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Lrp4 Is A Retrograde Signal For Presynaptic Differentiation At Neuromuscular Synapses

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Abstract

Motor axons receive retrograde signals from skeletal muscle that are essential for the differentiation and stabilization of motor nerve terminals [1]. Identification of these retrograde signals has proved elusive, but their production by muscle depends upon the receptor tyrosine kinase, MuSK, and Lrp4, a LDLR family member that forms a complex with MuSK, binds neural Agrin and stimulates MuSK kinase activity [2–5]. Here, we show that Lrp4 also functions as a direct muscle-derived retrograde signal for early steps in presynaptic differentiation. We demonstrate that Lrp4 is necessary, independent of MuSK activation, for presynaptic differentiation *in vivo*, and we show that Lrp4 binds to motor axons and induces clustering of synaptic vesicle and active zone proteins. Thus, Lrp4 acts bi-directionally and coordinates synapse formation by binding Agrin, activating MuSK and stimulating postsynaptic differentiation and in turn functioning as a muscle-derived retrograde signal that is necessary and sufficient for presynaptic differentiation.

Postsynaptic muscle cells provide signals to motor axons that regulate the formation, maturation, stabilization and plasticity of neuromuscular synapses [1]. During development, motor axons approach and form synapses with muscle in a prepatterned region, marked by elevated expression and clustering of key postsynaptic proteins, including acetylcholine receptors (AChRs) [6–11]. Muscle prepatterning depends upon MuSK and Lrp4, which forms a complex with MuSK and stimulates MuSK kinase activity [3–7,9,12–14]. Stabilization of developing synapses requires motor neuron-derived Agrin, which binds Lrp4, stimulates further association between Lrp4 and MuSK and increases MuSK kinase activity, leading to anchoring of key proteins in the postsynaptic membrane and elevated transcription of ‘synaptic genes’ in myofiber synaptic nuclei [4,5,13,15–17].

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Author Contributions

N.Y. designed and performed all of the experiments illustrated in Figures 1, 2 and 3. N.K. designed and performed the experiments illustrated in Figure 4. S.J.B. helped to design and interpret experiments. All authors wrote and edited the manuscript.

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Lrp4 and MuSK are each required for presynaptic as well as postsynaptic differentiation, since motor axons grow beyond the prepatterned region and fail to cluster synaptic vesicles in mice deficient in either gene [2,3]. How Agrin, Lrp4 and MuSK control presynaptic differentiation is poorly understood. Because Lrp4 activates MuSK, the presynaptic defects in *lrp4* mutant mice could be a consequence of inadequate MuSK activation and a failure to produce novel retrograde signals. Alternatively, Lrp4 may have a direct role in regulating motor axon growth and differentiation. To distinguish between these possibilities, we established a cell culture assay to determine whether Lrp4 is sufficient to induce presynaptic differentiation. First, we co-cultured motor neurons, dissected from *HB9::GFP* transgenic mice, with skeletal muscle cells and established culture conditions that were permissive for presynaptic differentiation. Under these conditions, Synapsin, a protein that is associated with synaptic vesicles, accumulated in motor axons at sites that were apposed to AChR clusters in muscle (Figure 1a,b). We then co-cultured motor neurons with NIH 3T3 cells or 3T3 cells expressing Lrp4 and stained for Synapsin. Figure 1 shows that Synapsin is distributed homogenously in axons of motor neurons co-cultured with control 3T3 cells, whereas Synapsin accumulated in motor axons at sites of contact with Lrp4-expressing 3T3 cells (Figure 1c). We also co-cultured motor neurons with HEK 293 cells that expressed a Flag-tagged version of Lrp4, allowing us to visualize cell surface Lrp4. Lrp4 was clustered on the cell surface, and Synapsin accumulation in motor axons was often apposed to these clusters of Lrp4 (Figures 1d, S2, see below). In addition, we transfected 293 cells with truncated forms of Lrp4 and found that the LDLa repeats from the extracellular region of Lrp4, in the absence of the EGF-like and β-propeller domains, are sufficient to induce presynaptic differentiation (Figure S2).

These experiments suggested that Lrp4 is sufficient to trigger presynaptic differentiation but left open the possibility that Lrp4 acted together with other proteins expressed in 3T3 and 293 cells to induce presynaptic differentiation. Therefore, we treated *HB9::GFP* motor neurons with a Lrp4-Fc fusion protein, containing the LDLa repeats, attached to polystyrene microspheres, and stained for Synapsin. We found that Synapsin, as well as Synaptophysin and SV2, bona-fide synaptic vesicle proteins, were clustered at contact sites with Lrp4-LDLa-Fc beads (Figures 2a, S3). We also stained for Bassoon, a protein that is concentrated at synaptic vesicle fusion sites in nerve terminals, termed active zones, and found that Bassoon was similarly clustered by Lrp4-LDLa-Fc beads (Figure 2b). In contrast, neither Fc alone nor the LDLa repeats of Lrp1, another Lrp-family member, induced clustering of Synapsin or Bassoon, indicating that presynaptic differentiation is induced selectively by the LDLa repeats from Lrp4 (Figures 2b,c, S3). Moreover, addition of soluble, dimeric Lrp4-LDLa-Fc, unattached to beads, failed to induce presynaptic differentiation (Figure S4), suggesting that a large number of interactions, conferred by the attachment of ecto-Lrp4 to polystyrene microspheres, cooperate to mediate presynaptic differentiation.

Because MuSK, like Lrp4, is required for presynaptic differentiation *in vivo*, and because MuSK activation causes clustering of MuSK as well as Lrp4 at synapses, we determined whether the extracellular region of MuSK could also induce presynaptic differentiation. Although microspheres with ecto-MuSK-Fc or ecto-Lrp4-Fc attached equally well to motor axons (Figure S5), only ecto-Lrp4-Fc induced clustering of synaptic vesicle and active zone

proteins (Figure 2b). Moreover, myc-MuSK, expressed in 293 cells, failed to induce clustering of Synapsin (Figure S2). Thus, although Lrp4 and MuSK are each required for the differentiation of motor nerve terminals *in vivo*, only Lrp4 is sufficient to stimulate presynaptic differentiation.

Because Lrp4 binds neural Agrin, we asked whether Agrin was required for Lrp4 to induce presynaptic differentiation. We crossed *agrin*$^{-/-}$and *HB9::GFP* mice and treated *agrin*$^{-/-}$explants with Lrp4-LDLa-Fc. *Agrin*$^{-/-}$and wild-type motor neurons were equally responsive to Lrp4-LDLa-Fc beads (Figure 2d), indicating that Lrp4 induces presynaptic differentiation in a manner that does not depend upon Agrin.

To determine whether Lrp4 induced functional release sites, we measured recycling of synaptic vesicles using FM 4-64FX. Depolarization of motor neurons, treated with Lrp4-LDLa-Fc beads, caused uptake of FM 4-64FX, and further depolarization lead to the release of dye (Figure 2e,f). In contrast, depolarization of motor neurons, treated with Lrp1-Fc beads, led to low and uniform axonal uptake of FM 4-64FX (Figure 2e). Thus, Lrp4 induced both morphologically and functionally specialized neurotransmitter release sites.

Our experiments suggest that Lrp4 interacts with a protein(s) expressed by motor axons to promote presynaptic differentiation. To determine whether motor axons express an Lrp4-binding protein, we cultured explants from the ventral neural tube, which contains motor neurons, and probed the explants with an alkaline phosphatase (AP)-ecto-Lrp4 fusion protein. We stained for AP activity and found that AP-ecto-Lrp4 bound strongly to motor axons and preferentially along distal rather than proximal segments (Figure 3a,b), indicating that motor neurons express an Lrp4-binding protein(s) that is enriched ~30-fold on distal motor axons (Figure S6). The gradual and linear increase in binding from proximal to distal regions is likely due to an increase in number rather than affinity of Lrp4-binding sites, as preferential binding to distal segments is evident at the highest concentration (25 nM) of AP-ecto-Lrp4 that we tested (Figure S6). Binding of ecto-Lrp4 to motor axons is independent of Agrin and mediated by the LDLa repeats from Lrp4 (Figure 3b,c), mirroring the manner in which Lrp4 induces clustering of synaptic vesicle and active zone proteins. AP-ecto-Lrp4 also bound to axons emanating from dorsal neural tube explants, which lack motor neurons, though staining was less intense and more uniform, compared to motor axons (Figure S7).

We next sought to determine whether Lrp4 is essential for motor axons to terminate and differentiate *in vivo*. Previously, we showed that increasing *MuSK* expression in muscle of *agrin* mutant mice is sufficient to rescue AChR clustering and presynaptic differentiation, preventing the neonatal lethality of *agrin* mutant mice [18]. These experiments demonstrated that a modest increase in *MuSK* expression can bypass the requirement for Agrin and suggested that Agrin normally acts to insure for sufficient MuSK kinase activity to stabilize presynaptic and postsynaptic differentiation.

To determine whether *MuSK* over-expression could bypass the requirement for Lrp4 in synapse formation, we crossed *HSA::MuSK-L* transgenic mice, which express three-fold more *MuSK* in muscle than wild-type mice, with *lrp4* mutant mice and analyzed diaphragm

muscles from E18.5 mice. In the absence of Lrp4, AChRs fail to cluster, and motor axons grow without terminating or differentiating (Figure 4) [3]. *MuSK* over-expression fully restored AChR clustering in *lrp4* mutant mice (Figures 4, S8), indicating that *MuSK* over-expression can by-pass the normal requirement for Lrp4 in postsynaptic differentiation. *MuSK* over-expression, however, failed to rescue presynaptic differentiation in *lrp4* mutant mice. Instead, motor axons grew throughout the muscle and rarely contacted AChR clusters (Figure 4, S9). Moreover, *MuSK* over-expression did not rescue the neonatal lethality of *lrp4* mutant mice, which is caused by a failure to form neuromuscular synapses [19]. These findings demonstrate that Lrp4 has an essential and early role, independent of MuSK activation, in presynaptic differentiation *in vivo*, as Lrp4 is required to arrest motor axon growth and induce clustering of synaptic vesicles.

We have a good, though incomplete understanding of the signals and mechanisms for postsynaptic differentiation at neuromuscular synapses, and this knowledge has led to the identification of genes responsible for congenital myasthenia and the synaptic proteins that are targeted in autoimmune myasthenia gravis [20,21]. In contrast, discovery of the signals and mechanisms by which muscle cells control the differentiation of motor nerve terminals has proved more challenging and remains one of the glaring gaps in our understanding of neuromuscular synapses.

Here, we show that Lrp4 acts in a bidirectional manner, coordinating synaptic development, as Lrp4 not only binds Agrin and regulates postsynaptic differentiation but also functions as a muscle-derived retrograde signal for early steps in presynaptic differentiation. This dual role of Lrp4 in presynaptic and postsynaptic differentiation represents a parsimonious means for mediating reciprocal signaling between adjacent cells and resembles the dual roles that Eph and ErbB receptors play in responding to their respective ligands and stimulating signaling in ligand-presenting cells [22,23]. Our findings suggest Lrp4 functions as a critical check-point at three steps during synapse formation (Figure S1): first, prior to innervation, Lrp4 forms a complex with MuSK to establish muscle prepatterning; second, as motor axons approach muscle, Lrp4, clustered as a consequence of MuSK activation, acts as a retrograde signal to promote their differentiation; third, once motor axons establish contact with muscle, Lrp4 binds Agrin, released from motor nerve terminals, stimulating further MuSK phosphorylation and stabilizing neuromuscular synapses.

Other ligands, including members of the FGF7/10/22 family, laminin β2, collagen IV and SIRP-α, stimulate clustering of synaptic vesicles in cultured motor neurons and have a role in synaptic maturation *in vivo* [24]. Nonetheless, motor axons terminate and differentiate to a considerable extent in the absence of these signaling components, indicating that additional retrograde organizers regulate earlier steps in presynaptic differentiation [24]. Because motor axons fail to stop and display any signs of presynaptic differentiation in mice lacking Lrp4, Lrp4 acts at an early stage in presynaptic differentiation

Auto-antibodies to AChRs, MuSK or Lrp4 are responsible for myasthenia gravis [25]. The clinical and pathological manifestations of anti-Lrp4 myasthenia have not been described in detail, but our studies suggest that auto-antibodies to Lrp4 have the potential to obstruct synaptic function not only by blocking binding between Lrp4 and Agrin or Lrp4 and MuSK,

but also by interfering with binding between Lrp4 and Lrp4 receptors on nerve terminals. Because the premature withdrawal of motor nerve terminals, which causes muscle denervation, is an early step in amyotrophic lateral sclerosis and a characteristic feature of muscle wasting during aging [26,27], defects in retrograde signaling may underlie or contribute to neuromuscular diseases and sarcopenia.

Lrp4, like *MuSK*, is expressed in the cerebellum, cortex, hippocampus and olfactory bulb (www.brainatlas.org), raising the possibility that Lrp4 may regulate synaptic differentiation in the central nervous system (CNS). Although *lrp4* mutant mice die at birth [3], well before the peak period of synapse formation in the CNS, *lrp4* mutant mice, rescued for Lrp4 expression in muscle, survive as adults, and should provide a good model system for studying the role of Lrp4 in synapse formation in the CNS [19].

Methods Summary

Muscles from wild-type, *agrin* mutant and *lrp4* mutant mice were stained with antibodies to Synapsin, SV2, Synaptophysin or Bassoon to assess presynaptic differentiation and with α-BGT to measure postsynaptic differentiation. Explants of neural tube, containing motor neurons, were grown in cell culture together with muscle, control non-muscle cells or non-muscle cells expressing Lrp4 or MuSK. Alternatively, motor neurons were treated with polystyrene microspheres, which had the extracellular region of Lrp4, Lrp1 or MuSK attached to the beads. Presynaptic differentiation was measured by staining with antibodies to presynaptic proteins and by quantifying vesicle recycling with the styryl dye FM4-64FX. Binding of AP-ecto-Lrp4 to the cell surface of motor axons was visualized and quantitated by staining for AP activity.

Full Methods

Co-culture of motor neurons and muscle or non-muscle cells

Explants of neural tube from E11.5–13 *HB9::GFP* transgenic mice were dissected and cultured in Neuro Basal (NB) medium, supplemented with B27 and GlutaMax (Invitrogen), 2 ng/ml BDNF, 2 ng/ml GDNF (Cell Sciences), 2 ng/ml CTNF, 1 ng/ml NGF (Sigma) and antibiotics. The ventrolateral portion of the neural tube, containing motor neurons, was dissected and isolated based on *HB9::GFP* expression; the dissected dorsal region of the neural tube lacked GFP expression. Explants were cultured on poly-L-ornithine- and laminin-coated tissue culture dishes for 4 to 6 days before application of microspheres or addition of myotubes or non-muscle cells (NIH 3T3 or HEK 293 cells). Mouse myotubes were generated from primary myoblasts in a separate culture dish and transferred to explant cultures by non-enzymatically detaching myotubes, as described previously [28]. Non-muscle cells were transfected with Flag-Lrp4, Flag-Lrp4-mCherry or mCherry [4], sorted by flow cytometry for mCherry or cell surface Flag expression, using M2 antibodies (Sigma). We monitored Lrp4 expression either by viewing mCherry expression in cells transfected with Flag-Lrp4-mCherry (Figure 1c) or by staining for Flag in cells transfected with Flag-Lrp4 (Figures 1d, S1). Non-muscle cells were co-cultured with explants for 20–24 h in supplemented NB medium together with conditioned medium from rat Schwann cells or E12.5 mouse neural tube cells. One half of the medium was replaced every other day.

Assays for presynaptic differentiation

Co-cultures were fixed with 3.7% formaldehyde and stained with antibodies to Synapsin (Synaptic Systems), GFP (Abcam), Bassoon (Stressgen), SV2 (Developmental Studies Hybridoma Bank), Synaptophysin (Invitrogen) and Alexa 647-conjugated α-bungarotoxin (α-BGT) (Invitrogen). Human-Fc (Jackson ImmunoResearch), Lrp1-LDLa-Fc (Cluster II of Lrp1 from R&D Systems) or Lrp4-LDLa-Fc [14] were attached to Protein A microspheres (Bangslabs) and incubated with explants for 20–24 h in the co-culture growth medium described above. Some Lrp4-beads are inadvertently removed during washing, which may explain the absence of beads at some Synapsin clusters. Uptake of the styryl dye FM4-64FX, a tracer for recycling synaptic vesicles, was assessed by incubating cells for 2 min in a depolarizing buffer (90 mM KCl, 64mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose, 20 mM HEPES, pH 7.2) containing 10μM FM4-64FX (Invitrogen). After washing in a non-depolarizing buffer, dye release was monitored by further depolarizing cells for 2 min, largely as described by others [29,30]. Images were acquired on a Zeiss 510 confocal microscope and analyzed using Volocity 3D imaging software (Perkin Elmer). We defined synaptic puncta as Synapsin clusters that were ≥3 μm^2 in size for co-cultures of motor neurons and HEK 293 or NIH 3T3 cells and ≥1.5 μm^2 in size for motor neurons treated with microspheres. FM4-64FX clusters that were ≥1.5 μm^2 in size were designated as puncta. We determined the number of puncta in a field of 1.44×10^4 μm^2.

Staining with AP-Lrp4

AP-Lrp4 fusion proteins were generated as described previously [14], and their concentrations were determined by measuring AP activity. Explants were incubated for 90 min at room temperature with culture medium containing AP-fusion proteins (10nM) in binding buffer (150 mM NaCl, 2mM CaCl2, 1 mM MgCl2, 0.2% BSA, 20 mM HEPES, pH 7.2, 0.1% NaN3). After washing five times in binding buffer, the explants were fixed for 10 min in 3.7% formaldehyde, washed three times in HBS (150 mM NaCl, 20 mM HEPES, pH 7.2) and incubated for 30 min at 65°C to inactivate endogenous AP activity. Following three washes in reaction buffer (100 mM NaCl, 50mM MgCl2, 100 mM Tris, pH 9.5), AP activity was revealed by overnight incubation in reaction buffer with NBT/BCIP (Roche) at room temperature. Images were acquired with a CCD camera (Princeton Instruments) and images were analyzed with MetaMorph or Image J. To quantitate binding along the proximal-distal axis, we measured staining along short axon segments at varying distances from the soma, and we subtracted the values for binding of AP alone from the values for AP-ecto-Lrp4.

Mice

Mice that are null for *agrin*, mutant (*mitt*) for *lrp4*, or carry an *actin::MuSK* transgene (*MuSK-L*), which increases *MuSK* expression by three-fold, have been described previously [9318]. Similar results were found in mice that over-express *MuSK* by twenty-fold (*MuSK-H*) [18]. Diaphragm muscles from E18.5 mice were dissected and stained with antibodies to Neurofilament (NF) and Synaptophysin (Syn) and with α-BGT, as described previously [18]. We examined at least seventy AChR clusters from three or more mice of each genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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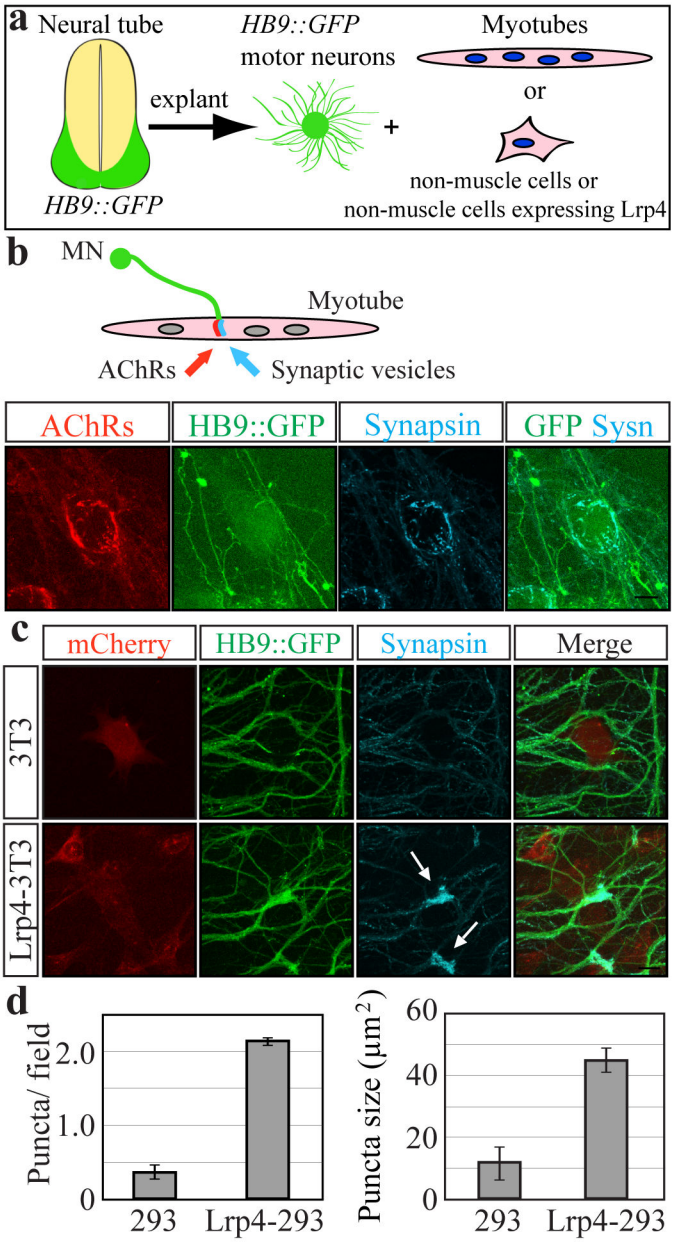

Figure 1. Lrp4-expressing non-muscle cells induce clustering of Synapsin in motor axons

a, Explants from the ventral neural tube of *HB9::GFP* transgenic mice, containing GFP-expressing motor neurons (MN), were co-cultured with primary muscle cells or non-muscle cells. **b**, Synapsin (blue) accumulates in motor axons (green) in apposition to clusters of AChRs (red) that form in muscle, marking synaptic sites. **c**, Synapsin is homogenously distributed in motor neurons that are co-cultured with control 3T3 cells, expressing mCherry alone, but clustered (arrows) in motor axons that contact 3T3 cells expressing Flag-Lrp4-mCherry (Lrp4-3T3). **d**, The number and size of Synapsin puncta are five-folder greater in axons contacting 293 cells expressing Flag-Lrp4 than control cells (m ± s.e.m., n=3). The bar = 10 μm.

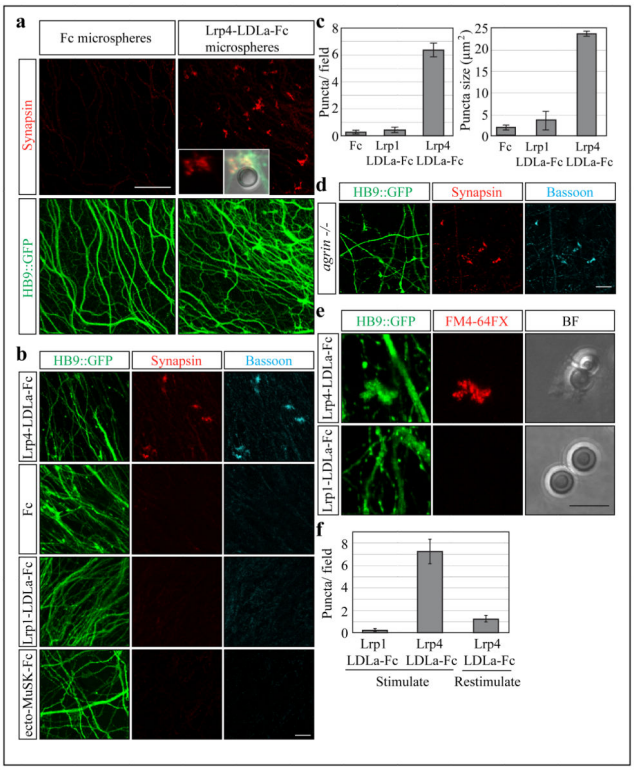

Figure 2. Lrp4, attached to polystyrene beads, induces presynaptic differentiation in motor neurons

a, Lrp4-LDLa-Fc, attached to polystyrene beads, induces clustering of Synapsin (red) in motor axons (green). Many Synapsin clusters are in close apposition with Lrp4-beads (inset). **b**, Lrp4 specifically induces clustering of Bassoon (blue) as well as Synapsin (red). **c**, Lrp4-beads induce a ~12-fold increase in the number of Synapsin puncta (m ± s.e.m., n=3). **d**, Lrp4-beads induce synaptic puncta, marked by Synapsin and Bassoon, in *agrin* mutant motor neurons. The response of *agrin* mutant motor neurons is not significantly different (106%) from wild-type motor neurons. **e,f**, Depolarization stimulates uptake (Stimulate) and release (Restimulate) of FM 4-64FX in motor axons at contact sites with Lrp4-LDLa beads, visualized by bright field (BF) microscopy (m ± s.e.m., n=3). The cartoon shows the LDLa repeats and β-propeller domains in Lrp4. The bar = 50 μm in a, 10 μm in b and d and 5 μm in e.

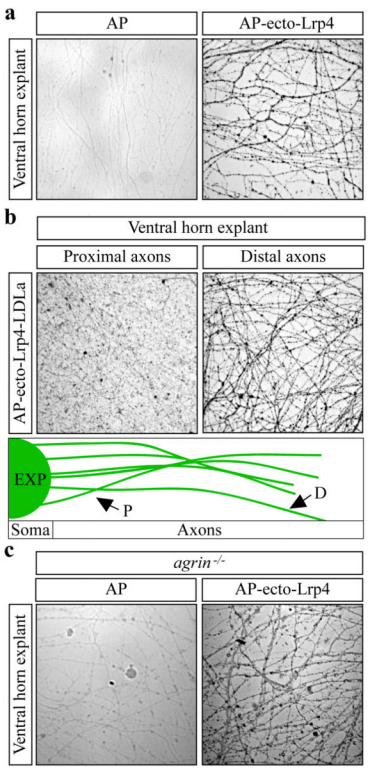

Figure 3. Lrp4 binds to motor axons

a, AP-ecto-Lrp4 binds to motor axons. **b**, AP-ecto-Lrp4-LDLa binds preferentially to distal (D) rather than proximal (P) segments of motor axons. **c**, AP-ecto-Lrp4 binds to distal segments of *agrin* mutant motor axons.

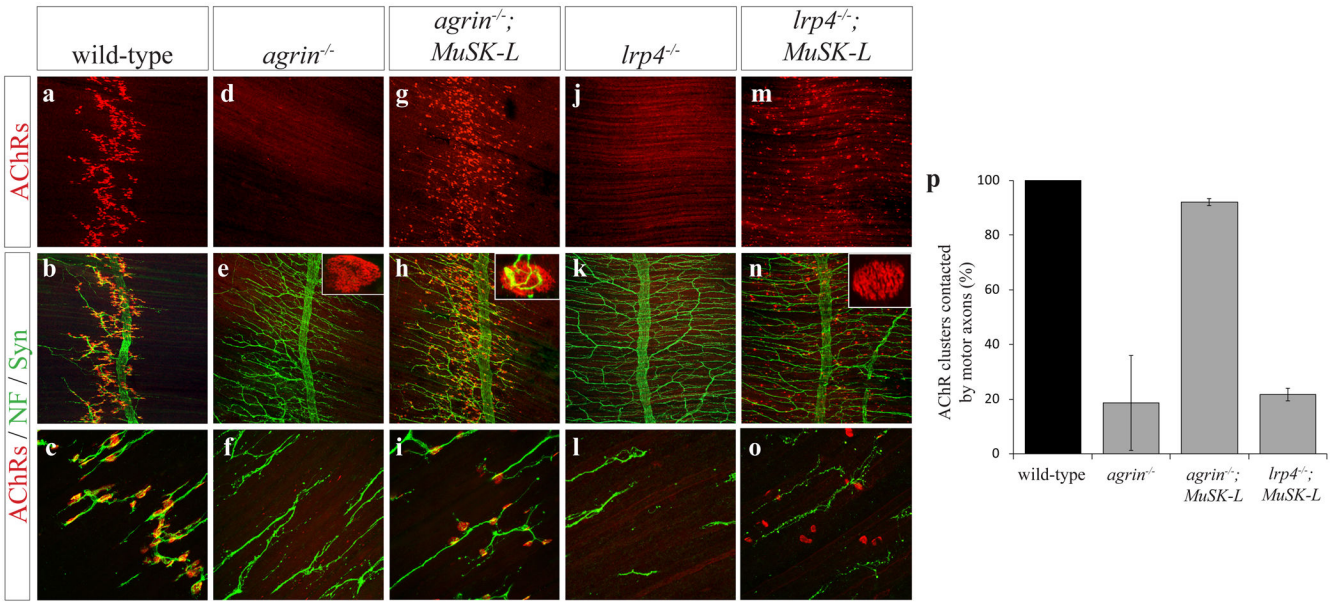

Figure 4. Lrp4 is essential for presynaptic differentiation independent of MuSK activation

a–i, In *agrin* mutant mice, a three-fold increase in *MuSK* expression, conferred by the *MuSK-L* transgene, restores AChR clusters and presynaptic differentiation [18]. **j–o**, In *lrp4* mutant mice, *MuSK-L* restores AChR clusters but not nerve terminal differentiation; instead, motor axons continue to grow beyond the prepatterned zone and fail to contact AChR clusters. **p**, In *agrin* mutant mice carrying *MuSK-L*, ~90% of AChR clusters are contacted by motor axons. In *lrp4* mutant mice that carry *MuSK-L*, ~15% of AChR clusters are contacted by motor axons; these contacts may be incidental, as motor axons grow and branch extensively throughout muscle of *lrp4* mutant mice, inevitably placing axons in the vicinity of AChR clusters (m ± s.e.m., n=3).