Molecular mechanisms underlying maturation and maintenance of the vertebrate neuromuscular junction

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The vertebrate neuromuscular junction (NMJ), a peripheral synapse formed between motoneuron and skeletal muscle, is characterized by a protracted postnatal period of maturation and life-long maintenance. In neuromuscular disorders such as congenital myasthenic syndromes (CMSs), disruptions of NMJ maturation and/or maintenance are frequently observed. In particular, defective neuromuscular transmission associated with structural and molecular abnormalities at the pre- and postsynaptic membranes, as well as at the synaptic cleft, has been reported in these patients. Here, we review recent advances in the understanding of molecular and cellular events that mediate NMJ maturation and maintenance. The underlying regulatory mechanisms, including key molecular regulators at the presynaptic nerve terminal, synaptic cleft, and postsynaptic muscle membrane, are discussed.

Introduction

Structural and functional maturation of established synapses during development controls the consolidation and refinement of neural circuits. For example, experience-dependent neuronal plasticity in the brain requires maturation and maintenance of a subset of newly formed synapses, while others are eliminated [1]. The peripheral synapse neuromuscular junction (NMJ) has long been used as a model system for studying the general principles of synapse development owing to its large size and experimental accessibility [2]. The vertebrate NMJ utilizes acetylcholine (ACh) as its neurotransmitter, which binds to ion-channel ACh receptors (AChRs) highly concentrated on the postsynaptic membrane. Notably, the NMJ goes through a unique series of maturation processes that differ from those of the central synapse [2,3]. First, whereas maturation of the central synapse normally occurs within hours, the NMJ takes weeks to refine its molecular and structural organization to achieve its mature form, which exhibits efficient neurotransmission. Furthermore, the stability of the NMJ increases on maturation, and the synaptic structure, once mature, persists throughout postnatal life, thus enabling life-long effective motor performance [2,3]. This is in contrast to the rapid turnover of central synapses; for example, mature dendritic spines (where excitatory synapses reside) can be rapidly eliminated and replaced by newly formed ones when neural circuits are reconstructed [1].

Abnormalities in NMJ maturation or maintenance are among the most common pathological causes of congenital myasthenic syndromes (CMSs), a heterogeneous group of hereditary neuromuscular diseases characterized by muscle weakness and a reduced safety margin for synaptic transmission [4]. Thus, delineation of the molecular mechanisms underlying NMJ maturation and maintenance is important for an understanding of the pathogenesis of CMSs and to provide significant insights for potential therapeutic approaches. Here we summarize the molecular and cellular events mediating NMJ maturation and maintenance, and review current knowledge on the regulation of these processes.

Structural and molecular changes during NMJ maturation and maintenance

Remarkable changes occur at the postsynaptic membrane during postnatal maturation of the NMJ [2,3]. First, alterations in AChR clusters take place at the levels of morphology, subunit composition and stability. The neonatal plaque-like AChR clusters with uniform receptor density are transformed into multi-perforated elaborate branches that display a pretzel-like shape (Figure 1a and Box 1) [5–7]. The embryonic-specific γ-subunit of AChR is replaced by the ε-subunit during early postnatal stage, leading to increased calcium conductance of the receptor [8,9]. The half-life of the synaptic AChRs increases on maturation, and insertion of newly synthesized AChRs and recycling of internalized AChRs contribute to maintenance of the high density of synaptic receptors [10–12]. Second, remodeling of AChR clusters is accompanied by a change in the topological organization of the postsynaptic membrane, which forms invaginations called junctional...
folds (Box 2). Finally, the postsynaptic molecular composition becomes specialized. A distinct set of postsynaptic proteins and signaling molecules is selectively transcribed in the subsynaptic myofiber nuclei and aggregates at high density in the postsynaptic membrane, accompanied by reorganization of the cytoskeleton at the postsynaptic submembrane.

Concurrent with the postsynaptic maturation, pronounced alterations also occur at motor nerve terminals, the synaptic cleft and terminal Schwann cells. Newborn polyinnervated NMJs transform into singly innervated ones through axon withdrawal during early postnatal weeks (Figure 1b) [13]. The axon terminals subsequently differentiate and become perfectly aligned with the AChR clusters, and synaptic vesicles loaded with readily releasable neurotransmitters are concentrated at the active zone. Furthermore, the molecular constituents of the extracellular matrix (ECM; the basal lamina) become specialized at the synaptic cleft during NMJ maturation [14]. Terminal Schwann cells, which cap the nerve terminals, retract from the synaptic cleft after NMJ formation and contribute to growth and long-term maintenance of the synapse [15].

**Presynaptic regulation during NMJ maturation and stabilization**

**Agrin: a master organizer of the NMJ**

Before the arrival of approaching motoneuron terminals, AChR clusters and postsynaptic-like structures are spontaneously formed at the center of muscle fibers, a process known as muscle prepatterning (Box 3) [16,17]. Although the initial formation of prepatterned structures is independent of nerve terminals, subsequent postsynaptic differentiation requires trans-synaptic activity. Nerve-derived agrin, a large heparin sulfate proteoglycan synthesized and released from the nerve terminal, is a master organizer that governs postsynaptic differentiation and stabilization [18]. Functional NMJ is absent in mice deficient in neural agrin [19]. The action of agrin on postsynaptic assembly and stabilization is primarily through activation of the muscle-specific kinase (MuSK) receptor on the muscle membrane [20]. Agrin also mediates MuSK-independent signaling through binding to dystroglycan, a transmembrane protein important for NMJ maturation and maintenance [21–24]. Furthermore, it has been suggested that proteolytic cleavage-mediated downregulation of agrin influences the development of junctional folds and the topological transformation of AChR clusters [25].

**The postsynaptic ubiquitin system and integrity regulate postsynaptic maturation**

Ubiquitin proteasome-dependent degradation of proteins is essential for controlling precise synaptic function through targeted protein degradation and clearance. Two ubiquitin-regulating proteins, ubiquitin speciﬁc peptidase 14 (Usp14) and ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), regulate the development of presynaptic terminals and postsynaptic maturation of the NMJ [26,27]. Presynaptic defects, including nerve terminal swelling and accumulation of phosphorylated neurofilament, and postsynaptic abnormalities, including defective plaque-to-prezetel transition of AChR clusters and imprecise synaptic apposition, are observed in mice lacking Usp14 or UCH-L1 [26,27]. Thus, maintenance of a proper ubiquitin pool in motoneurons is crucial for the development of presynaptic terminals and postsynaptic maturation of the NMJ.

The effect of presynaptic function on NMJ maturation is also evident in molecular pathological studies of spinal muscular atrophy (SMA), a motor neuron disease caused by mutations of the survival motor neuron 1 (SMN1) gene. In mouse models of SMA, impaired structure and function...
Box 2. Junctional fold: a unique postsynaptic structure of vertebrate NMJ

As the NMJ matures during the postnatal stage, the postsynaptic membrane sinks to form a gutter (the primary fold), and the gutter membrane invaginates to form a number of secondary folds (the junctional folds, Figure 1a) [2,3]. The junctional folds run parallel to each other, spaced at regular intervals, and are oriented orthogonal to the long axis of the muscle fiber (Figure 1b) [7]. Abnormalities of the fold architecture are the most common phenotypes associated with neuromuscular diseases, highlighting the junctional fold as a structural determinant for efficient neuromuscular transmission [140].

Here, we summarize several functional properties of junctional folds that are important for governing synaptic transmission and orchestrating postsynaptic signaling:

- AChRs are highly enriched (~10 000/μm²) at the crests of junctional folds, where ACh-releasing sites are in close proximity, whereas voltage-gated Na⁺ channels are concentrated in the depths of the folds. It has been suggested that this mutually exclusive arrangement of neurotransmitter receptors and Na⁺ channels amplifies the synaptic current by causing a voltage drop across the electrical resistance of the interfolds, and thus lowers the threshold for action potential generation [141].

- Different subdomains of the junctional folds serve as platforms for spatial segregation of postsynaptic receptors and membrane-associated proteins (Figure 1b,c) [142–144]. Furthermore, the constituents of the synaptic basal lamina (BL) are also segregated. Laminin α2, α5 and β2 are present at both the synaptic cleft and fold BL, whereas α4 only shows high levels between folds at the synaptic cleft BL [36].

Figure 1. Schematic illustrating development of the vertebrate NMJ, with a particular emphasis on junctional folds. (a) Formation of synaptic gutters and junctional folds during postnatal maturation of NMJ. P, postnatal day; Ad, adult. (b) An adult vertebrate NMJ. (c) Molecules expressed at the tops and troughs of adult junctional folds, as well as those expressed at the synaptic basal lamina. Such molecules include glycoproteins (e.g., laminins), receptors (e.g., MuSK and ErbB receptors), and scaffolding proteins (e.g., dystrophin, syntrophins, and utrophin).
of the NMJ are among the initial pathological signs that become evident during the first postnatal week [28,29]. Presynaptic abnormalities include decreased synaptic vesicle release and swollen nerve terminals with accumulation of neurofilament. Postsynaptic maturation is also impaired in these SMA mouse models, as indicated by the aberrant pretzel transition of AChR clusters and delayed switch of the fetal AChR γ-subunit to the adult ε-subunit. This finding provides evidence that the integrity of presynaptic structure and function is a determining factor for postsynaptic maturation.

ECM proteins at the synaptic cleft control both pre- and postsynaptic maturation

The synaptic basal lamina, composed of a distinct set of ECM proteins, extends through the synaptic cleft into the junctional folds. The synaptic basal lamina comprise four main types of ECM proteins: heparan sulfate proteoglycans (e.g., the neural agrin), laminins, collagens and nidogens [14]. As described above, neural agrin is an indispensable organizer for postsynaptic assembly. The other three core species, synthesized and deposited by muscle fibers, play important roles in the maturation and maintenance of both pre- and postsynaptic structures.

Three laminin heterotrimers, laminin 221 (α2β2γ1), 421 (α4β2γ1) and 521 (α5β2γ1), are present at the synaptic cleft [30]. Genetic deletion studies of different laminin chains revealed that synaptic laminins play multiple roles in NMJ maturation. First, synaptic laminins regulate the maturation of presynaptic terminals. Laminin β2 controls the proper distribution of active zones and synaptic vesicles through binding to voltage-gated calcium channels at the nerve terminal membrane [31–33]. Laminins also regulate differentiation of nerve terminals by inhibiting excessive outgrowth of terminal branches [30,34,35]. Second, synaptic laminins guide the development of junctional folds and the topological maturation of AChR clusters. Laminin-β2-null mice display markedly reduced junctional folds [35,36]. Studies on individual laminin heterotrimers show that laminin 421 and 521 are important for the plaque-to-pretzel transition of AChR clusters [36,37]. Third, synaptic laminins ensure the precise apposition of pre- and postsynaptic structures of the NMJ by preventing the intrusion of Schwann cell processes into the synaptic cleft [35]. Furthermore, laminin 421 is specifically required for the precise apposition of presynaptic active zones and openings of postsynaptic junctional folds [36]. In line with the morphological defects observed, electrophysiological studies revealed that laminin β2 mutant mice exhibit dramatically reduced evoked and spontaneous neuromuscular transmission from the first postnatal week onwards, providing direct evidence that synaptic laminins are crucial for NMJ maturation at the functional level [38]. It is interesting to note that deletion of individual synaptic laminin α chains results in relatively subtle but non-overlapping NMJ defects compared to the severe NMJ defects exhibited by laminin-β2-deficient mice. Indeed, different laminin α chains have distinct distribution patterns at the synaptic basal lamina, suggesting that the three laminin heterotrimers act collaboratively to coordinate the process of NMJ maturation [36].

Collagens comprise another group of core constituents of synaptic basal lamina. Collagen IV chains (α3–α6) accumulate at the mature NMJ and are important for maintenance of adult motoneuron terminals [32]. Collagen XIII, a transmembrane collagen concentrated at the postnatal NMJ, regulates topological maturation of AChR clusters and precise apposition of active zones and junctional folds [39]. Moreover, collagen Q (ColQ) is a synaptic collagen that binds to the ACh-hydrolyzing enzyme acetylcholinesterase (AChE) and regulates ACh accumulation at the synaptic basal lamina [40]. AChE deficiency due to ColQ mutations is commonly associated with CMSSs [4]. The synaptic anchorage of AChE by ColQ is partly dependent on the association of ColQ with MuSK receptor [40]. Reciprocally, ColQ influences MuSK-dependent AChR clustering [41]. Perlecan, a synaptic heparan sulfate proteoglycan linked to both ColQ and dystroglycan, is also important for synaptic recruitment of AChE [42].

Nidogen-2, the synaptic isoform of nidogen, is the third main group of synaptic ECM proteins. Nidogen-2 becomes concentrated at the NMJ from the second postnatal week, and is essential for topological maturation of AChR clusters and long-term postsynaptic maintenance [43].
maintenance of the postsynaptic apparatus. Agrin activates MuSK through binding to the MuSK co-receptor low-density lipoprotein receptor-related protein 4 (LRP4) [44,45]. Structural analysis has revealed that a tetrameric complex formed by two agrin–LRP4 heterodimers mediates MuSK dimerization and activation [46,47]. Activation of MuSK also requires its interaction with the cytoplasmic adaptor docking protein-7 (Dok-7) [48]. Like agrin-deficient mice, mice lacking either MuSK receptor, LRP4, or Dok-7 show a complete absence of postsynaptic NMJ structure and die perinatally because of respiratory failure [48–51]. The MuSK–LRP4–Dok-7 complex is also required for pre-patterning of AChR clusters (Box 3). It is noteworthy that CMSs caused by mutations in components of MuSK signaling can manifest after birth or later during postnatal development [4]. Thus, insufficiency or misregulation of MuSK signaling may exert a minimal effect on initial NMJ assembly, but mainly impacts subsequent synapse maturation or maintenance.

The role of MuSK in NMJ maintenance has been characterized in mice with conditional inactivation of MuSK during early postnatal stages [52]. AChR clusters disperse on postsynatal MuSK inactivation, and the mutant mice show severe muscle weakness and significantly shortened lifespan. Several mutations of MuSK that lead to reduced MuSK expression and impaired interaction with Dok-7 have been identified in CMS patients [53–55]. Reduced AChR clusters and simplified junctional folds are found in these patients, indicative of impaired NMJ maturation. A mouse model of CMS with mutations in MuSK (a missense mutation on one allele and deletion of the kinase domain on the other allele) shows progressive neuromuscular impairments during postnatal development, including fragmentation of AChR clusters with reduced receptor density, aberrant plaque-to-pretzel transition, simplified junctional folds, and defective neuromuscular transmission [54]. It has been suggested that both the kinase activity and the integrity of the extracellular domain of MuSK are important for the laminin-induced formation of topologically complex AChR clusters [56].

Several mechanisms that modulate the extent of MuSK activation are important for postsynaptic maintenance. First, association of MuSK with the adapter protein Dok-7 directly leads to activation of the receptor (see below). Tid1 (tumorous imaginal disc 1), a heat shock protein that binds to MuSK, promotes the MuSK–Dok-7 interaction and is important for AChR clustering [57]. Second, surface expression of MuSK is regulated by the postsynaptic ubiquitin–proteasome system or endocytic pathway. E3 ubiquitin ligase PDZ-domain-containing RING finger 3 (PDZRN3), which binds to MuSK and promotes its ubiquitination or degradation, is implicated in NMJ growth and maturation [58]. Furthermore, endocytosis of MuSK by N-ethylmaleimide-sensitive factor (NSF) is required for mediation of the effect of agrin–MuSK signaling [59]. Finally, MuSK activity can be modulated via its phosphorylation by cytoplasmic kinases, including serine/threonine kinase casein kinase 2 (CK2) and tyrosine kinase Abl [60,61].

Dok-7, a muscle adaptor protein associated with MuSK, acts as a co-activator of MuSK in both agrin-dependent and -independent manners [48,62]. Multiple mutations of Dok-7 have been reported in a major form of CMS that affects proximal muscle strength [63]. Smaller NMJ size and simplified junctional folds are two major abnormalities identified in these patients, although significant clinical heterogeneity is associated with Dok-7 mutations [4]. Dok-7 comprises an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain and a C-terminal region containing several tyrosine sites. Whereas Dok-7 binds to the juxtamembrane region of MuSK through its PTB domain, it self-dimerizes through its PH-PTB domain, and thus promotes dimerization and trans-autophosphorylation of MuSK [48,64]. Notably, mutations that truncate the C-terminal region of Dok-7 are the most common Dok-7 mutations found in CMSs, suggesting that the intact C-terminal domain is required for precise NMJ function in vivo. Agrin stimulation triggers phosphorylation of Dok-7 at two C-terminal tyrosine sites, leading to subsequent recruitment of adaptor proteins Crk and Crk-L, which are required for proper NMJ formation [65]. The C-terminus of Dok-7 is also required for agrin-independent transformation of AChR clusters into a pretzel shape on maturation [63]. The mechanism underlying the requirement of Dok-7 in this agrin-independent regulation awaits further characterization.

MuSK recruits a plethora of signaling molecules to guide both the aggregation and stabilization of AChR clusters [20]. Rapsyn, a membrane-associated cytoplasmic protein that directly interacts with AChRs, is indispensable for MuSK-induced AChR clustering and stabilization [49]. On agrin stimulation, the AChR–rapsyn interaction is strengthened, thereby enhancing the linkage of AChRs to the cytoskeleton [66]. Several rapsyn-interacting molecules, including heat shock protein 90 (HSP90), α-actinin and calpain, are important for stabilizing AChR clusters [66–68]. Rapsyn is also involved in agrin-stimulated targeting of AChRs to lipid rafts, which are membrane micro-domains serving as a platform for AChR clustering [69,70]. Moreover, MuSK activation leads to reorganization of the AChR-associated actin cytoskeleton through modulation of Rho family GTPases or their downstream molecules [71–74]. Caveolin-3, a component of caveolae lipid rafts, regulates MuSK-induced Rac1 activation and AChR clustering [75]. Interestingly, several components of the Wnt signaling pathway participate in MuSK-dependent AChR clustering, including β-catenin, dishevelled, and adenomatous polyposis coli (APC) [71,76,77].

A subset of molecules downstream of MuSK is specifically important for AChR stabilization. The non-receptor tyrosine kinase Src-family kinases (SFKs) phosphorylate the AChR β-subunit in response to agrin, which facilitates cytoskeletal anchorage and stabilization of AChR clusters [78]. Interestingly, SFK activity is not important for NMJ formation, but is required for postsynaptic maintenance and stabilization of AChR clusters [79,80]. It has been reported that SFKs enhance the association of postsynaptic components with cholesterol-rich lipid rafts of the postsynaptic membrane, which in turn maintain the phosphorylation of these components by SFKs [81].
Muscle erythroblastic leukemia viral oncogene homolog B (ErbB) receptors modulate AChR clustering and synapse maintenance

ErbB receptors, a group of receptor tyrosine kinases, are expressed at the postsynaptic muscle membrane and the surface of terminal Schwann cells. Neuregulins, the ligands for ErbB receptors, can be synthesized and released into the basal lamina by both motoneurons and muscles. Whereas Schwann cell ErbB receptors are essential for the survival and maintenance of Schwann cells [82], muscle ErbB receptors play modulatory roles in postsynaptic gene transcription and AChR clustering. Although early studies reported that neuregulin-activated ErbB signaling promotes the transcription of synaptic genes using in vitro cultured myotubes, later in vivo evidence confirmed that muscle ErbB receptors are dispensable for synaptic gene expression and synapse formation [83]. Nevertheless, muscle ErbB receptors are associated with the MuSK receptor via the adaptor protein Erbin, and it has been suggested that they potentiate MuSK-dependent AChR clustering [84,85]. Furthermore, muscle ErbB receptors regulate AChR stabilization and efficient neuromuscular transmission via phosphorylation of α-dystrobrevin-1, a cytoplasmic protein that is important for NMJ maturation and maintenance [86].

The neurotrophin receptors

The neurotrophins and their cognate receptors, which play multi-functional roles in synapse development, regulate postsynaptic maturation and maintenance of the NMJ. Tropomyosin receptor kinase B (TrkB)-mediated postsynaptic signaling is important for long-term stabilization of AChR clusters during postnatal life. Disruption of TrkB-mediated signaling in muscle or either early postnatal stages or adulthood by overexpression of a dominant-negative TrkB mutant causes disassembly of AChR clusters [87]. Furthermore, a precocious age-like decline in neuromuscular function including decreased muscle strength is evident in trkB+/- mice, which is associated with fragmentation of postsynaptic specializations in these mutants [88]. TrkB-dependent regulation of postsynaptic maintenance is probably mediated, at least in part, by its muscle-released ligand neurotrophin-4 (NT-4) in an autocrine fashion [89]. NT-4 and brain-derived neurotrophic factor (BDNF), another cognate ligand of TrkB, are also involved in regulating the maturation of presynaptic nerve terminals [90].

AChR clusters themselves are required for postsynaptic maintenance

A high synaptic density of AChRs is the hallmark of mature NMJs. Interestingly, AChRs themselves also play crucial roles in guiding synapse maturation and maintenance. First, replacement of the embryonic AChR γ-subunit by the postnatal ε-subunit not only increases the calcium permeability of the receptor but is also important for synaptic maintenance of AChR clusters and the development of junctional folds [91–93]. Second, phosphorylation of the AChR β-subunit by SFKs enhances the association of AChRs with rapsyn and regulates the integrity of AChR morphology and junctional folds [94,95]. Third, AChR clusters are important for the accumulation and maintenance of other synaptic regulators, including rapsyn and actin skeletal-associating proteins [96,97].

Molecules that regulate the postsynaptic cytoskeleton

The dystrophin–glycoprotein complex controls postsynaptic maturation and maintenance

The dystrophin–glycoprotein complex (DGC) is a multi-molecular complex linking AChR clusters to both the extracellular basal lamina and the intracellular cytoskeleton [98]. The main components of the DGC include a transmembrane module containing α- and β-dystroglycan, the associated cytoplasmic actin-binding proteins dystrophin and utrophin, and two groups of cytoplasmic proteins, α-dystrobrevins and syntrophins [98]. Some of the components are expressed along the entire muscle fiber, whereas others have synaptic isoforms that are present exclusively or concentrated at the NMJ. Whereas extrasynaptic DGC provides structural support for the integrity and stability of muscle fibers [99], synaptic DGC mainly contributes to postsynaptic maturation and long-term maintenance of the NMJ.

The dystroglycan module, containing extracellular α-dystroglycan, which binds to agrin and laminins, and its associated transmembrane β-dystroglycan, which binds to rapsyn and dystrophin/utrophin, is a key component that links other DGC proteins to the synaptic basal lamina and to AChR clusters. Dystroglycan is aggregated by synaptic laminins and acts as a laminin receptor to mediate laminin-dependent clustering and topological maturation of AChRs [37,100]. Moreover, dystroglycan participates in agrin-regulated AChR stabilization in a MuSK-independent manner. Dystroglycan-deficient myotubes fail to form stable AChR clusters in response to agrin, although the detailed mechanism of how agrin modulates the function of DGC is not clear [101,102].

Dystrophin and its synaptic homolog utrophin constitute a group of actin-binding proteins that interact with β-dystroglycan. Studies on mice with single or double mutation of dystrophin or utrophin have revealed that the two proteins play similar but non-overlapping roles in regulating AChR maintenance and junctional fold formation [102–105]. Deficiency of both dystrophin and utrophin leads to failed synaptic localization of two cytoplasmic DGC components, α-dystrobrevins and syntrophins, which are crucial downstream molecules in DGC signaling. α-Dystrobrevin-null mice exhibit fragmentation of AChR clusters and abnormal distribution of receptors to the troughs of junctional folds [102]. Two isoforms of α-dystrobrevin, α-dystrobrevin-1 and α-dystrobrevin-2, are present at the NMJ, whereas only α-dystrobrevin-1 is important for the synaptic function of α-dystrobrevins [106]. Tyrosine phosphorylation of α-dystrobrevin-1 at its C terminus by ErbB receptors is a regulatory mechanism for dystrobrevin-dependent stabilization of AChR clusters [86].

Two syntrophins, α- and β2-syntrophin, are present at the NMJ [107]. α-Syntrophin is indispensable for the maturation and stabilization of AChR clusters, as well as...
junctional fold development [108,109]. β2-Syntrophin plays additional roles in synaptic maintenance, because deficiency of both syntrophins results in more severe postsynaptic abnormalities than those observed in mice with a single α-syntrophin mutation [110]. Detailed analysis of AChR dynamics further revealed an increased turnover rate and a decreased recycling rate for receptors in α-syntrophin-null mice, leading to a reduction in synaptic AChR density [108]. Interestingly, this α-syntrophin-dependent regulation of AChR stability is mediated in part through the recruitment of α-dystrobrin-1 [108]. Neuronal nitric oxide synthase (nNOS), another molecule recruited by syntrophin, is also important for syntrophin-mediated signaling in synaptic maintenance [111,112].

**Cytoplasmic regulators for the actin cytoskeleton modulate plaque-to-pretzel transformation**

The aggregation, movement, stabilization and disassembly of AChR clusters are tightly controlled by the local dynamics of the postsynaptic membrane-associated cytoskeleton [113,114]. The use of aneurally cultured myotubes as an in vitro model to study postsynaptic NMJ maturation (Box 1) has begun to unravel the molecular mechanisms underlying cytoskeletal regulation during the plaque-to-pretzel transition of AChR clusters [115]. Concentration of F-actin with elevated turnover rate occurs at sites where perforations of AChRs subsequently occur, suggesting that elevated actin cytoskeletal dynamics guides the loss of AChRs [116,117]. Locally increased endocytosis may also play a role in the removal of AChRs [115–117]. Moreover, AChRs that are newly added to perforations are rapidly incorporated into existing AChR branches, indicating that AChR redistribution is another mechanism for maintaining previously formed perforations [117]. A group of actin-regulating proteins are present at perforations, among which actin-depolymerizing factor (ADF)/cofilin appears to be important for AChR dynamics [117]. Moreover, perforation-associated proteins form a podosome-like structure (a dynamic actin-rich, adhesive organelle) that is capable of remodeling ECM localization [116]. LL5β, a phosphatidylinositol-binding protein associated with podsomes, is implicated in the regulation of AChR clustering in cultured myotubes [116,118]. Thus, it would be interesting to further explore the in vivo function of podsomes in NMJ maturation.

Ephexin1, a guanine nucleotide exchange factor (GEF) of RhoA GTPase, has recently been identified as an essential actin regulator for both structural and functional maturation of the NMJ [119,120]. Whereas NMJ formation is normal in ephexin1-null mice, both the plaque-to-pretzel transition of AChR clusters and junctional fold development are impaired when ephexin1 is absent. Ephexin1 destabilizes AChR clusters through activation of RhoA, which is important for receptor loss during AChR perforation. Given the upregulation of ephexin1 activity by EphA receptors and cyclin-dependent kinase 5 (Cdk5) at central synapses [121], it would be of interest to investigate whether a similar mechanism underlies ephexin1-dependent postsynaptic maturation in the NMJ.

**Protein kinases and other synaptic regulators implicated in NMJ maturation and maintenance**

Two serine/threonine kinases, protein kinase C (PKC) and Ca2+/calmodulin-dependent protein kinase II (CaMKII), are involved in NMJ maturation and maintenance. PKC activity is important for the plaque-to-pretzel transition of AChRs and for motor axon withdrawal during synapse elimination [122]. CaMKII promotes the insertion of recycled AChRs into synaptic sites, and thus contributes to the maintenance of synaptic receptor density [123]. PKC and CaMKII are also implicated in suppression of the extrasynaptic expression of AChR subunits [124,125].

Neural cell adhesion molecule (NCAM) is a group of membrane-associated adhesion molecules concentrated at both pre- and postsynaptic sites and terminal Schwann cells. Whereas NMJ formation is normal in mice lacking the three synaptic isoforms of NCAM, presynaptic maturation, including synaptic vesicle cycling and neurotransmitter release, is disrupted in these mutants [126–128]. Moreover, delayed formation of junctional folds is found at the postsynaptic side of the mutant NMJ [129].

The P2X2 receptors are ligand-gated ion channels activated by extracellular ATP released by the nerve terminal. Mice lacking the P2X2 receptor subunit exhibit delayed plaque-to-pretzel transition, AChR fragmentation and reduced junctional folds [130].

**Synaptic transcription during NMJ maturation and maintenance**

Synapse-specific genes are locally transcribed at specialized nuclei located beneath motoneuron terminals (known as subsynaptic nuclei). A number of synaptic genes, including those encoding AChR subunits, utrophin and AChE, contain an Ets-binding promoter sequence (the N-box element) to target their synaptic transcription through binding to Ets transcription factors. A mutation of the N-box of the gene encoding AChRα is associated with CMSs, suggesting that N-box-directed subsynaptic transcription is important for NMJ development [131]. Erm (Ets-related molecule), an Ets transcription factor enriched postsynaptically, is important for the N-box-dependent subsynaptic transcription and maintenance of AChR clusters [132]. Another Ets transcription factor, growth-associated binding protein (GABP), is implicated in NMJ maturation, although it remains unclear whether GABP regulates subsynaptic transcription in vivo [133–136].

N-box-dependent subsynaptic transcription is activated by MuSK receptors through a Rac/Cdc42-mediated JNK pathway [137]. Interestingly, this MuSK–Rac/Cdc42–JNK pathway also upregulates the transcription of genes without the N-box element, such as musk [137]. Which transcription factors are involved in this N-box-independent transcription awaits further investigation. The neuregulin–ErbB pathway, although not directly acting on subsynaptic transcription, may have modulatory roles in MuSK-regulated synaptic gene expression.

**Concluding remarks**

Studies on the prototypic synapse NMJ have shed light on the general principles of synapse assembly. Nonetheless, the molecular mechanisms underlying NMJ maturation...
### Table 1. Molecules involved in NMJ maturation and maintenance

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<tr>
<td>MuSK</td>
<td>Aggregation, maintenance, PPT</td>
<td>CMS, myasthenia gravis</td>
<td>Activation of multiple signaling pathways</td>
<td>[20,50,53–56]</td>
</tr>
<tr>
<td>Dok-7</td>
<td>Aggregation, maintenance, PPT</td>
<td>CMS</td>
<td>MuSK</td>
<td>[48,62–65]</td>
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<td>Rapsyn</td>
<td>Aggregation, maintenance, PPT</td>
<td>CMS</td>
<td>Linking AChRs to cytoskeleton and membrane lipid rafts</td>
<td>[49,69]</td>
</tr>
<tr>
<td>SFKs</td>
<td>Maintenance</td>
<td></td>
<td></td>
<td>[78–80]</td>
</tr>
<tr>
<td>AChR β</td>
<td>Maintenance</td>
<td>CMS</td>
<td>Tyr phosphorylated of AChR β</td>
<td>[94,95]</td>
</tr>
<tr>
<td>AChR ε</td>
<td>Maintenance</td>
<td></td>
<td></td>
<td>[9,91–93]</td>
</tr>
<tr>
<td>TrkB</td>
<td>Maintenance</td>
<td>Presynaptic maturation</td>
<td></td>
<td>[87–90]</td>
</tr>
<tr>
<td>ErbB</td>
<td>Maintenance</td>
<td></td>
<td></td>
<td>[86]</td>
</tr>
<tr>
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<td>Maintenance</td>
<td>Synthetic apposition</td>
<td></td>
<td>[130]</td>
</tr>
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<td>Synaptic DGCb</td>
<td>Maintenance, PPT</td>
<td>Suppression of extrasynaptic gene transcription</td>
<td></td>
<td>Linking AChRs to the synaptic basal lamina and cytoskeleton [102–106, 108–110,112]</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Synthetic recycling</td>
<td>Suppression of extrasynaptic gene transcription</td>
<td></td>
<td>[123,124]</td>
</tr>
<tr>
<td>Ephexin1</td>
<td>PPT, destabilization</td>
<td>Synthetic apposition</td>
<td></td>
<td>RhoA activation [119]</td>
</tr>
<tr>
<td>ADF/cofilin</td>
<td>PPT, vesicular trafficking</td>
<td></td>
<td></td>
<td>Depolymerization of F-actin; regulated by 14-3-3ζ [117]</td>
</tr>
<tr>
<td>PKC</td>
<td>PPT</td>
<td>Suppression of extrasynaptic gene transcription</td>
<td></td>
<td>[122,125]</td>
</tr>
<tr>
<td>Erm</td>
<td>PPT, maintenance</td>
<td>AChR positioning at the muscle central band</td>
<td>Activation of subsynaptic transcription</td>
<td>[132]</td>
</tr>
<tr>
<td>GABP</td>
<td>PPT</td>
<td></td>
<td></td>
<td>[133,134]</td>
</tr>
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</table>

aAbbreviations: JFD, junctional fold development; PPT, plaque-to-pretzel transition.
bNCAM is expressed at nerve terminals, muscle membranes, and Schwann cells. Modulation of presynaptic maturation is more consistent with regulation by presynaptic NCAM [126–128].
cComponents of synaptic DGC include α- and β-dystroglycan, dystrophin/utrophin, α-dystrobrevin, α- and β2-syntrophin, and nNOS.
**Figure 2.** Postsynaptic maturation is regulated by positive and negative signaling pathways for AChR stabilization. Positive signaling pathways – including those activated by the MuSK receptor, the dystroglycan-glycoprotein complex, and ErbB and TrkB receptors – act on the assembly and maintenance of AChR clusters. CaMKII, a serine/threonine kinase activated by electrical activity, promotes insertion of AChRs into synaptic sites. Synapse-specific gene transcription, such as that mediated by the Ets transcription factor Erm, is important for AChR maintenance. By contrast, ephexin1-mediated upregulation of RhoA and activation of PKC and the actin modulator ADF/cofilin regulate AChR removal during plaque-to-pretzel transition. Cdk5-dependent dispersal of AChR clusters, enclosed in a dotted square, is a signaling pathway that has only been demonstrated in embryonic stages. In addition, dashed arrows indicate regulatory pathways established in vitro or in systems other than the NMJ. Abbreviations: Abl, v-abl Abelson murine leukemia viral oncogene homolog; ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; ADP, actin depolymerizing factor; APC, adenomatous polyposis coli; CaMKII, Ca2+/calmodulin-dependent kinase II; Cdk5, cyclin-dependent kinase 5; CK2, casein kinase 2; ColQ, collagen Q; DB, dystrobrevin; DG, dystroglycan; Dvl, dishevelled; Eph, erythropoietin-producing human hepatocellular carcinoma; ErbB, erythroblastoid leukemia viral oncogene homolog; Erm, Ets-related molecule; GABP, growth-associated binding protein; GGT, geranylgeranytransferase; JNK, c-Jun N-terminal kinase; LP4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; nNOS, neuronal nitric oxide synthase; NRG, neuregulin; NT-4, neurotrophin-4; NSF, N-ethylmaleimide sensitive factor; PDZRN, PDZ-domain-containing RING finger 3; PKC, protein kinase C; PTP, protein tyrosine phosphatase; SFKs, Src-family kinases; Tid1, tumorous imaginal disc 1; Trk, tropomyosin receptor kinase.

**Box 4. Outstanding questions**

- **What signaling pathways control the plaque-to-pretzel transition of AChR clusters?**
  - How are signals transduced from laminins to AChR maturation? The DGC transmembrane protein dystroglycan may act as a laminin receptor to regulate this process. However, most of the components of the DGC have more direct roles in the maintenance of synaptic receptors, and not in the plaque-to-pretzel transformation per se. The MuSK receptor is required for the formation of pretzel clusters induced by laminin-containing substrates [46,115]. It would be of interest to dissect whether laminin directly activates MuSK or indirectly activates MuSK via the regulation of other factors, such as Wnt or LRPs.
  - What signals initiate receptor loss within AChR plaques? An enigmatic issue is how selective regions of AChRs clusters are dispersed during receptor maturation. Cytoplasmic actin regulators, such as ephexin1 and ADF/cofilin, act as AChR-dispersing factors during pretzel formation. However, how these factors are activated is not clear. Another important question is whether AChR-dispersing factors and stabilizing factors have spatially differentiated regulation within AChR plaques.
  - What is the role of presynaptic input in the plaque-to-pretzel transition of AChR clusters? Although topologically complex AChR clusters can form independent of nerve innervation, agrin and normal presynaptic development influence AChR maturation physiologically. Their roles are worthy of further investigation.

- **What are the molecular mechanisms that underlie junctional fold formation?**
  - The structural integrity of the folds is tightly associated with the synaptic basal lamina and cytoskeleton network in subsynaptic areas, although how these molecules are regulated is not fully understood. The presynaptic input seems essential for the formation of junctional folds, because denervated muscles and aneurally cultured myotubes fail to develop folds [7,115]. Notably, forced expression of neural agrin in the extrasynaptic regions of innervated muscle fibers is sufficient to induce junctional fold-like membrane invaginations [160]. It will thus be important to elucidate the signaling pathways regulated by agrin or other trans-synaptic inputs that control junctional fold development.

- **What roles does muscle electrical activity play in NMJ development?**
  - Neurally evoked muscle electrical activity has been implicated in AChR subunit switch, in suppression of extrasynaptic gene transcription and in muscle development. However, the molecular events mediated by muscle activity remain elusive. Moreover, because numerous molecules regulate NMJ development, it is important to verify whether the roles of these molecules in NMJ development involve primary effects on NMJ structure and/or function, or secondary effects via modulation of muscle activity.
and maintenance are only beginning to be understood. We have reviewed molecular regulators at pre- and postsynaptic sites and the synaptonic cleft, discussed the regulation of postsynaptic maturation and maintenance (Table 1 and Figure 2), and summarized some outstanding questions that remain to be addressed (Box 4). It is noteworthy that there are other important molecular events during NMJ maturation and maintenance. These include the molecular mechanism by which synapse elimination is regulated [13], retrograde signals for presynaptic development [20], and the roles of terminal Schwann cells in NMJ maintenance [15]. A thorough understanding of these molecular mechanisms will potentially contribute to the development of targeted therapeutic approaches for CMSs.

Acknowledgments

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