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Challenging the Neurocentric View of Neuromuscular Synapse Formation

Minireview

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Precision of synaptic connectivity is essential for proper function of the nervous system. This is achieved by the projection of axons to correct targets within a region of the nervous system as well as by synapse formation on discrete domains of individual cells. This precision is widely thought to involve molecules that guide axons to their targets, followed by a period of experiential fine tuning of synaptic connectivity. The recent past has been rife with new information about the molecular machinery of synapses, though work on the neuromuscular junction (NMJ) has provided the conceptual framework for study of the more complex synapses in the brain. In particular, these studies have provided a detailed understanding of the assembly of the postsynaptic apparatus of NMJs.

Even a casual observer would notice that NMJs are commonly found within a narrow band in the midline of skeletal muscles. More detailed investigation reveals that these synapses are exquisitely organized with postsynaptic membrane folds that are aligned with vesicle release sites in the nerve terminal. Acetylcholine receptors (AChRs) are found at the tops of the folds and, together with acetylcholinesterase (AChE) in the extracellular matrix, are tightly restricted to the 0.1% of the muscle cell surface occupied by the synapse. Indeed, the NMJ constitutes a structure, on an otherwise uniform myotube, that extends from the nerve terminal, through synaptic extracellular matrix, to postsynaptic muscle membrane, muscle cytoskeleton, and even the subsynaptic nuclei that specifically express mRNAs for variety of synapse-specific genes.

A host of studies in culture and in vivo support neural control of neuromuscular synapse formation (Burden, 1998; Sanes and Lichtman, 1999). One particularly vivid example comes from studies of nerve muscle cocultures. Aneural muscle cells develop clusters of AChRs, but when neurons are added to muscle cultures, growth cones typically ignore these clusters to innervate other regions of the cell (Anderson and Cohen, 1977; Frank and Fischbach, 1979). The noninnervated clusters of AChRs disperse during this process. Subsequent work has identified a number of molecules key to the assembly and stabilization of the postsynaptic specialization. A widely accepted scenario (Figure 1A), supported by both in vivo and in vitro data, would have the motoneuron axon growing into embryonic muscles in which myoblasts are fusing into multinucleate myotubes. Once fused, these myotubes upregulate expression of a number of genes, including those for AChRs and AChE. The growth cones of motoneurons contact these myotubes and release agrin. Neural agrin activates the muscle-specific receptor tyrosine kinase (MuSK), whose function seems at least in part to be to reorganize AChRs, AChE, and other cell-surface proteins into a synaptic complex. This process is modulated by rapsyn, an intracellular protein that can self-associate and at least in heterologous cells appears to be able to mobilize surface AChRs into small clusters. Subsequently, these small clusters of AChRs coalesce into synaptic densities by anchoring to a transmembrane complex of dystrophin-associated proteins assembling in the postsynaptic membrane and extracellular matrix to help anchor AChE. In addition to this sequence of postynaptic events, contact of motoneuron growth cones with their targets also stops axonal growth and induces differentiation of nerve terminals. Interestingly, mice null for agrin and especially for MuSK have exuberant axonal growth as well as reduced nerve terminal differentiation. Thus, this ligand-receptor pair is necessary to induce a retrograde signal from muscle that regulates axon growth and presynaptic differentiation.

A later phase of synaptogenesis involves specific regulation of gene expression in the synaptic region to consolidate the earlier steps in synapse formation. For example, ongoing release of acetylcholine from differentiating nerve terminals activates AChRs in the muscle and triggers electrical activity. Propagation of this activity can downregulate the expression of AChR genes within all nuclei of the myofiber (Burden, 1998; Sanes and Lichtman, 1999). In the face of this, neuregulin/glial growth factor from the nerve and muscle may bind to and activate the erB-2/4 family of receptor tyrosine kinases, which in turn would increase transcription of AChR genes. These more conventional receptor tyrosine kinases increase the transcription of AChR genes. Activation could also contribute to the spatial restriction of AChR transcription since neuregulin would become concentrated within synaptic basal lamina and erb receptors are localized in subsynaptic plasma membrane.

It is fair to say that this neurocentric model of synapse formation does not take fully into account studies of Harris and coworkers (Braithwaite and Harris, 1979; Harris, 1981) who had shown that developing muscle rendered aneural with neurotoxins still expressed AChRs in a localized region of the muscle. In a recent paper, Burden and coworkers (Yang et al., 2000) confirmed and extended these data in a series of insightful studies of mice null for the topoisomerase IIβ gene. In these mice, motor nerves extended to the vicinity of developing skeletal muscles but failed to invade the diaphragm and innervate the myofibers. These workers found, however, that AChRs were found in clusters localized to the midline of the muscle, suggesting that muscle is patterned in the absence of innervation. Questions remained, however, as to whether the phrenic nerve might have transiently innervated the diaphragm and retracted within the 2-day intervals in which innervation was assayed, or if nerve-derived agrin or neuregulin diffused into the muscle to organize the AChRs in the

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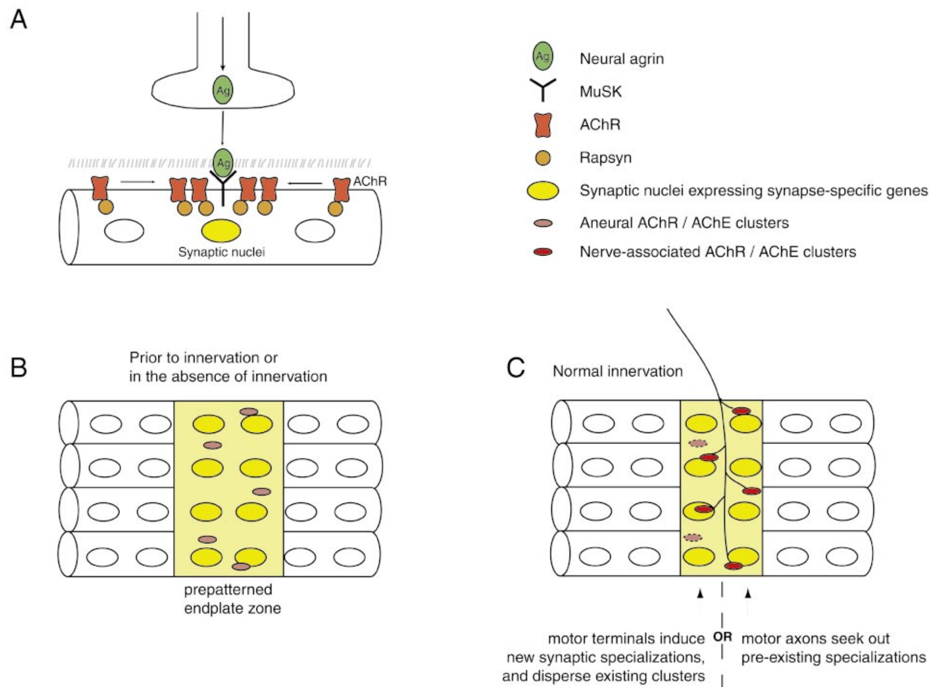

Figure 1. Models Showing Regulation of Neuromuscular Synapse Formation by the Nerve (A) and the Possible Contribution of Muscle Patterning (B and C).

midline. Now, two related papers by Lin et al. (2001) in *Nature* and Yang et al. (2001 [this issue of *Neuron*]): (1) remove any lingering doubts that aneural muscle is patterned; (2) eliminate the possibility that a nerve-derived agent, such as agrin or neuregulin, may have diffused into the muscle to establish this aneural compartment; and (3) show that the compartment includes clusters of AChE in the extracellular matrix and, more importantly, the restricted transcription of AChR mRNA. (Here we use the term "protosynapse" to refer to this aneural structure, which includes dense clusters of AChRs in the plasma membrane, AChE in the extracellular matrix, and localized expression of AChR mRNAs in the muscle nuclei.)

In both reports, genetic approaches have been used to prevent motor innervation or to eliminate motor neurons. To do this, both groups utilized HB9-deficient mice, in which many motoneurons fail to differentiate normally and the phrenic motor nerve to the diaphragm fails to form (Lin et al., 2001; Yang et al., 2001). In a second approach (Yang et al., 2001), motoneurons were eliminated entirely by selective expression of diphtheria toxin in the postmitotic motoneurons, further eliminating the possibility of any transient innervation of the diaphragm. In both cases, no motor projections were detected at any stage in the vicinity of the diaphragm muscle, yet distinct aggregates of AChRs and AChE were still observed on the aneural muscle cells in the central region of the muscle (Figure 1B). The central band of AChR clusters was somewhat wider than in innervated muscle (Figure 1C), however, and the clusters were initially smaller in size. This patterning also extended to transcription of the AChR genes by muscle nuclei where AChR α and γ subunit mRNAs were found to still be selectively expressed in the central muscle region, although again in a more diffuse band than in normal muscle (Lin et al. 2001; Yang et al., 2001). Together, these findings show that muscle is spatially patterned prior to innervation and that significant synaptic differentiation can occur in the absence of the nerve. Innervation clearly refines this pattern, however, and restricts the postsynaptic specializations and synapse-specific transcription to a narrow central band (Figures 1B and 1C).

Do protosynapses form during normal neuromuscular development? To address this, both groups assayed the distribution of AChR clusters in relation to motor nerve terminals at early stages of innervation (E14.5), just after the nerve reaches the muscle (E12.5). Although most AChR clusters were observed in close apposition to nerve terminals, some clusters in the central region of the muscle appeared to be aneural (Figures 1B and 1C). The occurrence of nonsynaptic AChR clusters in innervated muscle was transient, and by E18.5 all AChR clusters were found at synaptic sites. This suggested to both groups that motor innervation disperses aneural clusters, and this idea was elucidated further by examining agrin null mice that have diffuse, aberrant innervation and essentially no well-differentiated NMJs. A central zone of AChR clusters was present at early stages in these mice, but they progressively diminished in number and size. In contrast, in agrin/HB9 double mutants that lack any muscle innervation, AChR clusters were initially smaller and fewer in number, but they persisted as the muscle matured and even grew to the size of clusters in innervated muscle. This indicates that an agrin-independent signal from motoneurons is responsible for eliminating protosynapses.

Neural agrin, MuSK, rapsyn, and neuregulin have all been shown to be essential for neuromuscular synapse formation (Burden, 1998; Sanes and Lichtman, 1999). It was therefore tested whether these proteins are also re-

quired for the formation of protosynapses. As noted above, clustering of AChRs occurred at early stages in mutants lacking either neural agrin specifically or lacking all agrin. It was surprising that AChR clustering was completely absent in mice null for MuSK in which muscle is innervated and in MuSK/HB9 double mutants in which muscle is not innervated (Lin et al., 2001; Yang et al., 2001). Similarly, AChR transcription was uniformly distributed among muscle nuclei, rather than centrally localized in MuSK single mutants, and in MuSK/HB9 or MuSK/topoisomerase-IIβ double mutants. In contrast, the patterning of mRNA expression was retained in mice lacking neuregulin in their motoneurons (Yang et al., 2001), despite a wealth of data indicating that synapse-specific transcription of AChRs is regulated by neuregulin (Burden,1998). Future studies on the role of neuregulin will need to address whether muscle neuregulin is active at ectopically induced NMJs (Sanes and Lichtman, 1999), and is found along with erbs B 2,4 within aneural clusters of AChRs.

Clustering of the AChR protein was also absent at early stages in rapsyn mutants, although this was more expected given the known requirement for rapsyn in clustering (Lin et al., 2001). The more interesting issue of whether rapsyn is required for other aspects of patterning in aneural muscle, such as transcription, has yet to be addressed. These findings suggest distinct mechanisms for nerve-independent as compared with nerve-induced postsynaptic differentiation. The requirement for MuSK but not agrin raises the possibility that an alternative, muscle-derived ligand could activate MuSK to induce postsynaptic differentiation. Alternatively, some level of ligand-independent activation of MuSK may be sufficient to initiate postsynaptic patterning. Consistent with the latter idea, the level of AChR clustering was found to be significantly decreased in HB9 null/MuSK heterozygous mice, where the levels of MuSK and consequently its activation are presumed to be decreased (Lin et al., 2001). Activation of MuSK has also been reported to be enhanced by rapsyn in heterologous cells (Gillespie et al., 1996), although this finding is controversial (Apel et al., 1997). Thus, another possibility is that ligand-independent activation of MuSK is mediated in part by rapsyn.

How are protosynapses formed in the absence of molecular signals from ingrowing growth cones? One possibility is that the central zone of postsynaptic differentiation simply reflects the temporal-spatial pattern of myofiber differentiation. Myotubes generally extend in length by myoblast fusion at their ends. As a result, central myonuclei are the first to differentiate and therefore may express higher levels of synaptic proteins like rapsyn and MuSK than more peripheral nuclei. Alternatively, the central nuclei could be derived from a separate, specialized myoblast lineage. There is precedent for this in vertebrates, where fast and slow myotubes appear to derive from distinct populations of myoblasts (Stockdale, 1992). Also in *Drosophila*, specialized founder cells have been shown to pattern muscle, and to regulate the targeting of motor axons (Landgraf et al., 1999). Finally, naive muscle cells could be patterned by interactions with connective tissue elements (Harris, 1981), which are thought to be involved in sculpting individual muscles from embryonic muscle cell masses.

Clearly, the muscle is not the naive partner it was previously thought to be, but to what extent does it regulate innervation? One attractive idea is that the protosynaptic zone primes the muscle for innervation and increases the efficiency and speed of synaptogenesis (Figure 1B). Moreover, muscle-derived signaling proteins may be concentrated in this zone and could restrict innervation to the muscle midline and stimulate nerve terminal differentiation. This could include guidance molecules or synaptic differentiation factors, similar to neuroligin (Scheiffele et al., 2000). Support for this idea comes from the observation that nerve terminals branch more exuberantly in the muscles of MuSK mutants than in agrin mutants, which could reflect the lack of protosynapses in MuSK-deficient muscle. The main nerve bundle, however, still seems to make its way along the midline of the muscle in MuSK mutants, suggesting that its position is not regulated by the MuSK-dependent band of protosynapses, unlike the regulation of branching from the main nerve.

Are protosynapses important for the positioning and formation of postsynaptic specializations (Figure 1C)? In adult muscles, regenerating motor axons selectively innervate vacant synaptic sites, but in nerve-muscle coculture experiments, motor axons show no preference for preexisting AChR clusters. An important question, then, is to determine whether axons preferentially contact postsynaptic specializations in developing muscle. Alternatively, axons may extend into the protosynaptic zone, ignoring AChR clusters and inducing new specializations. In the former instance, axons must show some preference for clusters closer to the muscle midline. In the latter, axons must induce NMJs closer to the midline and disperse more peripherally located ones. Both of these scenarios require that motor innervation refines the pattern of postsynaptic specializations by dispersing some or all preexisting AChR clusters. Clusters of AChRs on cultured muscle cells can be rapidly reorganized by ingrowing neurites (Anderson and Cohen, 1977), and the experiments of Lin et al. (2001) and Yang et al. (2001) demonstrate that motor nerves disperse aneural AChR clusters by an agrin-independent mechanism. In contrast, reorganization of AChR mRNAs in the central muscle region is likely is be regulated transcriptionally. This may involve nerve-evoked electrical activity that downregulates the transcription of synaptic proteins by nonsynaptic nuclei (Sanes and Lichtman, 1999). Finally, it is possible that in reorganizing protosynapses, axons may contact all clusters but disperse more peripherally located ones by releasing some locally acting factor such as a protease (Champanaria et al., 1992) and then withdrawing.

It will be critical to test whether the formation of a muscle-autonomous zone is actually required for normal innervation. In HB9 null/MuSK heterozygous mice, there appears to be significantly decreased AChR clustering, yet the endplate zone in single MuSK heterozygotes has been reported to be normal (DeChiara et al., 1996). This is an important issue that should be reinvestigated to determine whether the location and timing of neuromuscular synapse formation is abnormal. If there are no such defects, it would suggest that protosynapses may not be required for formation of normal NMJs.

Together, these interesting studies (Lin et al., 2001; Yang et al., 2001) solidify and extend the notion that muscle is prepatterned and that this pattern is instructive in NMJ formation. Both groups propose a two-step

process wherein protosynapses form in the absence of any neural influence through a mechanism that requires MuSK and rapysn. In a second step, motoneurons form synapses in this compartment and refine it by eliminating protosynapses. What remains to be determined is the relative balance of influences from nerve and muscle that dictate the final pattern of innervation.

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