiently receive ACh released from motor neurons [6–10].

BOX #1

Active zone organization and active zone proteins

Active zones are a multiprotein complex accumulated at the presynaptic plasma membrane where synaptic vesicles accumulate, fuse with the plasma membrane, and release neurotransmitters into the synaptic cleft [1–5, 162, 204–208]. Using freeze-fracture electron microscopy, active zones were identified as two parallel arrays of 10–12 nm intramembranous particles arranged in two to four rows with each active zone containing 20 of these intramembranous particles [93, 96, 209, 210]. The density of active zones at the presynaptic membrane of NMJ is 2.4–2.7 active zones/μm^2 in adult humans and mice [4, 93, 96, 97], and this density is maintained during postnatal maturation periods when NMJs enlarge [175]. A collection of proteins make up the active zones (also known as the cytoskeletal matrix of the active zone (CAZ)) including Bassoon, CAST/ELKS2/Erc2, CAST2/ ELKS/Erc1, Munc13, Piccolo, Rab3 interacting protein-1/2 (RIM1/2), as well as Brunchpilot in**Drosophila** that is a homolog of CAST/ ELKS/Erc (Figure 3) [211–223]. These active zone proteins are involved in accumulation of synaptic vesicles to the plasma membrane and neurotransmitter release upon stimulation by calcium influx through VGCCs. It is important to note that active zone density is independent of nerve transmission. In acetylcholine transferase knockout mice, ACh cannot be properly synthesized therefore synaptic transmission is absent. However, active zone density is normal in these mice [224]. Thus, proper nerve transmission is not necessary for proper active zone organization and formation.

The active zone proteins Bassoon and Piccolo were discovered in screenings to determine structural proteins in rat brain synaptic junctions, and these two proteins share many structural similarities [212, 215, 219, 225]. In NMJs of rodents, Bassoon and Piccolo display a punctate staining pattern by fluorescent immunohistochemistry (Figure 4), with these puncta localizing to presynaptic active zones [63, 175, 189]. Mice without functional Bassoon have normal synapse formation, but impaired synaptic transmission, abnormal dendritic branches, ectopic formation of active zones, and lack proper anchoring of photoreceptor ribbon synapses to active zones with otherwise normal retinal anatomy [226, 227]. Piccolo has been shown to aid in synaptic vesicle exocytosis, and when absent, synaptic vesicle trafficking is disrupted [228]. Importantly, Bassoon helps to position VGCCs near the synaptic vesicle release sites [223]. Although disruption of Bassoon reduces synaptic functionality in some regions of the central nervous system, the disruption of active zone proteins may or may not result in aberrant NMJ formation and needs to be further tested.

The role of other active zone specific proteins in the formation of NMJ active zones is not as well established. CAST family of scaffolding protein CAST2/ELKS/Erc1 along with Piccolo, but not CAST/ELKS2/Erc2, are detected at NMJs [229]. In addition, CAST/ELKS2/Erc2, similar to Bassoon, has been linked to VGCC functionality [230]. The role of CAST/ELKS2/Erc2 in active zone organization was confirmed in photoreceptor synapses and inhibitory synapses due to the loss of CAST/ELSK2α [231, 232]. Munc13 has three homologous family members (Munc13-1/2/3) and has an essential role in neurotransmitter release and synaptic vesicle priming in synapses of the central nervous system [217, 233, 234]. Munc13-1/2 double knockout mice totally lack spontaneous and evoked synaptic transmission in excitatory and inhibitory synapses of hippocampal neurons [235], but exhibited residual amount of synaptic transmission at NMJs [236]. At the NMJ, these mice exhibit normal apposition between motor neuron terminals with endplates, and active zones with docked vesicles were detected [236, 237]. Therefore, Munc family of proteins may not be essential for the structural assembly of active zones. RIMs are multi-domain proteins consisting of three isoforms (α, β, γ) that also play essential roles in connecting active zone specific proteins to active zone structures and synaptic vesicles [238–244]. For example, direct interactions of PDZ domains in RIM and N- and P/Q-type VGCCs tether these VGCCs to presynaptic active zones [244]. RIM1 interacts directly with VGCC β subunit and suppresses voltage-dependent inactivation of neuronal VGCCs [242, 243]. RIM1/2 knockout mice have a reduced number of docked vesicles at active zones with reduced density of VGCCs in the calyx of Held synapse without loss of active zone density [245]. Further work is needed to elucidate the molecular mechanisms that underlie changes in active zone protein content during aging and other neuromuscular diseases.

During development, motor neurons must seek out and find nascent endplates making up only approximately 0.1% of the sarcolemmal surface area [11]. Individual components of the skeletal muscle fiber basement membrane help to guide the process of innervation by motor neurons, and also proper organization of pre- and post-synaptic NMJ morphology. Laminins play a large role in this process and individual laminin subunits are responsible for organizing different components of the NMJ structure [11, 12]. Laminin receptors, including basal cell adhesion molecule/Lutheran blood group antigen (Bcam), dystroglycan, integrins, and voltage-gated calcium channels (VGCCs), assume different roles influenced by each laminin chain (see Box 2 for expanded description of VGCCs at NMJs) [10–20]. These interactions are disturbed during some diseases and aging, thus influencing the morphology and function of pre- and post-synaptic NMJ structures. In this review, we will highlight the relationship of laminins and laminin receptors in the organization of the pre- and post-synaptic components of NMJs and how these molecular mechanisms relate to some disease processes.

BOX #2

Voltage-gated calcium channels

VGCCs localize at presynaptic active zones and are responsible for calcium influx upon sensing depolarization of the presynaptic plasma membrane. The rise of calcium

concentration in nerve terminals results in fusion of synaptic vesicles and exocytosis of neurotransmitters into the synaptic cleft. This process is essential for communication between neurons as well as between motor neurons and skeletal muscle fibers. A number of VGCC types exist, including P/Q-, N-, L-, and R-type VGCCs ($Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, and $Ca_v1.2$, respectively), but the P/Q- and N-type VGCCs are enriched specifically at motor nerve terminals [64, 246–248]. Synaptic transmission in NMJs at perinatal stages depends on both N-and P/Q-type VGCCs; however, the electrophysiological dependence shifts towards P/Q-type VGCCs for synaptic transmission in adulthood [64]. These calcium channels have two transmembrane subunits (α1 and α2δ) and a cytosolic β subunit [246]. Interestingly, N- and P/Q-type VGCCs are receptors for laminins containing the β2 chain. A leucine-arginine-glutamic acid (LRE) segment in laminin β2 binds directly to the 11th extracellular loop of the α subunit of P/Q- and N-type VGCCs [63]. The 11th extracellular loop of the P/Q-type VGCC has structural homology with the L5III loop of L-type VGCC ($Ca_v1.1$), whose 3-dimensional structure has been elucidated recently using single-particle cryo-electron microscopy [249]. The L5III domain formed one of the most exposed domains of the α subunit. The interaction between laminin β2 chain and P/Q- and N-type VGCCs is essential for NMJ function as well as presynaptic active zone formation and organization [63, 90].

P/Q-type VGCCs localize at motor terminal active zones [89, 189]. These VGCCs directly interact with synaptic vesicle related proteins syntaxin, synaptosomal associated protein of 25 kDa (SNAP-25), and synaptotagmin, and in turn SNAP-25 and syntaxin modify VGCC function [250–255]. In addition, a number of active zone proteins interact with different subunits of the VGCCs (Figure 3)[63, 90, 223, 242–244, 256–258]. Bassoon and CAST/ELKS2/Erc2 interact with the P/Q-type VGCC β subunits [90, 258], Bassoon interacts indirectly with P/Q-type VGCCs [223], and RIM1/2 interacts with P/Q- and N-type VGCC α subunits and β subunit [242–244, 256]. Bassoon and RIM1 have also been shown to inhibit the inactivation properties of P/Q-type VGCCs, prolonging the opening of these calcium channels localized with Bassoon and RIM1 compared to VGCCs without interacting with these active zone proteins [189, 242, 243]. Piccolo has been shown in pancreatic beta cells to interact with L-type VGCC α subunits aiding in vesicle secretion [257]. Thus, binding of active zone proteins to the VGCCs appears to be important for properly positioning these channels within active zones and regulating calcium influx. Alternatively, evidence supports that laminin β2 in the synaptic cleft binds to and organizes the locations of VGCCs, and it is this interaction that results in proper positioning of the active zones in front of junctional folds [63, 65]. Disruption of the VGCC-active zone protein relationship may reduce calcium influx during repeated depolarization and aged NMJs.

The basal lamina of NMJs

Skeletal muscle fibers are surrounded by a layer of connective tissue, the basement membrane, which can be further subdivided into an internal basal lamina layer connecting to the sarcolemma and an external reticular lamina layer [11, 12]. The basal lamina envelops

the entire muscle fiber including the synaptic cleft between the nerve terminal and endplate, but the reticular lamina is excluded from the synaptic cleft of NMJs [12]. The basal lamina is made up largely of collagen IV molecules, laminins, and other non-collagenous proteins including entactin/nidogen, perlecan, and fibronectin [11, 12, 21–23]. The network of laminins and collagen are connected to one another by the glycoprotein entactin/nidogen, but also anchored to the sarcolemma and intracellular cytoskeleton by binding to laminin receptors integrin and dystroglycan [14, 17, 24, 25]. The basal lamina provides strength and structure to the skeletal muscle fibers, but rather than being a static structure, the basal lamina plays a direct role in key biological functions.

Components of the basal lamina have important regulatory roles in myogenesis and synaptogenesis, as these molecules help guide cell growth, cell migration, and adhesion of motor neuron axons [12, 26–30]. Localization of specific collagen and laminin chains to regions of the endplate basal lamina plays a role in organizing the pre- and post-synaptic structures of the NMJ. In mammals, collagen IV α2, α3, and α6 chains, as well as collagen XIII, are concentrated at mouse NMJs [12, 31–34]. Collagen molecules are important for development of NMJs. For instance, collagen IV α2 chains are necessary for formation of NMJs and differentiation of motor neuron terminals during embryonic development, and collagens IV α3 and α6 are required for maintenance of mature NMJs [33]. As will be described below, both laminin α and β chains play a prominent role in the development of pre- and post-synaptic structures of NMJs, with each laminin chain playing a specific role. Disruption of individual components of the basal lamina or the connections of the basal lamina to the muscle fiber itself results in muscular dystrophy and degeneration of muscle fibers and NMJs [35–38].

The role of laminins in NMJs and active zone organization

Laminins are important components of the basal lamina. These large, multi-armed glycoproteins (~400–900 kDa) assemble into heterotrimers composed of three different chains: α, β, and γ [11, 12, 21, 39–43]. In mice and humans, five different α chains, three different β chains, and three different γ chains have been identified. To date, 19 laminin trimers have been described (laminin 1–15, as well as laminin 5B) with laminin-111 (α1β1γ1, formerly laminin-1) being the first characterized in Engelbreth-Holm-Swarm (EHS) tumors. The different laminin chains assemble into cross or T-shaped molecules. C-terminal ends of laminin α chains wrap into a coiled structure containing laminin globular (LG) domains. N-terminal ends, known as “short arms,” are free and contain N-terminal globular (LN) domains [11, 12, 21, 39–43].

Laminins have been shown to play an essential role in myogenesis and synaptogenesis as these molecules guide cell differentiation, migration, and adhesion [12, 26–29, 44, 45]. Specific heterotrimers of laminin chains assemble in specific regions of different tissues. In the case of skeletal muscle, laminin-211 (α2β1γ1, laminin-2) predominates throughout the extrasynaptic basal lamina, but laminin-421 (α4β2γ1, laminin-9), laminin-521 (α5β2γ1, laminin-11), and laminin-221 (α2β2γ1, laminin-4) are expressed specifically at the synaptic cleft of NMJs (Figure 1A) [46]. Laminin γ1 chains are found throughout the sarcolemma and are essential for formation of basement membranes as knockout of laminin γ1 is lethal

[45]. Both laminin α and β chains have been described to play specific roles in different aspects of development and maintenance of NMJs, as well as organization of presynaptic active zones.

Laminin β chains

Laminin β1 and β2 chains are homologous with nearly identical structures [31, 47]; however, localization patterns of these laminin chains vary even between different locations on the same cellular basal lamina. Laminin β2, formerly known as s-laminin due to its synaptic localization, is located at NMJs (Figure 1A and Figure 2), and also in developing brain, spinal cord, retina, and lens, capillaries, and kidney glomerulus [47–53]. In skeletal muscles, laminin β2 chains assemble with γ1 chains and either α2, α4, or α5 chains to form specific laminin molecules that are secreted from muscle and incorporated into the synaptic basal lamina [46, 47, 49, 54, 55]. Importantly, laminin β2 is specifically enriched at the basal lamina of NMJs but is not found at the extrasynaptic basal lamina (Figure 1A) [46, 55]. On the other hand, laminin β1 is found throughout the extrasynaptic basal lamina but excluded from NMJs [46, 55].

Early on, laminin β2 was shown *in vitro* to guide neurite outgrowth from motor neurons and promote differentiation into mature nerve terminals [30, 46, 56, 57]. The generation of a mouse model lacking laminin β2 proved insightful in the precise role of laminin β2 in synaptic development at the NMJ. Laminin β2 knockout mice die between days P15 and P30 due to failure of the renal glomerular filtration barrier [58, 59], and display malformed NMJs [58, 60, 61]. Despite being innervated initially, NMJs of laminin β2 knockout mice lack appropriate formation of pre- and post-terminal structures including a lack of junctional folds, reduced number of active zones, and Schwann cell infiltration of the synaptic cleft (Figure 1B) [58, 62, 63]. Different molecules have been shown to sequentially play a role in the development of NMJs from early embryonic stages to maintenance of adult NMJs [33]. Laminin β2 is important for maintenance and maturation of NMJs during the first few weeks after birth [33, 63]. On a functional level, NMJs of laminin β2 knockout mice have lower miniature endplate potential (mEPP) frequency and a higher likelihood of failure of synaptic transmission compared to wild-type mice [58, 60]. The reduction in neurotransmitter release in these mice relates to a reduced number of active zones and synaptic vesicles near the presynaptic membrane, as well as reduced protein levels of the active zone specific protein Bassoon and synaptic vesicle related proteins [58, 60, 61, 63]. Furthermore, laminin β2 knockout mice also have a failure in maturation of VGCC types that are present at presynaptic terminals of NMJs [61]. Laminin β2 knockout mice retained N-type VGCCs at presynaptic terminals, and fail to transition from expressing N- and P/Q-type VGCCs at presynaptic terminals shortly after birth to expressing predominately P/Q-type VGCCs during the first 2–3 weeks following birth that typically occurs in wild-type mice [61, 64]. Due to the loss of laminin β2, these knockout mice also have a loss of laminin-421 (laminin-9) and laminin-521 (laminin-11) at NMJs, which leads to investigation of the role of laminin α chains in organization of NMJs [58]. In summary, the loss of laminin β2 influences NMJ morphology and functionality partly by disrupting active zone formation and organization, and changing the composition of VGCC types at NMJs.

Laminin α chains

Laminin α2, α4, and α5 chains are essential for proper muscle development, as well as specifically establishing and maintaining the structure of NMJs and alignment of presynaptic active zones [20, 65–67]. Each of these laminin α chains plays a specific role in these processes. Laminins containing the laminin α2 chain are collectively known as merosin. Laminin α2 subunits assemble into laminin-211 (laminin-2) throughout the extrasynaptic skeletal muscle basal lamina [67–69], and assemble into laminin-221 (laminin-4) that is specific to NMJs. Laminin-211 promotes axon growth, migration of muscle cells, and Schwann cell functionality [56, 70, 71]. Mice harboring mutations of the laminin α2 gene display a muscular dystrophy-like syndrome [67], which is also observed in humans harboring genetic mutations in the gene encoding laminin α2 (*lama2*) [37, 72–75]. Laminin α2 mutant mice with a spontaneous hypomorphic allele (i.e., *dy* and dy^{2J} mice) and other laminin α2 mutant models (dy^{W} and dy^{3K} mice) do not develop post-synaptic junctional folds at motor endplates, display partial detachment of motor neuron terminals from the endplate, have demyelination of motor axons, and minor Schwann cell infiltration into synaptic cleft (Figure 1E) [35, 36, 76–86]. However, these mutant mice do assemble active zones, which properly oppose AChRs that accumulate at endplates [11, 77]. Thus, laminin α2 is essential for skeletal muscle maintenance and formation of structures at muscle endplates, but may not be required for formation of presynaptic structures of NMJs.

Laminin α4 and α5 chains appear to be directly responsible for organizing presynaptic active zones and endplate structures in NMJs, with each of these laminin chains playing an individual role. Laminin α4 chains are part of laminin-421 (laminin-9) molecules and concentrate to the synaptic cleft of NMJs [46]. Laminin α4 localizes within the basal lamina where active zones are absent, and does not localize at the crest of junctional folds (Figure 1A) [65]. This locational specificity may serve as a mechanism by which laminin α4 aligns presynaptic active zones and post-synaptic endplate structures. Consistent with this hypothesis, laminin α4 knockout mice have a normal number of active zones and junctional folds; however, the alignment of these pre- and post-synaptic structures is disrupted (Figure 1C) [65]. In wild-type mice, 78% of active zones align with the junctional folds; however, this was reduced to 23% in laminin α4 knockout mice [65]. Furthermore, laminin α4 knockout mice suffer a neuromuscular defect and display a phenotype that resembles premature aging of NMJs; however, these mice do not display signs of myopathy or muscular dystrophy [65, 87].

Laminin α5 is expressed throughout the basement membrane of developing skeletal muscle during embryonic stage, but becomes concentrated at the synaptic cleft shortly after birth [46]. In postnatal stages, laminin α5 chains follow an expression pattern paralleling laminin β2 chain location, but the role of laminin α5 is different from laminin β2. Complete loss of laminin α5 is embryonically lethal due to multiple developmental defects [88]. However, mice with a muscle-specific laminin α5 knockout display arrested postsynaptic maturation of NMJs to the early postnatal stage [20]. To further investigate the role of laminin α4 and α5 in skeletal muscle, laminin α4 knockout mice were crossed with muscle-specific laminin α5 knockout mice to generate double knockout mice of laminin α4/α5 [20]. These double mutants are smaller and weaker than either single mutant and survived to three months of

age [20]. In these double knockout mice, synapse elimination occurs normally. However, the remaining axon does not take over the AChR territory that was previously innervated by the losing axons (Figure 1D) [20]. In wild-type mice, the vacated area of an endplate will be occupied by the remaining winning axon. These results suggest a role for laminin α4 and α5 in presynaptic maturation. Furthermore, maturation of AChR clusters is arrested in laminin α4/α5 double knockout mice, and levels of the laminin receptor dystroglycan are reduced at NMJs [20]. Dystroglycan conditional knockout mice also have arrested maturation of AChR clusters on the post-synaptic membrane [20]. In support of these *in vivo* observations, primary myotube cultures prepared from laminin α4/α5 double knockout mice or muscle-specific laminin α5 knockout mice fail to form mature AChR clusters appropriately; however, myotube cultures prepared from laminin α4 or laminin β2 knockout mice are still capable of forming mature AChR clusters *in vitro* [20]. These results implicate the laminin α5-dystroglycan interaction is important for the maturation of AChR clusters in postnatal stages.

The role of laminin receptors in NMJ organization

A number of laminin receptors, including Bcam, dystroglycan, integrins, and VGCCs are localized to NMJs and participate in organizing pre- and post-synaptic structures as described briefly for dystroglycan above [10–20].

Voltage-gated calcium channels

In adult NMJs, P/Q-type VGCCs localize at presynaptic terminals specifically at active zones. For a more detailed description of VGCCs, please refer to Box #2. P/Q-type and N-type VGCCs have been identified as receptors for laminin β2, and the interaction of these two molecules is important for organizing presynaptic active zones as cytosolic domains of VGCCs also bind with active zone proteins (see Box #2, Figure 3, and [63]). A leucine-arginine-glutamic acid (LRE) segment in laminin β2 binds to the 11th extracellular loop of the α subunit of P/Q- and N-type VGCCs, but does not bind to other VGCC types (R- and L-type VGCCs) expressed in motor neurons [63]. The LRE sequence mediates cell adhesion and neurite outgrowth of ciliary ganglion neurons (muscle innervating neuron) induced by laminin β2 [28, 30]. Laminin β1 does not bind to P/Q-, N-, R-, or L-type VGCCs [63]. Disruption of the interaction between VGCCs and laminin β2 results in a loss of active zone proteins without influencing NMJ area or morphology [63].

Direct evidence for the role of VGCCs in organizing NMJ active zones comes from VGCC knockout mice. P/Q-type VGCC knockout mice show decreased active zone formation and reduced protein levels of Bassoon in NMJs [63]. These knockout mice display a compensatory increase in NMJ localization of other VGCC isoforms (i.e., N- or R-type VGCCs) [63, 89]. This is problematic as typically during postnatal maturation there is a decrease in the dependence on N-type VGCCs and a shift towards dependence on P/Q-type VGCCs for synaptic transmission at NMJs [64]. Thus, NMJs from these mice display delayed maturation of NMJs as they fail to properly switch from N-type to P/Q-type VGCCs with compensatory upregulation of N-type VGCCs [61] that also bind laminin β2 chains confounding the interpretation of these results. To overcome the confluence of this

compensation, P/Q- and N-type VGCC double knockout mice were generated [90]. These double knockout mice display proper NMJ innervation, but a loss of synaptic transmission and reduced levels of active zone specific proteins Bassoon, CAST2/ELKS/Erc1, and Piccolo [90]. Furthermore, VGCC $\alpha_2\delta$ subunit in *Drosophila* plays a role in synapse formation and loss of the $\alpha_2\delta$-3 subunit results in disorganized active zones [91]. These findings support the role of VGCCs in active zone organization in NMJs.

Disruption of the interaction between laminin β2 and P/Q-type VGCCs also results in loss of active zone structures. Soluble recombinant proteins generated from P/Q-type VGCCs containing only the 11th extracellular loop block the binding of P/Q-type VGCCs with laminin-421 (laminin-9) or a recombinant protein of LRE segment [63]. This indicates that the binding site for laminin in P/Q-type VGCCs is contained in the 11th extracellular loop of the α subunit. In contrast, recombinant protein containing the 11th extracellular loop of L-type VGCCs did not block this binding [63]. Consistently, injecting mice with the recombinant protein of P/Q-type VGCC's 11th extracellular loop to block the binding of laminin β2 with P/Q-type VGCCs results in a greater than 50% decrease in active zone number without altering postsynaptic AChR clustering [63]. Similarly, LEMS patients develop autoantibodies against P/Q-type VGCCs and cause internalization of P/Q-type VGCCs into nerve terminals [92–94]. Interestingly, some LEMS patients develop autoantibodies specifically against the laminin β2-binding domain on P/Q-type VGCCs, which may disrupt the binding of these molecules [95]. This loss of interaction between laminin β2 and VGCCs may lead to a reduced number of presynaptic active zones and muscle weakness [63, 96]. Consistently, mice injected with LEMS patient IgGs display reduced active zone number similar to patients [97]. Thus, VGCCs and their interaction with laminin β2 play an essential role in organizing the presynaptic active zones.

Dystroglycan

Dystroglycan is a laminin receptor that anchors the basement membrane laminin chains to the intracellular cytoskeleton of skeletal muscles by binding with dystrophin/utrophin/syntrophins/dystrobrevin [18, 98–104]. Dystroglycan is a component of the dystrophin-glycoprotein complex and is composed of α- and β-dystroglycan subunits encoded by a single gene [18]. α-dystroglycan functions as an extracellular receptor that binds to laminins but it also binds to perlecan, agrin, and neurexin. β-dystroglycan subunit is a transmembrane protein that links the extracellular α-dystroglycan to the cytoskeletal protein dystrophin [18]. Although dystroglycan is expressed throughout the sarcolemma, this receptor has higher concentration at NMJs [105]. Laminins (α4 and α5 chains) appear to be responsible for accumulating dystroglycan receptors to the post-synaptic membrane and this relationship is important for the maturation of AChR clusters [20, 106, 107]. Loss of dystroglycan in mice is embryonically lethal [108], and embryonic myotubes from dystroglycan null mice have poor AChR clustering and NMJ formation [109–111]. Arrested maturation of AChR cluster was confirmed in postnatal skeletal muscles of muscle-specific dystroglycan knockout mice [20]. As described earlier, mice lacking both laminin α4 and a muscle-specific knockout of laminin α5 (laminin α4/α5 knockout mice mentioned previously) also have arrested maturation of AChR clustering and reduced dystroglycan content at NMJs [20]. Thus, the

relationship between laminin α4/α5 and dystroglycan is important for appropriate endplate development.

Integrins

Integrins are a well-characterized family of laminin receptors. Cooperation between laminin and integrins help to link the interior cytoskeleton of muscle cells to the extracellular laminin molecules of the basal lamina [14]. These transmembrane proteins mediate cell-to-cell and cell-to-matrix interactions, and these receptors also possess the ability to activate intracellular signaling pathways [14]. Integrins form heterodimers consisting of integrin α and β chains, each consisting of a number of different variants. Six members of the β1 family of integrins (α1β1, α2β1, α3β1, α6β1, α7β1, and α9β1) and three α_v family members ($\alpha_v\beta3$, $\alpha_v\beta5$, $\alpha_v\beta8$), as well as α6β4, have been shown to interact with different laminin chains [14, 39, 112–117]. Laminin α1, α2, α3, and α5 chains have integrin binding sites located mainly in the C-terminal LG domain, although binding sites in the “short arm” N-terminus have been shown for integrin α1β1 and α2β1 [116–119].

Integrin α7 is enriched at NMJs and remain present at NMJs following denervation [13]. Through alternative splicing, integrin α7 is cleaved into three cytoplasmic domain variants (α7A, α7B, and α7C) [120–122]. Variants of integrin α7 are expressed differentially during development with the integrin α7A variant appearing immediately postnatally at NMJs, while the integrin α7B variant does not concentrate at NMJs until 2 weeks postnatally [13]. The integrin α7C variant is expressed both at NMJs and extrasynaptically [13]. In humans, genetic mutations of the gene encoding integrin α7 result in myopathy [123]. In mice, knockout of integrin α7 result in a loss of postsynaptic junctional folds, detachment of the myotendinous junction through loss of binding with laminin-211 (laminin-4), and myopathies similar to muscular dystrophy [124–126]. Dimers of integrin α7 and integrin β1 are predominately expressed in skeletal muscle [121, 127]. Both integrin α7β1 and dystroglycan are required for polymerization of laminins on the cell surface of cultured myotubes and these two receptors link actin filaments of the skeletal muscle fiber cytoskeleton to extracellular laminins [14, 71, 128]. Dystroglycan links to actin by binding to a complex of dystrophin/utrophin/syntrophins while integrin α7β1 links to actin by binding to a complex of talin and vinculin/metavinculin, and these two structures are linked to one another extracellularly by laminin [14]. Thus, cooperation between integrin α7β1 and dystroglycan link the cytoskeleton to laminins in the basal lamina in skeletal muscle, anchoring interior components of the muscle fiber with the basement membrane.

Other integrin subunits are important for muscle and active zone formation. Integrin β1 regulates skeletal muscle fiber development and is essential for myotube fusion and assembly of sarcomeres [129]. Furthermore, integrin β1 in skeletal muscle, but not in motor neurons, is required for innervation of NMJs [130]. Similar to integrin α7, integrin β1 is spliced and the integrin β1D variant appears to be the dominant integrin variant expressed in adult skeletal muscle [131, 132]. Integrin α3β1 has also emerged as important laminin receptor for active zone organization aiding laminin α4 in this process. Both integrins α3 and β1 are located at NMJs and expressed in motor neurons [13]. Integrin α3 is found at nerve terminal of *Torpedo californica* electric organs and concentrates near active zones of

nerve terminals in *Xenopus laevis* [133–135]. Laminin molecules containing laminin α4 chains bind with integrin α3, α6, and α7 chains, which complex with integrin β1 chains [136–138]. Integrin α3β1 dimers have been reported to complex with laminin 421 (laminin-9), active zone proteins Bassoon and Piccolo, and VGCCs in *Torpedo californica* electric organ synapses, which is a modified neuromuscular synapse [135, 139]. Thus, it has been hypothesized that this interaction between laminin α4 and integrin α3β1 is important for laminin α4's role as an active zone organizer in Torpedo electric organ synapses [135]. In summary, interactions between laminin receptors such as VGCCs, dystroglycan, and integrins with specific laminin chains are important for multiple aspects of NMJ development and maturation, especially development of presynaptic active zones.

Diseases associated with the loss of laminins or laminin-receptor interactions

Disorders that alter the NMJ organization and muscle function have been associated with the loss of specific laminin chains or disruption of the interaction between laminins and laminin receptors. In humans, laminin β2 gene (*lamb2*) has been localized on chromosome 3p21 and consists of 32 exons spanning 12 kb of genomic DNA making a protein consisting of 1,798 amino acids [140]. Recently, 49 different mutations in *lamb2* gene have been described resulting in recessive genetic disorders of varying degrees of severity including Pierson syndrome [140]. Pierson syndrome gives rise to a disruption of NMJs, ocular anomalies (i.e., microcoria, abnormal lens shape, and retinal abnormalities), and severe congenital nephrotic syndrome [140–144]. Pierson Syndrome patients usually pass away shortly after birth due to kidney disease. Individuals that do survive for longer can display developmental and neurological defects, including disrupted function and organization of NMJs [140, 142, 143, 145]. A patient suffering from a congenital myasthenic syndrome with a mutation of *lamb2* showed reduced mEPP amplitude, decay, and frequency, reduced quantal content, reduced endplate area, reduced number of active zones, reduced number of synaptic vesicles, and Schwann cell infiltration of NMJs [144]. The symptoms of Pierson syndrome patients are consistent with what is observed in laminin β2 knockout mice.

Loss of laminin α2 results in a muscular dystrophy-like syndrome. In humans, recessive familial disruption of the gene encoding laminin α2 protein (*lama2*), located on chromosome 6q22-q23, results in merosin-deficient congenital muscular dystrophy type 1A (MDC1A) [66, 146]. At birth or shortly thereafter, patients are hypotonic becoming non-ambulatory, require respiratory assistance, and pass away in the first decade of life or soon after in severe cases. Cerebral white matter abnormalities have been found by magnetic resonance imaging (MRI) in many cases, and seizures and mental retardation have been reported in a small portion of cases [66]. A number of different mutations have been observed resulting in a complete or partial loss of laminin α2 protein [37, 66, 72–75, 146–150]. Some patients have only a partial decrease in laminin α2 protein levels, and these patients have better prognosis becoming ambulatory with longer life expectancy although a progressive muscle weakness may persist [72, 149]. The human form of MDC1A mimics animal models where reduced levels of laminin α2 protein results in muscular dystrophy. In these animal models, severity of reductions in laminin α2 protein levels correlates with the

severity of phenotype [66, 67]. As mentioned previously, these mice have reduced junctional folds, detachment of motor neuron from the endplate, demyelination, and infiltration of Schwann cells into the synaptic cleft.

LEMS is a form of myasthenia resulting from autoimmune attack of VGCCs leading to neuromuscular failure [92–97, 151]. These patients have muscle weakness and fatigue, reduced synaptic transmission, and reduced active zone number. Over half of the patients have small cell lung carcinoma and the treatment of cancer reduces auto-antibodies [152–155], which can be the source of the VGCC antigens [156, 157]. The auto-antibodies produced against P/Q-type VGCCs result in internalization of VGCCs into nerve terminals, and potentially disrupt binding between laminin β2 and VGCCs, both of which leads to a loss of presynaptic active zones and muscle weakness [96]. The most common treatment for LEMS patients is the use of 3,4-diaminopyridine (3,4-DAP), a potassium channel blocker that prolongs the length of action potentials, leading to modest improvements in symptomology [158]. Recently, combining 3,4-DAP with a novel calcium channel agonist (GV-58) has led to a reversal of symptomology in a mouse model of LEMS [159, 160]. GV-58 acts specifically towards N- and P/Q-type VGCCs, not L-type VGCCs, increasing calcium influx during depolarization [159]. Use of this pharmacological combination restores neurotransmitter release in a LEMS mouse model [160]. These findings highlight the importance of VGCCs in the organization of presynaptic active zones, but also introduce this interaction as a favorable treatment target in some neuromuscular disorders.

Laminins, aging, and exercise

Aging skeletal muscle undergoes a progressive loss of muscle mass (sarcopenia) and strength leading to decreased quality of life and increased risk of falling, fractures, and mortality [161–163]. During aging, the pre- and post-synaptic NMJ morphology becomes disrupted. On the presynaptic side, motor neuron terminals atrophy during aging compared to young humans and rodents although the number of nerve terminal branches increases [164–170]. In addition, there is altered synaptic transmission, increased quantal release, decreased synaptic vesicle number, and a decrease in the number of active zones and content of active zone proteins [164, 171–175]. On the post-synaptic side, the endplate area expands despite AChR cluster area becoming fragmented ultimately leading to a progressive denervation of the NMJ [164, 166, 170, 172, 176–185]. These findings have been complicated by the examination of different muscle fiber types as fast and slow muscle fibers display differences in the influence of aging on NMJ morphology. Fast muscle fibers (i.e., extensor digitorum longus, EDL, muscle) typically show age-related changes in morphology prior to slow muscle fibers (i.e., soleus, diaphragm muscles) [179, 181, 186, 187]. Denervation during aging may be different between muscle fiber types as one report showed that denervation was greater in 29 month old mice in fast-twitch muscle fibers (i.e., EDL muscle) compared to slow twitch muscle fibers (i.e., soleus) [181]; however, another report shows that denervation differences between muscle fiber types was not different in two year old mice [188]. Thus advanced aged beyond two years may be necessary to compare denervation patterns between muscle types.

The loss of NMJ innervation precedes the degradation of spinal motor neurons [180, 181], which suggest a dying back neuropathy or distal axonopathy. In mice, partial denervation within individual NMJs begins to occur at approximately 18 months of age, and becomes more pronounced by 24 months of age and older [180]. In addition, we have observed that aging results in a reduced number of presynaptic active zones and a reduced level of Bassoon protein at presynaptic terminals [175, 189]. The loss of Bassoon will impair VGCC functionality and depress nerve transmission potentially leading to a loss of muscle strength or denervation [189]. One study has shown that protein levels of laminin α4, α5, and β2 chains are not reduced in NMJs during aging; however, the pattern of laminin localization in NMJs changes in parallel to changes of NMJ morphology [87]. This opens the possibility of changes in localization of specific laminin chains are partly causative of NMJ degeneration during aging.

Exercise has been shown to positively influence NMJs in aging muscle [168, 180, 183–185, 190–193]. Voluntary wheel running and treadmill running in aged mice and rats has been shown to reduce aging induced post-synaptic AChR fragmentation and help maintain NMJ innervation [180, 184, 193]. Even just one month of voluntary wheel running in 22-month-old mice reduces denervation of aged NMJs [180]. In rodents, endplate area expands and AChR clusters become fragmented during aging. However, forced treadmill running reduces post-synaptic endplate area and decreases fragmentation of AChR clusters in aged animals [168, 183, 191, 194]. Importantly, resistance training has also been shown to positively influence NMJ morphology and reduce AChR fragmentation in both adult and aged humans and rodents [185, 195].

Exercise has also been shown to modulate presynaptic active zone protein content. Active zone protein levels in NMJs are reduced during aging, but resistance training of 24-month old rats restores Bassoon to young adult levels [189]. No other reports, to our knowledge, have investigated active zone proteins at the NMJ with exercise training. More work is needed to elucidate the molecular mechanisms by which exercise modulates active zones and active zone proteins during aging. It is possible that exercise stimulates expression of activity dependent genes, synaptic laminin expression, and laminin-receptor interactions that in turn positively influence pre- and post-synaptic gene expression level, NMJ morphology, and innervation. Exercise activates AMPK by phosphorylating AMPKα (Thr172) in muscles [196–199] and directly phosphorylates and activates peroxisome-proliferator-activated receptor gamma co-activator 1α (PGC-1α) [200]. PGC-1α enhances transcription of NMJ genes including laminin β2 in C2C12 muscle cells [201]. However, laminin β2 mRNA expression did not increase *in vivo* in gastrocnemius muscle from transgenic mice overexpressing PGC-1α [201]. In a single human study, four hours after a single bout of resistance exercise laminin β2 gene expression was increased in subjects that previously performed 12 weeks of resistance training [202]. Elsewhere, Ogasawara *et al.* showed that resistance exercise increases integrin β1 levels without changing laminin α2 or integrin α7, however other laminin chains were not examined [203]. Thus, upstream regulators that govern the response to exercise may have a direct influence over synaptic laminins and NMJ organizers.

Conclusion

Synapse specific laminin chains and their interaction with laminin receptors are essential for NMJ formation and organization of pre- and post-synaptic structures. Loss of specific laminin chains or laminin receptors, as well as blocking the interaction between laminins and laminin receptors results in changes in NMJ innervation, morphology, and organization of pre- and post-synaptic structures including the active zones and junctional folds. Increasing levels of laminins, especially laminin β2, or increasing the signaling pathways stimulated by synaptic laminins are a viable molecular target to improve neuromuscular disease outcomes.

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Abbreviations

3,4-DAP	3,4-diaminopyridine
ACh	acetylcholine
AChR	acetylcholine receptor
AMPK	adenosine monophosphate-activated protein kinase
Bcam	basal cell adhesion molecule/Lutheran blood group antigen
CAZ	cytomatrix of the active zone
CAST	CAZ-associated structural protein
EHS	Engelbreth-Holm-Swarm
EDL	extensor digitorum longus
LEMS	Lambert-Eaton myasthenic syndrome
LRE	leucine-arginine-glutamic acid
MDC1A	segment, merosin deficient congenital muscular dystrophy type 1A
mEPP	miniature end plate potential
NMJ	neuromuscular junction
RIM	Rab3-interacting molecule
SNAP-25	synaptosomal associated protein of 25 kDa
VGCC	voltage-gated calcium channel

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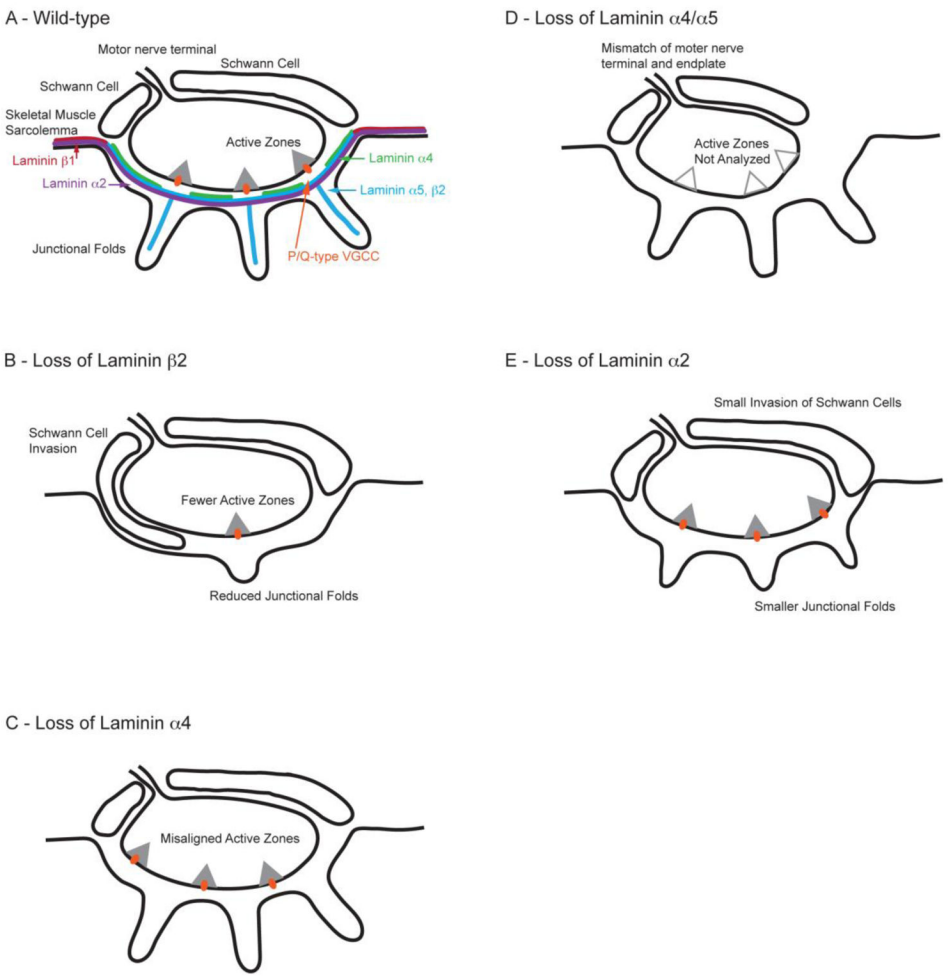

Figure 1.

The role of laminins at the NMJ. Laminin chains have specific roles in organizing the presynaptic active zones and endplate morphology. **(A)** Represents an NMJ of adult wild-type mouse. **(B)** Represents an NMJ of laminin β2 knockout mouse. There is a loss of active zones, reduced number of synaptic vesicles and junctional folds, and infiltration of the synaptic cleft by Schwann cells. **(C)** Represents an NMJ of laminin α4 knockout mouse. There is a misalignment between active zones and junctional folds. **(D)** Represents an NMJ of laminin α4 and α5 double knockout mouse where the nerve terminal size is small. **(E)** Represents an NMJ laminin α2 knockout mouse where there is a reduced number of junctional folds and moderate infiltration of synaptic cleft by Schwann cells.

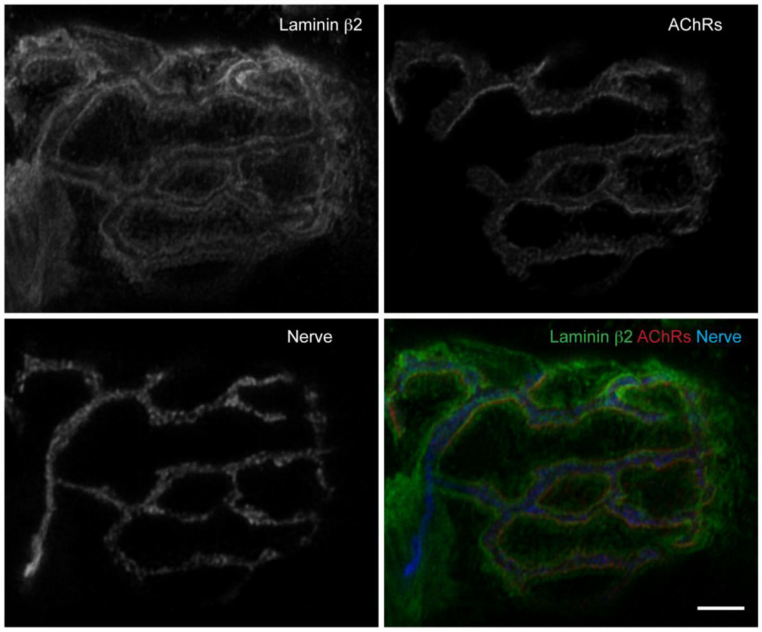

Figure 2.

Laminin β2 localization at an NMJ. Immunohistochemistry detection of laminin β2 in an NMJ of an adult (4 month old) wild-type C57Bl/6 mouse. Longitudinal sections of gastrocnemius muscle were stained with primary antibodies against laminin β2 (green), synaptic vesicle protein-2 (SV-2) and neurofilament to visualize the nerve terminal (Nerve, blue), and Alexa Fluor 594-labeled α-bungarotoxin to visualize acetylcholine receptors (AChRs, red). An NMJ was imaged on a confocal microscope and deconvolved. Scale bar represents 5 μm.

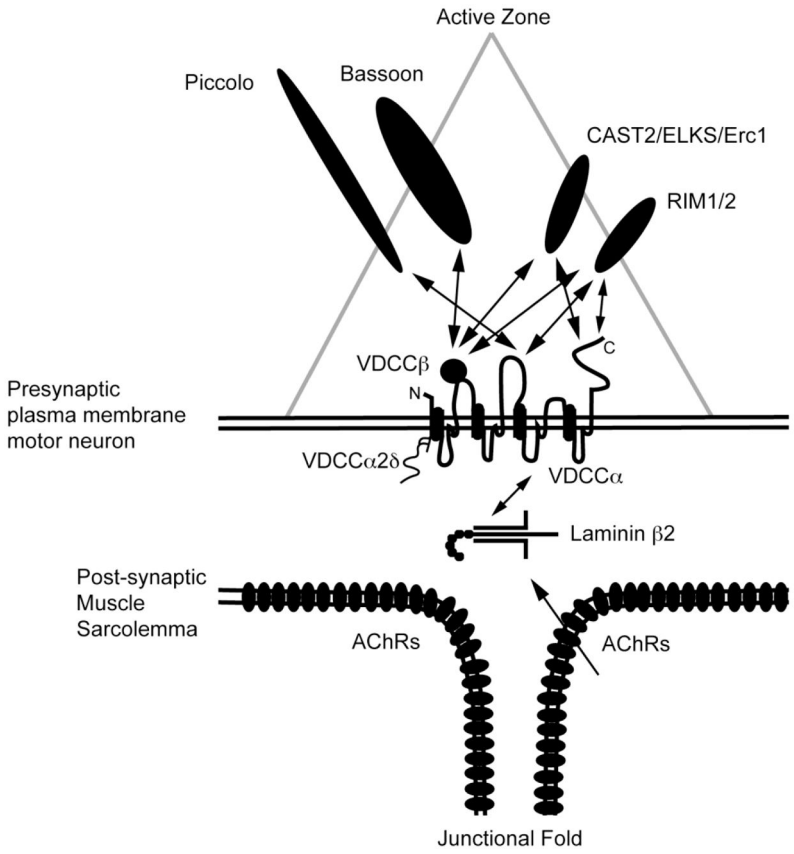

Figure 3.

Schematic of active zone organization in mammalian neuromuscular junctions. Laminin β2 is a muscle derived active zone organizer that binds with presynaptic voltage-gated calcium channels (VGCC). Active zone proteins, Bassoon, CAST2/ELKS/Erc1, Piccolo, and Rim1/2, bind with VGCCs at the cytosolic domains and subunits. This link of laminin β2 of the basal lamina with active zone proteins organizes the active zones.

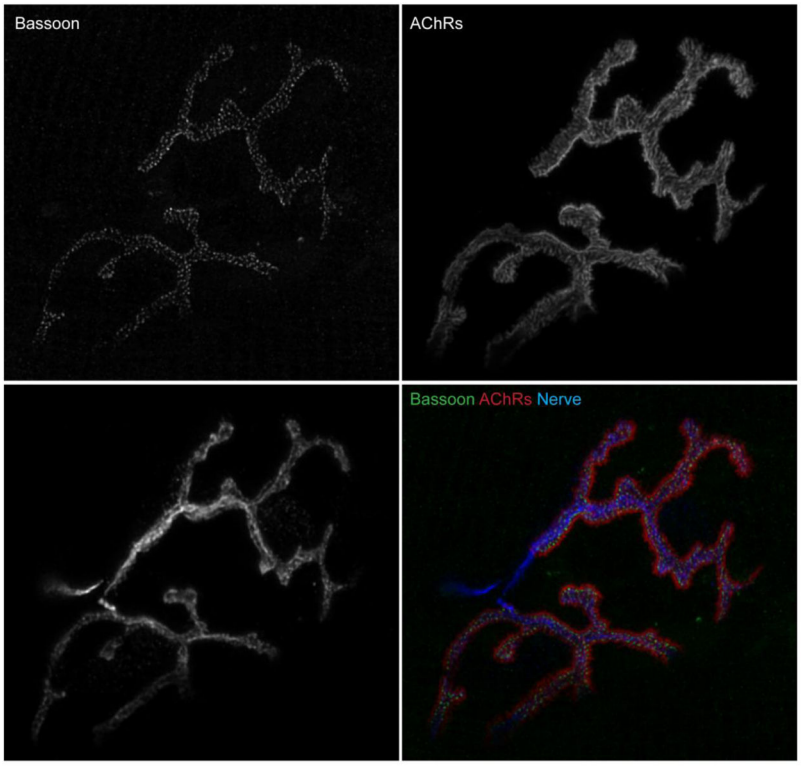

Figure 4.

Bassoon localization at an NMJ. Immunohistochemistry detection of active zone specific protein Bassoon in an NMJ of an adult (4 month old) wild-type C57Bl/6 mouse. Longitudinal sections of gastrocnemius muscle were stained with primary antibodies against Bassoon (green), SV-2 and neurofilament to visualize nerve terminal (Nerve, blue), and Alexa Fluor 594-labeled α-bungarotoxin to visualize acetylcholine receptors (AChRs, red). An NMJ was imaged on a confocal microscope and deconvolved. Scale bar represents 5 μm.