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The Extracellular Region of Lrp4 is Sufficient to Mediate Neuromuscular Synapse Formation

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Abstract

Neuromuscular synapse formation requires an exchange of signals between motor neurons and muscle. Agrin, supplied by motor neurons, binds to Lrp4 in muscle, stimulating phosphorylation of MuSK and recruitment of a signaling complex essential for synapse-specific transcription and anchoring of key proteins in the postsynaptic membrane. Lrp4, like the LDLR and other Lrp-family members, contains an intracellular region with motifs that can regulate receptor trafficking, as well as assembly of an intracellular signaling complex. Here, we show that the intracellular region of Lrp4 is dispensable for Agrin to stimulate MuSK phosphorylation and clustering of acetylcholine receptors in cultured myotubes. Moreover, muscle-selective expression of a Lrp4-CD4 chimera, composed of the extracellular and transmembrane regions of Lrp4 and the intracellular region of CD4, rescues neuromuscular synapse formation and the neonatal lethality of *lrp4* mutant mice, demonstrating that Lrp4, lacking the Lrp4 intracellular region, is sufficient for presynaptic and postsynaptic differentiation.

Keywords

acetylcholine receptor; MuSK; low-density lipoprotein-related receptor; synaptogenesis; skeletal muscle

Introduction

Agrin, a ligand that is supplied by motor neurons and deposited in the synaptic basal lamina, stimulates postsynaptic differentiation by binding to the low-density lipoprotein receptor-related protein 4 (Lrp4), leading to activation of MuSK, a receptor tyrosine kinase expressed by muscle (Kim et al., 2008; Zhang et al., 2008; Burden, 2011). Once tyrosine phosphorylated, MuSK recruits Dok-7, a muscle-specific adapter protein (Okada et al., 2006). The steps that follow Dok-7 recruitment and phosphorylation are poorly understood but lead to an accumulation of key proteins, including acetylcholine receptors (AChRs), in the postsynaptic membrane, and synapse-specific transcription (Okada et al., 2006; Hallock et al., 2010). These two pathways contribute to a high level of AChR expression in the postsynaptic membrane, which is necessary to insure for robust and reliable synaptic transmission (Sanes and Lichtman, 2001). Mice that are deficient in Agrin, Lrp4, MuSK or Dok-7 die at birth due to a failure to form neuromuscular synapses. Moreover, mutations that impair these signaling pathways lead to a reduced number of AChRs at synapses and are responsible for congenital myasthenia in humans (Engel et al., 2003; Beeson et al., 2006; Burden, 2011).

Like the low-density lipoprotein receptor (LDLR) and other LDLR-related protein (Lrp) family members, Lrp4 contains multiple LDLa repeats, as well as EGF-like and β -propeller domains in the extracellular region, which are required to bind Agrin and MuSK (Herz and Strickland, 2001; Kim et al., 2008; Zhang et al., 2008; Goldstein and Brown, 2009). In addition, the intracellular region of Lrp4 contains sequence motifs, shared with other family members, which have the potential to regulate receptor function (Herz and Strickland, 2001; Goldstein and Brown, 2009). In the LDLR, the intracellular region plays a critical role in receptor trafficking and delivering cholesterol to cells (Goldstein and Brown, 2009), and the intracellular regions of other Lrp family members, including Lrp1, Lrp5/6 and Lrp8, contain sequence motifs for tyrosine phosphorylation and cytoskeletal anchoring, which play important roles in transducing binding of Lrp ligands, PDGF, Wnts and Reelin, respectively, to intracellular signaling events (Herz and Strickland, 2001).

To understand how Lrp4 regulates neuromuscular synapse formation, we sought to determine whether the intracellular region of Lrp4 plays an important role in activating MuSK or stimulating synaptic differentiation. We introduced mutant forms of Lrp4, lacking notable motifs in the intracellular region, or a chimeric receptor in which the intracellular region of Lrp4 was replaced with the intracellular region of CD4, into *lrp4* mutant myotubes or muscle of *lrp4* mutant mice, and we studied synaptic differentiation. Here, we show that the intracellular region of Lrp4 is dispensable for activating MuSK and stimulating postsynaptic differentiation in cultured myotubes as well as forming neuromuscular synapses in mice, highlighting the crucial role for the Lrp4 extracellular region in stimulating synaptic differentiation.

Results

The extracellular region of Lrp4 rescues postsynaptic differentiation in cultured myotubes

Lrp4 is a ~220 kDa transmembrane protein, composed of LDLa, EGF-like and β -propeller (YWTD) domains in the extracellular region and a 158 amino acid intracellular region (Herz and Strickland, 2001). The extracellular region of Lrp4 binds Agrin and associates with MuSK (Kim et al., 2008; Zhang et al., 2008). The importance of the Lrp4 intracellular region in postsynaptic differentiation, including clustering AChRs at neuromuscular synapses, however, is not understood. We established a rescue assay with *lrp4* mutant myotubes to determine whether the Lrp4 intracellular region is necessary for Agrin-stimulated postsynaptic differentiation.

Lrp4 mutant myotubes express MuSK and AChRs, but the absence of Lrp4 renders these myotubes unresponsive to Agrin (Kim et al., 2008). We introduced wild-type or mutant forms of Lrp4, containing mutations in the Lrp4 intracellular region, into *lrp4* mutant myotubes and assessed their ability to restore Agrin-stimulated MuSK phosphorylation and AChR clustering (Figure 1A). The Lrp4 intracellular sequence was altered either by mutating individual motifs, which have the potential to regulate intracellular trafficking, tyrosine phosphorylation and anchoring of Lrp4, or by replacing the entire intracellular region of Lrp4 with the intracellular region from CD4, an unrelated transmembrane protein (Littman, 1987). Agrin fails to stimulate MuSK phosphorylation and AChR clustering in *lrp4* mutant myotubes (Weatherbee et al., 2006; Kim et al., 2008). The single NPSY motif in Lrp4, which functions as a motif for endocytosis in the LDLR and a site for phosphorylation in MuSK (Chen et al., 1990; Zhou et al., 1999; Herbst and Burden, 2000), is not required for Agrin to stimulate MuSK phosphorylation or AChR clustering (Figures 1, S1). Likewise, the C-terminal PDZ-binding site in Lrp4 is not necessary for Agrin-stimulated signaling (Figures 1, S1). Further, a Lrp4-CD4 chimera, composed of the extracellular and transmembrane domains from Lrp4 and the intracellular region of CD4, restored Agrin-stimulated MuSK phosphorylation and AChR clustering (Figure 1). Because the intracellular

regions of Lrp4 and CD4 are unrelated (1.7% identity; 3.9% similarity), these data provide strong evidence that the intracellular region of Lrp4 is not required for Agrin-stimulated postsynaptic differentiation. Moreover, these data extend experiments showing that a soluble form of Lrp4, lacking the transmembrane and intracellular regions, can restore Agrin-stimulated MuSK phosphorylation in *lrp4* mutant myotubes (Zhang et al., 2011).

Muscle-derived Lrp4 is sufficient to mediate neuromuscular synapse formation *in vivo*

Motor axons terminate and differentiate at neuromuscular synapses (Sanes and Lichtman, 2001). Lrp4 is expressed by muscle and concentrated in the postsynaptic membrane, where it responds to Agrin and activates MuSK (Kim et al., 2008; Zhang et al., 2008). In addition, Lrp4 self-associates, when expressed in the same cell or adjacent cells (Kim et al., 2008), raising the possibility that Lrp4, expressed by motor neurons, could associate with muscle-derived Lrp4 and regulate presynaptic differentiation. Although *in situ* hybridization failed to detect *lrp4* RNA expression in embryonic motor neurons (Weatherbee et al., 2006), low or transient expression may have escaped attention. To determine whether neuromuscular synapse formation depends upon Lrp4 expression in motor neurons, we generated transgenic mice that express wild-type Lrp4 selectively in skeletal muscle and crossed these transgenic mice with *lrp4* mutant mice. We established that the transgenes, expressed from the *human skeletal actin* (*HSA*) promoter, were expressed in skeletal muscle and not in spinal cord (Figure S2), allowing us to analyze neuromuscular synapses in mice expressing Lrp4 selectively in skeletal muscle (*HSA::lrp4*, *lrp4*^{-/-}) as well as in *lrp4*^{-/-} and wild-type control mice.

In *lrp4* mutant mice, motor axons fail to stop and differentiate, and AChRs fail to cluster (Weatherbee et al., 2006). We found that Lrp4, expressed selectively from skeletal muscle, fully rescued neuromuscular synapse formation. Restoring Lrp4 expression selectively in muscle caused motor axons to terminate in the central region of the muscle, apposed to AChR clusters, which were normal in size and AChR density (Figure 2). These findings demonstrate that Lrp4, supplied by muscle, is sufficient to promote both presynaptic and postsynaptic differentiation (Figure 2). Further, *lrp4* mutant mice die at birth, likely due to their inability to breathe, whereas *lrp4* mice carrying the *HSA::Lrp4-3* transgene survive postnatally (Figure 2C).

The extracellular region of Lrp4 mediates neuromuscular synapse formation *in vivo*

Mice mutant for Lrp4 display numerous and diverse developmental abnormalities, including defects in kidney, lung and bone development, aberrant digit formation and a failure to form neuromuscular synapses (Weatherbee et al., 2006). The defects in neuromuscular synapse formation in *lrp4* mutant mice are due to a failure of Agrin to activate MuSK (Figure 1) (Kim et al., 2008; Zhang et al., 2008). Although our experiments with cultured myotubes indicate that the intracellular region of Lrp4 is dispensable for Agrin-stimulated postsynaptic differentiation, the intracellular region of Lrp4 could have a role in a retrograde signaling pathway that is required for presynaptic differentiation (Sanes and Lichtman, 2001). To determine whether the intracellular region of Lrp4 plays a role in neuromuscular synapse formation *in vivo* we generated transgenic mice that express the Lrp4-CD4 chimera selectively in muscle. We established that the transgenes were expressed selectively in skeletal muscle (Figure S2), and we crossed the transgenes, encoding the chimeric receptor, into *lrp4* mutant mice. We found that the Lrp4-CD4 chimera, composed of the extracellular and transmembrane regions from Lrp4 and the intracellular region of CD4, rescued neuromuscular synapse formation, including presynaptic differentiation (Figure 3). These data indicate that the extracellular region of Lrp4 is sufficient to promote both presynaptic and postsynaptic differentiation.

The extracellular region of Lrp4 is sufficient to confer postnatal maturation of the neuromuscular synapse

During the first few weeks after birth, the neuromuscular synapse undergoes a transformation from a simple plaque-like shape to an elaborate pretzel-like structure (Slater, 1982; Kummer et al., 2004), which contributes to an increase in the safety factor and reliability of synaptic transmission (Wood and Slater, 2001). The postnatal changes in the arrangement of synaptic AChRs can be subdivided into stages of increasing complexity: (1) plaque-shaped, (2) perforated, and (3) branched or pretzel-like (Kummer et al. 2004). To determine whether the intracellular domain of Lrp4 is required for these structural changes, we analyzed P30 mice, which express the Lrp4-CD4 chimera selectively in muscle, and assigned individual synapses to one of these three stages.

First, we analyzed postnatal (P30) *lrp4* mutant mice expressing full-length Lrp4 selectively in muscle and found that these mice survive after birth, indicating that Lrp4 expression in kidney, lung, bone and other tissues is not essential for postnatal survival. *Lrp4* mutant mice, rescued with full length Lrp4 or the Lrp4-CD4 chimera, are fertile and live a normal life span. As expected for mice expressing Lrp4 only in skeletal muscle, the rescued mice display syndactyly and have defects in bone and tooth development (Johnson et al., 2005; Weatherbee et al., 2006). Neuromuscular synapses in postnatal mice expressing full-length Lrp4 selectively in muscle were indistinguishable from synapses in wild-type mice (Figure 4). Moreover, synapses in the rescued postnatal mice were structurally complex, similar to wild-type control mice (Figure 4). We next examined mice expressing the Lrp4-CD4 chimera selectively in muscle and found that these rescued mice also survived postnatally. Moreover, synapses in P30 mice rescued with the Lrp4-CD4 chimera matured like wild-type mice (Figure 4). Further, the rescued mice show no overt signs of muscle weakness, consistent with the idea that the rescued synapses function normally. The rescued mice display an uneven gait, likely caused by the defects in bone development, including the fusion of bones in distal limbs and ankles. For this reason, we have not studied whether the rescued mice fatigue more rapidly following exercise. Together, these results demonstrate that the formation and maturation of the neuromuscular synapse depends upon Lrp4 expression from skeletal muscle and not from other cell types. Moreover, the maturation as well as the formation of neuromuscular synapses requires the extracellular but not the intracellular region of Lrp4. Further, although Lrp4 is expressed in multiple organs, our findings indicate that Lrp4 is not required in tissues other than muscle for postnatal survival.

Discussion

The function of the LDLR and most Lrp family members depends upon by their intracellular regions, which regulate receptor trafficking and downstream signaling (Herz and Strickland, 2001; Goldstein and Brown, 2009). Here, we show that the intracellular region of Lrp4 is dispensable for Lrp4 to mediate the formation and maintenance of neuromuscular synapses. Although the intracellular region of Lrp4 may play important roles in other contexts, such as digit formation, our studies demonstrate that the extracellular and transmembrane domains of Lrp4 are sufficient to regulate both presynaptic and postsynaptic differentiation. Moreover, we show that muscle-selective expression of Lrp4 rescues neuromuscular synapse formation in *lrp4* mutant mice, revealing that synapse formation requires Lrp4 expression by muscle but not by motor neurons.

Our findings demonstrate that the intracellular region of Lrp4 does not possess an essential signaling function for synaptic differentiation and therefore suggest that Lrp4 controls neuromuscular synapse formation by binding Agrin and activating MuSK. Activated MuSK stimulates postsynaptic differentiation by engaging signaling cascades that regulate the expression and clustering of key postsynaptic proteins (Burden, 2011). Presynaptic

differentiation is triggered by the production and/or reorganization of muscle-derived retrograde signal(s) (Fox et al., 2007). These retrograde signals may be novel proteins or Lrp4 itself, as Lrp4, clustered in the muscle membrane as a consequence of MuSK signaling, may function directly as a muscle-derived retrograde signal. At present, our studies do not allow us to distinguish between these two possibilities.

Mutations in Lrp4 cause Cenani-Lenz syndrome, with exhibit abnormalities in craniofacial, bone, and digit development (Li et al., 2010). Because *lrp4* mutant mice, rescued for Lrp4 expression selectively in muscle, survive as adults and display these same abnormalities, the rescued mice should provide a good model for studying Cenani-Lenz syndrome.

Lrp4 is expressed in the central nervous system (CNS) and concentrated in postsynaptic densities (Tian et al., 2006). MuSK is expressed by excitatory neurons in the CNS (Ksiazek et al., 2007), and Agrin is widely expressed in the nervous system (Stone and Nikolics, 1995; Burgess et al., 2000). It will be interesting to learn whether these three proteins are co-expressed at synapses and whether Agrin/Lrp4/MuSK signaling has a role in synapse formation and function in the CNS. Because *lrp4* mutant mice die at birth, and synapse formation in the CNS largely occurs postnatally, mice that are rescued for Lrp4 at neuromuscular synapses and survive postnatally should be particularly useful to uncover the roles for Lrp4 at CNS synapses.

Experimental Procedures

Lrp4 expression constructs

Point mutations in *lrp4* were generated by site-directed mutagenesis, as described previously (Friese et al., 2007). NPSY was mutated to NPSA, and the three base pairs encoding valine at the carboxy terminus of Lrp4 were deleted. The Lrp4-CD4 chimera was generated by deleting amino acids 1748-1905 from murine Lrp4 and replacing this sequence with the 33 amino acid intracellular region of murine CD4, extending from K422 to I457. The structures of the constructs were confirmed by DNA sequencing. The intracellular regions of Lrp4 and CD4 were aligned according to Needleman-Wunsch/Needle EMBOSS algorithm.

MuSK tyrosine phosphorylation

Lrp4^{-/-} myoblasts were infected with a pBABE retrovirus expressing either wild-type or mutant forms of Lrp4, which included a FLAG tag, and myoblasts were allowed to differentiate to form myotubes (Friese et al., 2007; Kim et al., 2008). Myotubes were treated with neural Agrin (1 nM) for thirty minutes, and MuSK was immunoprecipitated with rabbit antibodies to the C-terminal region of MuSK (Herbst and Burden, 2000). Lrp4 was isolated with antibodies to FLAG (Sigma Aldrich, St. Louis, MO). Western blots of the immunoprecipitates were probed with antibodies to phosphotyrosine, MuSK or FLAG, as described previously (Herbst and Burden, 2000; Hallock et al., 2010). Expression levels were determined by LI-COR (Hallock et al., 2010), and we calculated the level of MuSK tyrosine phosphorylation relative to Lrp4 expression.

Generation of *HSA::Lrp4* and *HSA::Lrp4-CD4* transgenic mice

The *HSA* promoter/exon1/intron cassette (-2000/+234) was excised from *pHSA2000CAT* (Muscat and Kedes, 1987; Brennan and Hardeman, 1993) by digestion with HindIII, and the blunt-ended fragment was introduced into the StuI site of pSG5 (Agilent Technologies, Santa Clara, CA). cDNA encoding FLAG-tagged murine Lrp4 or the Lrp4-CD4 chimera was subcloned into pSG5 containing the *HSA* promoter cassette. The *HSA::Lrp4* transgenes, together with the linked rabbit β -globin intron and SV40 polyadenylation signal, were injected into zygotic male pronuclei, as described previously (Kim and Burden, 2008).

Founder mice and F1 progeny were genotyped by PCR, utilizing the FLAG epitope to distinguish the transgenes from wild-type *lrp4* (*HSA::Lrp4*: 5'-GCCAGCAGTCTGGAGGATTA-3' and 5'-TGCCACTGGGCGGGGATACA-3'). *Lrp4* mutant mice were genotyped by PCR, as described previously (Weatherbee et al., 2006). We obtained five *HSA::Lrp4* and nine *HSA::Lrp4-CD4* founder mice and generated lines from all fourteen mice. We analyzed *lrp4* expression from each line and chose two *HSA::Lrp4* and two *HSA::Lrp4-CD4* lines for detailed analysis (Figure S2). All experiments were approved by the Animal Care and Use Committee at NYU Medical School.

Histology

Whole-mounts of diaphragm muscles from E18.5, P0 and P30 mice were stained with Alexa-594-conjugated-alpha-bungarotoxin (alpha-BGT) (Invitrogen, San Diego, CA) to label AChRs and with antibodies against Neurofilament-L (SYnaptic SYstems, Goettingen, Germany) and Synaptophysin (Zymed, San Francisco, CA) or Synapsin (SYnaptic SYstems, Goettingen, Germany) to visualize motor axons and nerve terminals, respectively (Kim and Burden, 2008). The number and size of AChR clusters, as well as the density of AChRs within clusters, were measured as described previously (Friese et al., 2007). Synaptic complexity was determined by examining at least thirty synapses in each of three mice at P30 and assigning a complexity score, ranging from one to three, for each synapse, where 1 represents an immature, plaque-like arrangement of AChRs, 2 indicates a perforated organization and 3 represents the complex, pretzel-like shape (Kummer et al., 2004).

Quantitative real time PCR

Diaphragm muscles were homogenized on ice in RNA Isolation Reagent (Thermo Scientific, Rockford, IL) using a PT 10/35 Polytron (Kinematica AG, Littau-Lucerne, Switzerland), and total RNA was isolated according to the manufacturer's instructions. After treatment with DNase I for 15 min at 37°C, 200 ng of RNA was reverse transcribed (RT) using a Verso cDNA synthesis kit (Thermo Scientific, Rockford, IL). Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) on an Opticon 2 real-time thermocycler (Bio-Rad Hercules, CA), using 5 percent of each RT reaction. The primers for PCR amplification, which amplify RNA transcribed from the endogenous *lrp4* gene and the *lrp4* transgene, were: 5'-CCCTGCAAGTGAAGATGAAA-3' and 5'-TTCAAGAACTGCGATTCTGG-3' for *Lrp4* and 5'-CGTGTACCTCTGCTGCT-3' and 5'-CCTTCATATTGCCTCCCTTCT-3' for *muscle creatine kinase (mck)*, as described previously (Kim et al., 2008). *Lrp4* expression was normalized to *mck* expression. The linearity of the real time PCR reaction was confirmed by analyzing serial dilutions of samples, and each reaction was performed at least in duplicate. Expression levels were determined from at least four animals for each genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The extracellular domain and transmembrane domains of Lrp4 are sufficient to rescue postsynaptic differentiation in *lrp4* mutant myotubes

(A) The cartoon depicts the structure of wild-type Lrp4 and Lrp4 proteins with mutations in the intracellular domain. A FLAG epitope tag was inserted near the N-terminal terminus of Lrp4. (B) Wild-type Lrp4 and the Lrp4-CD4 chimera restore Agrin stimulated tyrosine phosphorylation of MuSK in *lrp4*^{-/-} myotubes. (C) Quantitation of MuSK phosphorylation in *lrp4* mutant myotubes expressing wild-type Lrp4 or Lrp4-CD4 (n=3; mean ± sem). (D) Wild-type Lrp4 and Lrp4 mutant proteins restore Agrin stimulated AChR clustering in *lrp4*^{-/-} myotubes.

Figure 2. Muscle-selective expression of *Lrp4* rescues neuromuscular synapse formation in *lrp4* mutant mice

(A) Schematic representation of the *HSA::Lrp4* transgene. (B) *lrp4* RNA expression in muscle from P0 mice. *HSA::Lrp4-3* and *HSA::Lrp4-9A* express 3- and 9-fold more *lrp4*, respectively, than wild-type mice. (C) Motor axons (green) arborize and form nerve terminals (green), apposed to AChRs (red) in the central region of muscle from wild-type mice. In the absence of *Lrp4*, motor axons fail to stop and differentiate, and AChR clusters fail to form. Restoring *Lrp4* expression in muscle of *lrp4* mutant mice rescues these defects in presynaptic and postsynaptic differentiation. (D) Mice lacking *lrp4* die at birth; *lrp4* mutant mice carrying the *HSA::Lrp4-3* transgene survive postnatally. Arrows mark syndactyly, a known *lrp4* mutant phenotype. (E) Synaptic size and the density of synaptic AChRs is normal in *lrp4* mutant mice carrying the *HSA::Lrp4-3* transgene. $n=3$; mean $\pm$ sem.

Figure 3. Muscle-selective expression of an *Lrp4*-CD4 chimera rescues neuromuscular synapse formation in *lrp4* mutant mice

(A) Schematic representation of the *HSA::Lrp4-CD4* transgene. (B) *lrp4* RNA expression in muscle from P0 mice. *HSA::Lrp4-CD4-2* and *HSA::Lrp4-CD4-9* express 2- and 9-fold more *lrp4*, respectively, than wild-type mice. (C) Motor axons (green) arborize and form nerve terminals (green), apposed to AChRs (red) in the central region of muscle from wild-type and *lrp4* mutant mice carrying *HSA::Lrp4-CD4-2*. (D) Synaptic size and the density of synaptic AChRs is normal in *lrp4* mutant mice carrying the *HSA::Lrp4-CD4-2* transgene. n=3; mean $\pm$ sem. xx The *Lrp4*-CD4 chimera, expressed from the *HSA::Lrp4-CD4-2* transgene in *lrp4*^{-/-} mice, is concentrated at synapses.

Figure 4. Synaptic maturation is normal in *lrp4* mutant mice that express an Lrp4-CD4 chimera selectively in muscle

(A) The shape and complexity of AChR clusters reaches full maturity in postnatal *lrp4* mutant mice carrying either HSA::Lrp4-3 or HSA::Lrp4-CD4-2 transgene. (B) Synaptic size and the density of synaptic AChRs is normal in postnatal *lrp4* mutant mice carrying either HSA::Lrp4-3 or HSA::Lrp4-CD4-2 transgene. (C) A majority of synapses in P30 *lrp4* mutant mice carrying the HSA::Lrp4-3 transgene and nearly all synapses in *lrp4* mutant mice carrying the HSA::Lrp4-CD4-2 transgene are fully mature.