To build a synapse: signaling pathways in neuromuscular junction assembly

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Summary
Synapses, as fundamental units of the neural circuitry, enable complex behaviors. The neuromuscular junction (NMJ) is a synapse type that forms between motoneurons and skeletal muscle fibers and that exhibits a high degree of subcellular specialization. Aided by genetic techniques and suitable animal models, studies in the past decade have brought significant progress in identifying NMJ components and assembly mechanisms. This review highlights recent advances in the study of NMJ development, focusing on signaling pathways that are activated by diffusible cues in the brain and contribute to a better understanding of muscular dystrophy.

Key words: Neural development, Neuromuscular junction, Retrograde signaling, Synapse formation

Introduction
The brain contains billions of nerve cells, or neurons, which receive and integrate signals from the environment, and which govern the body’s responses. Nervous system activity is made possible by synapses, contacts formed either between neurons or between a neuron and a target cell. Synapses are asymmetric structures in which neurotransmitter molecules are released from the presynaptic membrane and activate receptors on the postsynaptic membrane, thus establishing neuronal communication. As such, synapses are fundamental units of neural circuitry and enable complex behaviors. The neuromuscular junction (NMJ) is a type of synapse formed between motoneurons and skeletal muscle fibers. Large and easily accessed experimentally, this peripheral synapse has contributed greatly to the understanding of the general principles of synaptogenesis and to the development of potential therapeutic strategies for muscular disorders. The NMJ uses different neurotransmitters in different species; for example, acetylcholine (ACh) in vertebrates and glutamate in Drosophila, both of which are excitatory and cause muscle contraction. In Caenorhabditis elegans, there are two types of NMJs: at excitatory NMJs, ACh causes muscle contraction, whereas inhibitory NMJs release γ-aminobutyric acid (GABA) to cause muscle relaxation. Motor nerve terminals differentiate to form presynaptic active zones, where synaptic vesicles dock and release neurotransmitters. On the apposed postsynaptic membranes, neurotransmitter receptors are packed at high densities. Aided by genetic techniques and by the use of suitable animal models, including rodents, zebrafish, Drosophila and C. elegans, studies in the past decade have brought significant progress, not only in identifying components present in pre- and postsynaptic membranes, but also in understanding the mechanisms that underpin NMJ assembly. This review highlights recent advances in the study of NMJ development, focusing on signaling pathways that are activated by diffusible cues from motor nerves and muscle fibers. Readers are referred to other outstanding reviews for a broad view of NMJ development (see Froehner, 1993; Hall and Sanes, 1993; Kummer et al., 2006; Salpeter and Loring, 1985; Schaeffer et al., 2001).

NMJ formation
A chicken-and-egg problem: motoneurons and muscle fibers
A fundamental riddle in NMJ assembly is whether the motoneurons or the muscle fibers determine where and how NMJs are formed. In mouse aneural muscle fibers, ACh receptors (AChRs) are initially evenly distributed and subsequently accumulate in the middle, where innervation occurs; this happens, for example, between embryonic day 12.5 (E12.5) and E13.5 in the diaphragm (Bevan and Steinbach, 1977; Braithwaite and Harris, 1979; Creazzo and Sohal, 1983; Ziskind-Conhaim and Bennett, 1982). In vitro studies of synapse formation indicated, however, that spinal neuron axons ignore such pre-existing, primitive AChR clusters on co-cultured muscle fibers and instead form synapses at new locations (Anderson and Cohen, 1977), which indicates a dominant role for motoneurons in determining where NMJs are formed. Careful in vivo studies revealed, however, that primitive AChR clusters are located in the central region of muscle fibers prior to the arrival of motoneuron axons (Lin et al., 2001; Yang et al., 2001) (Fig. 1A). This phenomenon, called prepatterning, appears to be nerve independent, as it also occurs in mutant mice that lack phrenic or motor nerves (Yang et al., 2000). At E13.5, nerve terminals overlap some but not all, AChR clusters in the middle region of muscle fibers, and at E18.5 innervated clusters are enlarged, whereas primitive clusters have disappeared in both synaptic and extrasynaptic regions (Lin et al., 2001; Vock et al., 2008; Yang et al., 2001). These findings indicate that muscle fibers might play an active role in NMJ formation, and that some of the aneural, primitive AChR clusters are modified to form large, nerve-induced clusters (reviewed by Kummer et al., 2006) (Fig. 1A).

Aneural AChR clusters mark axon guidance activity and agrin responsiveness
Recent studies challenge the importance of aneural AChR clusters for postsynaptic differentiation. When rodent embryonic diaphragms are cultured in vitro, primary myotubes form synapses in regions without aneural AChR clusters (Lin et al., 2008). Mouse embryos that lack a certain AChR subunit do not form aneural AChR clusters, but are able to form neural AChR clusters at later stages, although these are distributed more broadly (Liu et al., 2008). Studies of NMJ formation in zebrafish, a model system that allows aneural and neural AChR cluster formation to be separated genetically (Jing et al., 2009), reached similar conclusions. Zebras
aneural clusters are formed on the medial surface of adaxial muscle cells prior to the arrival of motoneuron growth cones (Fig. 1B) (Flanagan-Steet et al., 2005; Panzer et al., 2005; Zhang et al., 2004). As growth cones arrive, adaxial muscle fibers migrate radially to the lateral surface and are replaced by fast muscle fibers. Motor axons innervate fast fibers to form neural clusters precisely where aneural clusters used to be. The muscle-specific receptor tyrosine kinase Musk/unplugged is required for both aneural and neural AChR cluster formation (Zhang et al., 2004). The induction of Musk/unplugged expression after prepatterning unexpectedly rescued neural AChR clusters on a null mutation background: zebrafish embryos formed normal NMJs and were fully motile (Jing et al., 2009). These observations suggest that NMJs can form in the absence of prepatterned AChRs. However, at least in zebrafish, this axon guidance activity apparently does not require neural AChR clusters per se, because fish with mutations in the gene encoding rapsyn, an intracellular scaffold protein that interacts with and aggregates AChRs (Burden et al., 1983; Gautam et al., 1995; LaRochelle and Froehner, 1986), lack prepatterned AChRs, but exhibit normal axon pathway finding (Zhang et al., 2004). Second, mouse embryonic diaphragms form AChR clusters confined to the central region in response to agrin, a motoneuron-derived factor (see below) (Lin et al., 2008), which suggests that the innervation of the central muscle region might result from a spatially restricted responsiveness to agrin. Thus, aneural AChR clusters seem to mark the middle region of muscle fibers, which itself is important for guiding motoneuron growth cones and for the responsiveness to neural agrin through as-yet unknown mechanisms.

It is worth noting that fundamental species differences exist in NMJ formation. For example, in rodents, primitive aneural and neural AChR clusters form on the same muscle fibers (Fig. 1A).
Motoneuron, muscle fiber and glial cell interactions
NMJ formation appears to require interactions among motoneurons, skeletal muscle fibers and glial cells. Factors released from motoneurons control postsynaptic differentiation directly by stimulating receptors on muscle cells or indirectly by promoting glial cell differentiation and function. In *Drosophila*, glutamate receptors preferentially cluster opposite to sites of high glutamate release, suggesting a role for glutamate in synaptic receptor clustering (Marrus and DiAntonio, 2004). However, an increase in extracellular glutamate appears to suppress receptor clustering at synapses through constitutive desensitization (Augustin et al., 2007). In rodents, muscle depolarization suppresses AChR subunit gene transcription and increases AChR degradation (Salpeter et al., 1986) (reviewed by Schaeffler et al., 2001). Furthermore, AChR clusters grow faster and larger in mutant mice that lack choline acetyltransferase (Chat), an enzyme that is crucial for ACh biosynthesis (Brandon et al., 2003; Misgeld et al., 2002), suggesting that ACh might negatively regulate aneural AChR clustering. Thus, muscle activity inhibits the three key mechanisms that contribute to the high density of AChRs at rodent NMJs (Fig. 2). Downstream mechanisms include activation of the serine/threonine kinase cyclin-dependent kinase 5 (Cdk5) or of Ca$^{2+}$/calmodulin-dependent protein kinase II (Camk2α – Mouse Genome Informatics) (Fu et al., 2005; Lin et al., 2005; Tang et al., 2001). The negative effect of ACh is global because ACh-mediated activation affects the entire muscle fiber; the high AChR density at the NMJ probably results from motoneuron-derived positive signals counteracting the inhibitory effect (Fig. 2). One such factor, agrin, is discussed in detail in the next section.

The importance of glial cells for NMJ formation is only beginning to be appreciated. A recent study demonstrates that muscle fibers express neurotrophin 3 to modulate the number of Schwann cells, the myelinating glial cells of the peripheral nervous system, in developing NMJs (Hess et al., 2007). Motoneurons release neuregulin 1 to promote Schwann cell survival and development (Hayworth et al., 2006; Trachtenberg and Thompson, 1996) (reviewed by Fischbach and Rosen, 1997; Lemke, 1993). Mice lacking neuregulin 1 or its receptors Erb2 or Erbb3 lack Schwann cells (Lin et al., 2000; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999; Wolpowitz et al., 2000), and their motoneurons form transient synapses with muscle fibers that fail to be maintained, indicating a crucial role for Schwann cells in NMJ formation and maintenance. Although the mechanisms involved remain unclear, experimental evidence indicates several possibilities. For example, Schwann cells might guide motoneuron growth cones (Reddy et al., 2003), as they sprout and guide nerve terminal regeneration after nerve injury (Reynolds and Woolf, 1992; Son and Thompson, 1995a; Son and Thompson, 1995b). They have also been shown to generate diffusible signals, one of which might be transforming growth factor β (TGFβ) (Feng and Ko, 2008), to promote NMJ development or function (Cao and Ko, 2007). This is similar to astrocytes, which regulate CNS synaptogenesis through diffusible factors (Christopherson et al., 2005). In *Drosophila*, glial cells have been shown to release axotactin, a neurexin-related molecule, to control the electrical properties of target axons and...
Intriguingly, Ruegg and Bixby, 1998). These observations demonstrate a mutation in the gene encoding agrin (Bezakova et al., 2001; Gesemann et al., 1995; Herbst and Burden, 1998). Agrin–/– mice lack NMJs, and Agrin is sufficient to induce ectopic AChR clusters in adult muscles (Jones et al., 1997) and to elicit the formation of a postsynaptic apparatus in denervated muscles (Bezakova et al., 2001; Gesemann et al., 1995; Herbst and Burden, 2000; Jones et al., 1997). Importantly, mice that carry a mutation in the gene encoding agrin (agrin–/) lack NMJs, and synaptic proteins, including AChRs, are distributed throughout the mutant muscle fibers (Gautam et al., 1996) (reviewed by Ruegg et al., 1992). Agrin is sufficient to induce ectopic AChR clusters in adult muscles (Jones et al., 1997) and to elicit the formation of a postsynaptic apparatus in denervated muscles (Bezakova et al., 2001; Gesemann et al., 1995; Herbst and Burden, 2000; Jones et al., 1997). Importantly, mice that carry a mutation in the gene encoding agrin (agrin–/) lack NMJs, and synaptic proteins, including AChRs, are distributed throughout the mutant muscle fibers (Gautam et al., 1996) (reviewed by Ruegg and Bixby, 1998). These observations demonstrate a pivotal role for agrin in AChR clustering and NMJ formation. Intriguingly, agrin– mice are able to form aneural AChR clusters prior to innervation, which suggests that agrin is not essential for prepatterning (Lin et al., 2001; Yang et al., 2001). Muscle fibers and Schwann cells also produce agrin, but neural agrin is 1000-fold more effective in stimulating AChR clustering because it contains certain key splice inserts at the C terminus (Gesemann et al., 1995; Reist et al., 1992).

**Musk as master organizer of NMJ development**

Musk was discovered owing to its abundance in the synapse-rich Torpedo electric organ (Jennings et al., 1993) and co-localizes with AChRs at NMJs (Valenzuela et al., 1995). In Musk–/– mice, muscle fibers form neither aneural clusters nor prepatterns prior to innervation, and no NMJs are formed either (DeChiara et al., 1996; Lin et al., 2001; Yang et al., 2001). Instead, AChRs are unevenly distributed along Musk–/– muscle fibers, suggesting a crucial role of Musk for both prepatterning and nerve-induced AChR clusters. Indeed, neuronal agrin is unable to induce AChR clusters in Musk–/– muscle cells (Glass et al., 1996), but agrin sensitivity can be restored through expressing wild-type Musk (Herbst and Burden, 2000; Zhou et al., 1999). Thus, muscle fiber prepatterning requires Musk, but not agrin, whereas the formation of nerve-induced AChR clusters and NMJs requires both.

Correspondingly, ectopic Musk expression stimulates synapse formation in the absence of agrin and rescues the lethality of mutations in the gene that encodes agrin (Kim and Burden, 2008). Finally, motoneuron terminals become highly branched and innervate a broader region in Musk–/– mice, suggesting that Musk plays a role in presynaptic differentiation (DeChiara et al., 1996; Lin et al., 2001; Yang et al., 2001).

As a receptor tyrosine kinase, it is not surprising that Musk interacts with a plethora of proteins that regulate its activity or activate downstream pathways (see below; see also Fig. 3). Interestingly, however, Musk also associates with scaffold proteins implicated in NMJ assembly, the regulation of gene expression and nuclear location (see Box 2). Together, these observations suggest that Musk might form a signalosome crucial for NMJ formation (reviewed by Luo et al., 2003a).
**Lrp4 as an agrin co-receptor**

Although agrin and Musk are essential for NMJ formation, the two proteins do not interact directly (Glass et al., 1996). How signals are transmitted from agrin to Musk has, until recently, been a fundamental gap in the understanding of NMJ formation. A hypothetical molecule, myotube-associated specificity component (MASC), was proposed to serve as an agrin receptor (Glass et al., 1996), but despite extensive studies, its identity remained unknown until recently.

Genetic studies of digit development indicated that the single-pass transmembrane protein low-density lipoprotein receptor-related protein 4 (Lrp4) is required for NMJ formation, as well as for the development of limbs, lungs, kidneys and ectodermal organs (Johnson et al., 2005; Simon-Chazottes et al., 2006; Weatherbee et al., 2006). Mice that lack Lrp4 die at birth with NMJ defects that resemble those of *Musk*<sup>−/−</sup> mutant mice (Weatherbee et al., 2006). Recently, two independent studies reported that Lrp4 is an agrin co-receptor (Kim et al., 2008; Zhang et al., 2008). Lrp4 binds selectively to neural agrin (Kim et al., 2008; Zhang et al., 2008); this interaction is of a high affinity and direct (Zhang et al., 2008). Moreover, Lrp4 is necessary for agrin-induced Musk activation and AChR clustering in cultured muscle cells, and is sufficient to reconstitute Musk signaling in non-muscle cells (Kim et al., 2008; Zhang et al., 2008). Furthermore, Lrp4 is expressed specifically in myotubes and is concentrated at the NMJ (Zhang et al., 2008). These findings indicate that Lrp4 is probably the missing link that couples agrin to Musk activation.

Lrp4 is a member of the low-density lipoprotein receptor (LDLR) family. It contains a large extracellular N-terminal region, a single transmembrane domain and a short C-terminal region without an identifiable catalytic domain (Johnson et al., 2005; Lu Y et al., 2007; Tian et al., 2006; Yamaguchi et al., 2006). How Lrp4 regulates Musk activity remains unknown. Intriguingly, Lrp4 self-associates (Kim et al., 2008) and can also interact with Musk through the extracellular domains of the two proteins (Kim et al., 2008; Zhang et al., 2008), in agreement with an earlier finding of binding activity on the myotube surface for the Musk extracellular domain as involved in AChR clustering (Wang, Q. et al., 2008). Therefore, Lrp4 could function in basal Musk activation in the absence of agrin, as well as in agrin-induced activation. Observations that Lrp4 co-expression alone is able to increase Musk activity support this notion (Kim et al., 2008; Zhang et al., 2008). In addition, the Lrp4 intracellular domain becomes tyrosine phosphorylated in agrin-stimulated myotubes (Zhang et al., 2008) and contains a typical NPxY motif and five additional tyrosine residues that may interact with phospho-tyrosine binding (PTB) or Src homology 2 (SH2) domains. In the related proteins Ldfr, Lrp1 and Lrp2, the NPxY motif serves as a docking site for adapter proteins (reviewed by Herz and Bock, 2002). The juxtamembrane domain of
Musk appears to be a master organizer of postsynaptic development at the NMJ. Mice with mutations in the gene that encodes Musk have deficiencies in forming primitive AChR clusters or prepatterned muscle fibers, and they do not form nerve-induced AChR clusters or NMJs. Evidence suggests that Musk does not only act as a receptor and tyrosine kinase for agrin, which initiates pathways leading to postsynaptic differentiation (see Fig. 3). By interacting with additional proteins, of which a growing number is being identified, Musk might also serve as a scaffold organizer that is crucial for compartmentalized signaling. Based on their function, Musk-interacting proteins can be classified into four groups (see figure).

The first group (orange) is necessary for Musk activity or downstream signaling. The second group (purple) controls agrin/Musk signaling. The function of the proteins in these two groups is discussed in Fig. 3. By interacting with additional proteins, of which a growing number is being identified, Musk might also serve as a scaffold organizer that is crucial for compartmentalized signaling. Based on their function, Musk-interacting proteins can be classified into four groups (see figure).

The third group (blue) consists of scaffold proteins, including rapsyn (Antolik et al., 2006; Apel et al., 1997), ColQ [a protein for acetylcholinesterase (AChE) enrichment in the synaptic cleft (Cartaud et al., 2004)], the MAGUK protein MAGI-1c (Strochlic et al., 2001) and AChR (Fuhrer et al., 1997). The fourth group (pink) includes proteins that might regulate gene expression, including 14-3-3-γ, a protein thought to regulate synaptic gene expression at the NMJ (Strochlic et al., 2004), and synaptic nuclear envelope 1 (Syne1), a nuclear envelope protein enriched in synaptic nuclei (Apel et al., 2000). This interaction was thought to help anchor synaptic nuclei in the synaptic region of NMJs, but although muscle nuclei in both synaptic and non-synaptic regions are disorganized in Syne1-null mutant mice, their NMJs are apparently normal (Zhang X. et al., 2007). These results indicate that the proper position of synaptic nuclei might not be as crucial as previously thought. It is worth pointing out that, unless otherwise discussed, the suggested functions of many of the Musk-interacting proteins have not been tested in vivo. See text for details.

Musk contains a similar motif (Y553) that becomes tyrosine phosphorylated in response to agrin stimulation and that is necessary for agrin-induced AChR clustering (Adams et al., 1995; Herbst and Burden, 2000; Zhou et al., 1999). Its interaction with the PTB domain of the adapter protein downstream-of-tyrosine-kinase-7 (Dok7) is required for Musk activation and downstream signaling (Okada et al., 2006). Mice that carry a mutation in Dok7 lack NMJs, and mutant muscle cells do not form AChR clusters in response to agrin, whereas forced expression of Dok7 activates Musk and induces aneural AChR clusters, indicating that Dok7 is also able to activate Musk in the absence of agrin (Okada et al., 2006). It would be interesting to investigate whether Lrp4 interacts with Dok7, and whether this interaction is crucial for agrin function.

Taken together, Lrp4 could mediate or regulate Musk signaling in three different ways: through maintaining basal activity by direct interaction; through serving as an agrin receptor; or through transducing signals via its intracellular domain.

**Agrin/Lrp4/Musk signaling**

Recent studies have shed light on the mechanisms controlling the agrin signaling pathway (Fig. 3). As discussed above, Musk activity is regulated by Dok7 and Lrp4. Musk also interacts with tumorous imaginal discs (Tid1; also known as Dnaja3), which is necessary for Dok7 binding to Musk in response to agrin (Linnola et al., 2008). Upon activation, Musk becomes rapidly internalized, which is required for AChR clustering (Zhu et al., 2008). This ligand-dependent endocytosis is regulated by the ATPase N-ethylmaleimide sensitive fusion protein, which interacts directly with Musk (Fig. 3; see also Box 2). Endocytosed Musk might also undergo proteasomal degradation, probably mediated by the E3 ligases putative Ariadne-like ubiquitin ligase (Paul) and PDZ domain containing RING finger 3 (Pdzrn3) (Bromann et al., 2004; Lu, Z. et al., 2007). Thus, agrin-Musk signaling is tightly controlled.

The signals that lead from Musk activation to AChR clustering have been extensively investigated in cultured muscle cells and in Xenopus neuron-muscle co-culture, and several pathways have been identified (Fig. 3).

First, AChR redistribution and anchoring are thought to involve cytoskeletal reorganization (Bloch, 1986; Dai et al., 2000), and numerous studies have investigated how Musk activation might trigger this. The underlying mechanism probably involves the tyrosine kinase Abl (Finn et al., 2003) and the metalloenzyme geranylgeranylation transferase 1 (GGT), which activates GT-Pases by prenylation (Luo et al., 2003b); both interact with Musk (see also Box 2). Endocytosed Musk might also undergo proteasomal degradation, probably mediated by the E3 ligases putative Ariadne-like ubiquitin ligase (Paul) and PDZ domain containing RING finger 3 (Pdzrn3) (Bromann et al., 2004; Lu, Z. et al., 2007). Thus, agrin-Musk signaling is tightly controlled.

One target of the activated Rho GT-Pases is the serine/threonine kinase Pak1, which is associated with Musk through Dvl (Luo et al., 2002). Pak1 might regulate actin dynamics by phosphorylating cortactin (Webb et al., 2006), an actin-binding protein present at developing NMJs (Peng et al., 1997). Pak1 also suppresses myosin light chain kinase (MLCK) and thus reduces the phosphorylation of myosin light chains (MLCs) and the association of MLCs with actin filaments. In addition, Pak1 activates LIM kinase, which phosphorylates and inhibits actin depolymerizing factor (ADF)/cofilin (Edwards et al., 1999;
Soosairajah et al., 2005); this, in turn, regulates actin-dependent vesicular AChR trafficking to the postsynaptic membrane (Lee et al., 2009). Other targets of Rho GTPases include Wiskott-Aldrich syndrome protein (WASP) family proteins, which activate the actin-related protein 2 and 3 (Arp2/3) complex (reviewed by Millard et al., 2004) (Fig. 3), and Rho-associated protein kinase (ROCK), which increases MLC phosphorylation.

Second, rapsyn interacts with AChRs (Burden et al., 1983; Sealock et al., 1984) and is essential for aneural and neural AChR clusters both in vivo and in vitro (Apel et al., 1995; Gautam et al., 1996; Glass et al., 1984). Rapsyn has recently been shown to interact with α-actinin and β-catenin (Dobbins et al., 2008; Zhang, B. et al., 2007) (Fig. 3). α-Actinin is an actin crosslinker whereas, in this context, β-catenin is thought to regulate α-catenin-dependent actin polymerization. Suppressing the expression of either protein inhibits agrin-induced AChR clustering. Agrin regulates rapsyn function in at least two ways. First, it stimulates the interaction of rapsyn with surface AChRs (Moransard et al., 2003) and with α-actinin (Dobbins et al., 2008), and could thus lead to AChR clustering. Second, rapsyn is an extremely unstable protein (its half-life is ~6 hours in muscle cells) (Luo et al., 2008). Its interaction with the molecular chaperone heat-shock protein 90β (Hsp90β), which is enhanced by agrin, could prevent it from being degraded at the synapse (Luo et al., 2008). Intriguingly, Hsp90β has been implicated in cross-linking branched actin filaments (Park et al., 2007), establishing another link between AChR clustering and cytoskeletal dynamics.

Third, agrin induces the association of AChRs with adenomatous polyposis coli (Apc), which is necessary for AChR clustering (Wang et al., 2003) (Fig. 3). Apc is crucial for cell polarity and migration, and can bind directly to either actin filaments or microtubules (Moseley et al., 2007), or indirectly to microtubules via end-binding protein 1 (Eb1). Apc also associates with the GEF Asef and the IQ-motif-containing GTPase activation protein 1 (Gtap1) (Kawasaki et al., 2000; Watanabe et al., 2004) and might recruit them into the proximity of AChR clusters, contributing to cytoskeletal reorganization.

Fourth, agrin stimulates AChR tyrosine phosphorylation and might thus stabilize AChR clusters (Fig. 3; Box 2). This process appears to involve Src homologous collagen D (ShcD; also known as Shc4) (Jones et al., 2007) and several kinases, including Src family kinases (Borges and Ferns, 2001; Mittaud et al., 2001; Mohamed et al., 2001) and casein kinase 2 (CK2) (Cheusova et al., 2006). Mutant mice that lack these kinases form morphologically normal NMJs, but AChR clusters become unstable (Cheusova et al., 2006; Smith et al., 2001).

Finally, agrin enhances the interaction of rapsyn with calpain, an enzyme that is involved in Cdk5 activation, and thus inhibits calpain activity (Chen et al., 2007) (Fig. 3). Considering the synaptic localization of rapsyn, this result suggests that it acts locally to inhibit Cdk5 activity and thus counteract ACh-mediated AChR cluster dispersal.

Another exciting area of recent progress regarding the signaling pathways that regulate NMJ formation is the role of Wnt ligands, which is discussed next.

**Wnt signaling in NMJ development**

Wnts are a family of secreted glycoproteins that regulate diverse cellular processes, including cell proliferation and fate determination, cell polarity and movement, and programmed cell death through several intracellular pathways (see Box 3). A function for Wnt signaling in the regulation of synaptogenesis was first discovered in the developing rodent cerebellum, where Wnt7a is used by granule cells as a retrograde signal for axon and growth cone remodeling (Hall et al., 2000). Recent studies in various species provide converging evidence for a pivotal role of Wnt signaling in NMJ development.

**Wnts in the invertebrate NMJ**

In *Drosophila* NMJs, motoneurons secrete Wnt ligands that are necessary for both pre- and postsynaptic differentiation (Liebl et al., 2008; Mathew et al., 2005; Packard et al., 2002) (Fig. 4A). Loss-of-function mutations in the *Drosophila* Wnt gene *wingless* (*wg*) reduce the number of synaptic boutons and disrupt synaptic organization (Packard et al., 2002). In response to *Wg* stimulation, the *Drosophila* Wnt receptor *Fz2* is endocytosed postsynaptically and transported to the perinuclear area to be cleaved. Its C-terminal fragment is translocated into the nucleus through a mechanism that requires the adaptor protein Grip (Ataman et al., 2006; Mathew et al., 2005). This Frizzled nuclear import (FNI) pathway is thought to regulate the formation or stabilization of synapses via transcriptional regulation (Fig. 4A). A recent study reports that mutations in the genes encoding Wnt5 and Derailed (an atypical receptor tyrosine kinase) also reduce the number of synaptic boutons (Liebl et al., 2008). Cell type-specific rescue experiments suggest that Wnt5 is secreted by motoneurons and activates Derailed, which is located at the surface of muscle cells, to drive postsynaptic differentiation (Fig. 4A).

*Wg* also activates Fz on the presynaptic membrane to direct postsynaptic differentiation. The disruption of Armadillo (*Drosophila* homolog of β-catenin) and Pangolin (*Drosophila* homolog of TCF) has no significant effect on synaptic phenotypes (Miech et al., 2008), which suggests a limited role of the canonical Armadillo/Pangolin-dependent pathway in this process. Instead, the actions of Fz are probably mediated by the inhibition of Shaggy (*Drosophila* homolog of Gsk3β), a substrate of which is Futsch [(*Drosophila* ortholog of microtubule associated protein 1B (MAP1B))] (Franciscovich et al., 2008; Franco et al., 2004; Gogel et al., 2006; Roos et al., 2000) (Fig. 4A). Together, these studies indicate that Wnts might serve as both anterograde and retrograde signals to promote NMJ formation in *Drosophila*. In addition, Wnt signaling also controls target specificity by preventing synapse formation on nontarget, neighboring muscle cells (Inaki et al., 2007).

In *C. elegans*, Wnt signaling determines where axons form synapses by inhibiting NMJ formation. The DA9 motoneuron is located near the ventral midline, and its axon first projects posteriorly, then turns towards the dorsal region and ultimately projects anteriorly. Interestingly, it does not form ‘en passant’ synapses until it reaches the dorsal, anteriormost region of the worm (Fig. 4B). This antisynaptic effect is mediated by LIN-44/Wnt, secreted in a posterior-anterior gradient by four hypodermal cells in the worm tail (Klassen and Shen, 2007), which appears to localize LIN-17/Fz to the synaptic region of the axon to inhibit presynaptic assembly via Dvl.

**Wnt acts as a Musk ligand in vertebrates**

The phenotypes of Lrp4 and Musk mutant mice are more severe than those of agrin mutant animals. In particular, prepatterning and aneural AChR clusters disappear in Lrp4 and Musk mutant mice, but not in agrin mutants, which suggests the existence of a pathway that requires Musk and Lrp4, but not agrin. Vertebrate Musk is known to have a cysteine-rich domain (CRD) that shows homology to a crucial Wnt-binding domain of the Wnt receptor Fz (Valenzuela et al., 1995) (Box 3). It has been hypothesized that Musk binds and presents Wnts to motor axons to initiate NMJ remodeling (Hall et al., 2000). Recent studies in various species provide converging evidence for a pivotal role of Wnt signaling in NMJ development.
formation (reviewed by Burden, 2000). However, no experimental evidence supported a role for Wnts or Wnt signaling components in vertebrate NMJ formation, not until Luo and colleagues showed that Musk interacts with Dvl and that disrupting Dvl function causes both pre- and postsynaptic defects (Luo et al., 2002) (Fig. 5). Subsequently, Apc and β-catenin have been implicated in AChR clustering in vitro and/or in NMJ formation in vivo (Li et al., 2008; Wang et al., 2003). Moreover, as discussed above, Musk associates with the LDLR-family member Lrp4 (Kim et al., 2008; Zhang et al., 2008). Other members of the LDLR family, such as Lrp5 and Lrp6, interact with Fz and are crucial for Wnt function (Fig. 5). Based on these observations, it was speculated that Wnt could bind directly to Musk and could regulate Musk activity (Zhang et al., 2008). Indeed, a recent study indicates that the CRD domain of zebrafish Musk (unplugged) interacts with Wnt1r, and subsequent Dvl-dependent signaling is implicated in the formation of aneural AChR clusters and in guiding motor axons to form NMJs (Jing et al., 2009). Mouse Wnt11, which shows homology to zebrafish Wnt11r, is expressed in both the spinal cord and in skeletal muscles and can interact with the Musk extracellular domain (B. Zhang and L.M., unpublished). These exciting observations lead us to propose a working model for signaling events in postsynaptic assembly (Fig. 5). According to this model, Wnt binds to and activates Musk prior to innervation, when neural agrin is absent, and the resulting Wnt-Musk signaling regulates axon guidance and aneural cluster formation. After innervation, the Wnt-Musk complex interacts with the agrin/Lrp4/Musk pathway to regulate agrin-induced AChR clustering (see Fig. 3). In both scenarios, Wnts, via the Frizzled-Lrp5/Lrp6 complex, initiate both canonical and non-canonical pathways to regulate pre- and postsynaptic differentiation.

This model predicts that Wnt might be a Musk ligand in rodents prior to innervation and that it can also regulate innervation-induced AChR clustering (Fig. 5). As there are 19 different Wnt molecules in mice and humans, the regulation of NMJ formation by Wnts in these species is probably complex. The expression of Wnt1, Wnt4, Wnt6 or Wnt7b in mouse muscle cells had no significant effect on basal and agrin-induced AChR clustering (Luo et al., 2002; Zhang, B. et al., 2007). Two recent studies reported opposite effects of Wnt on AChR clustering in cultured mouse muscle C2C12 cells. In one study, Wnt5a was shown to inhibit agrin-induced AChR clustering by suppressing rapsyn expression via β-catenin-dependent signaling (Wang, J. et al., 2008). By contrast, the other study showed that Wnt3 induces AChR microclusters and promotes agrin-induced clustering, with the latter effect apparently mediated by a non-canonical pathway that requires Rac1 (Henriquez et al., 2008). Moreover Wnt signaling could increase Musk expression (Kim et al., 2003). These results indicate that the functions of Wnts and Wnt signaling components in mammalian NMJ formation are probably diverse and deserve systematic investigation.

In summary, emerging evidence supports diverse roles for Wnt ligands and signaling molecules in NMJ development in various species, including C. elegans, Drosophila, zebrafish and rodents. Some Wnts appear to promote, whereas others inhibit synapse formation, and they also differ in the intracellular responses they
trigger. Novel non-canonical signaling mechanisms have been identified as being important in NMJ assembly, such as the FNI pathway and the direct interaction between Wnt and MusK. Is Wnt binding sufficient to activate MusK? Does it initiate the same or a distinct cascade from that triggered by agrin binding to Lrp4? If the cascades are the same, how is differential input integrated; if they are distinct, how do they interact with each other? Is there cross-talk between the newly identified Wnt signaling mechanisms and the canonical and non-canonical pathways that are initiated by Wnt interacting with Fz and Lrp5/Lrp6? More work is necessary to answer these questions and to assemble a complete picture from the available and emerging puzzle pieces.

**Retrograde signals for presynaptic development**

After discussing how nerve-derived factors regulate postsynaptic development, we now address a question that has been studied for more than a century: how does muscle control presynaptic differentiation? In the 1930s, Viktor Hamburger observed hypoplasia in the spinal cord after the removal of a limb bud, which is now known to be caused by motoneuron apoptosis (Hamburger, 1934). This finding provided the initial evidence for the existence of a target-derived signal and led to the discovery of the nerve growth factor (NGF) family of neurotrophic factors (or neurotrophins) (Levi-Montalcini, 1987). Neurotrophins, however, appear to have only a limited role in motoneuron survival or differentiation (Lu and Je, 2003). For example, muscle-specific ablation of brain-derived neurotrophic factor (Bdnf) has no detectable effect on NMJ morphology or function (X. P. Dong and L.M., unpublished). Here, we consider additional retrograde factors that are implicated in NMJ formation, such as TGFβ, fibroblast growth factor (FGF), glial cell line-derived neurotrophic factor (Gdnf), and proteins dependent on muscle, β-catenin or β1 integrin.

**TGFβ**

The TGFβ family is a large family of proteins that includes TGFβ, bone morphogenetic proteins (BMPs) and activins. These proteins stimulate type I and type II serine/threonine-kinase receptors (TbRI and TbRII) to regulate Smad-dependent transcription and non-Smad downstream components (Derynck and Zhang, 2003) (Box 4). TGFβ signaling is pivotal in neural development, and genetic studies in *Drosophila* indicate that TGFβ signaling regulates NMJ development. Mutations in the genes that encode the TGFβ ligand Glass bottom boat (Gbb), the type I receptors Thickveins (Tkv) and Saxophone (Sax), the type II receptor Wishful thinking (Wit) and Smad transcription factors all result in presynaptic defects: reduced number of NMJs; disrupted T-bars (presynaptic high density areas where synaptic vesicles assemble); and impaired neurotransmitter release (Marques et al., 2002; McCabe et al., 2004; McCabe et al., 2003; Rawson et al., 2003). Moreover, postsynaptic markers, such as Discs large (Dlg) and glutamate receptors (GluRs), appear to be normal in Wit mutants, and NMJ phenotypes could be specifically rescued by the transgenic expression of Wit in motoneurons (Aberle et al., 2002). These observations provide evidence for the idea that Gbb acts as a retrograde signal from muscle tissue that is crucial for presynaptic development (Fig. 6A), although a recent study has suggested a postsynaptic mechanism (Dudu et al., 2006). The function of TGFβ signaling in *C. elegans* NMJ formation remains unclear, and the role of TIG-2, the *C. elegans* ortholog of *Drosophila* Gbb, has not yet been investigated.

In mice, all three isoforms of TGFβ are expressed in motoneurons, muscle and Schwann cells (Jiang et al., 2000; McLennan and Koishi, 2002), but there is no evidence for TGFβ being a retrograde signal in mouse NMJ formation. Instead, TGFβ causes Schwann cell apoptosis during development (Awatramani et al., 2002; Paterson et al., 2001) and might thus regulate NMJ formation indirectly. *Tgfb1-*
null mutant mice die prematurely owing to defects in vasculogenesis and angiogenesis or to wasting syndrome (Kulkarni and Karlsson, 1993; Shull et al., 1992), but whether NMJ development is impaired is unknown. Tgfb2-null mutant newborn mice are unable to breathe and die in cyanosis soon after birth; however, the morphology and function of their NMJs is grossly normal (Heupel et al., 2008). Their neonatal death is probably due to the aberrant transmission of signals from the respiratory center in the brain. A recent study suggests that TGFβ1 might act as a Schwann cell-derived factor to promote NMJ formation in Xenopus, probably by increasing agrin expression in motoneurons (Feng and Ko, 2008), highlighting the complexity of the cellular and molecular interactions in this relatively simple structure.

FGF, laminin and collagen

Screens for a synaptic vesicle clustering activity in cultured chick motoneurons led to the identification of novel proteins involved in presynaptic differentiation: fibroblast growth factors (FGFs) and signal regulatory proteins (SIRPs) isolated from mouse brains, and collagen from the electric organ of marine rays (Umemori et al., 2004; Umemori and Sanes, 2008) (Fig. 6B). Careful genetic studies of mutant mice suggest that the FGF family members Fgf22, Fgf7 and Fgf10 might be involved in the induction of synaptic vesicle clustering, but not in the maturation or maintenance of nerve terminals (Fox et al., 2007).

The extracellular matrix (ECM) plays an important role in NMJ formation. Once released from motoneurons, agrin is concentrated in the ECM of the synaptic cleft. In Drosophila, the N-glycosaminoglycan-binding protein Mind the gap (Mtg) is synthesized in motoneurons and subsequently deposited in the ECM to regulate the extracellular distribution of certain lectin-binding glycanas, as well as the localization of postsynaptic integrin receptors (Rushton et al., 2009). The ECM also appears to be key in presynaptic differentiation. Early stages of vesicle clustering are promoted by collagen α2 (IV), a collagen isoform present throughout the basalmila of adult muscle fibers (Fox et al., 2007). However, the maturation and maintenance of nerve terminals do not require collagen α2, but instead depend on laminin β2 and on the synaptic collagens α3 and α6 (IV) (Fox et al., 2007; Nishimune et al., 2004; Nishimune et al., 2008; Noakes et al., 1995). How these collagens act remains elusive, but laminin β2 probably binds directly to and clusters the P/Q-type calcium channels that flank active zones, which in turn recruit other presynaptic components (Nishimune et al., 2004). This hypothesis is supported by studies in mice that lack either laminin β2 or P/Q-type calcium channels, in which active zones form initially but cannot be maintained (Fig. 6B). Moreover, the disruption of the laminin β2-calcium channel interaction in vivo reduces active zones. These results support a model according to which muscle fibers release multiple factors to orchestrate presynaptic development.

Gdnf

Gdnf is one of the most potent factors for motoneuron survival in vitro (Oppenheim et al., 1995). It is expressed in muscle cells, whereas its receptor Ret tyrosine kinase is expressed in motoneurons (Baudet et al., 2008) (Fig. 6B). Treating frog neuron-muscle co-cultures with Gdnf increases the frequency as well as the amplitude of spontaneous synaptic currents (Wang et al., 2002), which suggests that it might serve as a retrograde factor. Indeed, the conditional ablation of Ret in mouse cranial motoneurons leads to a severely compromised maturation of presynaptic terminals (Baudet et al., 2008), and the number of endplates is also reduced. In addition, Gdnf overexpression in Myo-Gdnf transgenic mice or Gdnf injection causes multiple innervation and slows the process of synapse elimination (Keller-Peck et al., 2001; Nguyen et al., 1998). Together, these observations suggest that Gdnf might be a muscle-derived factor that regulates presynaptic differentiation.

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Muscle β-catenin- and β1 integrin-dependent retrograde signals

Recent genetic studies have identified novel retrograde pathways that direct presynaptic differentiation in mice. Luo and co-workers found that the inhibition of Dvl function in muscle cells not only attenuates AChR clustering, but also reduces the frequency of spontaneous synaptic currents in neuromuscular synapses in culture, which indicates that a retrograde signal downstream of muscle Dvl is necessary for NMJ formation (Luo et al., 2002). NMJ defects in Dvl1 mutant mice are mild, probably as a result of the redundant function of two other Dvl isoforms (Henriquez et al., 2008) (Q. Wang and L.M., unpublished). To overcome this redundancy, Li and colleagues studied the role of β-catenin, which is downstream of Dvl (see Box 3), in NMJ formation in vivo (Li et al., 2008). β-catenin expression was specifically suppressed in skeletal muscles to avoid embryonic lethality. Mutant mice died soon after birth with considerable presynaptic defects. The primary branches of phrenic nerves were no longer located in the central region of diaphragm muscle fibers. Secondary branches were extended to innervate larger AChR clusters, which are distributed in a wider area in the central region. Moreover, spontaneous and evoked neurotransmitter release were reduced (Li et al., 2008). By contrast, the NMJ appeared morphologically and functionally normal in motoneuron-specific β-catenin-deficient mice.

These observations provide convincing evidence that muscle, but not neuronal, β-catenin is crucial for NMJ formation, and in particular for presynaptic differentiation or function. The absence of NMJ defects in motoneuron-specific β-catenin-deficient mice indicates that β-catenin-dependent hemophilic adhesion is dispensable. In light of the function of β-catenin in controlling gene transcription, a role for muscle β-catenin in regulating the expression of a signal that is required for NMJ formation seems most likely (Fig. 6B). Intriguingly, whether this signal is downstream of Wnt, and what signaling pathway it triggers, remains to be investigated. Either way, it is anticipated that a powerful array of genes that are differentially expressed in the muscles of muscle-specific β-catenin mutant mice compared with in wild-type mice will identify such a signal.

In addition to binding to P/Q calcium channels, laminin β2 also activates the ECM receptor β1 integrin, which increases AChR clustering in the absence of agrin, suggesting that this mechanism is involved in aneural AChR cluster formation and prepatterning. However, in mice that lack β1 integrin, muscle fibers are prepatterned, and muscle cells form AChR clusters in response to agrin that are indistinguishable from those of wild-type control animals (Schwander et al., 2004), indicating that the laminin β2/β1 integrin pathway is dispensable for prepatterning (Fig. 6B). However, in muscle-specific β1 integrin mutant mice, which die soon after birth, motoneurons branch excessively and fail to terminate at the muscle midline, demonstrating a crucial role for muscle β1 integrin in presynaptic development, whereas its tissue-specific mutation in motoneurons does not cause obvious neuromuscular phenotypes (Schwander et al., 2004). These observations suggest a retrograde signal downstream of muscle β1 integrin that is necessary for presynaptic development. Whether this process requires laminin β2 remains to be investigated. Notably, however, this signal appears to be different from the one that is β-catenin-dependent because the two mutants show distinct presynaptic phenotypes.

Conclusions

In the past few years, much has been learned about how nerves control NMJ formation. Numerous muscle-derived retrograde signals that direct presynaptic differentiation have been identified. With an increasing number of presynaptic structural and functional proteins identified (reviewed by Jin and Garner, 2008), we anticipate that future work will reveal more about the mechanisms by which retrograde signals direct presynaptic differentiation. We now know more about pathways leading to postsynaptic differentiation. At present, however, very little is known about the signals that pass from Schwann cells to muscle cells or motoneurons in NMJ formation. This area of research seems set for rapid growth because of the increasing understanding of Schwann cell development and the availability of Schwann cell-specific markers and genetic tools.

It is worth noting that many new players in AChR clustering have been characterized in cultured muscle cells, and that their roles in NMJ formation are yet to be verified in vivo. This raises the question of whether ‘AChR clustering’, a cell-biological phenomenon observed in cultured muscle cells, is relevant to NMJ formation. AChR concentration at the postjunctional folds is thought to be mediated by AChR diffusing on muscle membrane (Edwards and Frisch, 1976). Yet, the area of postjunctional folds accounts for less than 0.1% of the entire muscle fiber surface area (reviewed by Burden et al., 1995). It would therefore seem more economical if synaptic proteins, including AChR, were synthesized locally or delivered in a more efficient manner, such as by endocytosis.
Fig. 6. Retrograde mechanisms in NMJ formation. (A) In Drosophila, muscle cells release TGFβ/Gbb for both post- and presynaptic assembly. Wnt released from presynaptic neurons activates the Frizzled-dependent pathway for presynaptic differentiation. (B) In rodents, muscle fibers release Gdnf, which activates the receptor tyrosine kinase Ret for presynaptic differentiation. FGF, synaptic laminin and synaptic collagens orchestrate the complex temporal control of presynaptic differentiation. The interaction of laminin β2 with P/Q type calcium channels might be required for nerve terminal maturation. Muscle fibers also regulate presynaptic differentiation via a pathway that requires muscle- but not motoneuron-generated β-catenin or β1 integrin.

(Akaaboune et al., 1999; Bruneau et al., 2005). Indeed, AChR mRNA is enriched at the NMJ, and the transcription of genes encoding synaptic proteins is active in synaptic, but not in extrasynaptic, nuclei (Merlie and Sanes, 1985) (reviewed by Schaeffer et al., 2001). This transcription was thought to be mediated by neuregulin 1 (reviewed by Fischbach and Rosen, 1997), but recent evidence suggests that neuregulin 1 regulates NMJ formation indirectly by promoting Schwann cell differentiation (Escher et al., 2005). The question then becomes: what signals direct synapse-specific transcription? This awaits further investigation. Interestingly, the mRNA-binding proteins Nanos and Pumilio have recently been found to regulate glutamate receptor expression and thus NMJ development in Drosophila (Menon et al., 2009). Finally, unlike intracellular scaffolds, less is known about the extracellular counterparts. Yet, some proteins essential for NMJ formation or function, in particular the ACh hydrolase AChE, are enriched in synaptic basal lamina in the synaptic cleft. This localization appears to be mediated by a mechanism distinct from AChR clustering (Cartaud et al., 2004; Peng et al., 1999). Mice lacking perlecan form normal AChR clusters, but lack AChE counterparts (Arikawa-Hirasawa et al., 2002). How AChE localization correlates to pre- and postsynaptic differentiation remains unknown. AChR clustering at the C. elegans NMJ requires its interaction with the extracellular region of LEV-10, a transmembrane protein that contains an LDLR domain, and LEV-9, a secreted complement-control-related protein (Gendrel et al., 2009).

Although our review focuses on the molecular mechanisms of diffusible signals, this does not mean that non-diffusible molecules are less important; NMJ assembly might also be regulated by cell-contact-dependent mechanisms. Motoneuron neurite-muscle adhesion increases for a few minutes after synaptic contact (Evers et al., 1989). Direct interactions between nerve terminals and muscle fibers might be mediated by adhesion molecules, including neural cell adhesion molecule [NCAM (Polo-Parada et al., 2004)], CD24 [a glycosylphosphatidylinositol (GPI)-linked protein (Jevesek et al., 2006)], the immunoglobulin proteins Syg1 and Syg2 (Shen and Bargmann, 2003; Shen et al., 2004), and embigin (Lain et al., 2009). However, the mechanisms by which NMJ development is regulated by cell adhesion remain to be elucidated.

Finally, recent studies have identified several genes the mutation of which leads to NMJ development defects, including the neuron-specific splicing factors Nova1 and Nova2 (Ruggiu et al., 2009), amyloid precursor protein (APP) (Wang et al., 2005), dystrophin-associated proteins (Adams et al., 2004; Banks et al., 2002; Grady et al., 2003; Grady et al., 2003), the glycosyltransferase Large (Herbst et al., 2009), the protein degradation components Fbxo45, Ned44, Usp14 and Uch11 (Chen et al., 2009; Chen et al., 2010; Liu et al., 2009; Saiga et al., 2009), the chromatin organization protein HP1 (Aucott et al., 2008) and meltrin β, a metalloprotease (Yimoto et al., 2008). These studies demonstrate that the complexity involved in the formation of this simple, large peripheral synapse is only beginning to be unravelled. For example, the NMJ defects in Nova1 and Nova2 mutant mice cannot be rescued by overexpressing neuronal agrin in motoneurons (Ruggiu et al., 2009), suggesting the existence of additional neuronal factors.

Mutations in and/or autoimmune reactions to some proteins essential for NMJ development cause muscular dystrophies, including myasthenia gravis and congenital myasthenic syndrome (reviewed by Engel et al., 2008). Studies of NMJ formation could identify potential culprits and therapeutic targets for these disorders. Finally, many, if not all, of the molecules involved in NMJ formation
are expressed in the brain, including agrin, Lrp4 and β1 integrin, as well as Wnt and its downstream signaling components. Further studies of NMJ assembly are therefore also likely to shed light on the mechanisms of synaptogenesis in the brain.

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Competing interests statement

The authors declare no competing financial interests.

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