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Myogenin-dependent nAChR clustering in aneural myotubes

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During development of the neuromuscular junction, nerve-derived agrin and the cell substrate laminin stimulate postsynaptic nAChR clustering. This clustering is dependent on activation of the tyrosine kinase, MuSK, which signals receptor clustering via a rapsyn-dependent mechanism. Myogenin is a muscle-specific transcription factor that controls myoblast differentiation and nAChR gene expression. Here, we used RNA interference to investigate if myogenin is also necessary for nAChR clustering. We find that myogenin expression is essential for robust nAChR clustering and cannot be compensated by the muscle regulatory factors MyoD, myf5, and MRF4. In addition, we show that clustering cannot be rescued in myogenin-depleted myotubes by simply overexpressing the essential clustering molecules MuSK, rapsyn, and nAChRs. These data suggest that myogenin controls the expression of molecules crucial to nAChR clustering in addition to its role in regulating nAChR gene expression.

Keywords: Muscle; Nicotinic acetylcholine receptor; Myogenin; Muscle-specific kinase; Rapsyn; Synapse; Neuromuscular junction; Muscle regulatory factors; RNA interference; Muscle cell culture

Introduction

The postsynaptic membrane (endplate) of the neuromuscular junction is a complex structure resulting from the expression and organization of no less than 12 distinct proteins (Sanes and Lichtman, 2001; Banks et al., 2003). Preeminent among these proteins are the nicotinic acetylcholine receptors (nAChRs) which achieve densities greater than 10,000/μm^2 and are the functional units that facilitate communication between the presynaptic motor neuron and the postsynaptic muscle. The highly dense localization of nAChRs to the endplate requires the expression of muscle-specific kinase (MuSK) and rapsyn (Gautam et al., 1995; Glass et al., 1996). These latter proteins also localize with nAChRs during formation of the neuromuscular junction (NMJ). Genetic ablation of either MuSK or rapsyn results in a diffuse nAChR distribution in muscle. Interestingly, in heterologous cells, rapsyn alone is sufficient to stimulate nAChR clustering (Froehner et al., 1990). Rapsyn is a 43-kDa membrane-associated protein that self associates, interacts with nAChRs, and links them to the cytoskeleton via its interaction with β-dystroglycan (Burden et al., 1983; Bartoli et al., 2001). MuSK is a receptor tyrosine kinase that mediates endplate formation (Glass et al., 1996). MuSK is activated by motor neuron-derived agrin, a heparan sulfate proteoglycan, and initiates a signaling cascade that results in rapsyn-dependent nAChR clustering (Sanes and Lichtman, 2001; Banks et al., 2003).

Although MuSK and rapsyn are crucial for nAChR clustering, it is not clear what controls their expression in developing muscle. Undoubtedly, factors inducing these genes will be critical for successful synapse formation. Recently, MuSK expression was shown to be induced by a Wnt-dependent signaling mechanism (Kim et al., 2003). In addition, high-level MuSK gene expression in developing myotubes appears to be mediated by a specific E-box sequence (CAGCTG) within its promoter region (Kim et al., 2003). Synapse-specific expression of MuSK in adult muscle is mediated, at least in part, by an N-box sequence (CCGGAA) residing in the MuSK gene's first intron (Lacazette et al., 2003). Although GABP mediates gene expression via the N-box, the proteins regulating MuSK gene expression via its critical E-box have not been reported.

Like MuSK, rapsyn also harbors E-box sequences in its promoter that are critical for its expression in muscle (Ohno et al., 2003). However, unlike MuSK, synapse-specific expression of rapsyn does not require an N-box but may be mediate through another sequence (CTGCNA) that binds Kaiso, a POZ-zinc finger transcription factor that localizes to the NMJ in adult muscle (Rodova et al., 2004).

It is interesting that the muscle-specific expression of these genes, including nAChRs (Berberich et al., 1993; Numberger et al., 1991; Prody and Merlie, 1991; Su et al., 1995; Walke et al., 1996), requires a specific E-box sequence (either CACCTG or CAGCTG) and may suggest that particular E-box binding proteins are necessary for the expression of these synapse organizing molecules. It seems reasonable to propose that genes whose products participate in synapse formation may be coordinately regulated during early development via their E-box sequence.

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Muscle development is regulated by four myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin, and MRF4, which are bHLH proteins that bind to E-box (CANNTG) consensus sequences (Molkentin and Olson, 1996). MyoD and Myf5, and more recently MRF4 (Kassar-Duchossoy et al., 2004), have been identified as factors involved in muscle cell fate specification, while the functions of myogenin as well as MRF4 has been ascribed to processes of cell differentiation and maturation. Genetic knockout studies have demonstrated that single null mutations of MyoD, Myf5, and MRF4 result in viable mice that have some altered morphologies but no catastrophic phenotypes (Braun et al., 1992; Rudnicki et al., 1992; Yoon et al., 1997), suggesting that either these transcription factors are not necessary for the expression of the constituent proteins involved in endplate formation, or that there is sufficient functional redundancy to allow for a compensatory effect. If this was not the case, these knockout mice would die at birth due to respiratory failure. In contrast, myogenin null mice do die at birth due to the lack of secondary muscle fiber development and consequent muscle insufficiency (Hasty et al., 1993). The small percentage of primary muscle fibers produced in myogenin null mice grossly appear to differentiate but have morphological abnormalities as well as altered patterns of gene expression. In addition, they showed some very limited signs of nAChR clustering that were attributed to reduced nAChR subunit expression (Brennan et al., 1996). Thus, myogenin may be a key regulator of synapse formation via its essential role in regulating the expression of genes whose products contribute to the synapse. However, it is not clear if myogenin regulates the expression of endplate proteins in addition to nAChRs.

Because the small number of primary muscle fibers that survive in myogenin knockout animals may not develop normally, it is difficult to interpret studies of nAChR clustering using them as a model system. To circumvent this limitation, we used RNA interference to knockdown myogenin expression in a conditionally immortalized muscle cell line (RMT) recently developed in our laboratory (Macpherson et al., 2004). This model system allows for normal muscle cell differentiation prior to myogenin knockdown, allowing us to evaluate myogenin's function following muscle differentiation.

One of the hallmarks of synaptogenesis is the clustering of nAChRs beneath the innervating neuron. One can mimic this clustering in vitro by adding agrin to the medium (McMahan, 1990). Recently, Sanes and colleagues demonstrated that structurally complex aneural aggregates of nAChRs associated with many of the proteins residing at endplates in vivo can be generated in vitro by plating muscle cells on laminin substrate (Kummer et al., 2004). Similar to agrin-mediated clustering, laminin substrate-mediated clustering also requires MuSK and rapsyn (Kummer et al., 2004).

We have taken advantage of this clustering system to determine the impact myogenin expression has on nAChR clustering in differentiated myotubes. Our data demonstrate that following cell fusion, inhibition of myogenin expression, but not MRF4, results in a dramatic decrease in the number of receptor clusters observed on differentiated myotubes. Furthermore, this loss of clustering capacity was not appreciably rescued by exogenous expression of the nAChR subunits, Rapsyn and MuSK, indicating that additional proteins whose expression is regulated by myogenin are required for aneural nAChR aggregate formation. These results suggest myogenin is crucial to synapse formation by regulating the expression of genes whose products orchestrate receptor clustering in the postsynaptic membrane.

Results

Global inhibition of myogenin expression reduces nAChR clustering

In order to inhibit myogenin expression without blocking cell fusion/differentiation, we made use of an immortalized rat muscle cell line (RMT) developed in our laboratory (Macpherson et al., 2004) that is easily transfected after the onset of muscle differentiation. To inhibit myogenin expression, we used a short hairpin RNA (shRNA) driven off the U6 promoter or synthetic short interfering RNAs (siRNAs) targeted to different regions of the myogenin molecule. The shRNA targeted residues 252–273 of the myogenin transcript. The three different regions of the myogenin transcript that were targeted by the siRNAs were nucleotides 137–161 (137-siRNA), 246–270 (246-siRNA), and 654–678 (654-siRNA). We found that transfection of differentiated RMT cells with siRNAs results in about an 85% transfection efficiency (see below and Fig. 2B), whereas shRNA transfection efficiency is significantly less. Therefore, one can readily assay siRNA effects without identifying transfected fibers, while shRNA effects require identification of transfected fibers using a GFP reporter plasmid.

Two days after transfecting differentiated muscle cells with control siRNA (similar GC content as myogenin-targeted siRNAs), myogenin-targeted siRNAs or myogenin-targeted shRNA co-transfected with a GFP reporter, plates were fixed and assessed for myogenin expression by immunohistochemical staining. Whereas the control siRNA appeared to have no effect on myogenin expression, as indicated by virtually complete overlapping signals for myogenin (red) and nuclear localized DAPI staining (blue) (Fig. 1A), transfection with either 246- or 654-siRNA resulted in significant inhibition of myogenin expression (Figs. 1B and C). The 137-siRNA had a less pronounced effect on myogenin expression (not shown) and therefore was not used for further experimentation. The effectiveness of the 246- and 654-siRNA in blocking myogenin expression was further confirmed by Western blot analysis (Fig. 1Q) Similarly, we confirmed our previously reported result (Tang et al., 2004) that myogenin-targeted shRNA plasmid co-transfected with a GFP reporter showed specific reduction of myogenin (red) in GFP+ myotubes (green) (Fig. 1D). Approximately 75% of the GFP+ fibers were completely negative for myogenin staining, and the intensity of myogenin staining in the remaining fibers tended to be inversely correlated with the magnitude of the GFP signal.

To ensure that the siRNAs and shRNA were specifically inhibiting myogenin expression and not other MRFs, we performed immunohistochemical analysis with antibodies against MyoD, MRF4, and Myf5. In each case, we saw no attenuation in the expression of these transcription factors when differentiated myotubes were transfected with either the control or the myogenin-targeted siRNAs and shRNA (Figs. 1E–P).

We next determined the global effect of myogenin knockdown on nAChR clustering in differentiated myotubes. For these experiments, myotubes were differentiated for 60 h and then transfected with either 246- or 654-siRNA. Four days after transfection, differentiated myotubes were assayed for the presence