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Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost

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Studies of the vertebrate skeletal neuromuscular junction led to an influential model of how neurotransmitter receptors accumulate in the postsynaptic membrane. In this model, motor axons organize postsynaptic development by secreting neuregulin to induce acetylcholine receptor gene transcription in specialized subsynaptic nuclei, agrin to cluster diffuse receptors in the postsynaptic membrane, and acetylcholine to evoke electrical activity that promotes synaptic maturation. However, new studies in this area have first, demonstrated that axons sometimes innervate pre-existing receptor clusters; second, recast the roles of agrin and neuregulin; third, revealed early effects of neurotransmission; fourth, questioned the role of subsynaptic myonuclei; fifth, shown that elaborately-branched postsynaptic structures can form aneurally; and sixth, raised the possibility that neurotransmitter affects receptor type as well as distribution. These recent studies challenge the widely-held paradigms, although not the results that led to them, and suggest a new model for neuromuscular synaptogenesis.

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Introduction

The vertebrate skeletal neuromuscular junction (NMJ) has been widely used for analyses of synaptic structure, function and development, owing to its large size, accessibility and relative simplicity. Developmental studies, on which we focus here, have led to the formulation of a widely accepted model for how the postsynaptic membrane is assembled. According to this model, ingrowing motor axons secrete two factors, agrin and neuregulin, that induce the local synthesis and aggregation of neurotransmitter receptors (nicotinic acetylcholine receptors, AChRs) beneath presynaptic axon terminals. These early steps occur in an activity-independent fashion, whereas neurotransmission plays modulatory roles during synapse maturation [1,2].

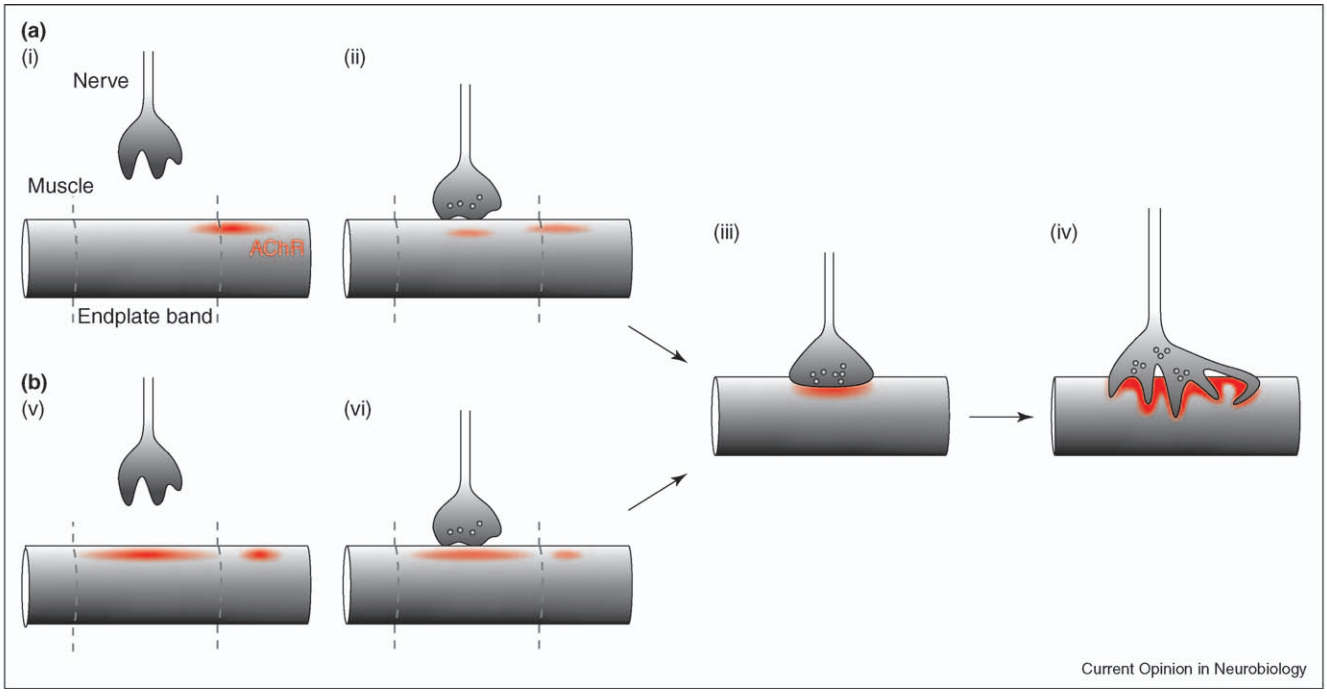
This paradigm has been influential. Several molecules initially characterized at the NMJ were subsequently implicated in formation of central synapses [3,4], and many investigations of synapse formation in the brain have been based on cellular and molecular mechanisms established at the NMJ [5,6]. Around 2000, however, several reports appeared that raised questions about the general roles of the nerve in postsynaptic development and the specific mechanisms by which agrin and neuregulin function. During the past year, a series of studies has begun to provide answers to these questions. Here, we summarize conventional views, discuss recent research that calls them into question, and propose a new model that accounts for both early and recent observations.

The paradigm

When myoblasts fuse to form myotubes, several synaptic genes, including those encoding AChR subunits, are activated. AChR subunits are produced, assembled into pentamers, and inserted in the myotube membrane where they reach a density of $\sim$1000/μm^2 (in rodents). In adults, by contrast, AChRs are concentrated at high density (10 000–20 000/μm^2) in the tiny fraction of synaptic muscle fiber membrane juxtaposed to the nerve terminal, whereas the remaining membrane (>99%) bears <10 AChRs/μm^2 [1,2]. Studies carried out in the 1970s on myotubes cultured aneurally showed that although some AChRs were diffusely distributed, others aggregated spontaneously into high-density clusters termed 'hot spots' [7,8]. This distribution suggested that ingrowing axons might seek out hot spots on the myotubes and incorporate them into NMJs. Surprisingly, when neurons were added to such cultures, their neurites ignored pre-existing hot spots. Instead, new AChR clusters formed at sites of nerve–muscle contact, and aneural hot spots dispersed [9,10]. Thus, although muscle cells can cluster AChRs (and many other components of the postsynaptic apparatus [2]) on their own, synapse formation *in vitro* entails nerve-directed assembly of postsynaptic sites (Figure 1a). An initial test of this model *in vivo* supported its conclusions, showing that AChR clusters in zebrafish form only in association with motor axons [11].

Further studies *in vitro* showed that motor neurons can both redistribute AChRs in the membrane and induce AChR synthesis [9,12,13]. These findings spurred a search during the 1980s for nerve-derived factors

Figure 1



Previous (a) and new (b) views of neuromuscular synaptogenesis. **(a)(i)** Numerous *in vitro* and *in vivo* observations suggested that nerves initiate postsynaptic development, rather than innervating spontaneously formed AChR clusters (red). The central location of the intramuscular nerve thus explained the localization of NMJs to the central 'endplate band'. **(ii)** In this model, spontaneous AChR clusters subsequently disappear and **(iii)** nerve-induced clusters grow and **(iv)** topologically mature as directed by the developing nerve terminal arbor, thus ensuring pre- and post-synaptic matching. **(b)(v)** New observations reviewed here suggest that muscles 'pre-pattern' AChRs independently of the ingrowing nerve. **(vi)** At least in some cases the nerve incorporates initially aneural clusters into NMJs, (iii) dispersing uninnervated clusters and remodeling innervated clusters to refine pre- and postsynaptic alignment. (iv) Formation of pretzel-shaped postsynaptic sites can occur nerve-independently *in vitro*, suggesting that some aspects of synaptic maturation might also be muscle-driven.

with these activities [14,15]. Several active factors were isolated from nervous system extracts [16], of which two have commanded the most attention. The first, agrin, organizes AChRs and other postsynaptic proteins into aggregates on cultured myotubes without immediately enhancing AChR production [17,18]. The second, neuregulin, induces the synthesis of new AChRs [19–21].

In the 1990s, gain- and loss-of-function studies bolstered the hypothesis that agrin and neuregulin stimulate postsynaptic development *in vivo*. In support of the function of agrin, AChRs and associated synaptic proteins cluster in extrasynaptic regions of muscles induced to express neural agrin [22,23]. Conversely, targeted mutation of the agrin gene dramatically impairs postsynaptic differentiation [24]. This result was difficult to interpret in that all three cells of the NMJ — neuron, muscle and Schwann cell — synthesize agrin. Neurons, however, produce an alternatively spliced isoform (called A^+ in chicks and Z^+ in mammals) that clusters AChRs *in vitro* ~1000 times more potently than muscle or Schwann cell-derived variants (A^- and Z^- agrin) [25,26]. Moreover, targeted mutation of just the Z^+ isoform, leaving the Z^- isoform intact, led to defects as severe as those seen in null mutants [27,28]. Additional genetic analysis demonstrated that neural agrin signals through a synaptically localized muscle-specific kinase (MuSK) and that rapsyn, a cytoplasmic protein, is essential for clustering of AChRs [29,30].

Neuregulin receptors, the ErbB receptor tyrosine kinases, are also concentrated in the postsynaptic membrane [31]. Genetic analysis of neuregulin proved difficult as mutants lacking neuregulin or ErbBs died early in embryogenesis because of cardiac defects. Synaptic AChR density was, however, decreased ~50% in neuregulin heterozygotes, and these animals were mildly myasthenic [32]. Independent studies revealed that genes encoding several postsynaptic components, including AChRs, are selectively activated in the few myonuclei lying directly beneath NMJs termed 'subsynaptic nuclei' [33–35]. Innervation (*in vivo*) and neuregulin (*in vitro*) enhanced transcription via identical regulatory elements in AChR subunit gene enhancers [36,37]. Thus, nerve-derived neuregulin appeared to promote AChR production through a local transcriptional effect.

Studies *in vivo* and *in vitro* showed that initial aspects of NMJ formation can occur in the absence of neurotrans-

mission or electrical activity [9,10,38], whereas activity regulates later aspects of synaptic maturation and remodeling. For example, AChR lifetime increases from less than one day to greater than one week as the NMJ matures postnatally [39]. Activity blockade reverses this process, whereas direct electrical stimulation mimics it [40]. Electrical activity is also responsible for down-regulating AChR levels extrasynaptically once the postsynaptic membrane has formed [39,41]. These results supported the view that early aspects of synaptic connectivity are activity-independent, whereas synaptic maintenance and maturation require activity [42,43].

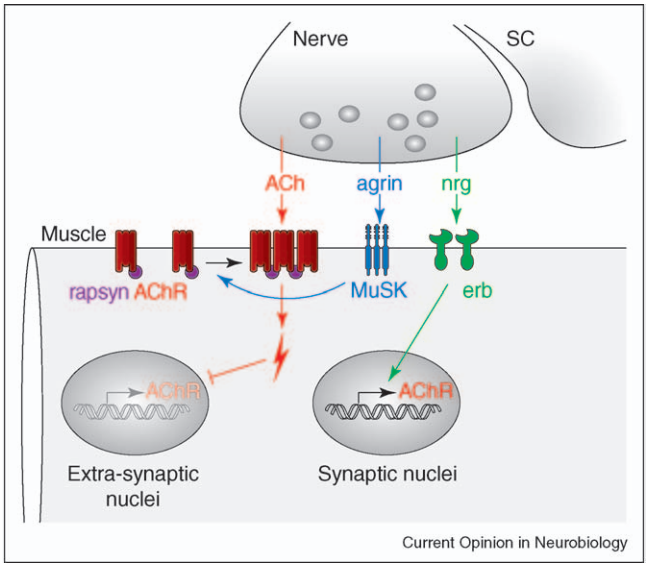
One striking aspect of postnatal NMJ maturation is the transformation of the initially plaque-shaped AChR cluster into an elaborate, pretzel-shaped array of branches in perfect register with the branches of the nerve terminal [1]. Denervation halts this transformation [44], and pretzel-shaped AChR aggregates were never observed in the absence of innervation *in vivo* or *in vitro*. Therefore, it appeared that neuronal activity was required for the topological maturation of the synapse.

On the basis of these studies a picture of neuromuscular synaptogenesis emerged in which axons released two localized signals to drive postsynaptic differentiation beneath their terminals: agrin to reorganize synaptic proteins in the plane of the membrane, and neuregulin to stimulate transcription in subsynaptic nuclei. These initial steps assemble a rudimentary postsynaptic apparatus, which is then remodeled by activity-dependent processes (Figure 2). Extensions of these processes maintain precise nerve–muscle apposition as axon branches pattern the maturing postsynaptic apparatus (see Figure 1).

Challenges to the paradigm

Despite the impressive amount of evidence in support of the model presented above, several reports appearing around 2000 called it into question. First, AChR aggregates formed, persisted and even grouped in a central endplate band of muscles that had been genetically rendered aneural [28,45–47]. Similar results were initially reported earlier [48], but methods available at the time could not rule out transient axon contact with muscle. Second, postsynaptic sites were present transiently in agrin mutant embryos, although they dispersed before birth [28]. Third, nuclei associated with AChR clusters became transcriptionally specialized in aneural muscles, casting doubt on the idea that nuclear specialization is a consequence of innervation [28,45]. Fourth, to enable neuregulin or erbB mutants to survive until birth, several groups generated mice in which cardiac defects were rescued [49–51], or neuregulin isoforms were selectively excised [52]. In all such analyses, neuromuscular development was disrupted but postsynaptic defects were surprisingly mild.

Figure 2



A molecular model of postsynaptic development at the NMJ, circa 1999. On the basis of numerous biochemical and genetic experiments, a core pathway of AChR clustering was formulated whereby neurally-released agrin (blue) binds MuSK to stimulate co-clustering of AChRs (red) and rapsyn (violet). Neuregulin signals via erb receptor kinases (green) to induce transcription of AChR and other synapse-specific mRNAs in subsynaptic nuclei. ACh-induced action potentials (red flash) repress extra-synaptic transcription of the same genes. SC, Schwann cell.

What, then, are the roles of pre-existing AChR clusters, neurotransmission, agrin, neuregulin, and synaptic nuclei? We now summarize new studies that shed light on these issues.

Aneural acetylcholine receptor clusters

Pre-patterned postsynaptic sites might play one of at least three roles in normal NMJ formation. One possibility is that nerves disperse pre-patterned sites and induce formation of their own, as occurs with hot spots *in vitro* [9,10]. Alternatively, pre-patterned sites might form only as a result of failed innervation, and not as part of a normal developmental program. More troubling for the neural organizer, agrin, and neuregulin paradigms is a third possibility: that they are recognized by axons and incorporated into synapses.

Direct observation of the earliest stages of synaptogenesis is the simplest way to distinguish among these alternatives. Unfortunately, mammalian embryos are inaccessible to imaging. Zebrafish embryos, by contrast, are transparent. Two studies recently revisited neuromuscular synaptogenesis in zebrafish using confocal imaging. Unexpectedly, and contrary to previous reports [11], both found that postsynaptic AChR aggregates formed in advance of the growing axon [53••,54••]. Some aggregates formed even before the axon left the spinal cord or if axon

extension was prevented. Although some of these aggregates dispersed before innervation, time-lapse imaging demonstrated that others were contacted by axons and incorporated into synapses [53••]. In a surprising turnaround, these postsynaptic sites influenced presynaptic development — contacting filopodia were stabilized and new filopodia selectively extended towards the contacted region. Axons were, however, required for maintenance of postsynaptic sites, as prevention of axon outgrowth resulted in the eventual loss of AChR aggregates [53••]. Thus, pre-formed postsynaptic specializations can participate in normal synaptogenesis, although their maintenance requires innervation.

Neurotransmission

The difficulty of completely and selectively eliminating synaptic transmission *in vivo* has long hindered detailed analysis of how neurotransmission affects early events in synaptogenesis. Generation of choline acetyltransferase (ChAT) mutant animals overcame this limitation [38,55]. Because ChAT is the sole synthetic enzyme for ACh, ChAT mutant NMJs are silent throughout development. Analysis of ChAT mutants revealed numerous defects in early postsynaptic differentiation; some had been noted previously but technical difficulties complicated their interpretation. Early nerve branching patterns were abnormal in the absence of neurotransmission and many myotubes bore more than one NMJ. In addition the endplate band, including its AChR clusters and their associated specialized nuclei, was widened in the absence of synaptic transmission. These data highlight features of early synaptogenesis that depend upon neurotransmission. Interestingly, at late embryonic stages, silent AChR aggregates grew larger than those at active synapses and acquired morphologically mature shapes precociously. This result was unexpected, given previous suggestions that activity promotes maturation, and raised the possibility that synaptic activity normally restricts otherwise muscle-autonomous differentiation.

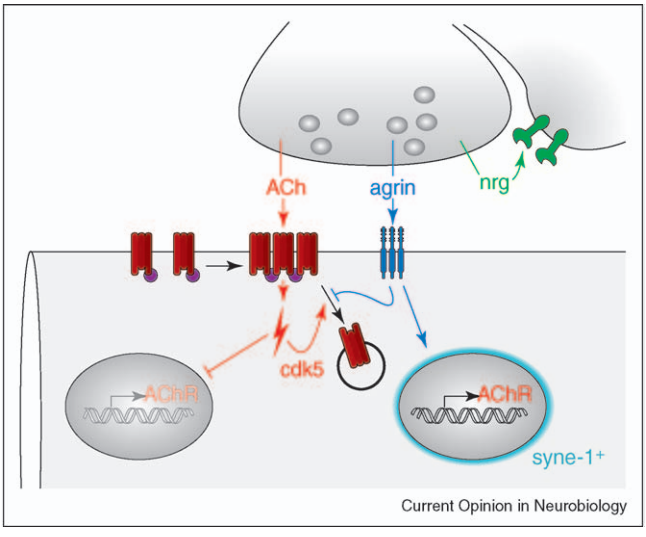
Agrin

Postsynaptic differentiation is clearly aberrant in the absence of agrin [24,27]. The discovery that AChR clusters formed in agrin mutant mice and even in aneural muscles, however, suggested that the role of agrin *in vivo* might relate more to synapse maintenance than to synapse formation. Importantly, AChR clusters persisted longer in aneural muscles than in innervated muscles of agrin mutants [28,45]. This finding indicates the possibility that agrin counteracts the effects of a second nerve-derived signal that disperses AChR clusters. The neurotransmitter acetylcholine was a plausible candidate for this second signal, based on its ability to down-regulate AChR gene expression [1,2].

To seek interactions between agrin and acetylcholine, two groups analyzed agrin–ChAT double knockouts (DKOs) [56••,57••]. Remarkably, NMJs in DKOs formed, underwent pre- and post-synaptic differentiation, and persisted until birth when the animals died. AChR density and cluster size were normal, although atrophy was severe and some DKO fibers were uninnervated. Thus, inactivity largely rescued the agrin phenotype or, put another way, agrin was at least partially dispensable in the absence of neurotransmission. These results imply that a primary function of agrin is to counteract a local dispersal effect of neurotransmission at synaptic sites (Figure 3).

Analysis *in vitro* supported this idea. Application of the cholinergic agonist carbachol dispersed AChR clusters formed spontaneously on cultured muscle cells, but this dispersal was blocked at sites of contact with heterologous cells that expressed Z^+ agrin [56••,57••,58]. Cells expressing Z^- agrin failed to counteract the effect of carbachol [56••]. Moreover, in co-cultures of wild type myotubes with motoneurons from agrin-mutant embryos, the incidence of AChR clusters at neurite–myotube contact sites was markedly enhanced when AChRs were blocked with α-bungarotoxin [56••]. This experiment independently demonstrated the agrin–acetylcholine interaction in a simplified system.

Figure 3



An updated molecular view of postsynaptic development at the NMJ. Although the core molecular players shown in Figure 2 remain unchallenged, the roles assigned to them are revised. Besides repressing extra-synaptic transcription of synaptic genes, acetylcholine also disperses AChR clusters via endocytosis. Agrin represses neurotransmission-induced loss of AChRs to promote postsynaptic development, rather than or in addition to directly clustering AChRs beneath the nerve. Although synaptic transcriptional specialization contributes to synaptic AChR accumulation, neuregulin signaling primarily affects muscle indirectly via its effects on perisynaptic Schwann cells. NMJ formation also occurs essentially normally in mice lacking subsynaptic nuclei because of disruption of syne-1 (cyan).

These data are consistent with previous reports of devastating neuromuscular defects in agrin mutants [24,27], but suggest that agrin functions as an 'anti-declustering' factor rather than (or perhaps as well as) a clustering factor. They also reveal an additional action of neurotransmission in early synaptogenesis — dispersing nascent postsynaptic sites that agrin has not stabilized. The dispersal mechanism remains to be elucidated, but involves activation of the cytoplasmic kinase Cdk5 [57••,59••], which was previously implicated in AChR gene expression [60] and endocytosis of AChRs [56••,61].

Neuregulin

As noted above, erbB2 or erbB3 mutants in which cardiac defects are rescued have modest but significant postsynaptic defects [49–51]. These results were difficult to interpret for at least three reasons. First, erbB receptors, especially erbB2 and erbB4, might have redundant functions. Second, these animals died at birth, preventing analysis of postsynaptic maturation. Third, neuregulin-dependent signaling from motor axons to Schwann cells is crucial for Schwann cell survival, and the presence of Schwann cells is crucial for axonal growth and differentiation [62]. Therefore, postsynaptic defects in these mutants might either result from a primary role of neuregulins or be secondary to impairment of presynaptic development.

To circumvent these limitations, Escher *et al.* [63••] conditionally deleted both erbB2 and erbB4 from skeletal muscle. This rendered muscle fibers insensitive to neuregulin, but preserved neuregulin signaling to Schwann cells and cardiac muscle. The resulting animals were viable, possessed normal strength, and had NMJs that were structurally and functionally normal in many respects, although AChR density was slightly decreased. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis from synaptic and extrasynaptic regions of muscle indicates that synapse-specific transcription was maintained with only a 20–30% reduction in synaptic AChR transcripts. Taken together, these results demonstrate that neuregulin signaling to muscle is dispensable for at least the principal aspects of postsynaptic development. Instead, defects observed in the absence of neuregulin–ErbB signaling are probably secondary to aberrant Schwann cell development.

How then is AChR transcription regulated? Neuregulin might play a modulatory role, or function redundantly with other nerve-derived factors that enhance AChR gene expression, such as calcitonin gene-related peptide (CGRP) [64]. Transcriptional specialization of synaptic nuclei can occur in the complete absence of innervation [28,45], although its maintenance appears to require agrin and MuSK [24,29,30,51,63••]. The mechanism by which agrin maintains transcriptional specialization remains to be elucidated, but it clearly does not involve autocrine neuregulin–ErbB signaling as proposed earlier [65,66].

Subsynaptic nuclei

Transcriptionally specialized myonuclei lie beneath the postsynaptic membrane where they locally synthesize AChRs and other postsynaptic proteins, thereby contributing to postsynaptic assembly [33,34]. In addition, synaptic nuclei are localized between the AChR-rich branches of the postsynaptic array, suggesting that such nuclei might also influence synaptic topology.

The discovery of Syne-1 provided an opportunity to test these ideas. Syne-1 is a large protein with an N-terminal actin-binding domain, spectrin repeats resembling those found in dystrophin and utrophin, and a C-terminal domain that mediates association with the nuclear envelope. Initially identified in a screen for proteins that interact with MuSK, Syne-1 is concentrated in the envelopes of synaptic nuclei at the NMJ [67]. Interestingly, Syne-1 is orthologous to ANC-1, a protein identified in *C. elegans* on the basis of its ability to control nuclear positioning [68]. This relationship raised the possibility that Syne-1 might deliver or anchor nuclei to the NMJ. Grady *et al.* [69••] tested this idea by transgenic expression of a dominant-interfering Syne-1 fragment in mouse muscle. Fewer nuclei were subsynaptic in mice bearing the dominant negative transgene than in controls, and some NMJs had no subsynaptic nuclei at all. No effects were seen on non-synaptic nuclei, but the number of nuclei at the periphery of synapses was increased. This finding suggests that Syne-1 is required for anchoring of synaptic nuclei, perhaps through interaction with MuSK, once other mechanisms bring them into the vicinity of the synapse. Importantly, despite the absence of synaptic nuclei from many fibers, there were no apparent NMJ defects. In particular, the size and the topology of the postsynaptic membrane were normal. Thus, subsynaptic nuclei are dispensable for formation and topological maturation of the NMJ, although transcriptional specialization of nuclei in the vicinity of the synapses might be required.

Maturation

Little is known about how the small, ovoid NMJs of neonates acquire their branched postnatal topology. The transformation was presumed to be nerve-dependent because it failed to occur on muscle fibers denervated *in vivo*, and because AChR clusters on myotubes cultured in isolation were generally plaque-shaped. A plausible idea was that as the motor axon formed its terminal arbor on the muscle fiber, it used agrin to shape the postsynaptic apparatus, thereby maintaining a precise apposition between pre- and post-synaptic structures. In this scenario, the shape of the postsynaptic array reflects the shape of the terminal arbor of the axon.

The finding that myotubes form topologically complex AChR aggregates when cultured aneurally on laminin or fibronectin challenged this view [70••]. These postsynaptic arrays appeared on myotubes derived from myogenic cells

carried *in vitro* for decades, ruling out the possibility of a neural imprint. 'Aneural pretzels' shared remarkable similarities to mature NMJs: they were oblong, broken at one side, elaborately branched and approximately the size of adult NMJs. Numerous postsynaptic markers were associated with these structures, including clusters of Syne-1-rich myonuclei. Repeated imaging showed that pretzels developed *in vitro* from simpler structures through a series of transitions paralleling those that occur in association with axons *in vivo*. Further work identified the protein LL5β, a synaptically enriched transcript, as a potential mediator of these transitions [71]. Thus, muscle-intrinsic machinery can produce the intricate pattern of the mature NMJ in the absence of the nerve, although it remains to be determined how muscle-intrinsic and nerve-derived factors collaborate to shape the mature synapse.

Non-cholinergic neuromuscular junctions?

It has long been assumed that neurotransmission at the NMJ is cholinergic under all circumstances. Shockingly, even this idea has recently been challenged. Borodinsky *et al.* [72••] demonstrated that the neurotransmitter phenotype of motor neurons depends on calcium fluxes during a critical developmental window. Genetic or pharmacologic enhancement of calcium spikes in *Xenopus* spinal neurons induced a shift to inhibitory neurotransmitters without altering expression of cell-specific markers. Decrements in activity increased the number of excitatory neurons, suggesting a homeostatic regulation of spinal output. On the basis of these results, Borodinsky and Spitzer [73] have now shown that aberrant neurotransmitter phenotypes induce cognate adaptations in muscle: when motor neurons synthesized GABA, myocytes synthesized GABA receptors; when motor neurons accumulated glutamate, myocytes accumulate glutamate receptors in response.

A second study extended these ideas to mammals. Brunelli *et al.* [74••] severed the nerve to an adult rat muscle, and implanted a peripheral nerve graft to induce reinnervation by central non-motor axons. They found that glutamatergic axons contacted muscle fibers, which responded by accumulating glutamate receptors and assembling functional glutamatergic NMJs. Taken together, these two studies show that in exceptional circumstances nerves can affect not only the density and distribution but also the type of neurotransmitter receptors in the postsynaptic membrane. Given the recent discovery that motor neurons are normally capable of glutamate release at their central synapses [75,76], it is possible that such modulation could even occur at normal NMJs under some circumstances.

Conclusions

Work reported during the past few years challenges our views of postsynaptic development at the NMJ. Major findings include the following (see Figure 1b and Figure 3). First, concentration of NMJs in a central end-plate band does not merely result from the central entry point of the motor nerve; instead, muscles possess a nerve-independent pre-pattern that targets postsynaptic sites to their centers. Second, ingrowing axons do not necessarily ignore pre-patterned AChR clusters; some are recognized and incorporated into synapses. Third, neurotransmitters play multiple roles in early synaptogenesis, including provoking the dispersal of pre-patterned AChR clusters. Fourth, nerve-derived agrin counteracts activity-induced dispersal, functioning at least in part as an 'anti-declustering' factor to stabilize nerve-contacted AChR aggregates. Fifth, neuregulin is crucial for NMJ formation as a Schwann cell trophic factor, but its direct effects on the postsynaptic membrane are subtle. Sixth, subsynaptic nuclei are dispensable for synaptogenesis. Seventh, nerve-independent machinery can pattern the mature, branched postsynaptic array. And eighth, presynaptic neurotransmitter phenotypes can reprogram muscle fiber neurotransmitter receptor expression wholesale, producing glutamatergic, GABAergic or glycinergic NMJs.

In summary, these studies show that nerve-derived signals function in large part to modulate muscle-intrinsic processes, but that muscle nonetheless remains exquisitely sensitive in surprising ways to presynaptic input. They furthermore suggest that numerous cell-autonomous and cell-cooperative processes collaborate to ensure construction of this fail-safe synapse.

What are the morals of this story? First, despite the unexpected complexities of neuromuscular synaptogenesis revealed in the studies described above, it remains better understood than interneuronal synapse formation. The interactions among organizing factors and neurotransmission at the NMJ probably presage related interactions in the CNS. The idea that the effects of agrin are modulatory and activity-dependent, might, for example, help explain the subtle interneuronal synaptic abnormalities seen in agrin mutants [77,78]. Second, the work we have reviewed here highlights the complexities inherent in analyzing mutant phenotypes. It is important to note that almost none of the facts upon which prior views of neuromuscular development were based are being challenged. New reports, rather, dispute their interpretation. Thus, although there is no doubt that agrin and neuregulin are crucial for postsynaptic development, their effects now appear conditional (agrin), or indirect (neuregulin). This is not to deny the value of genetic experiments, but rather to say that similar to all experiments, they are subject to interpretative uncertainties. For that reason, the model presented above will probably require significant revision at the hands of future scholars of the neuromuscular junction.

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