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Neuromuscular Junction Formation, Aging, and Disorders

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Abstract

Synapses, the fundamental unit in neuronal circuits, are critical for learning and memory, perception, thinking, and reaction. The neuromuscular junction (NMJ) is a synapse formed between motoneurons and skeletal muscle fibers that is covered by Schwann cells (SCs). It is essential for controlling muscle contraction. NMJ formation requires intimate interactions among motoneurons, muscles, and SCs. Deficits in NMJ formation and maintenance cause neuromuscular disorders, including congenital myasthenic syndrome and myasthenia gravis. NMJ decline occurs in aged animals and may appear before clinical presentation of motoneuron disorders such as amyotrophic lateral sclerosis. We review recent findings in NMJ formation, maintenance, neuromuscular disorders, and aging of the NMJ, focusing on communications among motoneurons, muscles and SCs, and underlying mechanisms.

INTRODUCTION

Synapses are fundamental units of neural circuitry in the nervous system that enable communications between neurons and between a neuron and its target cell. Synaptic transmission and plasticity enable brain functions, including sensation, cognition, memory, and motor control. The neuromuscular junction (NMJ) is a chemical synapse that is formed between motoneurons and skeletal muscles and is covered by Schwann cells (SCs). Upon the arrival of action potentials, the motoneuron terminals release acetylcholine (ACh), which activates ACh receptors (AChRs) of muscle fibers to depolarize the muscle cell and trigger calcium release from the sarcoplastic reticulum to initiate muscle contraction. The NMJ is thus essential for our physical mobility and daily life. Deficits in NMJ formation and maintenance cause neurological disorders, including congenital myasthenic syndrome (CMS) and myasthenia gravis (MG). Studies of the NMJ have also contributed to our understanding of the structure and function of synapses in the brain.

NMJ formation involves the differentiation of presynaptic nerve terminals, postsynaptic muscle membranes, and terminal Schwann cells (tSCs). Prior to the arrival of motor nerve terminals, muscle fibers form primitive, small, or thin AChR clusters that are distributed in the central region; this phenomenon is called muscle prepatterning (1) (**Figure 1*a***, **E11–E12**). As the nerve terminals innervate muscle fibers, they induce new clusters and disperse those in nonsynaptic areas;

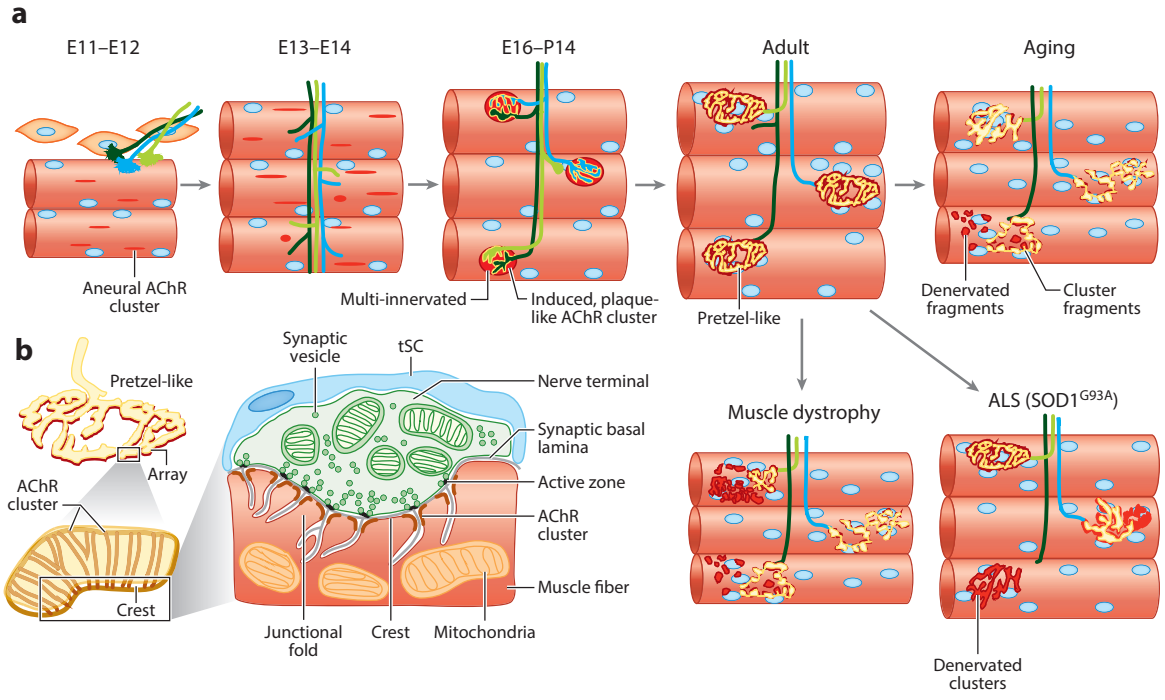

Figure 1

NMJ development in mice. (*a*) Prior to the arrival of nerve terminals, myotubes form primitive, small, thin AChR clusters that are distributed in a broad middle region (axons E11–E12; E13–E14). Nerve-induced clusters are initially oval plaques, often innervated by multiple axons (E16–P14). As NMJs mature, AChR clusters become perforated and complex, resembling pretzels with arrays or branches that are innervated by one axon per NMJ (adult). AChR clusters become fragmented and denervated in aged mice and in muscular dystrophic mice. In SOD1^{G93A} mice, some AChR clusters are denervated. (*b*) NMJ structures at different magnifications. Abbreviations: AChR, acetylcholine receptor; ALS, amyotrophic lateral sclerosis; NMJ, neuromuscular junction; tSC, terminal Schwann cell.

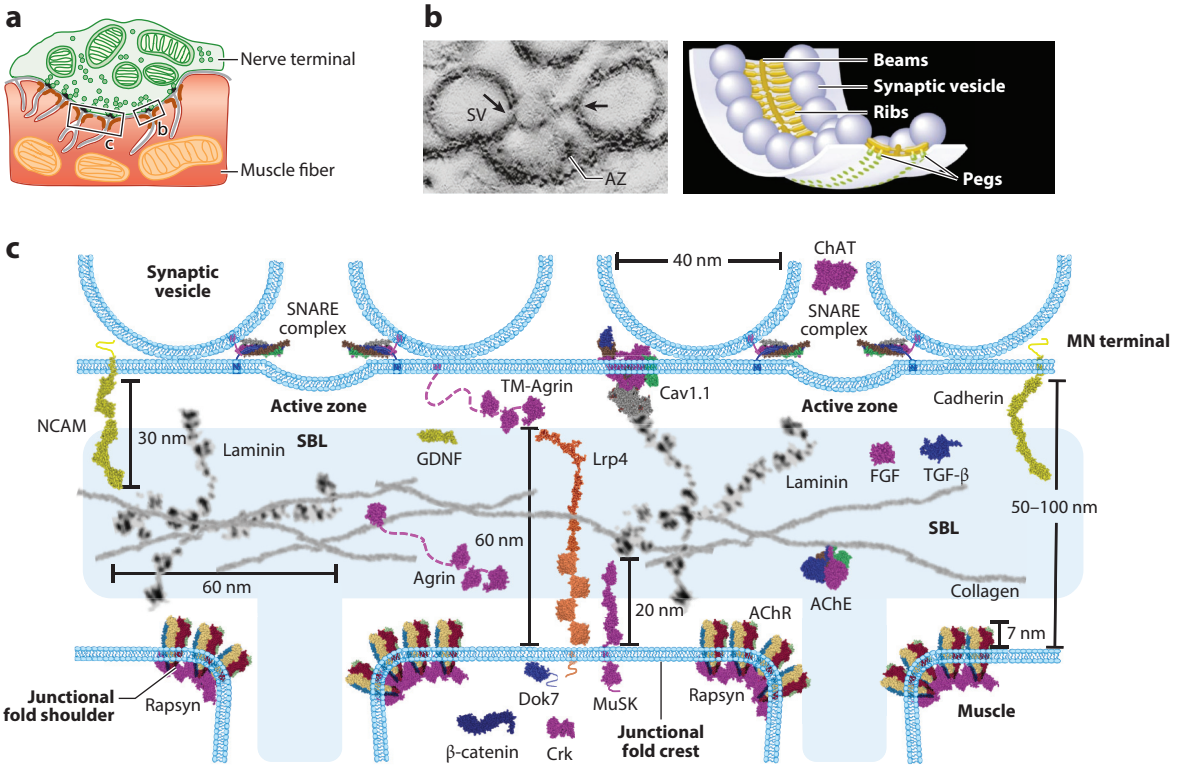

Figure 2

Structures and key molecules of the NMJ. (*a*) A synapse between motor nerve terminal and muscle fiber. Boxed regions in panel *a* are enlarged as panels *b*,*c*. (*b*) Electronic microscopic images of frog NMJ. (*Left*) Tomographic virtual slice taken from a series of virtual slices in a reconstructed three-dimensional volume. Arrows indicate the attachment site of vesicles to active zone components. (*Right*) Composite diagram of vesicles and active zone components. Panel reproduced with permission from Reference 164. Copyright 2001, Nature Publishing Group. (*c*) Key structural and signaling molecules at the NMJ. Shown are synaptic vesicles, synaptic cleft, and most molecules in real scale (structural images obtained from the Protein Data Bank). Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; AZ, active zone; ChAT, choline acetyltransferase; Crk, CT10 regulator of kinase; FGF, fibroblast growth factor; GDNF, glial cell line–derived neurotrophic factor; Lrp4, low-density lipoprotein receptor–related protein; MN, motoneuron; MuSK, muscle-specific kinase; NCAM, neural cell adhesion molecule; NMJ, neuromuscular junction; SBL, synaptic basal lamina; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; SV, synaptic vessel; TGF-β, transforming growth factor-beta; TM, transmembrane.

consequently, NMJs are formed in the middle region of muscle fibers. Initially, AChR clusters appear like oval plaques that are often innervated by more than one axon (E16–P14). As the NMJs mature, the plaques become perforated and eventually appear as pretzel-shaped arrays or branches of AChR (**Figure 1*a***, **adult**; **Figure 1*b***). Branches are filled with junctional folds that are usually perpendicular to the long axis of the arrays, and AChRs are concentrated at the shoulder areas of junctional fold crests (165), at an estimated concentration of 10,000/μm^2. Facing the AChR clusters are active zones of the presynaptic membrane (**Figures 1** and **2**). The NMJ is covered by tSCs that are necessary for its formation, maintenance, and regeneration. As NMJs age, AChR clusters break into fragments that could be poorly innervated and spread in a larger area than that of the original pretzel. Many disorders affecting motoneurons, muscles, and SCs are associated with NMJ decline (**Figure 1*a***, **aging**).

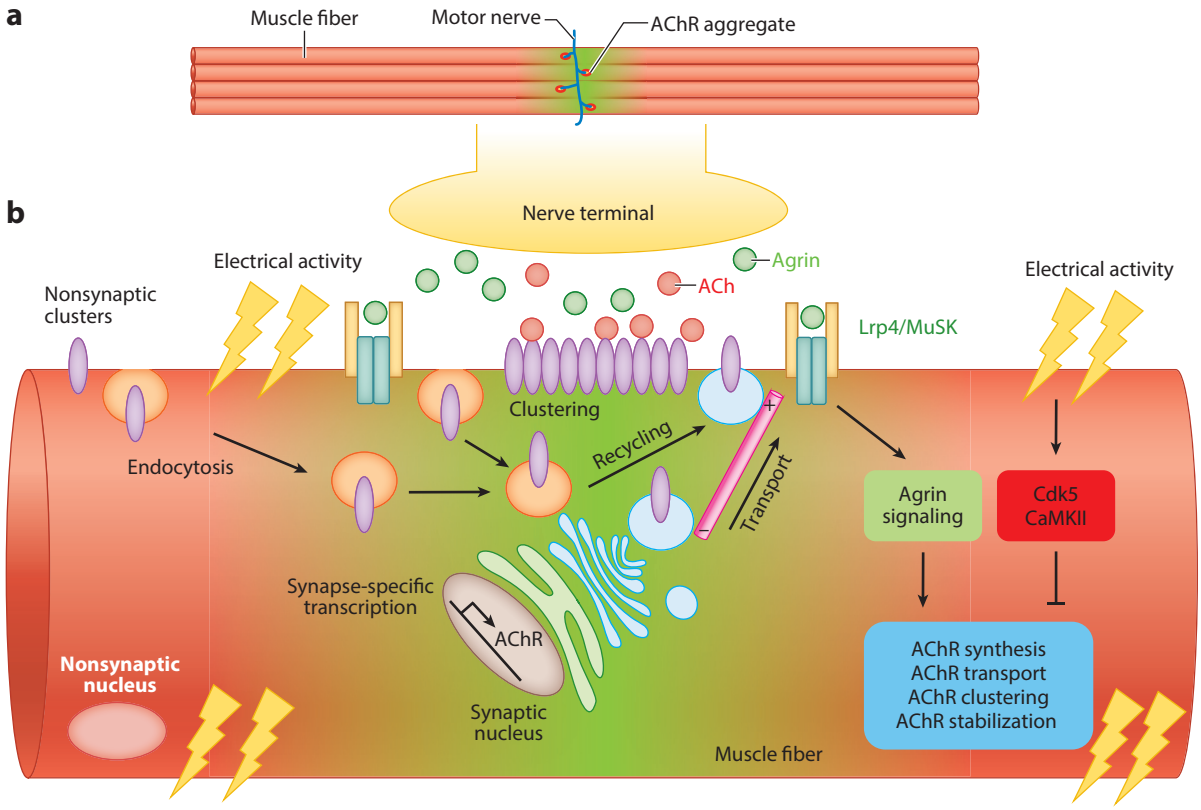

Figure 3

Mechanisms contributing to high AChR concentration at the NMJ. (*a*) A diagram of NMJs versus muscle fibers. An NMJ occupies <0.1% of the surface of a muscle fiber. Positive signaling for NMJ formation and maintenance is restricted at the NMJ (*green*). (*b*) Coordination of positive and negative signals in AChR concentration. Shown is the middle region of a single muscle fiber in panel *a*. Electrical activity induced by ACh, as a negative signal (*red*), suppresses AChR synthesis, transport to the cell membrane, clustering or anchoring, and stability in entire muscle fibers. These effects are counteracted by the positive signaling (*green*) of agrin, which is produced by nerve terminals and deposited in synaptic basal lamina. Abbreviation: AChR, acetylcholine receptor; CaMKII, calcium/calmodulin–dependent protein kinase II; Cdk5, cyclin-dependent kinase 5; Lrp4, low-density lipoprotein receptor–related protein; MuSK, muscle-specific kinase; NMJ, neuromuscular junction.

NMJ development requires extensive communication among the three components of the tripartite synapse: presynaptic motoneurons, postsynaptic muscle fibers, and SCs. In fact, the synaptic cleft is filled with synaptic basal lamina that is enriched with many proteins from these cells for NMJ development and maintenance (**Figure 2**). NMJs occupy less than 0.01–0.1% of the entire muscle surface (**Figure 3**). How this small area of muscle membrane becomes differentiated to possess a high concentration of AChRs, a hallmark of the NMJ, has riveted neuroscientists over many generations. Increasing evidence suggests the following working model. Nerve terminals secrete positive factors such as agrin to concentrate AChRs at the NMJ by promoting the transcription of genes of AChR subunits and other proteins for NMJ structure and function in synaptic nuclei, AChR transport to the postjunctional membrane, AChR clustering, or anchoring and AChR stability. They also release ACh, a negative signal that suppresses these machineries, to eliminate supernumerary AChR clusters from extrasynaptic regions (**Figure 3**). The past decade

has witnessed significant progress in understanding underlying mechanisms, as summarized in outstanding reviews (1–5). Here, we provide an update on NMJ assembly and a review of recent findings on NMJ maintenance, neuromuscular disorders, and NMJ aging, with a focus on communication among motoneurons, muscles, and SCs.

AGRIN-Lrp4-MuSK SIGNALING

Agrin was identified as an activity that induces AChR clusters in cultured myotubes from the electric organ of *Torpedo californica* in a heroic effort to search for proteins involved in NMJ formation (6). It is expressed in two splice variants at the N-terminus and several at the C-terminus with nine follistatin-like repeats, and two laminin-B-like modules, followed by three laminin globular (LG) domains. Splicing at the N-terminus generates two isoforms: The short isoform contains a transmembrane domain and is mainly expressed in the brain, whereas the long isoform, expressed in motoneurons, muscles, and other tissues, is secreted (7) (**Figure 2*c***). Splicing also occurs at three sites (X, Y, and Z sites in rodents) in the C-terminus with inserts of 3/12, 4, and 8/11/19 amino acids, respectively. The Z8 agrin is expressed only by motoneurons (thus named neural agrin), but not muscles, and is ~5,000-fold more active than muscle agrin in inducing AChR clusters. In fact, the laminin-like globular 3 (LG3) domain containing the Z8 insert is sufficient to trigger muscle-specific kinase (MuSK) activation and AChR clustering (7). Noticeably, LG3 and LG2/LG3 are monomeric, unlike many known ligands for receptor tyrosine kinases (8). The function of other domains remains unclear, but they are thought to concentrate agrin at the synaptic basal lamina. Agrin-null mice or motoneuron-specific knockout mice are unable to form the NMJ (1–5).

Lrp4 as Agrin Receptor

Agrin acts by binding to its receptor Lrp4 [low-density lipoprotein (LDL) receptor–related protein 4], a member of the LDL receptor family, in muscle cells to activate MuSK (9, 10). Both Lrp4 and MuSK are critically important for aneural as well as agrin-induced AChR clustering and NMJ formation (1–5). Lrp4 is a single-transmembrane protein with no identifiable enzymatic activity. It has an enormous extracellular domain (ECD) that contains 1,725 of its 1,905 residues in mice and humans, suggesting that it functions mainly as a receptor. Its intracellular domain (ICD) has a PDZ-interacting C-terminus and a characteristic NPXY motif (11) and becomes tyrosine phosphorylated by MuSK (9), but it is apparently dispensable in NMJ formation (12). Whether the ICD-dependent signaling is required for NMJ maintenance during aging remains unclear (see below). The ECD contains eight LDLa (LDL class A) repeats that are followed by two epidermal growth factor (EGF)-like modules and four YWTD-motif–containing β-propeller domains that are separated by an EGF-like module. The first β-propeller (β1) domain is necessary and sufficient to interact with agrin (8).

The Agrin-Lrp4 Tetrameric Complex Activates MuSK

Recent analysis of the crystal structure of the Z8-containing LG3-β1 propeller complex has revealed how agrin interacts with Lrp4 to activate MuSK (13). The agrin LG3 domain adopts a slightly curved β-sandwich structure, and the Z8 insert forms a long loop that engages Lrp4-β1. The six-bladed Lrp4-β1 domain has two concave surfaces perpendicular to the central pseudosixfold axis: One is covered by the two EGF modules flanking the β-propeller domain, whereas the opposite, positively charged surface is involved in binding to the negatively charged agrin Z8 loop (**Figure 4*a***). Via this Z8 interface, agrin and Lrp4 form a necessary binary complex, which subsequently dimerizes to form an agrin-Lrp4 tetramer, a reaction that requires two additional, albeit weak interfaces: the agrin-Lrp4 dimer interface and agrin-agrin dimer interface

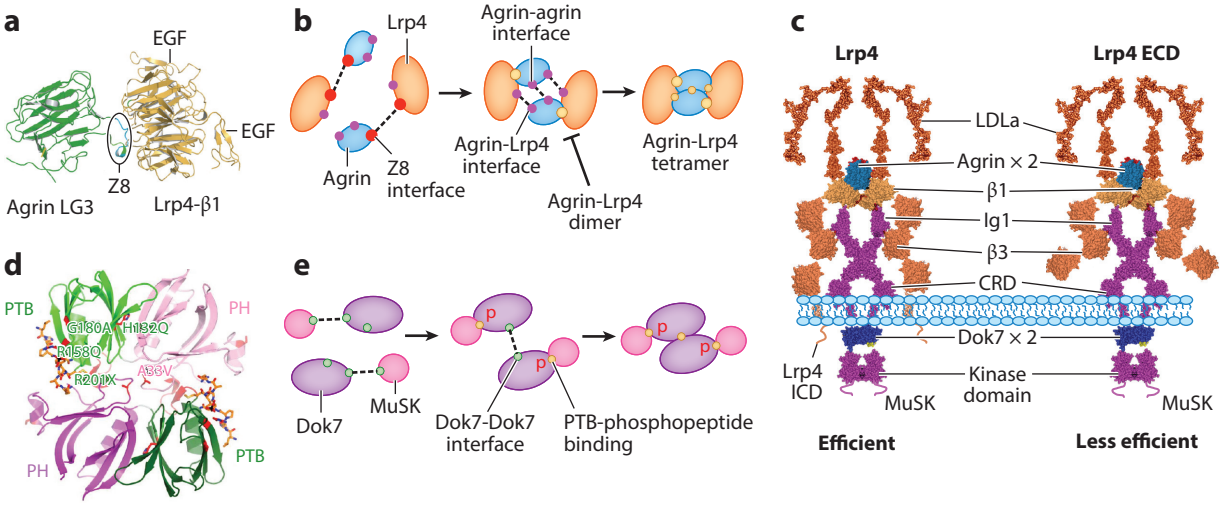

Figure 4

Molecular mechanisms of MuSK activation. (*a*) Z8-dependent agrin-Lrp4 heterodimeric complex. Panel reproduced with permission from Reference 13. Copyright 2012, Cold Spring Harbor Laboratory Press. (*b*) A tetrameric complex of two agrin-Lrp4 heterodimers is necessary for MuSK activation by agrin. (*c*) Activation of MuSK by full-length Lrp4 and its ECD. Full-length Lrp4 induces activation of MuSK more efficiently than its ECD (that lacks the transmembrane domain and ICD). (*d*) A Dok-MuSK heterodimeric complex. MuSK pY553 phosphopeptides (shown in stick representation) are associated with PTB domains of two molecules of Dok7. Panel reproduced with permission from Reference 23. Copyright 2010, Elsevier. (*e*) A tetrameric complex of MuSK and Dok7 is necessary for MuSK activation. Abbreviations: CRD, cysteine-rich domain; Dok7, downstream of tyrosine kinase 7; ECD, extracellular domain; EGF, epidermal growth factor; ICD, intracellular domain; Ig1, immunoglobulin 1; LDLa, low-density lipoprotein receptor class A repeat; LG3, laminin-like globular 3; Lrp4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; PH, pleckstrin homology; PTB, phosphotyrosine-binding.

(**Figure 4*b***). The Z8 loop is completely disordered in the absence of Lrp4 (14) but has a well-defined structure when bound to Lrp4, indicating an induced-fit recognition between agrin and Lrp4. Together with earlier biochemical and function studies, these observations support a working model of MuSK activation by Lrp4 and agrin (**Figure 4*b***). Lrp4 binds to MuSK, in the absence of agrin, to form a heterodimer to maintain basal activity (9, 10). Agrin, via the Z8 loop, binds with Lrp4 to form the binary complex, which then reconfigures the relatively weak agrin-Lrp4 and agrin-agrin dimer interfaces to assemble the tetrameric agrin-Lrp4 complex. This supercomplex promotes activation and transphosphorylation of MuSK, leading to AChR clustering and NMJ formation.

The crystal analysis reveals insight into mechanisms of agrin signaling. For example, because the Z8 interface is absolutely required to form the agrin-Lrp4 complex, the result explains why muscle agrin, which lacks the insert, is unable to activate MuSK and stimulate AChR clustering. Agrin induction of AChR clusters is known to be enhanced by calcium (14). Interestingly, the agrin-agrin dimer interface is in close proximity to a calcium ion (Ca^{2+})-binding site in agrin. Calcium binding may change the local conformation of agrin and facilitate the agrin-agrin dimerization. The formation of the tetrameric complex in the absence of MuSK raises a philosophical question about the nature of ligand and receptor. Perhaps the agrin-Lrp4 complex is the ligand, whereas MuSK is the receptor. Indeed, the soluble complex of agrin-Lrp4 ECD is sufficient to activate MuSK, albeit less efficiently (15). Mutant mice expressing only Lrp4 ECD are able to form NMJs (although they are abnormal) (16) (**Figure 4*c***). Considering that the ECD could be released by

extracellular cleavage of Lrp4 (15), the soluble agrin-Lrp4 complex may enable transactivation of MuSK or similar kinase in cells that do not express Lrp4. Less efficient activations of MuSK and abnormal mutant mice expressing Lrp4 ECD suggest an important role of the transmembrane and ICD of Lrp4.

Many questions remain unanswered. For example, it is unclear how the tetrameric agrin-Lrp4 complex activates MuSK, which is thought to be mediated by dimerization. Agrin binding may change the conformation of Lrp4 and/or MuSK for better interaction or may form a new composite surface on the agrin-Lrp4 complex for MuSK activation. Coprecipitation data suggest that the β3 propeller and fourth and fifth LDLa repeats are required for Lrp4 to bind MuSK (17). Future structural studies of the agrin-Lrp4-MuSK complex will reveal how the agrin-Lrp4 complex activates MuSK and induces MuSK phosphorylation.

MuSK Activation by Dok7

MuSK activation requires Dok7, an adaptor-like protein that contains a pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain, and a C-terminal region enriched with tyrosine residues for potential phosphorylation (18). Dok7 is indispensable for MuSK basal activity and activation by agrin and is thus required for muscle prepatterning and NMJ formation. In addition, Dok7 overexpression is sufficient to activate MuSK (19, 20), induce NMJs in agrin mutant mice, and enable them to survive for weeks (21). These results indicate that Dok7 is an activator of the kinase (i.e., as a cytoplasmic ligand).

Dok7 binds to MuSK Tyr553 in an NPXY motif in the juxtamembrane region (18). Autophosphorylation of Tyr553 is critical for MuSK activation (22). A study of the crystal structure of Dok7 PH-PTB domains in a complex with peptides containing phosphorylated Tyr553 provided insight into how Dok7 activates MuSK (23). The PTB domain binds directly to phosphorylated Tyr553, and the PH domain facilitates this interaction by binding to plasma membranes containing phosphatidylinositol phosphates (both PIP2 and PIP3) (23, 24) (**Figure 4*d***). The PH domain also mediates Dok7 dimerization, which then dimerizes MuSK to facilitate transautophosphorylation of tyrosines in the activation loop of the kinase (23) (**Figure 4*e***). Autophosphorylation of the activation loop is necessary to release the autoinhibition and thus activate MuSK (22). This process may be modulated by MuSK-dependent serine kinase (25). How Tyr553 is initially phosphorylated remains unclear. Because mutation of the conserved catalytic lysine (K608) eliminates all tyrosine phosphorylation on MuSK (26), this seems to exclude the involvement of a heterologous kinase. Therefore, basal (agrin-independent) MuSK activity may be induced by adventitious Lrp4-MuSK and MuSK-MuSK interactions, both of which occur in the absence of agrin (9, 10).

MuSK activity is also regulated by proteins that interact with Lrp4 and the kinase itself. For example, Tid1, a member of the Hsp-40 protein family, binds the juxtamembrane region of MuSK to promote the MuSK-Dok7 interaction (27). β-amloid precursor protein (APP) interacts with Lrp4 in *cis* and in *trans* to increase agrin-induced AChR clustering (16). Anti-MuSK and anti-Lrp4 antibodies can stimulate MuSK or induce AChR clusters (28, 29). Mesoderm development candidate 2 (Mesdc2) binds to Lrp4 and facilitates its glycosylation and surface expression. MuSK can act as a BMP co-receptor to regulate muscle development independent of kinase activity (166).

INTRACELLULAR AND EXTRACELLULAR MECHANISMS

Intracellular Pathways Downstream of MuSK

The intracellular pathways downstream of MuSK are not well understood. A plethora of molecules or cascades has been implicated. Most of them appear to be modulatory, except rapsyn whose

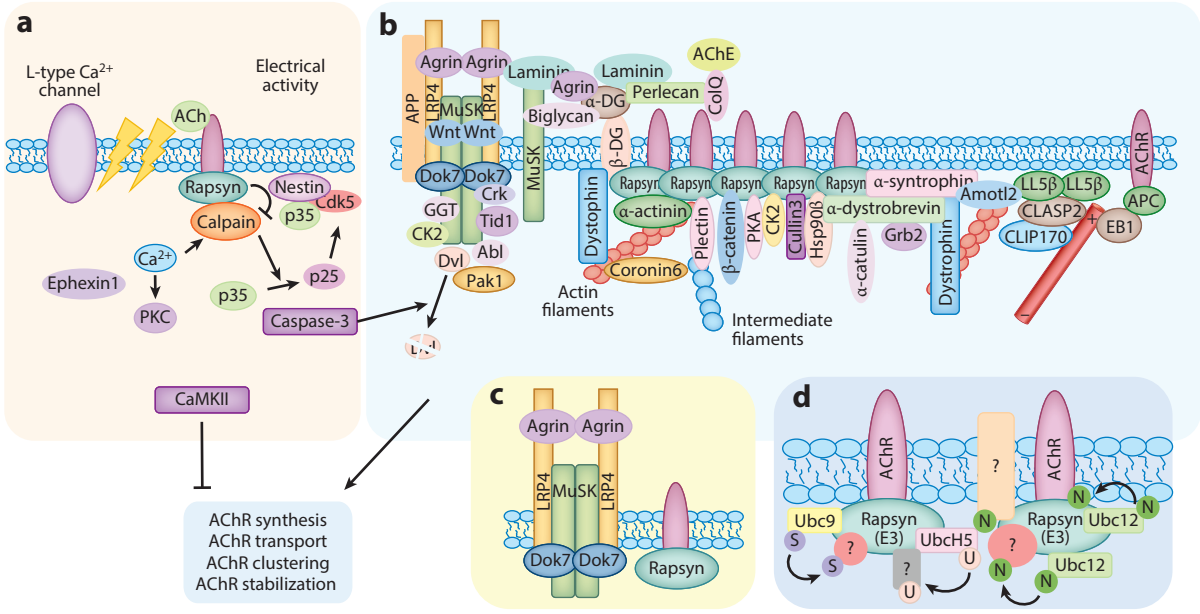

Figure 5

Intracellular pathways for AChR clustering. (*a*) Electrical activity in response to AChR activation and subsequent calcium influx inhibit AChR synthesis, transport, clustering, and stability in entire muscle fibers. This process requires Cdk5, CaMKII, and PKC and may involve ephexin1. Cdk5 is regulated by a calpain–rapsyn complex and membrane-associated nestin. ACh-activated caspase-3 can disperse AChR clusters by degrading Dvl. (*b*) Agrin binds to Lrp4 to activate MuSK, which can be activated by Dok7. Agrin signaling can be regulated by proteins interacting with Lrp4/MuSK, such as extracellular proteins APP, Wnt, laminin, and biglycan and intracellular proteins Abl, Tid1, Dvl/Pak1, GGT, CK2, and Crk (via Dok7). Rapsyn has two functions: as an adaptor protein to bridge AChRs to actin and intermediate filaments and to the dystrophin-dystroglycan complex; and as an E3 ligase whose enzymatic activity is required for AChR clustering (see panel *d*). The function and stability of rapsyn are regulated by PKA, Hsp90β cullin3, and α-syntrophin/α-dystrobrevin. LL5β and CLASP2 are required for microtubule capture to AChR clusters and for intracellular AChR trafficking. APC associates with AChR and EB1 at the microtubule plus-end to regulate AChR clustering. Many of the pathways or molecules are derived from in vitro studies and lack genetic evidence, except those depicted in panel *c* (3, 4). (*c*) Molecules critical for NMJ formation. (*d*) Rapsyn serves as an E3 ligase, whose substrates can include NMJ structure and function proteins. Modifications mediated by rapsyn may include neddylation, ubiquitination, and/or sumoylation. Question marks indicate unidentified substrate proteins of rapsyn. Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; APC, adenomatous polyposis coli; APP, amyloid beta precursor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; CK2, casein kinase 2; Crk, CT10 regulator of kinase; DG, dystroglycan; Dok7, downstream of tyrosine kinase 7; Dvl, Dishevelled; GGT, geranylgeranyltransferase; Lrp4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; N, nedd8; Pak1, P21-activated kinase 1; PKA, protein kinase A; PKC, protein kinase C; S, sumo; Tid1, tumorous imaginal discs protein tid56 homolog; U, ubiquitin.

mutation abolishes NMJ formation. In one model, the C-terminal region of Dok7 becomes tyrosine phosphorylated by agrin stimulation and binds to the adapter proteins Crk and Crk-L, which in turn associate with Sorbs1 and Sorbs2 (31). Double mutation of *Crk* and *Crk-L* impairs, but does not abolish, NMJ formation (unlike Dok7 null) (32). In agreement, the *Dok7* mutant without the C-terminal tyrosine-rich region is able to partially rescue the neonatal lethality of null mice (33). These results suggest the involvement of additional mechanisms to transduce signal from MuSK to AChR clustering. In vitro studies have suggested several enzyme effectors downstream of MuSK, including Abl, GGT, Rho GTPases, and Pak1, which have been reviewed previously (4) (**Figure 5*b***).

Recent data suggest that AChR trafficking plays an important role in NMJ formation. Agrin promotes the recycling of AChRs and thereby increases their metabolic stability (34). It enhances a vesicle-dependent transport of AChRs to the synaptic membrane (35). In this model, agrin activates PI3 kinase, which phosphorylates and thus inactivates GSK3β, leading to the synaptic capture of dynamic microtubules by microtubule plus-end tracking proteins CLASP2 and CLIP-170 (36). Agrin also recruits the PIP3-binding protein LL5β to AChR clusters; LL5β and filamentous actin are necessary for microtubule capture at AChR clusters (35) (**Figure 5*b***). CLASP2 ablation reduces microtubule plus-ends at the subsynaptic muscle membrane and AChR synaptic density and cluster size. An actin-depolymerizing factor/cofilin-dependent AChR endocytosis, in response to agrin, helps transport aneural AChR clusters and newly synthesized AChRs to the synaptic site (37) (**Figure 3**).

Rapsyn as a Key Effector

Rapsyn is an intracellular protein that is required for agrin-induced AChR clustering and NMJ formation (1–5). As observed in mutant mice of agrin, Lrp4, MuSK, or Dok7, rapsyn-null mice have no AChR clusters. These proteins are key proteins for NMJ formation (**Figure 5*c***). Rapsyn mutant mice survive a few hours after birth, probably because constitutive AChR expression in fetal myotubes is sufficient to sustain some transmission. It is generally believed that rapsyn serves as a downstream effector of MuSK signaling.

Rapsyn is an *N*-myristoylated protein with three domains [a domain with seven tetratricopeptide repeats, a coiled-coil (CC) domain, and a RING domain]. It is believed to anchor AChRs to the cortical cytoskeleton by associating with cytoskeletal proteins or regulators (**Figure 5*b***) (4). For example, rapsyn coaggregation with AChR and β-dystroglycan requires the CC and RING domains, respectively, and its self-aggregation depends on the tetratricopeptide repeats (38). The interaction between the CC domain and AChR is required for rapsyn targeting to the postjunctional membrane (39). Rapsyn also interacts with plectin isoform 1f to bridge AChRs and the intermediate filament network beneath the postsynaptic membrane (40). Unlike many scaffold proteins, however, rapsyn has a remarkable turnover rate, 4–6 times faster than AChRs, in cultured muscle cells and in the adult NMJ (41, 42). This may suggest that rapsyn could be a signaling protein instead of an adapter protein.

Rapsyn as an E3 Ligase for Posttranslational Modification

Mutations of rapsyn have been identified in CMS patients (38) (see below). Many of them cause rather mild NMJ deficits, in contrast to neonatal lethality of null mutation in mice, except for a partial deletion of the RING domain that causes fetal akinesia and premature termination of pregnancy (43). Intrigued by this, authors of a recent study demonstrate that the RING domain, which is conserved among vertebrates, possesses an E3 ligase activity (44). Mutation of an amino acid (cysteine 366) in the RING domain critical for this activity prevents rapsyn from clustering AChRs in heterologous cells and inhibits agrin-induced clustering in muscle cells. Importantly, knockin mice carrying this mutation do not form the NMJ, much like rapsyn-null mice. These observations suggest that E3 ligase activity plays a critical role in AChR clustering and NMJ formation.

As an E3 ligase, rapsyn may ubiquitinate partner proteins such as the AChR. However, rapsyn coexpression decreases, rather than increases, AChR ubiquitination (44). Evidence indicates that rapsyn promotes the conjugation of Nedd8 (neural precursor cell expressed, developmentally downregulated 8), a ubiquitin-like protein, to the AChR in a reaction called neddylation

(**Figure 5*d***). This reaction is known to be catalyzed by E3 ligases to regulate the function and/or localization of proteins (45). Interestingly, AChR neddylation could be stimulated by agrin in muscle cells and by coexpressing MuSK (44). Agrin-induced AChR clustering is inhibited by mutation of Lys397, a major neddylation site in the δ-subunit. Pharmacological inhibition of neddylation or genetic ablation of the Ubc12 E2 ligase attenuates agrin-induced AChR clustering in muscle cells, and inhibiting neddylation impairs NMJ formation in vivo. Together, these observations identify rapsyn as a novel enzymatic effector of the agrin/Lrp4/MuSK pathway. Thus, rapsyn not only serves as an adapter but also as an enzyme that is stimulated by agrin to promote AChR clustering and directs NMJ formation (**Figure 5*d***). The finding that rapsyn, a classic scaffold protein, is in fact an enzyme would call for revisits of other so-called scaffold proteins that have been implicated in synapse formation.

How rapsyn E3 ligase promotes clustering remains unclear. The C366A mutation does not change its own stability in HEK293T cells, localization at the NMJ, or coprecipitation with AChR or actin (44). In vitro, rapsyn is able to catalyze both neddylation as well as ubiquitination. In cells, however, coexpression increases AChR δ-subunit neddylation, which is associated with reduced ubiquitination. Conversely, inhibiting neddylation by the Nae I inhibitor MLN4924 increased δ-subunit ubiquitination (44). The inverse relationship between neddylation and ubiquitination of the AChR suggests that the neddylation may act by reducing ubiquitination of the AChR and thus increasing its stability. In addition to neddylation, rapsyn may modify the function or structure of proteins by K63-linkage polyubiquitination, monoubiquitination, and/or sumoylation (**Figure 5*d***). Because it is strategically located at the NMJ, rapsyn could have substrate proteins critical for NMJ formation, maintenance, and/or function, including MuSK, with which it is known to interact (**Figure 5*b***).

Extracellular Organizers

Synaptic basal lamina contains many proteins critical for NMJ formation and function, including agrin, laminin β2, and AChE (**Figure 2**). They are either delivered there specifically, as in the case of agrin, or synthesized locally by muscle fibers such as AChE and laminin β2. The NMJ localization of these proteins is likely maintained by interacting with scaffold proteins, such as postsynaptic α-dystroglycan and even Lrp4 for agrin, or with presynaptic calcium channels for laminin β2 and ColQ, a short collagen, for AChE (**Figure 5*a***). The synaptic basal lamina protein biglycan binds to MuSK to maintain MuSK levels at the NMJ (46). These enrichment mechanisms are likely to be downstream of MuSK, and in return, they strengthen MuSK activity as a positive feedback.

Interestingly, when myotubes are cultured on laminin-coated substrate, AChR clusters could transform from oval plaques into pretzel-like structures (47), suggesting that laminin could be an extracellular organizer of the NMJ. In agreement, this effect requires the MuSK ECD in addition to the kinase activity (48). The perforations in the clusters appear to resemble podosomes (dynamic actin-rich adhesive organelles) (49) and require Amotl2, a regulator of actin cytoskeleton, which interacts with LL5β (50), α-dystrobrevin-1, and associated proteins α-catulin, Grb2 (51), and coronin 6 (an actin-binding protein, which also requires agrin-induced clustering) (52) (**Figure 5*b***).

Perlecan is an extracellular organizer that specifically anchors AChE to the NMJ. In perlecan mutant mice, all NMJ components remain intact at the synapse except AChE (2, 4). Mice lacking muscle-derived collagen XIII, another extracellular protein, showed a delay in NMJ development (53).

Dispersing AChR Clusters by Muscle Activity

Muscle activity is known to suppress AChR expression (1–5). This is mediated by kinases that are activated by calcium influx via AChR and L-type calcium channels. For example, serine/threonine kinases, including PKC and CaMKII, phosphorylate myogenin and reduce its binding to *cis*-elements and reduce transactivation (54), which has been implicated in diminishing aneural AChR clusters. In agreement with this, reducing muscle activity by deleting the dihydropyridine receptor (an L-type calcium channel) increases expression of AChR and MuSK and thus disrupts muscle prepatterning (55). AChR clusters could be dispersed by the serine/threonine kinase Cdk5. This kinase could be activated by calpain that cleaves p35 to p25 (56) or could be recruited to synaptic membrane by forming a tertiary complex with p35 and nestin (57, 58). A Rho guanine nucleotide exchange factor protein, ephexin1, is required for AChR cluster dispersal, and in its absence, NMJs fail to mature into the pretzel shape (59). Muscle activity can also activate caspase-3 to cleave Dishevelled (Dvl) to disperse AChR clusters (60). Some of these machineries may contribute to synapse elimination.

RETROGRADE SIGNALING FOR PRESYNAPTIC DIFFERENTIATION

The tightly coordinated assembly of pre- and postsynaptic structures during NMJ development indicates strong reciprocal signaling between the two. Motor nerve terminals arborize extensively in mutant mice lacking genes critical for AChR cluster formation, including agrin, Lrp4, MuSK, and rapsyn, indicating the involvement of the agrin-Lrp4-MuSK axis in also controlling presynaptic differentiation. Accordingly, in MG mouse models with antibodies against Lrp4 and MuSK, presynaptic defects are abundant (61, 62). Dvl interacts with MuSK, and disrupting this interaction in muscles impairs presynaptic development in vivo (63). In zebrafish, Dvl-dependent signaling initiated by Wnt-MuSK interaction in muscle fibers restricts growth cone guidance to the central region of muscle fibers (64, 65) and restricts neural crest cells to the center of each somite (66). Many muscle proteins or muscle-derived factors have been implicated in presynaptic differentiation, including embigin (67), fibroblast growth factor (FGF) 7/10/22, collagen IV, glial cell line–derived neurotrophic factor (GDNF), signal regulatory protein-α, laminin β2, and integrin β1. Their roles were summarized in previous reviews (2, 4). Here, we provide an update on recent progress in studies of retrograde signaling in NMJ formation.

Lrp4 as a Transsynaptic Regulator

Lrp4 has 1,905 residues, 1,725 of which are in the ECD (**Figures 2** and **4**). Using different but complementary genetic approaches, two studies independently revealed that muscle Lrp4 is necessary for motor nerve terminal differentiation (15, 68). First, miniature endplate potential (mEPP) frequency, motor nerve terminals, active zones, and synaptic vesicles are compromised in muscle-specific *Lrp4* mutant mice (15, 68). Motor nerve terminals extend into nonsynaptic areas, bypassing AChR clusters, as if not knowing where to stop (15). Second, the presynaptic deficits cannot be rescued by MuSK overexpression in muscles, suggesting a MuSK-independent mechanism (68). Third, Lrp4-expressing HEK293 cells or ecto-Lrp4-beads are able to induce punctas of vesicle and active zone proteins in contacting axons (15, 68), suggesting that Lrp4 may be "synaptogenic" by binding to a partner in nerve terminals. These punctas could be labeled by FM dye, suggesting they may represent functional neurotransmitter release sites (68). These observations corroborate that muscle Lrp4 may have a novel function as a retrograde signal for presynaptic development, in addition to its well-established role as agrin's receptor (**Figure 6**). The identity of the binding partner on nerve terminals for postsynaptic Lrp4 remains unclear.

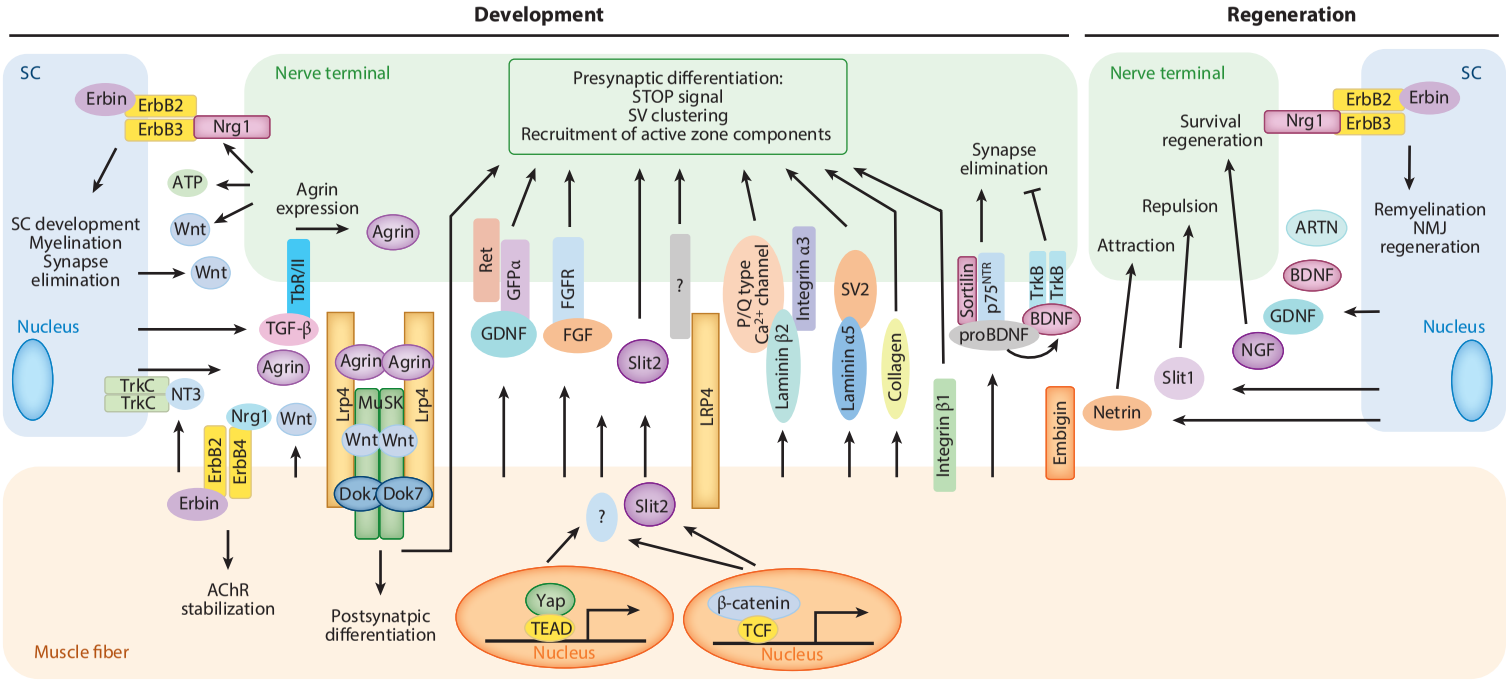

Figure 6

Interactions among motoneurons, muscles, and terminal SCs for NMJ formation. During development (*left*), in addition to agrin, motor nerve terminals release Nrg1, which binds to ErbB3 to activate ErbB2 for SC development, axon myelination, and AChR stabilization. Presynaptic differentiation is regulated by agrin signaling in muscle fibers and by muscle transmembrane proteins, including Lrp4, integrin β1, and embigin. In addition, muscle fibers produce various factors such as GDNF, FGF-family members (Fgf22, Fgf7, Fgf10), Slit2 (in a β-catenin–dependent manner), pro-BDNF, laminins, and collagen, which have been implicated in NMJ development. SCs can release Wnt, agrin, and TGF-β for both presynaptic and postsynaptic differentiation. After injury (*right*), SCs release factors for motor neuron survival and muscle reinnervation. Nrg1 from motoneurons is required for remyelination (see 3, 4). Abbreviations: AChR, acetylcholine receptor; ARTN, artemin; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; GDNF, glial cell–derived neurotrophic factor; MuSK, muscle-specific kinase; NGF, nerve growth factor; NMJ, neuromuscular junction; Nrg1, Neuregulin 1; SC, Schwann cell; Slit2, slit guidance ligand 2; TGF-β, transforming growth factor-beta; YAP, Yes-associated protein.

β-Catenin and Slit2

β-Catenin muscle-specific conditional knockout (cKO) causes morphologic defects in motoneuron terminals (69). The primary nerve branches of the mutant mice are mislocated; secondary or intramuscular nerve branches are fewer but elongated; and the AChR area covered by nerve terminals is reduced. Functionally, β-catenin–deficient NMJs display a reduction in spontaneous and evoked ACh release as well as compromised short-term plasticity and calcium sensitivity of neurotransmitter release. The presynaptic deficits do not appear to be secondary because the postsynaptic abnormality of muscle-specific β-catenin cKO is minor, with comparable AChR clusters (albeit larger and distributed across a wide region). Moreover, motoneuron-specific cKO of β-catenin has little effect on NMJ development. These findings indicate a necessary role for muscle β-catenin in presynaptic differentiation and function. Interestingly, when β-catenin is stabilized in muscles in a gain-of-function model, mice display phenotypes similar to those in β-catenin cKO mice, including increased width of the synaptic band, primary branch mislocation, and reduced mEPP frequency (70, 71). On the other hand, synaptic phenotypes include increased complexity of motor nerve terminal arborization without, however, a reduction in axon diameters, a reduction of the active zones and vesicle numbers, and an increase in AChR cluster size, unlike in cKO mice. Together, these data demonstrate that normal NMJ formation requires an intricate balance of β-catenin activity in muscles. YAP (Yes-associated protein) could be upstream of β-catenin because presynaptic deficits of muscle-specific *Yap* mutant mice could be rescued by inhibiting β-catenin degradation (72).

In addition to its role as a key transcriptional activator of the Wnt canonical pathway, β-catenin is a component of the cell-adhesion complex regulating aspects of the intracellular cytoskeleton. Transgenic rescue experiments indicate that the regulation of presynaptic differentiation requires the transcription activation activity, but not the cell-adhesion function (73), suggesting that muscle β-catenin likely regulates presynaptic differentiation by controlling the expression of muscle-derived proteins. Such a protein could be secretable, and its expression is downregulated in β-catenin mutant muscle. A screen for proteins that match these criteria led to the identification of Slit2. Slits serve as repulsive cues in axons and neurons and promote axonal and dendritic branching. Motoneurons use Slit2 to promote axon fasciculation by an autocrine or juxtaparacrine mechanism (74). Transgenic expression of Slit2 in muscles rescues presynaptic deficits in β-catenin mutants, including poor innervation, reduced vesicle density and active zones, and reduced mEPP frequency. Together, these observations suggest that Slit2 is a factor utilized by muscle β-catenin to direct presynaptic differentiation. However, phenotypes such as mislocation of the primary branches are not rescued, suggesting the involvement of additional retrograde factors (**Figure 6**).

Laminin β2 and P/Q-Type Voltage-Dependent Calcium Channel

Skeletal muscles produce various types of heterotrimeric laminins (α/β/γ subunits); β2-containing laminin (laminin β2) is enriched in the synaptic basal lamina. In vitro studies suggest that it acts as stop and differentiation signals for motor nerve terminals (2, 4). Laminin β2 can bind to P/Q-type calcium channels to recruit other presynaptic components (2, 4). Mutant mice lacking P/Q-type calcium channels have a reduction in active zones at the NMJ, in docked vesicles, and in presynaptic proteins such as Bassoon, Piccolo, and CAST/Erc2. These phenotypes are exacerbated by further mutation of the N-type calcium channel that also binds laminin β2 (75). Mice lacking laminin β2 display aberrant presynaptic development (2, 4) and calcium channel maturation at the NMJ (76). Because calcium channels could associate with Bassoon or CAST/Erc2, it is believed that they link the muscle-derived laminin β2 to presynaptic proteins to organize activate zones (**Figure 6**).

Glial Cell Line–Derived Neurotrophic Factor

GDNF is a member of the transforming growth factor-beta (TGF-β) family that acts by stimulating the GDNF family receptor alpha (GFRα)/Ret receptor complex (77). In transgenic mice overexpressing GDNF in muscles, the muscles are hyperinnervated, and motor units are enlarged (78). At the NMJ formed between hypoglossal motoneurons and tongue muscles, Ret receptors are in registry with α-bungarotoxin staining (79). Ret knockout in hypoglossal motoneurons (via Nkx6.2-Cre) compromises motoneuron survival, presynaptic maturation during development, and maturation of adult reinnervated nerve terminals. There is a significant reduction of NMJs in mutant mice, probably due to the loss of motoneurons. A recent study using microfluidic chambers demonstrates that GDNF facilitates axon growth and NMJ formation when applied only at the NMJ but not to the soma (80).

β-Amloid Precursor Protein Family as Transsynaptic Regulators

APP family members, including APP, APLP1, and APLP2, are necessary for NMJ formation. They could serve as transsynaptic regulators; for example, APP family members of motoneurons and muscles can form in-*trans* dimers (81), or motoneuron APP interacts with muscle Lrp4 (16). The latter interaction could also promote MuSK activation (16). APP in muscles was also shown to control the expression of GDNF (82).

WNT SIGNALING IN NEUROMUSCULAR JUNCTION FORMATION

Wnt signaling is critical for diverse developmental processes. Wnt binds to Frizzled and Lrp5/6 on plasma membrane to recruit the scaffold protein Dvl to initiate several pathways. In the canonical pathway, the β-catenin destruction complex, consisting of axin, adenomatous polyposis coli, casein kinase, and GSK3, becomes destabilized; this increases β-catenin in the nucleus to regulate gene expression. Noncanonical pathways of Wnts include the planar cell polarity pathway to regulate cytoskeletal organization and the calcium pathway that leads to increased intracellular Ca^{2+} levels. The first experimental evidence that Wnt signaling may regulate vertebrate NMJ formation appeared when Dvl1 was found to interact with MuSK (63). However, Wnt regulation of vertebrate NMJ is more complex because there are multiple Wnts in vertebrates, particularly in rodents, that are functionally heterogeneous. In addition to Frizzled and Lrp5/6, MuSK and Lrp4 may serve as Wnt receptors that may initiate different pathways. Overall, the function of Wnts appears to be modulatory, unlike that of agrin (**Figure 7**).

Regulation of AChR Clustering

The importance of endogenous Wnt in AChR clustering was revealed by the experiment that antagonist Sfrp1 reduces the number of AChR clusters in chick wing muscles (83). Injection of Wnt4/11, DKK1, and Sfrp4 in mouse embryos increases or decreases AChR clusters and their distribution area, respectively (84). Wnts have three types of effects on AChR clustering (**Figure 7**). Wnt9a, Wnt9b, Wnt10b, Wnt11, and Wnt16 can promote AChR cluster formation in cultured myotubes in the absence of agrin (83, 85, 86). This effect is dose dependent and saturable, with subnanomolar EC_{50} (85). Some Wnt proteins (such as Wnt3) can promote agrin-induced clustering, whereas others (Wnt3a, Wnt7a, Wnt8a, and Wnt10b) could be inhibitory to agrin induction (83, 85, 87). Because of the functional heterogeneity and redundancy, mutation of individual *Wnt* genes has not been informative or consistent. For example, depending on the mouse strain, *Wnt4* and/or *Wnt11* mutant mice exhibit NMJ defects or have normal NMJs (66, 84, 88).

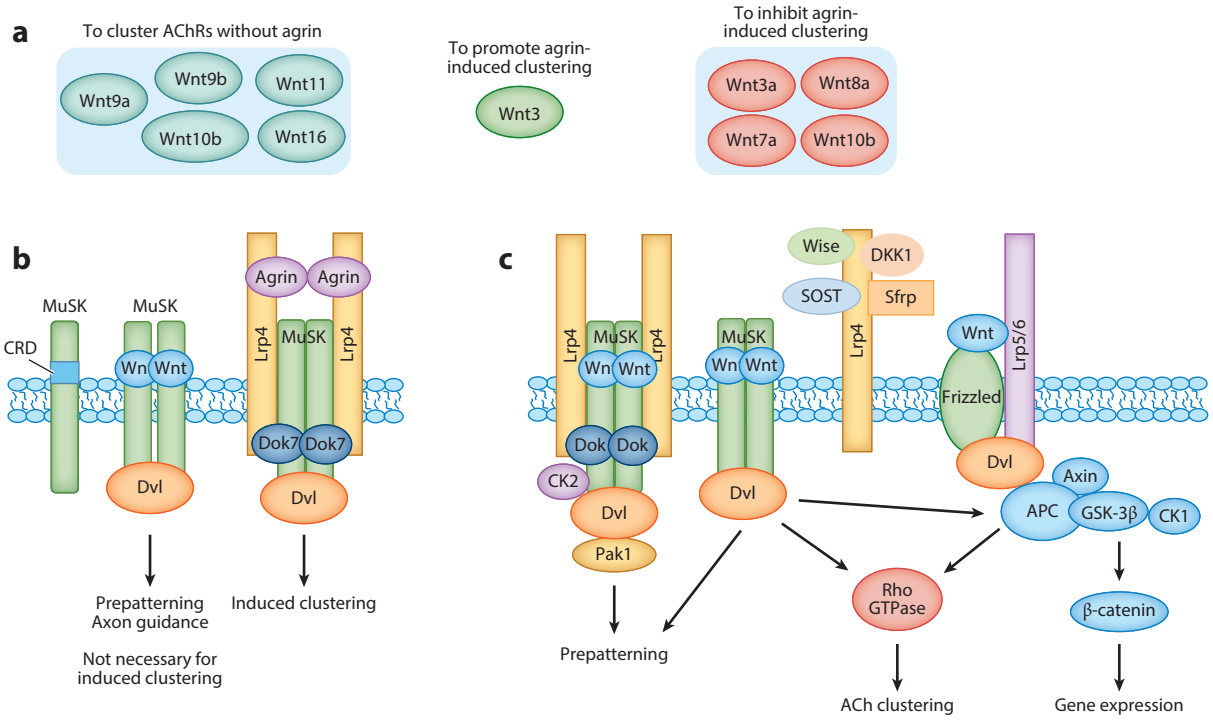

Figure 7

Wnt signaling in NMJ formation. (*a*) Three different effects of Wnt proteins on AChR clustering. (*b*) In zebrafish, MuSK, which can be activated by Wnt, is required for prepatterning and axon guidance. Prepatterning is not required for NMJ formation. (*c*) Wnt regulation of AChR clustering could be mediated by interacting with MuSK or Lrp4 or by activating Wnt pathways. Lrp4 interacts with Wnt signaling inhibitors (Wise, SOST, DKK1, Sfrp), whose functions in NMJ formation remain unknown. Abbreviations: AChR, acetylcholine receptor; APC, adenomatous polyposis coli; CK1/2, casein kinase 1/2; CRD, cysteine-rich domain; DKK1, Dickkopf-related protein 1; Dvl, Dishevelled; GSK-3 β, glycogen synthase kinase-3 β; GTPase, guanosine triphosphate hydrolase enzyme; Lrp4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; NGF, nerve growth factor; NMJ, neuromuscular junction; Pak1, P21-activated kinase 1; Sfrp, secreted frizzled-related protein; SOST, sclerostin.

However, NMJ development appeared to be normal in mice lacking Wls (Wnt ligand secretion mediator, a protein for Wnt maturation and secretion) in muscle cells (88). This suggests that Wnt secretion from muscles is not necessary for NMJ formation (88). Interpretation of this negative result needs caution, because there is thus far no evidence that Wls is required for secretion of all Wnts. Whether Wls is required for 19 rodent Wnts is unknown. Therefore, understanding the involvement of muscle Wnts in NMJ formation awaits future work. Adding to the complexity is that Wnts from SCs (89) or motoneurons (83) may contribute to NMJ development.

Wnt in Muscle Prepatterning

In zebrafish, muscle prepatterning does not require Lrp4 but does require MuSK and its cysteine-rich domain (CRD); it also requires Wnt11r and Wnt4a that interact with MuSK (64, 88, 90, 91) (**Figure 7*b***). In mice, the prepatterning requires Lrp4 or MuSK but not agrin or motor nerves. This suggests that MuSK activity in mouse can be maintained by Lrp4 (9) or by a yet to be identified ligand that binds MuSK. Such a ligand could be a Wnt protein because MuSK CRD is homologous to that in the Wnt receptor Frizzled. In line with this model, deletion of the CRD in

MuSK reduces AChR clusters in mutant mice, and remaining clusters are distributed in a wider region (92). The deficits were not observed in heterozygous mice, indicating the dependence on genotypes (84). Prepatterning deficits also occur in *Wnt4* and *Wnt11* mutant mice (83, 84, 86). These data support the model that Wnt proteins regulate muscle prepatterning via binding to MuSK CRD. However, the same CRD is found dispensable for muscle prepatterning in another strain of MuSK mutant mice (88). The discrepancy may be due to genetic backgrounds of the two mutant strains and calls for future work to delineate the function of MuSK CRD in muscle prepatterning and/or NMJ development.

Wnt Mechanisms of Action

What are the molecular mechanisms of Wnt regulation of NMJ formation? First, Wnts could stimulate Frizzled and Lrp5/6 to initiate the canonical and noncanonical pathways. Levels of β-catenin, the major effector of the canonical pathway, control the location, and to a lesser extent, the size of AChR clusters and presynaptic development (see section Retrograde Signaling for Presynaptic Differentiation) (69–71). Noncanonical pathways such as Rac1 activation explain well the potentiating effect of Wnt3 on agrin-induced AChR clustering (83). Mechanisms by which Wnt proteins inhibit agrin-induced AChR clustering are poorly understood. First, Wnt3a was shown to disperse AChR clusters by repressing the expression of rapsyn (87). Second, Wnts may bind to MuSK and/or Lrp4 to initiate pathways that are shared with agrin signaling. This notion is supported by the observation that the in vitro cluster-inducing effect of Wnt9a and Wnt11 is only ~50% of the efficacy of agrin and not additive to agrin-induced clustering (85). Finally, Wnt binding to MuSK may initiate Wnt canonical and noncanonical pathways. NMJ deficits in MuSKΔCRD mutant mice could be rescued by LiCl, an inhibitor of GSK3β, to increase β-catenin, suggesting the involvement of the canonical Wnt signaling (92). In zebrafish, MuSK activation by Wnt is able to initiate Dvl-dependent, noncanonical, or planar cell polarity signaling in muscle fibers that restricts aneural AChR clusters to the central region of muscle fibers (64, 65). As observed for agrin activation of MuSK (93), MuSK activation by Wnts stimulates MuSK translocation to recycling endosomes, which is a necessary step for AChR accumulation at the NMJ (90).

The cross talk between Wnt and agrin signaling could happen at several levels. First, it could be facilitated by shared molecules that have been implicated in Wnt and agrin pathways. For example, Dvl1 interacts with MuSK and recruits Pak1 to MuSK upon agrin stimulation (63). Adenomatous polyposis coli interacts with AChR β-subunit (94); rapsyn binds β-catenin (95); and casein kinase 2 interacts with and phosphorylates MuSK (96). These interactions are required for agrin-induced AChR clustering and NMJ formation in vitro. Second, Wnt7a binds to MuSK but inhibits agrin-induced clustering, perhaps by altering MuSK binding with agrin (97). Finally, Lrp4 is believed to be a receptor for various Wnt-negative regulators such as DKK1, Sfrp, sclerostin, and Wise (98). Such interactions may modulate those between Lrp4 and agrin or MuSK and/or intracellular pathways (**Figure 7*c***).

SYNAPSE ELIMINATION

During an embryonic (E) stage, a muscle fiber receives inputs from multiple axons, perhaps to ensure that all motoneurons find their target and that all muscle fibers are innervated. Within two weeks after birth, extra innervations are gradually lost, ~10% per day, leaving every muscle fiber innervated by one axon only. The process, called synapse elimination, is critical for generating motor units and thus for precise control of muscle contraction (**Figure 1*a***).

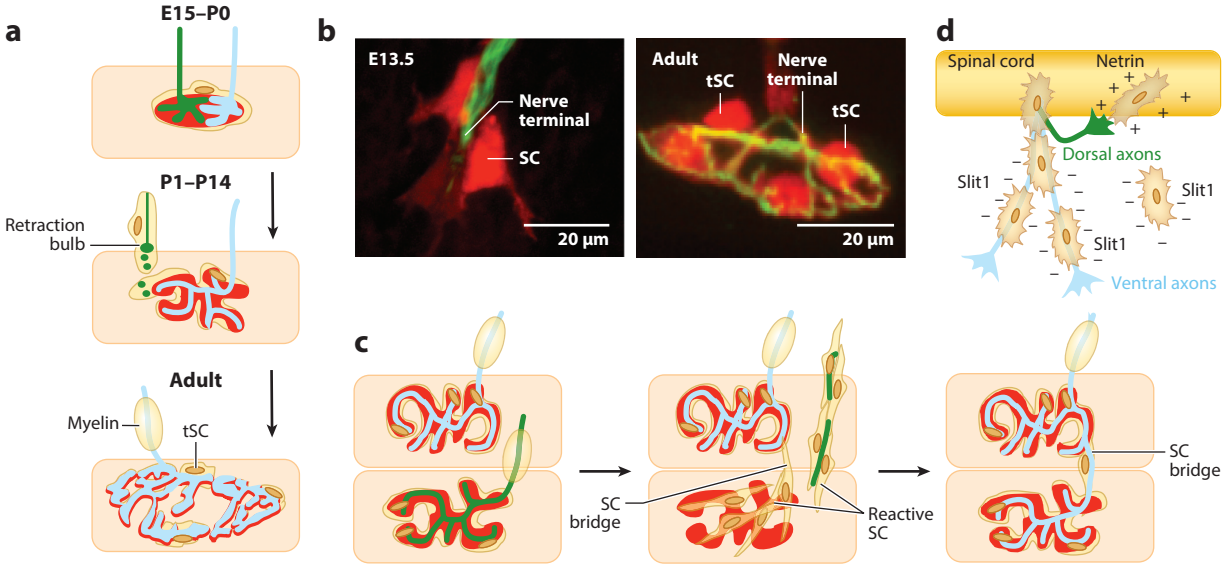

Figure 8

Function of SCs in NMJ development and regeneration. (*a*) tSCs engulf retracting MN terminals from eliminated NMJ. During E15 to P0, a muscle fiber may be innervated by two nerve terminals, which compete to occupy the NMJ. During P1 to P14, the winning axon innervates the entire NMJ, and the losing axon forms retraction bulbs. In adults, the mature NMJ appears as a pretzel-like structure that is covered by several tSCs. (*b*) Extension of SCs ahead of developing axons (E13.5), and adult NMJs are covered by properly tiled tSCs. (*Left*) SCs are visualized by tdTomato (*red*) and nerve terminals by green fluorescent protein (*green*) in tamoxifen-treated Plp::CreER;tdTomato;Hb9::GFP mice. (*Right*) tSCs are labeled by tdTomato in tamoxifen-treated Dhh::CreER;tdTomato mice, where nerve terminals are visualized by antibodies against synaptophysin and neurofilament (*green*). Panel *b* (*left*) was reproduced with permission from Reference 127; panel *b* (*right*) was provided by Tiankun Hui. (*c*) SC bridges lead reinnervation after injury. (*d*) SCs guide regenerating motor axons after injury in zebrafish. Abbreviations: MN, motoneuron; NMJ, neuromuscular junction; SC, Schwann cell; Slit1, slit guidance ligand 1; tSC, terminal Schwann cell.

Activity-Dependent Competition

Synapse elimination is a competitive process that is believed to be regulated by activity of axons as well as muscle fibers (99). Synapses formed by axons of less active motoneurons are preferably eliminated. In mouse extraocular muscles, NMJ elimination is delayed because of the delayed onset of muscle activity (100). In a rat strain where the soleus muscle is innervated by two nerves, asynchronous activity reduces polyinnervation, and more active terminals outcompete the less active ones (101). During synapse elimination, the reduction in AChR density seems to occur before the loss of presynaptic markers, suggesting that muscle cells play an important role in competitive synaptic reorganization. When an area of AChRs of the same pretzel is asymmetrically blocked, this leads to reduced AChR density and axon retraction from the blocked area (102).

Terminal Schwann Cells in Synapse Elimination

As axons withdraw, they shed axonal tips and synaptic organelles that are engulfed by tSCs, forming membrane-bound remnants called axosomes (103) (**Figure 8*a***). Eventually, the winning axon expands to occupy the territory of withdrawn axons (104). Intracellular mechanisms may involve the aforementioned AChR cluster-dispersing machineries. Although tSCs can differentially decode synaptic activity from competing nerve terminals (105), their phagocytosis involves all axons

nondiscriminatively, suggesting that tSCs do not distinguish between winning and losing axons during synapse elimination (106). Rather, they extend processes to separate nerve terminals from one another and separate them from muscle fibers (106, 107). A parsimonious explanation of these observations is that SCs may not initiate synapse elimination, but they do contribute to cleaning up synaptic debris.

Molecular Mechanisms in Synapse Elimination

Recent studies have begun to explore the molecular mechanisms of synapse elimination. A number of proteins for SC function are shown to regulate synapse elimination. For example, axonal Nrg1 type III promotes the actions of tSCs in synapse elimination (107). Mice lacking the glial isoform of neurofascin (Nfasc155) are slow in eliminating multi-innervated NMJs (108). These mutant mice display normal myelination, compaction, and nodal architecture, but they lose paranodal junctions. Because synapse elimination is normal in mice lacking the axonal paranodal protein Caspr, it was proposed that loss of Nfasc155 from SCs disrupts neuronal cytoskeletal organization and trafficking and thus delays synapse elimination. Synapse elimination may be mediated by branch-specific disassembly of microtubule and axonal transport (109). Synapse pruning in the brain is regulated by major histocompatibility complex, class I (MHCI) molecules. Interestingly, MHCI is expressed in neonatal NMJs, and unlike control mice in which multi-innervated NMJs disappeared within two weeks, ~20% of NMJs remain multi-innervated in mice lacking most surface MHCI proteins (110). This suggests a role of MHCI proteins in NMJ elimination, similar to their role in central nervous system synapses. It remains unknown whether MHCI expression or secretion is specific for axons, muscles, tSCs, or a subcellular domain (e.g., a region of AChR clusters whose activity is blocked) and if it is regulated by neuronal and/or muscle activity. A provocative model is activity-dependent processing of probrain-derived neurotrophic factor (BDNF) that may stabilize (via BDNF) or eliminate (via pro-BDNF) presynaptic terminals depending on its proteolytic conversion at the synapses (111) (**Figure 6**). Genetic evidence for this model has not yet been obtained.

NEUROMUSCULAR JUNCTION MAINTENANCE

Considering the tripartite nature of the NMJ, it is understandable that NMJ maintenance requires proper structure and function of motoneurons, muscles, and SCs. For example, mutations that alter motoneuron terminals destabilize the NMJ (112); likewise, ablating SCs in adult animals destabilizes the NMJ (see below). The NMJ becomes unstable when supported lipid bilayer proteins, such as laminin α4 or collagen XIII, are absent (53, 113). Although the dystrophin-glycoprotein complex is present at the NMJ, it is not required for NMJ formation. However, the NMJ becomes unstable in the absence of individual components of the complex, including α-syntrophin and α-dystrobrevin (114), possibly via increasing AChR turnover rate (115). As with NMJ formation, the AChR surface level is controlled by both electric activity and agrin signaling. It was estimated that the AChR's half-life at fully functioning synapses is quite long (~9–14 days) but is reduced to several hours when synaptic activity is blocked (114). Synaptic AChR density requires CaMKII and a related anchoring protein called Akap (116). Stimulation of protein kinase C or inhibition of protein kinase A accelerates the removal of synaptic AChRs and depresses AChR recycling (117). Protein kinase A is enriched at the NMJ by interacting with rapsyn (118).

As with NMJ formation, agrin signaling is required for NMJ stability. NMJs disintegrate in mice that lose agrin, Lrp4, or Dok7 after NMJ formation (113, 119, 120). Similar results were obtained in mice when MuSK was knocked out by a late-onset Cre expression or by shRNA (121, 122) and in mutant mice without biglycan that maintain the MuSK level at the NMJ (46). NMJs

could be induced in agrin mutant mice by overexpressing Dok7; however, these NMJs cannot be maintained in the absence of agrin, suggesting that agrin may have a function independent of Dok7-mediated MuSK activation (21).

Several other pathways that are not required for NMJ formation have been implicated in NMJ maturation and maintenance. Neural cell adhesion molecules are involved in the organization of presynaptic NMJ (167). AChRs become mobile and move rapidly from synaptic to perisynaptic regions in muscles in which ErbB kinases are abolished or inhibited (123). Evidence suggests that ErbB kinases phosphorylate α-dystrobrevin 1 to enhance the stability of AChR anchoring to synaptic membrane. NMJ maintenance also requires muscarinic AChR and sympathetic inputs to the synapse and β2-adrenoreceptor activation (124, 125).

SCHWANN CELLS

Besides motor nerve terminals and skeletal muscle fibers, a third component of the tripartite synapse is a special type of SC called tSCs (also called teloglia or perisynaptic SCs) (**Figure 1*b***). There are three types of SCs: Two of them—myelinating and nonmyelinating SCs—ensheathe motor axons with and without myelin, respectively, whereas tSCs do not ensheathe axons but envelop the nerve terminal and muscle surface in proximity of the synapse and cover the synaptic cleft (5). In mice, NMJs are covered by one tSC initially; the number increases to 3–5 in adults, perhaps via TrkC in response to muscle-released NT3. Young tSCs dynamically intermingle, but adult tSCs show static tile patterns (**Figure 8*a*,*b***).

SCs are derived from neural crest cells and comigrate with growth cones of motoneuron axons. Electron microscopic examinations indicate that SCs are present at early nerve-muscle contacts in mice (126). Recent data from immunostaining and genetic labeling of SCs and motoneurons indicate that SC processes are ahead of, and perhaps lead, developing axons before axons stop to induce AChRs (**Figure 8*b***) (97). It is unclear whether these leading SCs evolve into tSCs. Because of the intimate interaction of tSCs with nerve terminals and muscle fibers, they have been implicated in NMJ formation, modulation of synaptic activity, and NMJ regeneration after nerve injury, as summarized by an outstanding review (5). Here, we focus on tSCs in NMJ development and regeneration. It is worth pointing out that, due to the lack of a selective marker and Cre, certain tSC functions discussed below are inferred. This is because it is not currently possible to determine whether NMJ deficits are due to the loss of tSCs or other SCs.

Schwann Cells in Neuromuscular Junction Formation

The first genetic evidence showing that SCs are critical for NMJ formation emerged when NMJ deficits were observed in *Nrg1* and *ErbB* mutant mice (5) (**Figure 6**). Accordingly, *ErbB2* mutant mice do not have SCs; however, their motor nerves can project to muscle fibers, but nerve terminals are poorly developed and eventually retract. *ErbB2* mutation reduces junctional folds but has no effect on synapse-specific transcription. In the frog, growth of nerve terminals is reduced after the removal of tSCs. In mice, when SCs are killed before innervation (at E12.5), both the size and density of innervated AChR clusters are reduced (127). When SCs are ablated after NMJ formation, only the size, but not density, of AChR clusters is reduced, and NMJs become fragmented so that neuromuscular transmission is compromised. SC ablation at either time does not change the location of NMJs in the center of the muscle. When SCs are ablated after NMJ maturation (at P30 in mice), NMJs become fragmented, and neuromuscular transmission is compromised (127). The amplitude of mEPP was reduced three days after SC ablation, but both amplitude and frequency were reduced six days after. This result indicates that tSCs are important for NMJ formation and maintenance.

Terminal Schwann Cells in Neurotransmission

The tSCs are not electrically excitable but can sense ACh, adenosine 5′-triphosphate, and nerve trophic factors. In response, they increase their intracellular calcium levels, which in turn regulate neuromuscular transmission via a G protein–coupled receptor pathway (5).

Schwann Cells in Neuromuscular Junction Regeneration

During recovery after nerve injury, SCs are critical for NMJ regeneration. When nerve injury is minor, regenerating axons can retrace their original paths along SC tubes to reach original targets (128, 129). This specificity is lost when nerves are severed. After nerve section, SCs become active and extend into the synaptic cleft to surround fragments of nerve terminals. Nerve injury also causes tSCs of denervated NMJs to sprout into nearby intact NMJs to form SC bridges, which guide generating axons from intact NMJs to injured synapses to prevent loss of AChRs and to form new AChR clusters (130, 131) (**Figure 8*c***). This process may require nitric oxide because SCs fail to extend the processes after injury when nitric oxide synthase is inhibited (132). In mutant zebrafish lacking SCs, regenerating axons fail to cross the injury site and stray along aberrant trajectories, suggesting a necessary role of denervated SCs in directing regenerating axons (133). A group of SCs express netrin to attract regenerating axons that express DCC (deleted in colorectal carcinoma). Certain SCs increase their expression of glycosyltransferase lysyl hydroxylase 3 to present *collagen4a5*, which works together with Slit1a to destabilize and repel mistargeted axons (134) (**Figure 8*d***).

Factors from Schwann Cells

In principle, SCs may promote synaptogenesis by releasing synaptogenic factors or indirectly by regulating motoneurons (**Figure 6**). A conditioned medium of frog SCs enhances AChR clustering in cultured myotubes (135). Frog spinal neurons form fewer synapses in the absence of SCs compared to those cocultured with SCs (136). In vitro assays have identified several factors released from SCs. Agrin from SCs was implicated in target selection and synapse formation by regenerating axons. TGF-β1 was shown to enhance synapse formation in cultured systems (2, 4). In response to nerve injury, SCs produce many factors, including GDNF, artemin, BNDF, nerve growth factor, leukemia inhibitory factor, cytokines, and chemokines to promote motoneuron survival and axonal regeneration (137, 138).

NEUROMUSCULAR JUNCTION DISORDERS AND AGING

Myasthenia Gravis

MG is the most common acquired autoimmune disorder of the NMJ, affecting 400–600 patients per million people. MG can be diagnosed by identifying antibodies against the AChR and later MuSK (139, 140). However, ~10–15% of MG patients lack these antibodies (this condition is thus called double-negative MG). Recently, antibodies against agrin and Lrp4 have been identified in these patients (28, 141–145). Besides fixing complements and attracting macrophages, anti-agrin and anti-Lrp4 antibodies may disrupt the agrin signaling (28, 61, 140, 142, 144), further suggesting an important role of agrin signaling in NMJ maintenance.

Congenital Myasthenia Syndrome

CMS is less common than MG and usually has an early onset (146). Based on gene functions, CMS cases can be grouped into three examples: postsynaptic (~34%), synaptic (~7%), and presynaptic

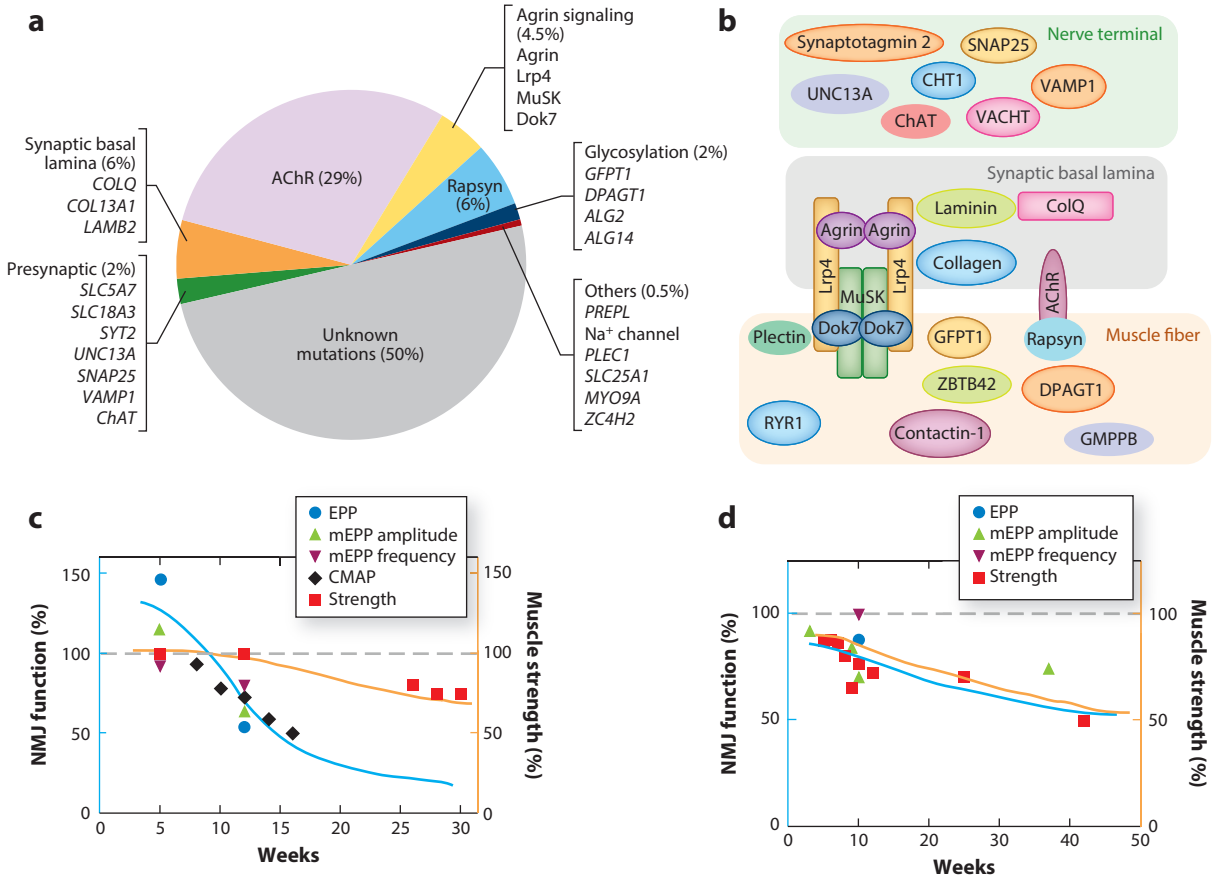

Figure 9

NMJ-related disorders. (*a*) Contribution (percentage) of mutant genes identified in congenital myasthenia syndrome (CMS). (*b*) Localization of CMS-related proteins in muscle fiber, nerve terminal, or synaptic basal lamina. (*c*,*d*) Function decline in NMJ transmission and muscle strength in (*c*) $SOD1^{G93A}$ mice and (*d*) muscular dystrophic mice. Abbreviations: CMAP, compound muscle action potential; EPP, endplate potential; mEPP, miniature EPP; NMJ, neuromuscular junction.

(~2%) (**Figure 9*a*,*b***). In line with the role of agrin signaling in NMJ formation, mutations of its key genes have been identified in CMS cases, including mutations of agrin itself, Lrp4, MuSK, and Dok7 (146). However, the majority of CMS cases are due to mutations in AChR subunits (~29% of CMS cases) and rapsyn (~6% of CMS cases). Other mutant genes include ryanodine receptor 1 (*RYR1*, a calcium release channel in the sarcoplasmic reticulum) and contactin 1 (*CNTN1*, a cell-adhesion molecule). Synaptic CMSs are caused by mutations in *AChE* and its anchor *ColQ*, collagen XIII, and laminin β2. Presynaptic CMS cases are caused by mutations in *ChAT*, choline transporter, and SNARE proteins. Except certain mutations in AChR subunit genes, most mutations are autosomal recessive.

Neuromuscular Junction in Aging Animals

During aging, there is a gradual loss of lean body mass and strength—a process called sarcopenia. Muscle fibers in aged animals are reduced in total number, have increased heterogeneity of fiber

size and types, and are infiltrated with adipocytes and connective tissue (147). The aging process is associated with NMJ fragmentation (148, 149). In aged mice (>2 years old), AChR clusters appear as small islands that are distributed in an area larger than pretzel-like clusters in young adults (**Figure 1**). This could be a consequence of degeneration and regeneration of muscle fiber segments beneath the synapse (150). Many clusters have reduced density of AChR (148). Presynaptically, motoneuron loss is observed in aged human subjects (147) but remains controversial in mice (148, 149). In aged mice, axons become thinner and disorganized and contain swollen varicosities. Some clusters are partially innervated or innervated by multiple axons in limb muscles (148) but not in sternomastoid muscles (151). Interestingly, on the individual NMJ level, postsynaptic changes in aging mice can occur suddenly as a consequence of degeneration and regeneration of muscle fiber segments beneath the synapse (150). A recent study did not reveal a decline of neuromuscular transmission between 12- and 24-month-old mice, although NMJ fragments were increased (152). It is unclear whether a functional decline occurs before or after the studied period.

The mechanisms of the NMJ that decline in aging animals are not well understood. Autophagy impairment in muscle induces NMJ degeneration and precocious aging (153). A critical question is whether the NMJ decline contributes to sarcopenia or results from it. Interestingly, NMJ fragmentation was observed in cKO of agrin or Lrp4 (113, 119) and in mice that produce or are injected with anti-Lrp4 antibodies (61). Accordingly, augmented proteolytic cleavage of agrin in neurotrypsin-overexpressing mice increases serum levels of a C-terminal agrin fragment, destabilizes NMJs, and induces denervation. The phenotype of those mice is reminiscent of sarcopenia (154), suggesting that compromised agrin signaling is involved in, and perhaps causal to, NMJ decline and subsequently sarcopenia. Preventing the NMJ decline and/or increasing neuromuscular transmission could be therapeutic to sarcopenia.

Comorbidity, Consequence, or Cause?

Because of the tripartite nature of the NMJ, its structure and function feature in various disorders that affect motoneurons, including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA); disorders of the muscles, including Duchenne muscular dystrophy (DMD); and disorders of the SCs, including Charcot-Marie-Tooth disease and Guillain-Barre syndrome. However, it remains unclear whether the NMJ structure and function alternations are a complication of these disorders or if they represent comorbidities of similar genetic mutations. NMJ decline in aging shares similarities with those of an ALS model (155). MicroRNAs that delay ALS progression also promote NMJ regeneration in mice (156). In fact, NMJ alternations occur long before clinical presentation and death in ALS mouse models (157), whereas NMJ and muscle strength declines display a similar time course in a DMD mouse model (**Figure 9*c*,*d***). SMA mouse models show profound NMJ defects even in the absence of motoneuron loss (158). It will be informative to carefully study the time courses of NMJ structural and functional decline and the primary morbidity of these disorders (**Figure 9*c*,*d***). Nevertheless, improving NMJ function may have therapeutic benefits for neuromuscular disorders. Administration of soluble agrin helps reverse sarcopenia-like phenotypes in neurotrypsin-overexpressing mice (159). Overexpressing agrin, MuSK, and Dok7 is beneficial for mouse models of SMA, ALS, Emery-Dreifuss muscular dystrophy, and MG (20, 160–162).

CONCLUSIONS

The NMJ is a chemical synapse that has been studied for more than a century. Classic research on the NMJ by neuroscience pioneers has contributed to our understanding of the structure and

function of synapses in the brain. This synapse continues to serve as a model for the molecular underpinning of synapse formation, maintenance, and disorders because of synaptic abnormality (synaptopathy). Although much has been learned about the molecular mechanisms of NMJ formation over the past decade, many questions remain. Various new enzymes and cytoskeletal proteins or regulators have been identified or implicated in NMJ formation or AChR clustering, but their roles appear to be modulatory, unlike those of key proteins in the agrin pathway, including agrin, Lrp4, MuSK, Dok7, and rapsyn. This may suggest that the pathway has to diverge at levels of Lrp4, MuSK, Dok7, or rapsyn to direct different aspects of NMJ development. A global nonbiased approach to identify MuSK substrates could provide new leads for future studies (163). The finding that rapsyn is an E3 ligase raises several questions. For example, what are its substrates? There could be many in light of its strategical location at the NMJ. In fact, numerous proteins have been shown to interact with rapsyn. Adding to the complexity is that rapsyn may catalyze sumoylation, monoubiquitination, polyubiquitination, and neddylation (**Figure 5*d***). A key to better understanding the mechanisms of action of the rapsyn E3 ligase is to identify its substrate proteins and the nature of the modifications. Other outstanding questions include how tSCs regulate muscles and nerve terminals at the molecular level; whether there is an agrin-like molecule for presynaptic differentiation; and how unnecessary synapses are eliminated. It is encouraging that NMJ studies are becoming more translational. Future studies of NMJ decline in aged animals and disorders of motoneurons, SCs, and muscles will contribute to better treatments of these conditions.

DISCLOSURE STATEMENT

L.M. and W.C.X. have a patent on the Lrp4 antibody (via Augusta University).

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