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(54) **Title**: DETECTION AND TREATMENT OF LRP4-ASSOCIATED NEUROTRANSMISSION DISORDERS

(57) **Abstract**: The present invention includes methods for the detection of neurotransmission or developmental disorders, including, but not limited to, myasthenia gravis that is seronegative for autoantibodies to the acetylcholine receptor (AChR) and/or muscle specific tyrosine kinase (MuSK), the method including detecting autoantibodies that bind to LRP4, or an epitope thereof. Also included are methods for the treatment of an individual suffering from a neurotransmission disorder, the method including detecting in a bodily fluid of the individual autoantibodies that bind to LRP4, or an epitope thereof, and administering to the patient an effective amount an immunosuppressant and/or another appropriate therapeutic modality. Also included are antibodies that bind to autoantibodies to LRP4 and kits for the detection of neurotransmission or developmental disorders.

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DETECTION AND TREATMENT OF LRP4-ASSOCIATED NEUROTRANSMISSION DISORDERS

CONTINUING APPLICATION DATA

10 This application claims the benefit of U.S. Provisional Application Serial No. 61/253,610, filed October 21, 2009, which is incorporated by reference herein.

BACKGROUND

15 Myasthenia gravis is an autoimmune disease that causes dysfunction of the neuromuscular synapses. Seventy percent of patients with myasthenia gravis carry autoantibodies to the acetylcholine receptor (AChR) and a separate 10% carry autoantibodies to muscle specific tyrosine kinase (MuSK) (Vincent and Leite, 2005, *Curr Opin Neurol*; 18(5):519-25). However, twenty percent of patients with
20 myasthenia gravis are seronegative for autoantibodies to AChR and MuSK. Thus, there is a need for improved diagnostic and treatment methods for neuromuscular disorders such as myasthenia gravis.

25

SUMMARY OF THE INVENTION

The present invention includes a method for diagnosing a neurotransmission or developmental disorder in a mammal, the method including detecting in a bodily fluid of the mammal autoantibodies that bind to the low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

30 The present invention includes a method for diagnosing a neurotransmission or developmental disorder associated with interference of agrin/MuSK/LRP4/AChR neuromuscular junction formation or function in a mammal, the method including detecting in a bodily fluid of the mammal autoantibodies to an epitope of low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

The present invention includes a method for diagnosing congenital and acquired muscle disorders associated with interference of agrin/MuSK/LRP4/AChR neuromuscular junction formation of functioning in a mammal, the method including detecting in a bodily fluid of the mammal autoantibodies to an epitope of low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

The present invention includes a method of diagnosing myasthenia gravis in a mammal, the method including detecting autoantibodies to an epitope of the low density lipoprotein receptor-related protein 4 (LRP4) in a bodily fluid of the mammal. In some aspects, the myasthenia gravis is seronegative for autoantibodies to the acetylcholine receptor (AChR) and/or muscle specific tyrosine kinase (MuSK).

In some aspects, the detection methods of the present invention include contacting the bodily fluid with a LRP4 polypeptide or antigenic determinant thereof, and detecting any antibody-antigen complexes formed between said LRP4 polypeptide or antigenic fragment thereof and antibodies present in the bodily fluid; wherein the presence of antibody-antigen complexes is indicative of said mammal suffering from a neurotransmission or developmental disorder. In some aspects, the antibody-antigen complex is detected using a LRP4, epitope, or antigenic determinant thereof tagged or labeled with a reporter molecule. In some aspects, the antibody-antigen complex is detected using an anti-IgG antibody tagged or labeled with a reporter molecule. In some aspects, the reporter molecule may include any of a heavy metal, a fluorescent or luminescent molecule, radioactive or enzymatic tag. In some aspects, the enzymatic tag may include horseradish peroxidase-protein A. In some aspects, the reporter molecule may be a radioactive label. In some aspects, the label may be ^{125}I.

The present invention includes an assay kit for diagnosing a neurotransmission disorder in a mammal, the kit including a LRP4 polypeptide or an epitope thereof. In some aspects of the assay kit, the LRP4 polypeptide or epitope thereof is immobilized on a solid surface. In some aspects, the assay kit may further include a means for contacting said LRP4 polypeptide or epitope thereof with a bodily fluid of said mammal. In some aspects, the assay kit may further include an acetylcholine receptor polypeptide, or fragment thereof, and/or a muscle specific tyrosine kinase (MuSK) polypeptide, or fragment thereof. In some aspects, the LRP4

polypeptide or epitope thereof has a detectable label thereon. In some aspects of the assay kit, the detectable label is ^{125}I. In some aspects, the assay kit may detect myasthenia gravis, muscular dystrophy, or a congential myasthenic syndrome. In some aspects the assay kit further includes a negative control and/or a positive
5 control.

The present invention includes an isolated or purified antibody specific for an anti-LRP4 autoantibody from a bodily fluid of a mammal. In some aspects, the antibody inhibits the binding of an anti-LRP4 autoantibody to LRP4. In some aspects, the antibody may be conjugated to a reporter molecule. The present
10 invention includes compositions including one or more such antibodies and a pharmaceutically acceptable carrier, diluent or excipient therefor. The present invention includes methods of treating a patient suffering from a neurotransmission disorder including administering to the patient an effective amount of such an antibody. The present invention includes diagnostic kits for detecting a
15 neurotransmission disorder in a mammal, the diagnostic kit including one or more such antibodies. In some aspects, the diagnostic kit may further including a means for contacting the antibody with a bodily fluid of the mammal.

The present invention includes a method of identifying compounds capable of alleviating or treating a neurotransmission disorder, the method including contacting a
20 candidate compound in the presence of LRP4 or an epitope thereof and an antibody capable of binding LRP4, wherein a compound that prevents binding of the antibody to LRP4 or an epitope thereof is a candidate for treating a neurotransmission disorder. The present invention includes compounds identified by such a method and methods of treating a patient suffering from a neurotransmission disorder including
25 administering to said patient an effective amount of one or more such compounds. In some aspects, the neurotransmission disorder is myasthenia gravis, muscular dystrophy, or a congential myasthenic syndrome. In some aspects, the neurotransmission disorder is seronegative for autoantibodies to the acetylcholine receptor (AChR) and/or muscle specific tyrosine kinase (MuSK).

30 The present invention includes a method of treating an individual suffering from a neurotransmission disorder, the method including detecting in a bodily fluid of the individual autoantibodies that bind to the low density lipoprotein receptor-related

protein 4 (LRP4), or an epitope thereof, and administering to the patient an effective amount an immunosuppressant and/or another appropriate therapeutic modality.

The present invention includes a method for diagnosing a neurotransmission or developmental disorder in a mammal, the method including detecting a genetic mutation in the low density lipoprotein receptor-related protein 4 (LRP4) gene. In some aspects of the method, the genetic mutation may be an intronic mutation, an exonic mutation, a splice junction mutation, a point mutation, a missense mutation, an insertion mutation, a deletion mutation, an insertion-deletion mutation, alters one or more amino acids, a read through mutation, a frameshift mutation, affects mRNA splicing, introduces a stop codon, affects mRNA half life, affects mRNA transcription, affects mRNA translation, reduces LRP4 mRNA and/or protein expression, and/or prevents LRP4 mRNA and/or protein expression.

In some aspects of the methods for diagnosing a neurotransmission or developmental disorder, the method may further include providing a report or print out summarizing the binding of autoantibodies to the low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

The present invention includes a method of treating an individual suffering from a neurotransmission disorder, the method including detecting a genetic mutation in the low density lipoprotein receptor-related protein 4 (LRP4) gene and administering to the patient an effective amount an immunosuppressant and/or another appropriate therapeutic modality.

In some aspects of the methods or kits of the present invention, the neurotransmission disorder is myasthenia gravis, muscular dystrophy, or a congenital myasthenic syndrome. In some aspects of the methods or kits of the present invention, the developmental disorder is muscle paralysis and/or fixed joints in newborn offspring due to maternal antibodies to LRP4. In some aspects of the methods or kits of the present invention, the neurotransmission or developmental disorder is seronegative for autoantibodies to the acetylcholine receptor (AChR) and/or muscle specific tyrosine kinase (MuSK).

In some aspects of the methods or kits of the present invention, a bodily fluid may include plasma, serum, whole blood, urine, sweat, lymph, feces, cerebrospinal fluid and nipple aspirate.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. LRP4 is specifically expressed in myotubes and concentrated at the NMJ. Fig. 1A shows the temporal expression pattern of LRP4 during muscle differentiation. C2C12 myoblasts were switched to the differentiation medium. Muscle cells were collected at indicated times and lyzed. Lysates (30 μg of protein) were resolved by SDS-PAGE and visualized by immunoblotting using indicated antibodies. Fig. 1B shows colocalization of LRP4 with R-BTX in muscle sections. Diaphragm sections were incubated with polyclonal antibodies against LRP4 or MuSK, which was visualized by Alexa Fluor 488-conjugated anti-rabbit antibody. R-BTX was included in the reaction to label postsynaptic AChRs. Arrows indicate co-localization of LRP4 or MuSK with AchRs. Fig. 1C shows enrichment of LRP4 in synaptic regions of muscles. Synaptic (S) and non-synaptic (NS) regions of hemi-diaphragms were isolated and homogenized. Homogenates (30 μg of protein) were analyzed for LRP4 or AChR (as control) using specific antibodies. Samples were also probed for ß-actin to indicate equal loading.

Figure 2. The LRP4 extracellular domain interacts with neuronal agrin. Figs. 2A-2C show interaction of LRP4 and neuronal agrin in solution. Beads were conjugated with Flag-nAgrin, which were subsequently incubated with condition media of HEK293 cells expressing LRP4N-Myc (Fig. 1A), MuSKect-Myc (Fig. 1B), LRP6N-Myc (Fig. 1C), or empty vector (control). Bound proteins were isolated by bead precipitation, resolved by SDS-PAGE and visualized by immunoblotting with anti-Myc antibody. Flag-nAgrin interacted with LRP4N-Myc (Fig. 1A), but not MuSKect-Myc (Fig. 1B) or LRP6N-Myc (Fig. 1C). Fig. 1D shows interaction of Wnt-1 and LRP6N. Beads were conjugated with Wnt-1-HA, which were subsequently incubated with LRP6N-Myc. Bound LRP6N-Myc was revealed by immunoblotting. Fig. 2E demonstrates no interaction between Wnt-1 and LRP4N. Beads were conjugated with Wnt-1-HA, which were subsequently incubated with LRP4N-Myc. Bound LRP4N-Myc was revealed by immunoblotting.

Figure 3. High-affinity and specific interaction between of LRP4-neuronal agrin. Fig. 3A presents schematic diagrams of AP constructs. Neuronal or muscle agrin was fused to AP in pAPtag-5. The fusion proteins contain a signal peptide (SS)

in the N-terminus, and two additional tags (Myc and His) in the C-terminus. Neuronal agrin contains 4- and 8-amino acid residue inserts at the Y and Z sites, respectively. Fig. 3B presents functional characterization of agrin-AP recombinant proteins. C2C12 myotubes were stimulated with AP alone, mAgrin-AP or nAgrin-AP for 18 hours (hr). AChR clusters were assayed as described in Experimental Procedures of Example 1. Data shown were mean ± SEM. n = 4; *, P < 0.05 in comparison with AP or mAgrin-AP. Fig. 3C presents differential binding activities of mAgrin-AP and nAgrin-AP to myoblasts and myotubes. C2C12 myoblasts and myotubes were incubated AP alone, mAgrin-AP or nAgrin-AP for 90 minutes (min) at room temperature. Endogenous AP was inactivated by heating and bound AP was assayed by staining with BCIP/NBT. Data shown were mean ± SEM. n = 6; *, P < 0.05. Fig. 3D shows direct interaction between LRP4 and neuronal agrin. LRP4-Myc was purified and coated on Maxi-Sorp Immuno Plates, which were incubated with nAgrin-AP or mAgrin-AP. AP activity was measured with pNPP as substrate. Control, condition medium of HEK293 cells transfected with the empty pAPtag-5. Data shown were mean ± SEM. n = 3; *, P < 0.05 in comparison with AP or mAgrin-AP. Fig. 3E presents dose-dependent interaction between LRP4 and neuronal Agrin. Purified LRP4-Myc was coated on Maxi-Sorp Immuno Plates, which were incubated with nAgrin-AP or mAgrin-AP. AP activity was measured with pNPP as substrate. Data shown were mean ± SEM. n = 4; *, P < 0.05. Fig. 3F is a Scatchard plot of data in Fig. 3E. Y axis represents the ratio of bound to free nAgrin-AP whereas X axis represents the concentration of bound nAgrin-AP.

Figure 4. Expression of LRP4 enables binding activity for neuronal agrin and MuSK signaling. Fig. 4A shows neuronal, but not muscle, agrin bound to intact C2C12 myoblasts transfected with LRP4. C2C12 myoblasts were transfected by empty vector (control), LRP4 and/or Flag-MuSK. 36 hr after transfection, myoblasts were incubated with AP alone, mAgrin-AP or nAgrin-AP for 90 min at room temperature. Endogenous AP was inactivated by heating and bound AP was visualized in cells by staining with BCIP/NBT. Fig. 4B is a quantification of data in Fig. 4A. Data shown were mean ± SEM. n = 6; *, P < 0.05 in comparison with mAgrin-AP of the same group or nAgrin-AP in the control group. Fig 4C and 4D show nAgrin-AP bound to HEK293 cells expressing LRP4, but not those expressing

LRP5. HEK293 cells were transfected without (control) or with LRP4-Myc (Fig. 4C) or LRP5-Myc (Fig. 4D). 36 hr after transfection, transfected cells were incubated with nAgrin-AP or mAgrin-AP. In some experiments, control cells were incubated with nAgrin-AP. After heat inactivation of endogenous AP, lysates were assayed for transfected AP using pNPP as substrate. Lysates were also subjected to immunoblotting to reveal the expression of different amounts of LRP4-Myc (Fig. 4C) and LRP5-Myc (Fig. 4D). Data shown were mean ± SEM. n = 6. Fig. 4E and 4F show LRP4 expression enabled MuSK and Abl activation by agrin in HEK293 cells. Cells were transfected with LRP4 and/or Flag-MuSK (Fig. 4E) or Flag-Abl (Fig. 4F). 36 hr after transfection, cells were treated without or with neuronal agrin for 1 hr and were then lyzed. In Fig. 4E, lysates were incubated with anti-Flag antibody, and resulting immunocomplex was analyzed with anti-phosphotyrosine antibody 4G10. In Fig. 4F, active Abl was revealed by immunoblotting with specific phospho-Abl antibody. Lysates were also blotted for Flag and/or Myc, LRP4, or ß-actin to indicate equal amounts of proteins. Fig. 4G is a quantitative analysis of data in E and F. MuSK and Abl phosphorylation was quantified by using the ImageJ software. Data shown were mean ± SEM. n = 3; *, $P < 0.05$ in comparison with control.

Figure 5. Suppression of LRP4 expression attenuates neuronal agrin binding, MuSK activation, and induced AChR clustering. Fig. 5A is a characterization of LRP4-miRNA constructs. HEK293 cells were transfected with LRP4 and LRP4-miLRP4 constructs or control miRNA that encoded scrambled sequence. Cell lysates were analyzed for LRP4 expression by immunoblotting with anti-LRP4 antibody. ß-Actin was used as loading control. miLRN4-1062 was most potent in inhibiting LRP4 expression. Fig. 5B shows repression of LRP4 expression reduced neuronal agrin binding to myotube surface. C2C12 myotubes were transfected with control (scramble) miRNA or miLRP4-1062. Cells were incubated with AP, mAgrin-AP or nAgrin-AP, which was visualized in cell as described in Fig. 3A. Fig. 5C is a quantitative analysis of data in Fig. 5B. Data shown were mean ± SEM. n = 6; *, $p < 0.05$ in comparison nAgrin-AP with control. Fig. 5D shows MuSK activation by neuronal agrin was diminished in C2C12 myotubes transfected with miLRP4-1062. C2C12 myotubes were transfected with control miRNA or miLRP4-1062. 36 hr later, myotubes were treated without or with agrin for 1 hr and

cells were then lyzed. MuSK was isolated by immunoprecipitation and blotted with the anti-phosphotyrosine antibody 4G10. Lysates were also blotted for MuSK, LRP4, GFP (encoded by miRNA constructs), and ß-actin to indicate equal amounts of proteins. Fig. 5E is a quantitative analysis of data in D by ImageJ software (mean ± SEM, n = 3; *, $P < 0.05$ in comparison with control). Fig. 5F shows neuronal agrin-induced clustering of AChRs was inhibited in C2C12 myotubes transfected with miLRP4-1062. C2C12 myotubes were transfected by control miRNA, miLRP4-1062, miMuSK-1161, or miLRP5-1490. AChR clusters were induced by neuronal agrin and quantified as described in Experimental Procedures (mean ± SEM, n = 5; *, $p < 0.05$ in comparison with control). miMuSK-1161 and miLRP5-1490 were able to suppress expression of respective proteins in transfected cells.

Figure 6. Direct interaction between LRP4 and MuSK. Fig. 6A shows increased LRP4-MuSK interaction in the presence of neuronal agrin. Flag-MuSKect immobilized on beads were incubated with condition media of cells expressing the extracellular domains of LRP4 (LRP4N-Myc) or the empty vector (control) in the presence or absence of neuronal agrin. Precipitated LRP4 was analyzed by immunoblot with anti-Myc antibody. Reaction mixtures were also blotted directly for Flag and Myc to demonstrate equal amounts of proteins. Fig. 6B is a quantitative analysis of LRP4N-Myc and Flag-MuSK. Data shown were mean ± SEM, n = 3; *, $p < 0.05$ in comparison with the no-agrin group. Fig. 6C shows a dose-dependent interaction between LRP4 and MuSK. Purified LRP4-Myc was coated on Maxi-Sorp Immuno Plates, which were incubated with MuSK-AP. Bound AP was measured with pNPP as substrate. Data shown were mean ± SEM. n = 4. Fig. 6D is a Scatchard plot of data in Fig. 6C. Y axis represents the ratio of bound to free MuSK-AP whereas X axis represents the concentration of bound MuSK-AP. Fig. 6E shows no interaction of LRP6 and MuSK extracellular domains. Experiments were done as in Fig. 6A except condition medium of cells expressing the extracellular domain of LRP6 was used. Fig. 6F shows co-immunoprecipitation of LRP4 and MuSK. HEK293 cells were transfected with LRP4 and/or Flag-MuSK. Lysates were incubated with anti-Flag antibody, and resulting immunocomplex was analyzed for LRP4 and Flag. Lysates were also probed to indicate equal amounts of indicated proteins. Fig. 6G shows interaction of LRP4 with MuSK in mouse muscles. Mouse

muscles of indicated ages were homogenized, and homogenates were incubated with rabbit anti-LRP4 antibody or rabbit normal IgG. Precipitates were probed for MuSK and LRP4. Homogenates were also probed directly for MuSK, LRP4, and ß-actin (bottom panels).

Figure 7. Agrin stimulates the LRP4-MuSK interaction and LRP4 tyrosine phosphorylation. Fig. 7A shows agrin stimulated the interaction between endogenous LRP4 and MuSK. C2C12 myotubes were stimulated without or with neuronal agrin. Lysates were subjected to immunoprecipitation with rabbit anti-LRP4 antibody (top panels) or rabbit normal IgG (middle panels). Resulting precipitates were probed for MuSK or LRP4. Lysates were also probed with antibodies against LRP4, MuSK, or ß-actin to demonstrate equal amounts (bottom panels). Fig. 7B is a quantitative analysis of data in Fig. 7A by using the ImageJ software (mean ± SEM, $n = 3$; *, $P < 0.05$ in comparison with the no-agrin group).

Fig. 7C shows agrin stimulated tyrosine phosphorylation of LRP4 in muscle cells. C2C12 myotubes were treated without or with agrin for 1 hr. Lysates were subjected to immunoprecipitation with antibodies against LRP4 and MuSK, respectively. Resulting precipitates were probed with anti-phospho-tyrosine antibody 4G10, or antibodies against LRP4 and MuSK, respectively, to indicate equal amounts of precipitated proteins. Fig. 7D is a quantitative analysis of data in Fig. 7C. Data shown were mean ± SEM, $n = 3$; *, $p < 0.05$ in comparison with no-nAgrin. Fig. 7E presents a working model. In the absence of neuronal agrin, LRP4 could interact with MuSK and this interaction is increased by agrin stimulation. Such interaction is necessary for MuSK activation and downstream signaling that leads to AChR clustering. P, phosphorylation.

Figure 8. Attenuation of agrin function by LRP4 extracellular domain. Fig. 8A shows attenuation of agrin-induced AChR clustering by the extracellular domain of LRP4. C2C12 myotubes were treated without (control) or with neuronal agrin (nAgrin) or nAgrin that was pre-incubated with LRP4N-Myc immobilized on beads for 18 hr. Representative myotubes were shown. Fig. 8B is a quantitative analysis of data in Fig. 8A. Data shown were mean ± SEM, $n = 5$; *, $p < 0.05$ in comparison with nAgrin. Fig. 8C shows inhibition of MuSK phosphorylation by the extracellular domain of LRP4. C2C12 myotubes were treated as in Fig. 8A, except for 1 hr.

Lysates were subjected to immunoprecipitation with rabbit anti-MuSK antibody. Resulting precipitates were probed with the anti-phospho-tyrosine antibody 4G10. Precipitates were also probed with anti-MuSK antibody to demonstrate equal amounts. Fig. 8D is a quantitative analysis of data in Fig. 8C. Data shown were
5 mean ± SEM, n = 3; *, $p < 0.05$ in comparison with the no-agrin group.

Figure 9. LRP4 antibodies are detected by ELISA. Antibodies to LRP4 were found in 12 of 31 AChR/MuSK seronegative MG patients compared with healthy controls.

Figure 10. Antibodies from AChR/MuSK seronegative MG patients bind to
10 LRP4. AChR/MuSK-antibody-negative MG IgGs bound to 293 cells expressing full-length, whereas healthy control IgG did not (normal serum). LRP4 immunoreactivity appeared as a speckled pattern, similar to that seen previously with rabbit anti-LRP4 antibodies.

Figure 11. Amino acid sequence of human low density lipoprotein
15 receptor-related protein 4 (LRP4) precursor (SEQ ID NO:11).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

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The present invention identifies the low density lipoprotein receptor-related protein 4 (LRP4) as the postsynaptic receptor for agrin, demonstrates that LRP4 and muscle specific tyrosine kinase (MuSK) associate, and provides the first demonstration of the association of autoantibodies to LRP4 with a subset of
25 seronegative myasthenia gravis (MG). Included in the present invention are methods for diagnosing a neurotransmission or developmental disorder in a subject by detecting autoantibodies that bind to the low density lipoprotein receptor-related protein 4 (LRP4), or an antigenic epitope thereof, in a sample obtained from the individual. Such a neurotransmission or developmental disorder may be associated
30 with interference of agrin/MuSK/LRP4/AChR neuromuscular junction formation or function in a mammal. A neurotransmission or developmental disorder may be a congenital or acquired muscle disorder associated with interference of

agrin/MuSK/LRP4/AChR neuromuscular junction formation or functioning.

Neurotransmission disorders include, but are not limited to, myasthenia gravis, muscular dystrophy, and congential myasthenic syndrome (CMS). Congenital myasthenic syndromes are heterogeneous disorders in which neuromuscular
5 transmission is compromised by one or more specific mechanisms. See, for example, Engel, 2008, *Handb Clin Neurol*; 91:285-331. A developmental disorder includes, but is not limited to, muscle paralysis and/or fixed joints in newborn offspring due to maternal antibodies to LRP4.

Myasthenia gravis (MG) is an autoimmune disease that causes dysfunction of
10 the neuromuscular synapses. Seventy percent of patients with myasthenia gravis carry autoantibodies to the acetylcholine receptor (AChR) and a separate 10% carry autoantibodies to muscle specific tyrosine kinase (MuSK). However, twenty percent of patients with myasthenia gravis are seronegative for autoantibodies to AChR and MuSK. A neurotransmission disorder may be seronegative for autoantibodies to the
15 AChR, including myasthenia gravis in patients who are anti-AChR autoantibody negative (AAAN). A neurotransmission disorder may be seronegative for autoantibodies to MuSK, including myasthenia gravis in patients who are anti-MuSK autoantibody negative. The MuSK protein has been sequenced and the protein characterized recently by Valenzuela et al. (International patent application number
20 PCT/US96/20696, published as WO97/21811). It is a receptor tyrosine kinase (RTK) located on the cell surface of muscle cells at the neuromuscular junction. Methods of detecting autoantibodies to the MuSK protein for the diagnosis of neuromuscular disorders are described, for example, in US Patent 7,267,820. A neurotransmission disorder may be seronegative for autoantibodies to both the AChR and MuSK,
25 including myasthenia gravis in patients who are anti-AChR autoantibody negative (AAAN) and anti-MuSK auto antibody negative. Such a neurotransmission disorder may be moderate or severe generalized MG in which a standard radio-immunoprecipitation assay for anti-AChR antibodies and/or anti-MuSK antibodies is negative on several occasions.

30 A neurotransmission disorder, such as MG, may be characterized by fatigable muscle weakness and may be confirmed, for example, by electromyographic evidence of a defect in neuromuscular transmission (a decrement of more than 10% in the

amplitude of the compound muscle action potential on repetitive nerve stimulation at 3 Hz and/or an increase in jitter on single fiber studies), or by a positive response to anticholinesterase medication (edrophonium or pyridostigmine).

LRP4 (or MEGF7, for multiple epidermal growth factor (EGF)-like domain 7)
5 is a member of the LDLR family, and contains a large extracellular N-terminal region that possesses multiple EGF repeats and LDLR repeats, a transmembrane domain and a short C-terminal region without an identifiable catalytic motif (Johnson et al., 2005, *Hum Mol Genet*; 14:3523–3538; Lu et al., 2007, *Brain Res*; 1177:19–28; Tian et al., 2006, *Eur J Neurosci*; 23:2864–2876; Yamaguchi et al., 2006, *Gene Expr Patterns*;
10 6:607–612). It was identified by a motif trap screen of genes encoding proteins with multiple EGF domains (Nakayama et al., 1998, *Genomics*; 51:27–34).

With the methods, antibodies, and kits of the present invention, a LRP4 polypeptide may be from a mammal, including, for example, human, mouse, or rat. A fragment of a LRP polypeptide may include an antigenic epitope and be bound by
15 an antibody. A fragment may include the extracellular domain. A fragment may include the intracellular domain. A fragment thereof may include one or more EGF repeats and/or one or more LDLR repeats of the LRP4 polypeptide. LRP4 genomic and amino acid sequences are available for a variety of mammals, including, but not limited to mouse (see, for example, informatics.jax.org/searches/
20 accession_report.cgi?id= MGI:2442252 on the worldwide web), rat (see, for example, RGD ID 619731; and rgd.mcw.edu/tools/genes/genes_view.cgi?id= 619731 on the worldwide web), and human (see, for example, Naayama et al., Genomics 1998, 51(1):27-34; GENBANK Accession No. NM_002334; and UniProtKB/Swiss-Prot O75096). Human lipoprotein receptor-related protein 4 (LRP4) polypeptide includes,
25 but is not limited to, the LRP4 polypeptide produced from the amino acid sequence shown in Fig. 11.

The present invention includes methods for diagnosing a neurotransmission or developmental disorder in a subject by detecting in a bodily fluid obtained from the individual autoantibodies that bind to the low density lipoprotein receptor- related
30 protein 4 (LRP4), or an antigenic fragment thereof (also referred to herein as "antigenic determinant" or "epitope thereof").

Autoantibodies may be detected by any of a variety of methods, including, but

not limited to, the methods described herein and any suitable method available to the skilled artisan. Immunoassays that can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as BIAcore analysis, FACS (Fluorescence activated cell sorter) analysis, immunofluorescence, immunocytochemistry, Western blots, radio-immunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art. With any of the methods of the present invention, the intensity of a signal from an anti-human immunoglobulin autoantibody may be indicative of the relative amount of the anti-LRP4 autoantibody in the bodily fluid when compared to a positive and negative control reading.

With any of the methods of the present invention, an antibody-antigen complex may be detected for example, by using a LRP4, or antigenic determinant thereof tagged or labeled with a reporter molecule or an anti-immunoglobulin antibody tagged or labeled with a reporter molecule. An anti-immunoglobulin antibody may include, but is not limited to, an anti-IgG, an anti-IgM, an anti-IgG1, or and anti-IgG4 antibodies. A reporter molecule may be, for example, a heavy metal, a fluorescent or luminescent molecule, a radioactive tag (such as, for example, said label is ^{125}I), and an enzymatic tag (such as, for example, horseradish peroxidase-protein A followed by reaction with o-phenylenediamine for subsequent measurement at A^{492})

The actual steps of detecting autoantibodies in a sample of bodily fluids may be performed in accordance with immunological assay techniques known in the art. Examples of suitable techniques include ELISA, radioimmunoassays and the like. An assay may use an antigen which may be immobilized on a solid support. A sample to be tested may be brought into contact with the antigen and if autoantibodies specific to the protein are present in a sample they will immunologically react with the antigen to form autoantibody-antigen complexes which may then be detected or quantitatively measured. Detection of autoantibody-antigen complexes may be carried out using a secondary anti-human immunoglobulin antibody, typically anti-IgG or anti-human

IgM, which recognizes general features common to all human IgGs or IgMs, respectively. The secondary antibody is usually conjugated to an enzyme such as, for example, horseradish peroxidase (HRP) so that detecting of autoantibody/antigen/secondary antibody complexes is achieved by addition of an enzyme substrate and subsequent calorimetric, chemiluminescent or fluorescent detection of the enzymatic reaction products.

Thus, in one embodiment the antibody/antigen complex may be detected by a further antibody, such as an anti-IgG antibody. Complexes may alternatively be viewed by microscopy. Other labels or reporter molecules which may be used in a method according to the invention. A reporter molecule or label may include any of a heavy metal, a fluorescent or luminescent molecule, radioactive or enzymatic tag. The label or reporter molecule may be such that the intensity of the signal from the anti-human IgG antibody is indicative of the relative amount of the anti-LRP4 autoantibody in the bodily fluid when compared to a positive and negative control reading.

An alternative method of detecting autoantibodies for LRP4 or an epitope thereof relies upon the binding of LRP4 or its epitope, together with a revealing label, to the autoantibodies in the serum or bodily fluid. This method may include contacting LRP4 or an epitope or antigenic determinant thereof having a suitable label thereon, with a bodily fluid, immunoprecipitating any antibodies from the bodily fluid and monitoring for label on any of the antibodies, wherein the presence of label may be indicative of a mammal suffering from a neurotransmission or developmental disorder. The label may be a radioactive label, such as for example, ^{125}I, or the like. Iodination and immunoprecipitation are standard techniques in the art.

Any of the diagnostic methods of the present invention may include the additional step of providing a report or print out summarizing the binding of autoantibodies in a sample to the low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof. For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The present invention includes assay kits for diagnosing a neurotransmission disorder in a mammal. Such kits may include a LRP4 polypeptide or an epitope

thereof. The LRP4 polypeptide or fragment thereof may be immobilized on a solid surface. Such kits may further include means for contacting the LRP4 polypeptide, or epitope thereof, with a bodily fluid from a mammal. Thus, an assay system for detecting neurotransmission disorders, including myasthenia gravis in patients who
5 are anti-AChR autoantibody negative (AAAN) and anti-MuSK auto antibody negative is provided. Prior to the present invention there was no basis for providing an immediate clinical diagnosis for such patients.

Such kits may further include a acetylcholine receptor polypeptide, or fragment thereof, and/or a muscle specific tyrosine kinase (MuSK) polypeptide, or
10 fragment thereof. Such polypeptides or fragments may be be immobilized on a solid surface. With the kits and methods of the present invention, LRP4, AchR, and/or MuSK polypeptides and fragments thereof may have a detectable label thereon, including, but not limited to, ^{125}I. The assay kits and methods of the present invention may also include appropriate negative controls and/or a positive controls.

15 Also provided by the invention is an isolated or purified autoantibody specific for LRP4. Such an antibody can be detected in bodily fluids of mammals and isolated or purified therefrom using techniques which would be known to the skilled practitioner, such as, immunoabsorption, or immunoaffinity chromatography or high pressure chromatography.

20 The present invention includes isolated or purified antibodies that specifically bind an anti-LRP4 autoantibody from a bodily fluid of a mammal. Such an antibody may inhibit the binding of an anti-LRP4 autoantibody to LRP4. Such an antibody may be conjugated to a reporter molecule. The present invention includes diagnostic kit for detecting neurotransmission disorders including one or more such antibodies.
25 The present invention includes methods of treating an individual suffering from a neurotransmission disorder by administering an effective amount of an antibody antibodies specific for an anti-LRP4 autoantibody.

As used herein, specific binding means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by
30 determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity, for example, an antibody that binds a distinct epitope or antigen. Specificity of binding

also can be determined, for example, by competition with a control molecule, for example, competition with an excess of the same molecule. In this case, specific binding is indicated if the binding of a molecule is competitively inhibited by itself. Thus, specific binding between an antibody and antigen is measurably different from
5 a non-specific interaction and occurs via the antigen binding site of the antibody.

An antibody may be an intact antibody, an antibody binding fragment, or a chimeric antibody. A chimeric antibody may include both human and non-human portions. An antibody may be a polyclonal or a moncoclonal antibody. An antibody may be a derived from a wide variety of species, including, but not limited to mouse
10 and human. An antibody may be a humanized antibody. An antibody may be linked to another functional molecule, for example, another peptide or protein, a toxin, a radioisotype, a cytotoxic agent, cytostatic agent, a polymer, such as, for example, polyethylene glycol, polypropylene glycol or polyoxyalkenes.

The antibodies of the present invention include various antibody fragments,
15 also referred to as antigen binding fragments, which include only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments include, for example, Fab, Fab', Fd, Fd', Fv, dAB, and F(ab')2 fragments produced by proteolytic digestion and/or reducing disulfide bridges and fragments produced from
20 an Fab expression library. Such antibody fragments can be generated by techniques well known in the art. Antibodies of the present invention can include the variable region(s) alone or in combination with the entirety or a portion of the hinge region, CH1 domain, CH2 domain, CH3 domain and/or Fc domain(s).
Antibodies include, but are not limited to, polyclonal antibodies, affinity-purified
25 polyclonal antibodies, monoclonal antibodies, human antibodies, humanized antibodies, chimeric antibodies, anti-idiotypic antibodies, multispecific antibodies, single chain antibodies, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F(ab') fragments, F(ab')2 fragments, Fv fragments, diabodies, linear antibodies fragments produced by a Fab expression library, fragments comprising
30 either a VL or VH domain, intracellularly-made antibodies (i.e., intrabodies), and antigen-binding antibody fragments thereof.

An antibody of the present invention can be of any type (e.g., IgG, IgE, IgM,

IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Immunoglobulins can have both heavy and light chains. An array of IgG, IgE, IgM, IgD, IgA, and IgY heavy chains can be paired with a light chain of the kappa or lambda form.

5 An antibody of the invention can be from any animal origin, including birds and mammals. In some embodiments, the antibodies are human, murine, rat, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin

10 libraries or from animals transgenic for one or more human immunoglobulins.

The term "polyclonal antibody" refers to an antibody produced from more than a single clone of plasma cells. In contrast "monoclonal antibody" refers to an antibody produced from a single clone of plasma cells. The preparation of polyclonal antibodies is well known. Polyclonal antibodies may be obtained by immunizing a

15 variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, hamsters, guinea pigs and rats as well as transgenic animals such as transgenic sheep, cows, goats or pigs, with an immunogen. The resulting antibodies may be isolated from other proteins by using an affinity column having an Fc binding moiety, such as protein A, or the like.

20 Monoclonal antibodies can be obtained by various techniques familiar to those skilled in the art. For example, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell. Monoclonal antibodies can be isolated and purified from hybridoma cultures by techniques well known in the art. Other known methods of producing transformed B cell lines that

25 produce monoclonal antibodies may also be used. In some embodiments, the antibody can be recombinantly produced, for example, produced by phage display or by combinatorial methods. Such methods can be used to generate human monoclonal antibodies.

A therapeutically useful antibody may be derived from a "humanized"

30 monoclonal antibody. Humanized monoclonal antibodies are produced by transferring one or more CDRs from the heavy and light variable chains of a mouse (or other species) immunoglobulin into a human variable domain, then substituting

human residues into the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with immunogenicity of murine constant regions. The constant region of a humanized monoclonal antibody of the present invention can be 5 that from human immunoglobulin belonging to any isotype. It may be, for example, the constant region of human IgG.

An antibody which is specific for anti-LRP4 autoantibodies may be used in an immunoadsorption therapy for the treatment of a neurotransmission disorder, including, but not limited to, seronegative myasthenia gravis.

10 An antibody which is specific for anti-LRP4 autoantibodies may be used in a diagnostic kit for detecting neurotransmission disorders, including, but not limited to, seronegative myasthenia gravis. Such a kit may include one or more isolated or purified antibodies specific for anti-LRP4 autoantibody. Such a kit may further include a means for contacting the antibody with a bodily fluid.

15 In accordance with the present invention a bodily fluid may be, for example, plasma, serum, whole blood, urine, sweat, lymph, feces, cerebrospinal fluid or nipple aspirate. In some embodiments, the methods of the invention will be performed on samples of serum or plasma.

The present invention includes methods of identifying compounds capable of 20 alleviating or treating a neurotransmission disorder. Such methods include contacting an anti-LRP4 antibody and a LRP4 polypeptide, or fragment thereof, in the presence a candidate compound and determining the amount of antibody-LRP4 polypeptide binding, and identifying compounds that prevent or inhibit the binding of the anti-LRP4 antibody to the LRP4 polypeptide or fragment thereof. The present invention 25 includes compounds identified by such methods.

Such methods of identifying compounds capable of alleviating or treating neurotransmission disorders may include the steps of contacting a candidate compound in the presence of LRP4, or an epitope thereof, and an antibody capable of binding LRP4, wherein a compound that prevents binding of the antibody to LRP4 or 30 an epitope thereof is a candidate for treating neurotransmission disorders. Such compounds may also be used in treating neurotransmission or developmental disorders or in the manufacture of a medicament for treating such disorders. The

compounds identified may also, as would be appreciated by those of skill in the art, serve as lead compounds for the development of analogue compounds. The analogues should have a stabilized electronic configuration and molecular conformation that allows key functional groups to be presented to the polypeptides of the invention in substantially the same way as the lead compound. In particular, the analogue compounds have spatial electronic properties which are comparable to the binding region, but can be smaller molecules than the lead compound, frequently having a molecular weight below about 2 kilodaltons (kD) and preferably below about 1 kD. Identification of analogue compounds can be through the use of techniques such as, for example, self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are available; e.g., Rein, Computer-Assisted Modelling of Receptor-Ligand Interactions (Alan Liss, New York, 1989). Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, N.Y., USA. Furthermore, derivatives and analogues can be tested for their effects according to methods known in the art. Furthermore, peptidomimetics and/or computer aided design of appropriate derivatives and analogues can be used.

The present invention includes compositions with one or more of the antibodies and/or compounds described herein. A composition may also include, for example, buffering agents to help to maintain the pH in an acceptable range or preservatives to retard microbial growth. Such compositions may also include a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" refers to one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The compositions of the present invention are formulated in pharmaceutical preparations in a variety of forms adapted to the chosen route of administration. Such compositions may be administered in an effective amount to a subject for the treatment of a neurotransmission or developmental disorder.

The present invention also includes pharmaceutically acceptable salts of

inhibitors. As used herein, "pharmaceutically acceptable salts" refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. The pharmaceutically acceptable salts of the present invention include the conventional non-toxic salts or the quaternary ammonium salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present invention can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods.

The agents of the present invention can be administered by any suitable means including, but not limited to, for example, oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal), intravesical, or injection into or around the tumor.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intraperitoneal, and intratumoral administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by the FDA. Such preparation may be pyrogen-free.

For enteral administration, the inhibitor may be administered in a tablet or capsule, which may be enteric coated, or in a formulation for controlled or sustained release. Many suitable formulations are known, including polymeric or protein microparticles encapsulating drug to be released, ointments, gels, or solutions which can be used topically or locally to administer drug, and even patches, which provide

controlled release over a prolonged period of time. These can also take the form of implants.

Therapeutically effective concentrations and amounts may be determined for each application herein empirically by testing the compounds in known in vitro and in vivo systems, such as those described herein, dosages for humans or other animals may then be extrapolated therefrom. The efficacy of the administration of one or more agents may be assessed by any of a variety of parameters well known in the art.

In some therapeutic embodiments, an "effective amount" of an agent is an amount that results in a reduction of at least one pathological parameter. Thus, for example, in some aspects of the present invention, an effective amount is an amount that is effective to achieve a reduction of at least about 10%, at least about 15%, at least about 20%, or at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%, compared to the expected reduction in the parameter in an individual not treated with the agent.

It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods.

An agent or antibody of the present invention may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to

be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods.

As used herein, the term “subject” includes, but is not limited to, humans and non-human vertebrates. In preferred embodiments, a subject is a mammal, particularly a human. A subject may be an individual. A subject may be a patient. Non-human vertebrates include livestock animals, companion animals, and laboratory animals. Non-human subjects also include non-human primates as well as rodents, such as, but not limited to, a rat or a mouse. Non-human subjects also include, without limitation, chickens, horses, cows, pigs, goats, dogs, cats, guinea pigs, hamsters, mink, and rabbits.

The methods of the present invention include in vivo and in vitro methods. As used herein "in vitro" is in cell culture and "in vivo" is within the body of a subject.

As used herein, "isolated" refers to material that has been either removed from its natural environment (e.g., the natural environment if it is naturally occurring), produced using recombinant techniques, or chemically or enzymatically synthesized, and thus is altered "by the hand of man" from its natural state.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

The present invention includes methods of treating a subject suffering from a neurotransmission disorder, the method including first determining that a bodily fluid of the individual contains autoantibodies that bind to the low density lipoprotein

receptor-related protein 4 (LRP4), or an epitope thereof, followed by providing one or more appropriate therapeutic modalities to the individual. The method may include additional steps determining whether or not the bodily fluid contains autoantibodies that bind to the AchR, and/or determining whether or not the bodily fluid contains
5 autoantibodies that bind to MuSK. Treatment may include, but is not limited to, administering an effective amount of one or more immunosuppressant agents, plasma exchange, antigen-specific immunoadsorption, and additional appropriate therapeutic modalities. This aspect of the invention is particularly advantageous because the identification of this new subclass or subtype of MG patients will allow for more
10 accurate and speedy diagnosis of individuals by medical practitioners. The method according to this aspect of the invention will allow for detection of neurotransmission abnormalities that are either congenital or acquired, for example, postnatally or prenatally from transmission from the mother to the fetus.

The present invention includes methods for diagnosing neurotransmission or
15 developmental disorders by detecting a genetic mutation in one or more alleles of the low density lipoprotein receptor-related protein 4 (LRP4) gene. A genetic mutation includes, but is not limited to, an intronic mutation, an exonic mutation, a mutation in a coding sequence, a mutation in a regulatory sequence, a splice junction mutation, a point mutation, a missense mutation, an insertion mutation, a deletion mutation, an
20 insertion-deletion mutation, a mutation altering one or more amino acids, a read through mutation, a frameshift mutation, a mutation affecting mRNA splicing, a mutation introducing a stop codon, a mutation affecting mRNA half life, a mutation affecting mRNA transcription, a mutation affecting mRNA translation, a mutation reducing LRP4 mRNA and/or protein expression, and a mutation preventing LRP4
25 mRNA and/or protein expression. The present invention includes assay kits that include one or more polynucleotide sequences for identifying such mutations. Such mutations may be identified using LRP4 genomic or RNA sequences, or primers derived from such sequences in methods including, but not limited to, those described in Engel et al., 2009, *J Mol Neurosci*; 40(1-2):143-53; Selcen et al., 2008, Ann
30 Neurol; 64(1):71-87; Shen et al., 2008, *J Clin Invest*; 118(5):1867-76; Di Castro et al., 2007, *J Physiol*; 579(Pt 3):671-7; and Ohno and Engel, 2005, *Acta Myol*; 24(2):50-4.

The present invention is illustrated by the following examples. It is to be

understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

LRP4 serves as a co-receptor of agrin

Formation of the neuromuscular junction (NMJ) requires agrin, a factor released from motoneurons, and MuSK, a transmembrane tyrosine kinase that is activated by agrin. However, how signal is transduced from agrin to MuSK remains unclear. This example demonstrates that LRP4, a low-density lipoprotein receptor (LDLR)-related protein functions as a co-receptor of agrin. LRP4 is specifically expressed in myotubes and is concentrated at the NMJ. The extracellular domain of LRP4 interacts with neuronal, but not muscle, agrin. Expression of LRP4 enables agrin binding activity and MuSK signaling in cells that otherwise does not respond to agrin. Suppression of LRP4 expression attenuates agrin binding activity, agrin-induced MuSK tyrosine phosphorylation and AChR clustering in muscle cells. LRP4 also interacts with MuSK in a manner that is stimulated by agrin. Finally, this example showed that LRP4 becomes tyrosine-phosphorylated in agrin-stimulated muscle cells. These observations identify LRP4 as a functional co-receptor of agrin that is necessary for agrin-induced MuSK signaling and AChR clustering.

Experimental Procedures

Reagents and antibodies. Taq DNA polymerase, T4 DNA ligase, and restriction enzymes were purchased from Promega. Horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit antibodies and enhanced chemifluoresent (ECL) reagents for Western blotting were from Amersham. Rhodamine-aBTX (R-BTX) was from Molecular Probes. Oligonucleotides were synthesized by Operon Biotechnologies. Unless otherwise specified, all chemicals were from Sigma-Aldrich. Antibodies were purchased from Sigma (Flag M2, F3165); Torrey Pines Biolabs (GFP, TP401); Upstate Biotechnology (4G10, 05–1050); Cell Signaling (Phospho-c-Abl, 2861); Novus (ß-actin, NB600-501).

Rabbit anti-MuSK antibodies were described previously (Luo et al., 2002, *Neuron*; 35:489–505). Rabbit anti-LRP4 antibody was described previously (Lu et al., 2007, *Brain Res*; 1177:19–28). Rat anti-AChR α-subunit antibody was mAb35 and rat anti-AChR ß-subunit antibody was mAb124.

5 Constructs. Agrin-AP constructs were generated by fusing neuronal and muscle agrin (aa 1145–1940) (Ferns et al., 1993, *Neuron (USA)*; 11:491–502) with AP in pAPtag-5. To generate Flag-MuSK, the MuSK DNA was generated by PCR and subcloned in EcoRI/XbaI sites in pFlag-CMV1 downstream of an artificial signal peptide sequence and a Flag epitope. LRP4-Myc was generated by subcloning the 10 full length LRP4 DNA into NheI and HindIII sites in pcDNA3.1-MycHis (Invitrogen) with 3 alanine insert after amino acid 1746. LRP4N-Myc was generated by subcloning LRP4 extracellular domain DNA into NheI and NotI sites in pcDNA3.1-MycHis. LRP5 DNA was amplified with pCMV-Sports6-LRP5 (Open Biosystems) as template and subcloned into XbaI and NotI sites in 15 pcDNA3.1-MycHis to generate LRP5-Myc. LRP4-, LRP5- and MuSK-miRNA constructs were generated using the BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogene, K4936-00). Oligonucleotide sequences for miRNA constructs were as follows:

For mi-MuSK-1161:

20 5'-TGCTG TAACA CAGCA GAGCC TCAGC AGTTT TGGCC ACTGA CTGAC TGCTG AGGCT GCTGT GTTA-3' (sense) (SEQ ID NO:1) and

5'- CTGTA ACACA GCAGC CTCAG CAGTC AGTCA GTGGC CAAAA CTGCT GAGGC TCTGC TGTGT TAC -3' (antisense) (SEQ ID NO:2);

For mi-LRP5-1490:

25 5'- TGCTG ATCAC AGGGT GCAAC ACAAT GGTTT TGGCC ACTGA CTGAC CATTG TGTCA CCCTG TGAT -3' (sense) (SEQ ID NO:3) and

5'- CCTGA TCACA GGGTG ACACA ATGGT CAGTC AGTGG CCAAA ACCAT TGTGT TGCAC CCTGT GATC -3' (antisense) (SEQ ID NO:4);

For mi-LRP4-1062:

30 5'-TGCTG TTAAC ATTGC AGTTC TCCTC AGTTT TGGCC ACTGA CTGAC TGAGG AGATG CAATG TTAA-3' (sense) (SEQ ID NO:5) and

5'-CCTGT TAACA TTGCA TCTCC TCAGT CAGTC AGTGG CCAAA ACTGA

GGAGA ACTGC AATGT TAAC-3' (antisense) (SEQ ID NO:6);

For mi-LRP4-2603:

5'-TGCTG AATAC ATGTA CCCGC CCATG GGTTT TGGCC ACTGA CTGAC CCATG GGCGT ACATG TATT-3'(sense) (SEQ ID NO:7) and

5 5'-CCTGA ATACA TGTAC GCCCA TGGGT CAGTC AGTGG CCAAA ACCCA TGGGC GGGTA CATGT ATTC-3' (antisense) (SEQ ID NO:8);

For mi-LRP4-5355:

5'-GCTGT AGCAC AGCTG ATTAT ACACG GTTTT GGCCA CTGAC TGACC GTGTA TACAG CTGTG CTA-3'(sense) (SEQ ID NO:9) and

10 5'-CCTGT AGCAC AGCTG TATAC ACGGT CAGTC AGTGG CCAAA ACCGT GTATA ATCAG CTGTG CTAC-3' (antisense) (SEQ ID NO:10).

The authenticity of all constructs was verified by DNA sequencing. The following constructs were described previously: MuSK-AP (Wang et al., 2008, *Neurosignals*; 16:246–253); pcDNA-LRP4 (Lu et al., 2007, *Brain Res*; 1177:19–28);

15 Wnt1-HA (Zhang et al., 2007, *J Neurosci*; 27:3968–3973); and Wnt1-Myc, LRP6-N-Myc, and mfz8CRD-IgG (Tamai et al., 2000, *Nature*; 407:530–535).

Cell culture and transfection. HEK293 cells and mouse C2C12 muscle cells were maintained and transfected as previously described (Zhang et al., 2007, *J Neurosci*; 27:3968–3973). In some experiments, myotubes were transfected with

20 lipofectamine 2000 (Invitrogen, 11668-019). The cells were incubated with a mixture of DNA, lipofectamine and serum-free medium for 8 hours before being switched to the fusion medium. The DNA: lipofectamine ratio in the mixture was 1 μg: 2μl. The optimal volume of the mixture for 24-well dishes was 200 μl per well with 2 μg plasmid DNA.

25 Recombinant protein production and purification. To produce recombinant proteins, HEK293 were transfected with respective plasmids. Twenty-four hours after transfection, cells were switched to Dulbecco's Modified Eagle Medium supplemented with reduced concentration (0.05%) of fetal bovine serum, and secreted proteins were harvested 24 hr later. nAgrin-AP, mAgrin-AP, or MuSK-AP

30 recombinant proteins, which contained 6-His-tags that were encoded by pAPtag-5, were purified by affinity chromatography using TALON Resins (BD Biosciences).

Solution binding assay. Flag-nAgrin was immobilized to protein A Sepharose

beads (that were preabsorbed with anti-Flag antibody), which were incubated with 1 ml (0.5 nM) of LRP4N-Myc, MuSKect-Myc, or LRP6N-Myc condition medium, and Flag-nAgrin-bound proteins were isolated by bead precipitation and resolved by SDS-PAGE and visualized by immunoblot with anti-Myc antibody. In some
5 experiments, LRP4N-Myc, LRP6N-Myc or MuSKect-Myc was incubated with Wnt-1-HA immobilized on beads. LRP4 and LRP6 that were co-precipitated with Wnt-1 were analyzed by immunoblot with anti-Myc antibody.

Solid phase binding assay. Maxi-Sorp Immuno Plates (Nunc) were coated with purified LRP4-Myc at 4°C overnight, and then incubated with 1% BSA in PBS
10 to block non-specific binding. Coated wells were incubated with purified AP fusion proteins and the AP activity was measured using pNPP as substrate.

Intact cell binding assays. Live C2C12 myoblasts or myotubes in 15-mm dishes were incubated at room temperature for 90 min with 500 μl of 5 nM nAgrin-AP, mAgrin-AP or AP. Cells were washed three times with the HABH buffer
15 (0.5 mg/ml bovine serum albumin, 0.1% NaN3, 20 mM HEPES, pH 7.0 in Hank's balanced salt solution) and fixed in 60% acetone, 3% formaldehyde in 20 mM HEPES (pH 7.0) for 15 sec. Fixed cells were washed once in 20 mM HEPES (pH 7.0), 150 mM NaCl, incubated at 65°C for 100 min to inactivate endogenous AP, washed again in the AP buffer (0.1 M Tris-HCl, pH 9.4/0.1 M NaCl/5 mM MgCl2) and stained at
20 room temperature overnight with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (165 μg/ml)/nitroblue tetrazolium (NBT) (330 μg/ml) in the AP buffer. Digital photographs of stained cells were analyzed by using the NIH ImageJ software. In some experiments, Agrin-AP-bound cells were lyzed in the lysis buffer (1% Triton-X100, 10 mM Tris, pH 8.0). After the inactivation of the endogenous AP,
25 lysates were assayed for AP activity using p-nitrophenyl phosphate (pNPP) as substrate.

Immunoprecipitation, immunoblotting, and AChR clustering assays. These assays were performed as previously described (Luo et al., 2002, *Neuron*; 35:489–505; Zhang et al., 2007, *J Neurosci*; 27:3968–3973; Zhu et al., 2008, *J*
30 *Neurosci*; 28:1688–1696). Unless otherwise indicated, the final concentration of recombinant neuronal agrin was 1 nM to stimulate muscle cells. Band intensity of immunoblot was analyzed by using the ImageJ software.

Statistical Analysis. Data of multiple groups was analyzed by ANOVA, followed by a student-Newman-Keuls test. Two-tailed Student's t test was used to compare data between two groups. Differences were considered significant at $P < 0.05$. Values and error bars in figures denote mean ± SEM.

5

Results

LRP4 is expressed specifically in myotubes and concentrated at the NMJ Because neuronal agrin binds only to myotubes, but not myoblasts (Glass et al., 1996, *Cold Spring Harb Symp Quant Biol*; 61:435–444), the expression of LRP4 in
10 developing myotubes was characterized. C2C12 myoblasts were switched fusion medium to induce muscle differentiation. Under these conditions, myotubes began to form 48 hr after medium switch (Luo et al., 2002, *Neuron*; 35:489–505; Luo et al., 2003, *Neuron*; 40:703–717; Si et al., 1996, *J Biol Chem*; 271:19752–19759). Developing myotubes were collected and LRP4 expressed analyzed by
15 immunoblotting with anti-LRP4 antibody. As shown in Fig. 1A, LRP4 was barely detectable in myoblasts, but its expression gradually increased as myotubes matured.

As control, expression of MuSK was examined in same preparations, whose expression was known to be regulated by muscle differentiation (Glass et al., 1996, *Cell*; 85:513–523; Ip et al., 2000, *Mol Cell Neurosci*; 16:661–673; Valenzuela et al.,
20 1995, *Neuron (USA)*; 15:573–584) (Fig. 1A). These results indicate that LRP4, like MuSK, is expressed in well differentiated myotubes, but not myoblasts. Next, LRP4 distribution in vivo was investigated by staining muscle sections with anti-LRP4 antibody. The immunoreactivity of LRP4, as well as MuSK, showed a pattern of labeling similar to that of rhodamine-conjugated a-bungarotoxin (R-BTX) that labels
25 AChRs (Fig. 1B), suggesting that LRP4, like MuSK, is enriched at the NMJ. This notion was supported by results from immunoblot analysis of LRP4 expression of muscles. Hemi-diaphragms were divided into three regions: the central, narrow region, where NMJs are enriched, as synaptic region; the region close to ligaments to the ribs as non-synaptic region; and the middle in between. The AChR was enriched
30 in the synaptic, but not non-synaptic, region (Fig. 1C). In agreement with results of immunostaining, LRP4 was readily detectable in the synaptic region where AChRs were enriched. However, little, if any, LRP4 was found in the non-synaptic region.

Together these results demonstrated that LRP4 is specifically expression in myotubes and is enriched at the NMJ, suggesting a role of LRP4 in NMJ formation.

The LRP4 extracellular domain binds to neuronal agrin. Next, it was determined whether agrin binds to LRP4. A secreted form of neuronal agrin, Flag-nAgrin, was generated that comprised the C-terminus of neuronal agrin fused with the Flag epitope, and a secreted form of LRP4 (i.e., LRP4N–Myc), which consisted of the LRP4 extracellular domain tagged by the Myc epitope. Flag-nAgrin was immobilized on beads and incubated with LRP4N-Myc. As shown in Fig. 2A, LRP4N–Myc was precipitated with neuronal agrin, suggesting that the two proteins interact in solution.

In contrast, Flag-nAgrin did not precipitate MuSKect–Myc, which consisted of the extracellular region of the kinase (Fig. 2B), in agreement with previous findings that agrin and MuSK do not directly bind to each other (Glass et al., 1996, *Cold Spring Harb Symp Quant Biol*; 61:435–444). Moreover, Flag-nAgrin did not interact with LRP6N-Myc, which comprised Myc-tagged extracellular domain of LRP6, a homologous member of the LRP family whose extracellular structural organization resembles that of LRP4 (Fig. 2C). As control, LRP6N-Myc was able to interact with Wnt-1-HA when the two proteins were incubated together (Fig. 2D), indicating proper folding and specific binding of LRP6N-Myc. Furthermore, LRP4N-Myc did not co-precipitate with Wnt-1-HA (Fig. 2E), suggesting that the two proteins do not interact. These results demonstrate that agrin binds specifically to the extracellular domain of LRP4, but not that of MuSK, or LRP6 and on the other hand, Wnt-1 interacts with LRP6, but not LRP4. In support of this notion, the extracellular domain of LRP4 was able to neutralize neuronal agrin and thus prevented it from stimulating MuSK tyrosine phosphorylation and AChR clustering (Fig. 8).

To determine whether the interaction is direct, the recombinant agrins nAgrin-AP and mAgrin-AP were produced, which contained the C-terminal region of neuronal and muscle agrin, respectively. They were fused with the heat-insensitive human placental isozyme of alkaline phosphatase (AP) (Flanagan et al., 2000, *Methods Enzymol*; 327:198–210) (Fig. 3A). The activity of the AP recombinant proteins was tested in AChR cluster assays. As shown in Fig. 3B, nAgrin-AP was able to stimulate AChR clustering in C2C12 myotubes, indicating proper folding of

the recombinant neuronal agrin protein. In contrast, mAgrin-AP or AP alone had little effect on AChR clustering.

Next, the binding activity of the AP proteins to muscle cells was characterized by in-cell assays, as described in Experimental Procedures. AP binding to myoblasts
5 or myotubes was minimal (Fig. 3C). mAgrin-AP binding to myoblasts was higher than that of AP alone, presumably because myoblasts express alpha-dystroglycan to which muscle agrin is known to interact (Bowe et al., 1994, *Neuron (USA)*; 12:1173–1180; Campanelli et al., 1996, *Development*; 122:1663–1672; Campanelli et al., 1994, *Cell*; 77:663–674; Gee et al., 1994, *Cell;* 77:675–686; Gesemann et al.,
10 1996, *Neuron (USA)*; 16:755–767; Hopf and Hoch, 1996, *J Biol Chem*; 271:5231–5236; Sugiyama etal., 1994, *Neuron (USA)*; 13:103–115). The mAgrin-AP binding to myotubes was higher in comparison with that in myoblasts because alpha-dystroglycan expression was increased during muscle differentiation. nAgrin-AP binding to myoblasts was similar to that of mAgrin-AP (Fig. 3C).
15 However, nAgrin-AP binding was significantly higher in myotubes than in myoblasts (Fig. 3C), in agreement with earlier reports (Glass et al., 1996, *Cell*; 85:513–523) and the LRP4 expression pattern in developing muscle cells (Fig. 1).

These results demonstrate differential ability of recombinant muscle and neuronal agrins in binding to myotubes. Having established that nAgrin-AP was able
20 to bind to myotubes and stimulate AChR clustering, the interaction between LRP4 and nAgrin-AP was characterized. LRPN4-Myc was purified and immobilized on plates and incubated with purified nAgrin-AP. After wash, the AP activity bound to immobilized LRPN4-Myc was assayed by a modified ELISA (enzyme-linked immunosorbent assay). In comparison with control (AP alone), there was a
25 significant increase in AP activity when nAgrin-AP were incubated with LRP4N-Myc (Fig. 3D), suggesting direct interaction between the two proteins, i.e., independent of a third protein. Quantitatively, the interaction between neuronal agrin and LRP4 was dose-dependent, saturable, and of high affinity (Kd values of 0.5 ± 0.053 nM) (Fig. 3E and 3F). This affinity is comparable to that (0.1–0.5 nM) of LRP6 for Dkk1 and
30 Dkk2 (Bafico et al., 2001, *Nat Cell Biol*; 3:683–686; Mao etal., 2001, *Nature*; 411:321–325; Semenov et al., 2001, *Curr Biol*; 11:951–961). In contrast, muscle agrin, which lacks four and eight amino acid inserts at the Y and Z sites, respectively

(Fig. 3A) and is 1000 times less potent than neuronal agrin in stimulating AChR clusters (Gesemann et al., 1995, *J Cell Biol*; 128:625–636; Reist et al., 1992, *Neuron (USA)*; 8:865–868), did not appear to bind to LRP4 (Fig. 3D). The binding of LRP4N-Myc to muscle agrin was minimal even at high concentrations (Fig. 3E).
5 Together, these results suggest LRP4 binds specifically to neuronal agrin with high affinity. These results indicate that LRP4 binds to neuronal, but not muscle, agrin in a manner that is concentration-dependent, saturable and of high affinity.

Reconstitution of neuronal agrin binding and signaling in transfected cells. To determine whether agrin binds to LRP4 in vivo, exogenous LRP4 was expressed in
10 C2C12 myoblasts that, unlike myotubes, do not bind neuronal agrin (Glass et al., 1996, *Cell*; 85:513–523) (Fig. 3C). Myoblasts were transfected with full length LRP4 or the empty vector (as control). Intact transfected myoblasts were incubated with AP alone, nAgrin-AP or mAgrin-AP. The AP activity bound to cell surface was measured in situ after heat inactivation of endogenous AP. As shown in Fig. 4A and
15 4B, when incubated with AP alone, control and LRP4-transfected myoblasts show no difference in AP activity. However, nAgrin-AP binding was significantly higher to LRP4-transfected myoblasts in comparison with control, indicating that LRP4 enables myoblasts to interact with neuronal agrin. By contrast, transfection of MuSK had no consistent effect on binding to nAgrin-AP, in agreement with earlier observations that
20 agrin does not bind to MuSK (Glass et al., 1996, *Cell*; 85:513–523) (Fig. 2B). In addition to myoblasts, HEK293 cells were able to bind to nAgrin-AP after LRP4 transfection (Fig. 4C).

The in situ binding activity generated by transfected LRP4 had the following characters. First, it was dose-dependent. Increase in LRP4 expression in transfected
25 HEK293 cells led to higher nAgrin-AP binding activity (Fig. 4C). Probably due to rate-limiting surface integration of overexpressed LRP4, nAgrin-AP binding was not further increased in cells transfected with 2 μg of DNA. Earlier studies have reported that overexpressed LRP4 is retained in the endoplasmic reticulum (Lu et al., 2007, *Brain Res*; 1177:19–28; Obermoeller-McCormick et al., 2001, *J Cell Sci*;
30 114:899–908). Notice that the blot reveals total, but not surface, LRP4 (Fig. 4C). Second, LRP4 binding was specific for neuronal agrin because the amount of mAgrin-AP bound to transfected myoblasts and HEK293 cells was minimal, and not

concentration-dependent (Figs. 4A to 4C). Notice that mAgrin-AP, like nAgrin-AP, also contained the AP and the Myc and His tags. Inability of mAgrin-AP to bind to LRP4-transfected cells indicate that binding to LRP4 does not involve the AP or tags. Third, the binding activity was LRP4 specific. Expression of LRP5, another member
5 of the LRP family (Herz and Bock, 2002, *Ann Rev Biochem*; 71:405–434), did not increase agrin binding in transfected cells (Fig. 4D). Last, nAgrin-AP binding was similar between cells transfected with LRP4 alone and those co-transfected with LRP4 and MuSK (Fig. 4A and 4B), indicating that the neuronal agrin binding activity is mainly contributed by LRP4 although LRP4 and MuSK could interact in muscle
10 cells (see below). Taken together, these results demonstrate the ability of LRP4 to reconstitute agrin binding in cells that otherwise do not interact with agrin.

Next, it was determined if LRP4 was able to reconstitute MuSK signaling in cells that do not respond to agrin. MuSK is a receptor tyrosine kinase whose activation has been shown to be upstream of all known agrin signaling cascades
15 (Fuhrer et al., 1997, *Embo J*; 16:4951–4960; Glass et al., 1997, *Proc Natl Acad Sci USA*; 94:8848–8853; Glass et al., 1996, *Cell*; 85:513–523; Herbst and Burden, 2000, *Embo J*; 19:67–77; Luo et al., 2002, *Neuron*; 35:489–505; Strochlic et al., 2005, *Bioessays*; 27:1129–1135; Wang et al., 2008, *Neurosignals*; 16:246–253; Zhou et al., 1999, *J Cell Biol*; 146:1133–1146). Therefore we first examined whether LRP4
20 expression enables MuSK activation by agrin in HEK293 cells that do not express LRP4 (Fig. 4E). Flag-MuSK was transfected into HEK293 cells with or without LRP4 and transfected cells were stimulated with neuronal agrin. As shown in Fig. 4E and 4G, agrin was unable to elicit MuSK tyrosine phosphorylation in HEK293 cells transfected with MuSK alone. Intriguingly, LRP4 co-expression enabled agrin to
25 activate MuSK, indicating that LRP4 could be an agrin receptor able to stimulate MuSK. Basal tyrosine phosphorylation of MuSK, i.e., in the absence of agrin, was increased by LRP4, which could suggest a role of LRP4 in MuSK auto-activation, presumably by its direct interaction with the kinase (see below). Agrin-induced AChR clustering requires the intracellular tyrosine kinase Abl (Finn et al., 2003, *Nat*
30 *Neurosci*; 6:717–723). To further investigate the role of LRP4, Abl activation was examined by anti-phospho-Abl antibody in cells co-expressing LRP4 and MuSK. As shown in Fig. 4F and 4G, active Abl was barely detectable in cells transfected with

Myc-MuSK alone, regardless of agrin stimulation. In contrast, agrin elicited a significant increase in phospho-Abl in cells co-expressing LRP4 and Myc-MuSK. Together, these results indicate that LRP4 expression enables binding activity for neuronal agrin, MuSK activation, and initiation of intracellular signaling in cells that otherwise do not respond to agrin. Decrease of LRP4 levels attenuates neuronal agrin binding, MuSK activation, and induced AChR clustering in muscle cells.

It was next determined if LRP4 is necessary for agrin/MuSK signaling by a loss-of-function approach. To this end, we generated several microRNA constructs of LRP4. As shown in Fig. 5A, miLRP4-1062 was most potent in inhibiting LRP4 expression. First, it was determined if repression of LRP4 affects agrin binding to intact muscle cells. C2C12 myoblasts were transfected with miLRP4-1062 or the control miRNA that encoded scramble sequence, and resulting myotubes were incubated with AP, mAgrin-AP or nAgrin-AP and assayed for AP activity by in-cell staining. In comparison with control miRNA, miLRP4-1062 did not appear to alter binding activity of AP and mAgrin-AP to myotubes (Fig. 5B and 5C). However, myotubes transfected with miLRP4-1062 had lower levels of nAgrin-AP staining in comparison with those transfected with the control vector (Fig. 5B and 5C), indicating a necessary role of endogenous LRP4 for neuronal agrin binding. Second, it was tested whether LRP4 is required for agrin to stimulate tyrosine phosphorylation of MuSK. MuSK was precipitated from myotubes transfected with control miRNA or miLRP4-1062 and assayed for tyrosine phosphorylation. Expression of miRNA constructs was indicated by the presence of GFP that was encoded by the parental vector. As shown in Fig. 5D, transfection of miLRP4-1062 reduced expression of endogenous LRP4, but not MuSK or ß-actin. Remarkably, agrin-induced MuSK tyrosine phosphorylation was attenuated in myotubes transfected with miLRP4-1062 in comparison with control miRNA (Fig. 5E). These results suggest that MuSK activation is impaired when LRP4 levels were reduced.

Finally, whether LRP4 is necessary for agrin-induced AChR clustering was investigated. Myoblasts were transfected with control miRNA or miLRP4-1062, or miRNA constructs against MuSK and LRP5 that reduced expression of MuSK and LRP5, respectively. Transfected myotubes were stimulated without or with agrin and AChR clusters in GFP-expressing myotubes scored as described previously (Zhang et

al., 2007, *J Neurosci*; 27:3968–3973). Expression of these miRNA constructs did not appear to alter basal AChR clusters. However, the number of agrin-induced AChR clusters was reduced in myotubes transfected with miLRP4-1062 (Fig. 5F), suggesting a necessary role of LRP4 in agrin-induced clustering. Similar reduction was observed in myotubes expressing miMuSK-1161, as expected. Transfection with miLRP5-1490, however, had no effect on agrin-induced AChR clustering, in agreement with the observation that LRP5 does not bind to neuronal agrin (Fig. 4D). Interaction between LRP4 and MuSK.

In a working model, LRP4 serves as a co-receptor that binds to agrin and, together with MuSK, stimulates AChR clustering. To examine the relationship among agrin, LRP4 and MuSK, it was determined whether LRP4 interacts with MuSK and if so, if the interaction is regulated by agrin. Secreted Flag-MuSKect, which comprised the entire extracellular region of MuSK fused with the Flag epitope, was incubated with LRP4N–Myc in the absence or presence of agrin. Flag-MuSKect alone was able to co-precipitate with LRP4N–Myc (Fig. 6A and 6B), indicative of direct binding between the extracellular domains of MuSK and LRP4. Quantitatively, the interaction between MuSK and LRP4 was dose-dependent and saturable, and of high affinity (Kd values of 0.45 ± 0.041 nM, Fig. 6C and 6D). Interestingly, the amount of LRP4 co-precipitated with Flag-MuSKect was increased by agrin (Fig. 6A and 6B). In contrast, as control, LRP6N-Myc failed to co-precipitate with Flag-MuSKect regardless of the presence or absence of agrin (Fig. 6E).

These observations suggest that LRP4 and MuSK form a complex in the absence of the ligand agrin; however, agrin, via binding to LRP4, enhances the LRP4-MuSK interaction. To test this hypothesis further, we examined if full length MuSK and LRP4 interact with each other in cells. LRP4 and Flag-MuSK were co-transfected into HEK293 cells. MuSK was precipitated from cell lysates by a Flag antibody and the resulting immunocomplex was analyzed for LRP4. As shown in Fig. 6F, LRP4 co-precipitated with MuSK in transfected cells, in support of the notion that the two proteins interact in transfected cells. Moreover, the LRP4-MuSK association was detectable in mouse muscle homogenates (Fig. 6G), suggesting in vivo interaction of the two proteins.

Neuronal agrin stimulates LRP4 interaction with MuSK and tyrosine

phosphorylation. To further investigator the role of LRP4 in agrin signaling, whether the LRP4-MuSK interaction in muscle cells is regulated by neuronal agrin was examined. C2C12 myotubes were treated without or with agrin for one hour. Myotubes were subjected to immunoprecipitation with anti-LRP4 antibody and resulting precipitates were probed for MuSK. As shown in Fig. 7A, MuSK co-precipitated with LRP4 from cells in the absence of agrin, suggesting basal interaction of the two proteins and in agreement with in vitro binding results (Fig. 6A–D). The co-precipitation was increased in agrin-stimulated myotubes (Fig. 7A and 7B). These observations indicate that LRP4 and MuSK form a complex in a manner that is up-regulated by agrin. LRP4 has a large intracellular domain containing six tyrosine residues.

Recent evidence indicates that LRP4, immunopurified from the brain, could be phosphorylated on serine residues presumably by CaMK II (Tian et al., 2006, *Eur J Neurosci*; 23:2864–2876). Other members of the LRP family, LRP5 and LRP6, become phosphorylated upon activation of the Wnt canonical pathway (Ding et al., 2008, *J Cell Biol*; 182:865–872). Unlike LRP5 and LRP6, LPR4 has a NPXY motif in the intracellular region that may be phosphorylated by a tyrosine kinase (Herz and Bock, 2002, *Ann Rev Biochem*; 71:405–434). Having demonstrated that LRP4 interacts with MuSK and the interaction is enhanced by agrin, we determined whether LRP4 itself becomes phosphorylated on tyrosine residues. C2C12 myotubes were stimulated with neuronal agrin for one hour and lysates were subjected immunoprecipitation of LRP4 and MuSK, respectively. Resulting precipitates were probed with the anti-phospho-tyrosine antibody 4G10. As shown in Fig. 7C and 7D, LRP4 as well as MuSK became tyrosine-phosphorylated in agrin-stimulated myotubes. This result suggests a role of LRP4 in agrin signaling.

Discussion

This example demonstrates the LRP4 is specifically expressed in myotubes, but not myoblasts and is concentrated at the NMJ (Fig. 1). Further, it is both necessary and sufficient to bind to agrin and to activate MuSK signaling that leads to AChR clustering. Using three different assays (in solution, on solid phase, and in cells), this example demonstrated that neuronal agrin was able to interact directly with

the extracellular region of LRP4 (Fig. 2, Fig. 3, and Fig. 4). The binding activity of LRP4 was specific because 1) LRP4 binding to muscle agrin was minimal; 2) the binding is concentration-dependent and of high affinity with a sub-nanomolar Kd; and 3) neuronal agrin did not bind to LRP5 or LRP6, two other members of the LRP
5 family that are highly homologous to LRP4. Further, expression of LRP4 enabled binding activity for neuronal agrin and MuSK signaling in cells that otherwise did not respond to agrin (Fig. 4). And, suppression of LRP4 expression attenuated agrin binding activity and agrin-induced MuSK phosphorylation and AChR clustering in muscle cells (Fig. 5). Further, LRP4 could interact with MuSK in a manner that is
10 increased by agrin (Fig. 6 and Fig. 7). Finally, LRP4 became tyrosine-phosphorylated in muscle cells in response to agrin stimulation (Fig. 7). These observations indicate that LRP4 can bind to agrin and transmit signals to MuSK, suggesting that it may serve as a functional receptor for agrin.

These observations indicate that LRP4 interacts with MuSK at basal levels in
15 the absence of the ligand. Upon agrin stimulation, the interaction was increased to activate MuSK and subsequent downstream signal cascades for AChR clustering (Fig. 7E). Despite the essential role of MuSK in NMJ formation, mechanisms of how it is activated and how it acts to control NMJ formation remain elusive. Recent studies have shed light on intracellular pathways downstream of MuSK. They are thought to
20 involve the adapter protein Dok-7 (Okada et al., 2006, *Science*; 312:1802–1805), and several enzymes including Src-family kinase (Ferns et al., 1996, *J Cell Biol*; 132:937–944; Mittaud et al., 2001, *J Biol Chem*; 276:14505–14513; Mohamed et al., 2001, *J Neurosci*; 21:3806–3818; Qu and Huganir, 1994, *J Neurosci*; 14:6834–6841; Wallace, 1991, *Philos Trans R Soc Lond Biol*; 331:273–280), Abl (Finn et al., 2003,
25 *Nat Neurosci*; 6:717–723), casein kinase 2 (Cheusova et al., 2006, *Genes Dev*; 20:1800–1816), geranylgeranyl transferase I (GGT) (Luo et al., 2003, *Neuron*; 40:703–717), GTPases of the Rho family (Weston et al., 2003, *J Biol Chem*; 278:6450–6455; Weston et al., 2000, *J Cell Biol*; 150:205–212), and Pak1, a serine/threonine kinase that is activated by Rho GTPases (Luo et al., 2002, *Neuron*;
30 35:489–505).

Although agrin is known to activate MuSK, the two proteins, however, do not interact directly. The MASC co-receptor was hypothesized that has to be myotubes

specific and is able to transmit signal from agrin to MuSK (Glass et al., 1996, *Cell*; 85:513–523). Remarkably, LRP4 is a protein specifically expressed in myotubes, not in myoblasts (Fig. 1), fulfilling a requirement of MASC. Second, LRP4 is able to reconstitute agrin binding and MuSK signaling in cells that otherwise do not respond to agrin (Fig. 4). Third, LRP4 is required for agrin binding and induced MuSK signaling and AChR clustering in muscle cells (Fig. 5). Fourth, genetic studies have demonstrated that phenotypes of LRP4 mutant mice are similar to those in MuSK mutant (Weatherbee et al., 2006, *Development*; 133:4993–5000). LRP4 mutants die at birth with defects in both pre- and post-synaptic differentiation and in particular, the rapsyn-dependent scaffold fails to assemble in LRP4 mutants.

These results provide strong evidence that LRP4 satisfies essential criteria of serving a functional co-receptor of agrin. The identification of LRP4 as a co-receptor for agrin could provide insight into mechanisms of how agrin stimulation leads to AChR clustering. First, bridging agrin and MuSK, LRP4 could transmit signal to MuSK and thus activate intracellular cascades that have been identified, leading to AChR clustering. Second, LRP4 may regulate MuSK activity. MuSK and LRP4 co-precipitate in vitro and in muscle cells in the absence of agrin (Fig. 6 and Fig. 7), and tyrosine phosphorylation of MuSK is increased in cells co-expressing LRP4 (Figs. 4E and 4G). These observations may suggest that LRP4 promotes MuSK auto-activation, presumably by regulating MuSK dimerization. Exactly how LRP4 regulates MuSK function and the stoichiometry of the LRP4-MuSK interaction warrant further investigation. Third and alternatively, LRP4 itself may function as a signal transducer. The juxtamembrane cytoplasmic region of LRP4 contains a NPXY motif. This motif in LDLR, LRP1 and LRP2 has been shown to serve as a docking site for cytoplasmic adaptor proteins through a phosphotyrosine binding (PTB) domain (Herz and Bock, 2002, *Ann Rev Biochem*; 71:405–434). Intriguingly, LRP4 becomes tyrosine phosphorylated upon agrin stimulation (Fig. 7C and 7D).

It would be interesting to investigate whether tyrosine phosphorylation of LRP4 is necessary for agrin signaling and AChR clustering and whether phosphorylated LRP4 binds to PTB domain-containing proteins. One such protein is Dok7, which is essential for NMJ formation (Okada et al., 2006, *Science*; 312:1802–1805). Wnt signaling is implicated in synapse formation (Ciani and

Salinas, 2005, *Nat Rev Neurosci*; 6:351–362). Wnt-7a released from granule cells induces axon and growth cone remodeling in mossy fibers (Hall et al., 2000, *Cell*; 100:525–535). In *C. elegans*, Wnt signaling positions NMJs by inhibiting synaptogenesis (Klassen and Shen, 2007, *Cell*; 130:704–716). NMJ formation in
5 Drosophila requires Wnt signaling (Mathew et al., 2005, *Science*; 310:1344–1347; Packard et al., 2002, *Cell*; 111:319–330). However, it remains unclear whether Wnt signaling regulates mammalian NMJ formation. Wnt ligands act by binding to the receptor complex of Frizzled and LRP5/6 (Cadigan and Liu, 2006, *J Cell Sci*; 119:395–402; He et al., 2004, *Development*; 131:1663–1677; Malbon and Wang,
10 2006, *Curr Top Dev Biol*; 72:153–166; Schulte and Bryja, 2007, *Trends Pharmacol Sci*; 28:518–525)

Subsequently, signal is believed to be transmitted to the adapter protein Dishevelled (Dvl), which interacts with Frizzled, to initiate intracellular canonical and non-canonical pathways. Intriguingly, MuSK, like Frizzled, interacts with both a
15 LRP protein (i.e., LRP4) and Dvl (Luo et al., 2002, *Neuron*; 35:489–505). In addition, MuSK contains an extracellular CRD domain that is highly homologous to that in Frizzled that interacts with Wnt (Glass et al., 1996, *Cell*; 85:513–523; Valenzuela et al., 1995, *Neuron (USA)*; 15:573–584). Moreover, a number of Wnt signaling molecules including APC and ß-catenin have been implicated in MuSK
20 cascades (Li et al., 2008, *Nat Neurosci*; 11:262–268; Wang et al., 2003, *Nat Neurosci*; 6:1017–1018; Zhang et al., 2007, *J Neurosci*; 27:3968–3973). These observations raise a question whether the agrin-LRP4-MuSK signaling is regulated by a Wnt ligand that may interact with LRP4 and/or MuSK. This example showed that LRP4 does not bind Wnt-1 (Fig. 2E). This, however, does not exclude possible involvement
25 of one of the 18 other Wnt proteins in mouse (Clevers, 2006, *Cell*; 127:469–480) (see the Wnt Homepage on the worldwide web at stanford.edu/~rnusse/ wntwindow).

On the other hand, the "Wnt signaling" molecules (including Dvl, APC, and ß-catenin) may simply function in a manner independent of Wnt signaling in mammalian NMJ formation. It is of interest to note that the phenotypes of MuSK and
30 LRP4 mutant mice are more severe than those of agrin mutant. In LRP4 or MuSK mutants, but not agrin mutants, AChR clusters are absent when clusters begin to assemble at E13.5 and the rapsyn-dependent scaffold fails to assemble (Lin et al.,

2001, *Nature*; 410:1057–1064; Weatherbee et al., 2006, *Development*; 133:4993–5000).

These observations could suggest the existence of a signaling pathway that requires MuSK and/or LRP4, but not agrin. This pathway may regulate the formation of aneuronal AChR clusters prior to the arrival of motoneuron terminals or assembly of rapsyn-dependent scaffold. It may be regulated by a ligand that could interact with MuSK and/or LRP4. In light of the above discussion, such ligand may be a Wnt protein. Agrin is expressed in the brain (Cohen et al., 1997, *Neuroscience*; 76:581–596; Mann and Kroger, 1996, *Mol Cell Neurosci*; 8:1–13; O'Connor et al., 1994, *J Neurosci*; 14:1141–1152). Suppression of its expression impairs dendritic development and synapse formation in cultured hippocampal neurons (Bose et al., 2000, *J Neurosci*; 20:9086–9095; Ferreira, 1999, *J Cell Sci*; 112(Pt 24):4729–4738). Agrin-deficient neurons appear to be resistant to excitotoxic injury and agrin heterozygous mice are less sensitive to kainic acid-induced seizure and mortality (Hilgenberg et al., 2002). Agrin is thought to bind to the a3 subunit of Na+/K+-ATPase in neurons and thus regulates their function (Hilgenberg et al., 2006, *Cell;* 125:359–369). LRP4 expression is enriched in the brain and could interact with postsynaptic scaffold proteins including PSD-95 and SAP97 (Lu et al., 2007, *Brain Res*; 1177:19–28; Tian et al., 2006, *Eur J Neurosci*; 23:2864–2876; Weatherbee et al., 2006, *Development*; 133:4993–5000). The identification of LRP4 as a co-receptor of neuronal agrin may shed light on molecular mechanisms of how agrin and LRP4 work in the brain.

Example 2

Auto-antibodies to LRP4 in patients with myasthenia gravis

Autoimmune myasthenia gravis (MG) is the most common disorder of the neuromuscular synapse, affecting 10 to 20 per 100,000 people. MG involves a fatiguing weakness of the voluntary muscles with a characteristic decrement in the compound muscle action potential (CMAP) on repetitive stimulation of the motor nerve. In approximately 80% of patients, auto-antibodies to the muscle nicotinic

acetylcholine receptor (AChR) are present. These antibodies cause loss of AChR numbers and function, and lead to failure of neuromuscular transmission with muscle weakness. Although most cases of MG are caused by autoantibodies against AChR, about 20% of cases display no anti-AChR antibodies. In 2001, antibodies against
5 MuSK (muscle specific tyrosine kinase) were identified in a proportion of patients with generalized MG (Hoch et al., 2001, *Nat Med*; 7(3):365-8). MuSK is a key organizer of NMJ formation. MuSK is required for clustering of AChR during the formation of NMJ and is expressed predominantly at the postsynaptic membrane in mature NMJ. In MuSK knockout mice, muscle fibers do not form aneural clusters or
10 "prepattern" prior to innervation and do not form the NMJ, and AChRs fail to cluster opposite to growing motoneuron terminals on the surfaces of myotubes, suggesting a critical role of MuSK for both muscle fiber prepatterning and nerve-induced AChR clusters. Additionally, a case of heteroalleric MuSK mutations that caused the reduction of MuSK expression has been associated with congenital myasthenic
15 syndrome (CMS).

Recent studies by Vincent and others showed that the frequency of MuSK antibodies in MG patients who were AChR seronegative (that is, lacked autoantibodies to the AchR) varied from 4 to 50%. MuSK antibodies interfere with the agrin/MuSK/AChR clustering in myotubes and alter MuSK function at the adult
20 NMJ. The observation that rabbits immunized with the MuSK ectodomain manifested muscular weakness typical of MG and diminished AChR clustering at the NMJ provided a direct proof for the pathogenic role of anti-MuSK antibodies. See, for example, ter Beek et al., 2009, *Am J Pathol*; Oct;175(4):1536-44 (Epub 2009 Sep 10); Lang and Vincent, 2009, *Curr Opin Pharmacol*; Jun;9(3):336-40 (Epub 2009
25 May 8); Littleton et al., 2009, *Mol Cell Proteomics*; Jul;8(7):1688-96 (Epub 2009 Mar 29); Beeson et al., 2008, *Ann N Y Acad Sci*; 1132:99-103; Vincent et al., 2008, *Ann N Y Acad Sci*; 1132:84-92; Leite et al., 2008, *Brain*; 131(Pt 7):1940-52; Farrugia et al., 2007, *J Neuroimmunol*; 185(1-2):136-44; Deymeer et al., 2007, *Neurology*; 68(8):609-11; Farrugia et al., 2007, *Clin Neurophysiol*; 118(2):269-77; Vincent, 2006,
30 *Acta Neurol Scand Suppl*; 183:1-7; Farrugia et al., 2006, *Muscle Nerve*; 33(4):568-70; Benveniste et al., 2005, *J Neuroimmunol*; 170(1-2):41-8; Vincent and Leite, 2005, *Curr Opin Neurol*; 18(5):519-25; Nemoto et al., 2005, *J Neurol Neurosurg*

Psychiatry; 76(5):714-8; Shiraishi et al., 2005, *Ann Neurol*; 57(2):289-93; Vincent et al., 2005, *Neurology*; 64(2):399; Vincent and Rothwell, 2004, *Autoimmunity*; 37(4):317-9; Vincent et al., 2004, *Semin Neurol*; 24(1):125-33; Zhou et al., 2004, *Muscle Nerve*; 30(1):55-60; McConville et al., 2004, *Ann Neurol*; 55(4):580-4;
5 Vincent et al., 2003, *Ann N Y Acad Sci*; 998:324-35; Vincent et al., 2003, *Lancet Neurol*; 2(2):99-106; Sanders et al., 2003, *Neurology*; 60(12):1978-80; McConville and Vincent, 2002, *Curr Opin Pharmacol*; 2(3):296-301; Liyanage et al., 2002, *Muscle Nerve*; 25(1):4-16; and Hoch et al., 2001, *Nat Med*; 7(3):365-8.

Although a number of studies have documented that AChR or MuSK
10 antibodies cause structural and functional damage to the NMJ of MG patients, the identity of autoantigen(s) in the more than 10% of MG patients without such antibodies to AChR or MuSK remain unknown.

The present invention demonstrates that a subset of seronegative MG patients have serum antibodies against LRP4. LRP4, or MEGF7 (for multiple epidermal
15 growth factor [EGF]-like domain 7), is a member of the LDLR family and contains a large extracellular N-terminal region that possesses multiple EGF repeats and LDLR repeats, a transmembrane domain, and a short C-terminal region without an identifiable catalytic motif. LRP4 is required for NMJ formation as well as the development of the limb, lung, kidney, and ectodermal organs. Mice lacking LRP4
20 die at birth with deficits that resemble the phenotype observed in MuSK mutant mice. Recent studies show that agrin binds LRP4, and LRP4 binds and activates MuSK through its extracellular domain. See, Zhang et al., 2008, *Neuron*; Oct 23;60(2):285-97, and Kim et al., 2008, *Cell*; Oct 17;135(2):334-42 (Epub 2008 Oct 9). The functional importance at NMJ and spatial proximity with AChR and MuSK
25 make LRP4 one promising autoantigen for AChR and MuSK seronegative MG patients.

This example shows that 40% of AChR/MuSK-antibody-seronegative MG patients have serum autoantibodies against LRP4. The LRP4 antibodies were specific for the extracellular domains of LRP4 expressed in transfected HEK293 cells and
30 strongly inhibited LRP4 function in cultured myotubes. The results of this example indicate the involvement of LRP4 antibodies in the pathogenesis of AChR/MuSK-antibodies-seronegative MG, thus defining a novel immunological

form of the disease. Measurement of LRP4 antibodies will substantially aid diagnosis and clinical management.

Methods

5 *Patients.* Samples were obtained from male and female patients with moderate or severe generalized MG in whom the standard radio- immunoprecipitation assay for anti-AChR antibodies was negative on several occasions. All had typical fatigable muscle weakness. The diagnosis was confirmed by electromyographic evidence of a defect in neuromuscular transmission (a decrement of more than 10% in 10 the amplitude of the compound muscle action potential on repetitive nerve stimulation at 3 Hz and/or an increase in jitter on single fiber studies), or by a positive response to anticholinesterase medication (edrophonium or pyridostigmine). In some cases, plasma was obtained during therapeutic plasmapheresis, which improved muscle strength. Some sera were taken on first examination. Some patients had received 15 corticosteroids for up to two months before sampling. Sera or plasmas were also obtained from healthy volunteers, from patients with AChR-Ab-positive MG, and from patients with other immune-mediated neurological disorders. IgG preparations were made using a ImmunoPure (G) IgG purification kit (Pierce, Rockford, IL).

LRP4 and agrin expression constructs. Constructs encoding full-length and 20 entire extracellular domain LRP4 and the neural agrin were prepared as described in Example 1. All constructs were transiently transfected into HEK293 cells. For the production of soluble agrin and LRP4 constructs, cells were switched to low-serum medium (0.5% FBS) the second day after transfection. Conditioned media, containing LRP4 or agrin fragments, were removed 24 hours later and analyzed by western 25 blotting to confirm expression.

Immunostaining of LRP4-transfected HEK293 cells. COS7 cells were plated onto chamber slides the day after transfection. Two days later, cells were fixed with 4% paraformaldehyde and stained as described. Plasmas of MG patients and controls were analyzed at various dilutions (between 1:50 and 1:100). Bound antibodies were 30 visualized with secondary antibodies conjugated to FITC (anti-human IgG, Southern Biotech, Birmingham, AL). In all experiments, expression of transfected LRP4 constructs was confirmed by staining parallel slides with rabbit antibodies against

LRP4.

ELISA detection of antibodies to LRP4. Conditioned medium from LRP4-transfected HEK293 cells or from control cells mock-transfected with empty vector, was diluted 1:1 with 100 mM NaHCO3 buffer (pH 9.5) and applied overnight to ELISA plates. Plasmas were first tested at 1:50 in triplicates and subsequently at 1:100 in duplicates. Bound antibodies were detected by alkaline phosphatase labeled Goat anti human IgG, IgM and IgA antibody (Abcam, Cambridge, MA) followed by p-nitrophenyl phosphate and measuring A405. For each sample, nonspecific immunoreactivity, determined by incubation of plates coated with conditioned medium from mock-transfected HEK293 cells, was subtracted. The efficient immobilization of LRP4 was confirmed by ELISA with an antibody directed against the myc-epitope.

Results

AChR/MuSK-antibodies-seronegative MG is thought to represent about 10-15% of all MG patients, but the true prevalence is difficult to assess because of differences in patient ascertainment and referral to specialist centers. To establish an assay that could be used for future diagnosis and epidemiological studies, sera and plasmas were tested in an ELISA using plates coated with fragments of the extracellular domains of LRP4, expressed in secreted form from HEK293 cells. A cut-off (1.25 optical density units [OD]) was calculated on the basis of the mean ±3 s.d. of the values with healthy control plasmas or sera. Raised levels of IgG antibodies to LRP4 were found in 12 of 31 samples from patients with AChR/MuSK-Ab seronegative MG. As shown in Fig. 9, LRP4 antibodies are detected by ELISA. Antibodies to LRP4 were found in 12 of 31 AChR/MuSK seronegative MG patients compared with 6 healthy controls.

Analysis of patients information. Overall, there is 98% homology between rat LRP4 and the published human sequence, and they differ by only 68 out of 1905 amino acids. Although the use of rat LRP4 yielded significant results, it is expected that the use of human LRP4, as well as further refinement of the assay, may increase the sensitivity of the test.

Coded plasmas from AChR/MuSK-antibodies-seronegative MG patients and

healthy individuals were tested using HEK293 cells transfected with rat LRP4 constructs. IgG from all five AChR/MuSK-antibodies-seronegative MG plasmas, but not from the three healthy control plasmas, bound to LRP4 aggregates on the cell surface at dilutions of up to 1:50. Each of the AChR/MuSK-antibody-seronegative MG plasmas recognized the extracellular domains of LRP4. As shown in Fig. 10, antibodies from AChR/MuSK seronegative MG patients bind to LRP4. AChR/MuSK-antibodies-negative MG IgGs bound to 293 cells expressing full-length, whereas healthy control IgG did not (normal serum). LRP4 immunoreactivity appeared as a speckled pattern, similar to that seen previously with rabbit anti-LRP4 antibodies.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Sequence Listing Free Text

SEQ ID NO:1-10	Synthetic oligonucleotide sequences for miRNA constructs
SEQ ID NO:11	Amino acid sequence of human low density lipoprotein receptor-related protein 4 (LRP4) precursor.

What is claimed is:

1. A method for diagnosing a neurotransmission or developmental disorder in a mammal, the method comprising detecting in a bodily fluid of the mammal autoantibodies that bind to the low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

2. A method for diagnosing a neurotransmission or developmental disorder associated with interference of agrin/MuSK/LRP4/AChR neuromuscular junction formation or function in a mammal, the method comprising detecting in a bodily fluid of the mammal autoantibodies to an epitope of low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

3. A method for diagnosing congenital and acquired muscle disorders associated with interference of agrin/MuSK/LRP4/AChR neuromuscular junction formation of functioning in a mammal, the method comprising detecting in a bodily fluid of the mammal autoantibodies to an epitope of low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

4. A method of diagnosing myasthenia gravis in a mammal, the method comprising detecting autoantibodies to an epitope of the low density lipoprotein receptor-related protein 4 (LRP4) in a bodily fluid of the mammal.

5. The method of claim 4, wherein the myasthenia gravis is seronegative for autoantibodies to the acetylcholine receptor (AChR) and/or muscle specific tyrosine kinase (MuSK).

6. The method according to any one of claims 1-5, wherein the method comprises:

contacting the bodily fluid with a LRP4 polypeptide or antigenic determinant thereof; and

detecting any antibody-antigen complexes formed between said LRP4 polypeptide or antigenic fragment thereof and antibodies present in the bodily fluid;

wherein the presence of antibody-antigen complexes is indicative of said mammal suffering from a neurotransmission or developmental disorder.

7. The method according to claim 6, wherein the antibody-antigen complex is detected using a LRP4, epitope, or antigenic determinant thereof tagged or labeled with a reporter molecule.

8. The method according to claim 6, wherein the antibody-antigen complex is detected using an anti-IgG antibody tagged or labeled with a reporter molecule.

9. The method according to claim 7 or claim 8, wherein said reporter molecule includes any of a heavy metal, a fluorescent or luminescent molecule, radioactive or enzymatic tag.

10. A method according to claim 9, wherein said enzymatic tag comprises horseradish peroxidase-protein A.

11. A method according to claim 9, wherein said reporter molecule is a radioactive label.

12. The method according to claim 11, wherein said label is ^{125}I.

13. The method according to any one of claims 1-12 wherein the neurotransmission disorder is myasthenia gravis, muscular dystrophy, or a congenital myasthenic syndrome (CMS) .

14. The method according to any one of claims 1-12, wherein the developmental disorder is muscle paralysis and/or fixed joints in newborn offspring due to maternal antibodies to LRP4.

15. The method of claim to any one of claims 1-14, wherein the neurotransmission or developmental disorder is seronegative for autoantibodies to the acetylcholine

receptor (AChR) and/or muscle specific tyrosine kinase (MuSK).

16. An assay kit for diagnosing a neurotransmission disorder in a mammal, the kit comprising a LRP4 polypeptide or an epitope thereof.

17. The assay kit of claim 16 wherein the LRP4 polypeptide or epitope thereof is immobilized on a solid surface.

18. The assay kit of claim 16 further comprising a means for contacting said LRP4 polypeptide or epitope thereof with a bodily fluid of said mammal.

19. The assay kit of any one claims 16-18 further comprising an acetylcholine receptor polypeptide, or fragment thereof, and/or a muscle specific tyrosine kinase (MuSK) polypeptide, or fragment thereof.

20. An assay kit according to any one of claims 16-19, wherein the LRP4 polypeptide or epitope thereof has a detectable label thereon.

21. An assay kit according to claim 20 wherein the detectable label is ^{125}I.

22. The assay kit according to any one of claims 16-21 for detecting myasthenia gravis, muscular dystrophy, or a congential myasthenic syndrome.

23. The assay kit according to any one of claims 16-22 further comprising a negative control and/or a positive control.

24. An isolated or purified antibody specific for an anti-LRP4 autoantibody from a bodily fluid of a mammal.

25. The antibody of claim 24, wherein the antibody inhibits the binding of an anti-LRP4 autoantibody to LRP4.

26. The antibody of claim 24 or 25 conjugated to a reporter molecule.

27. A composition comprising an antibody of any one of claims 24-26 and a pharmaceutically acceptable carrier, diluent or excipient therefor.

28. A diagnostic kit for detecting a neurotransmission disorder in a mammal, the diagnostic kit comprising an antibody according to any one of claims 24-27.

29. The diagnostic kit of claim 28 further comprising a means for contacting said antibody with a bodily fluid of the mammal.

30. The method of any one of claims 1-15, a kit of any one of claims 16-22 or 27-39 or an antibody of any one of claims 23-27, wherein the bodily fluid is selected from the group consisting of plasma, serum, whole blood, urine, sweat, lymph, feces, cerebrospinal fluid and nipple aspirate.

31. A method of identifying compounds capable of alleviating or treating a neurotransmission disorder, the method comprising:

contacting a candidate compound in the presence of LRP4 or an epitope thereof and an antibody capable of binding LRP4,

wherein a compound that prevents binding of the antibody to LRP4 or an epitope thereof is a candidate for treating a neurotransmission disorder.

32. A compound identified by the method of claim 31.

33. A method of treating a patient suffering from a neurotransmission disorder comprising administering to said patient an effective amount of an antibody according to any one of claims 23-27 or a compound of claim 32.

34. The method of claim 33, wherein the neurotransmission disorder is myasthenia gravis, muscular dystrophy, or a congential myasthenic syndrome.

35. The method of claim 33 or 34, wherein the neurotransmission disorder is seronegative for autoantibodies to the acetylcholine receptor (AChR) and/or muscle specific tyrosine kinase (MuSK).

36. A method of treating an individual suffering from a neurotransmission disorder, the method comprising:

detecting in a bodily fluid of the individual autoantibodies that bind to the low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof; and

administering to the patient an effective amount an immunosuppressant and/or another appropriate therapeutic modality.

37. A method for diagnosing a neurotransmission or developmental disorder in a mammal, the method comprising detecting a genetic mutation in the low density lipoprotein receptor-related protein 4 (LRP4) gene.

38. The method of claim 37, wherein the genetic mutation is an intronic mutation, an exonic mutation, a splice junction mutation, a point mutation, a missense mutation, an insertion mutation, a deletion mutation, an insertion-deletion mutation, alters one or more amino acids, a read through mutation, a frameshift mutation, affects mRNA splicing, introduces a stop codon, affects mRNA half life, affects mRNA transcription, affects mRNA translation, reduces LRP4 mRNA and/or protein expression, and/or prevents LRP4 mRNA and/or protein expression.

39. A method for diagnosing a neurotransmission or developmental disorder of any one of claims 1-15, 31, 37, or 38, the method further comprising providing a report or print out summarizing the binding of autoantibodies to the low density lipoprotein receptor-related protein 4 (LRP4), or an epitope thereof.

40. A method of treating an individual suffering from a neurotransmission disorder, the method comprising:

detecting a genetic mutation in the low density lipoprotein receptor-related protein 4 (LRP4) gene; and

administering to the patient an effective amount an immunosuppressant and/or another appropriate therapeutic modality.

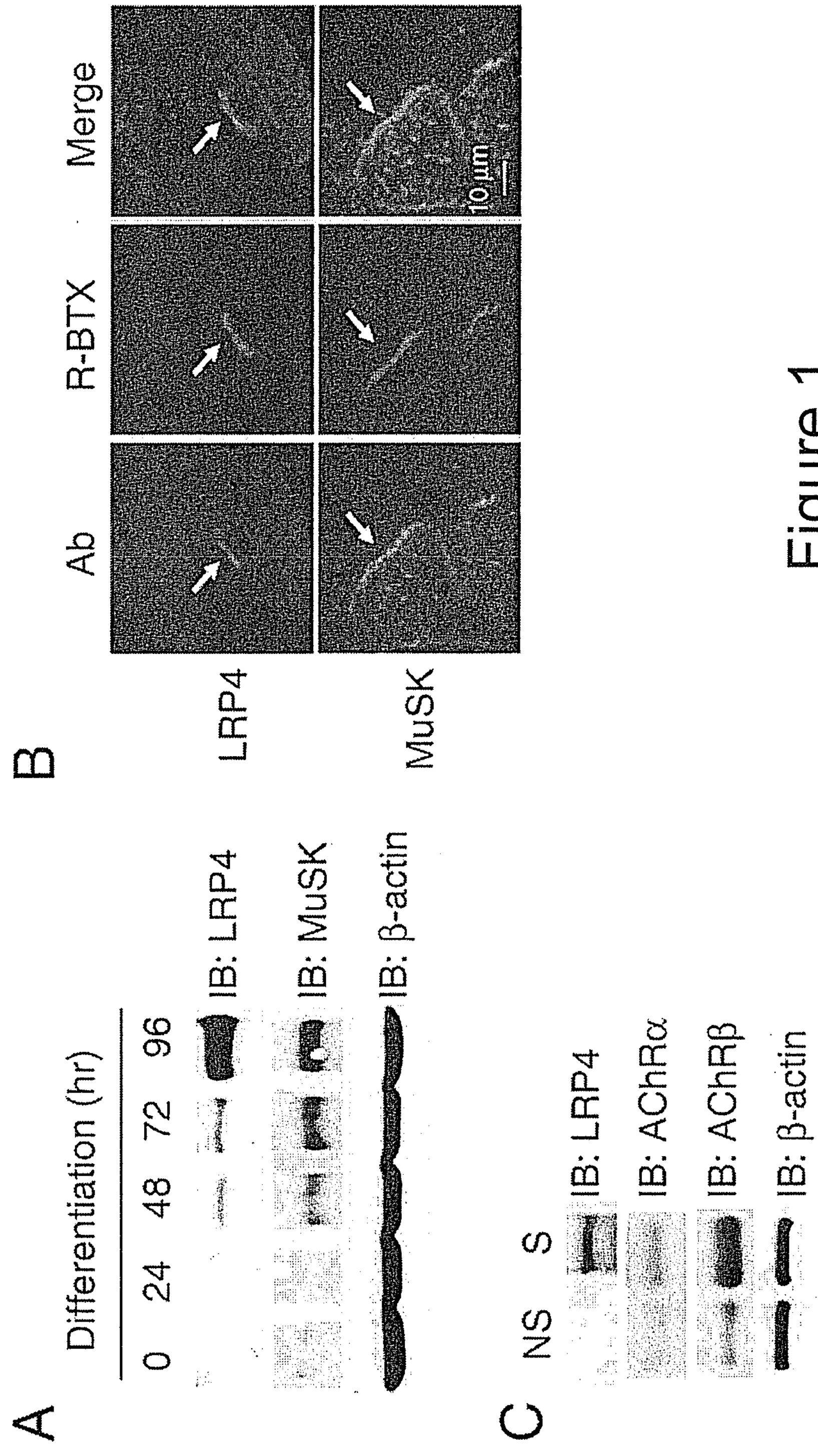


Figure 1

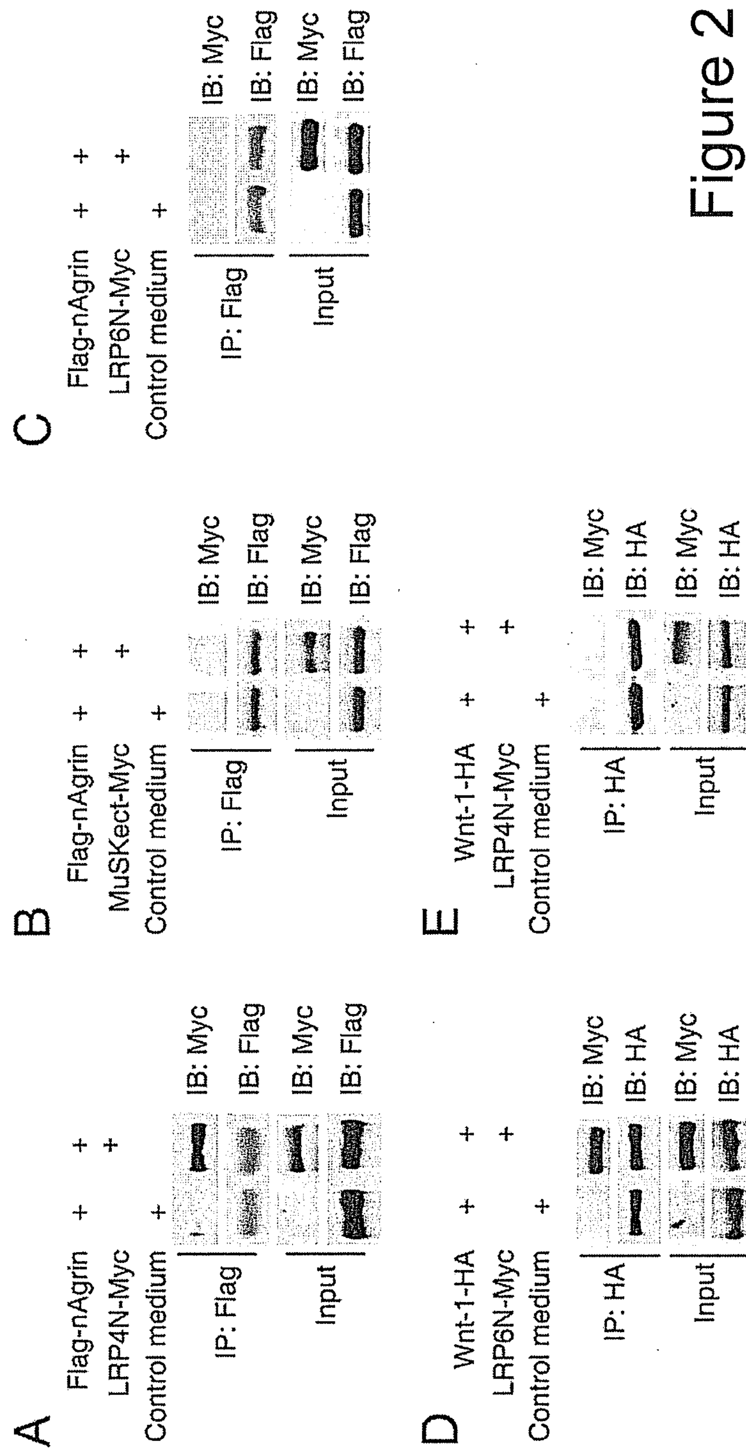


Figure 2

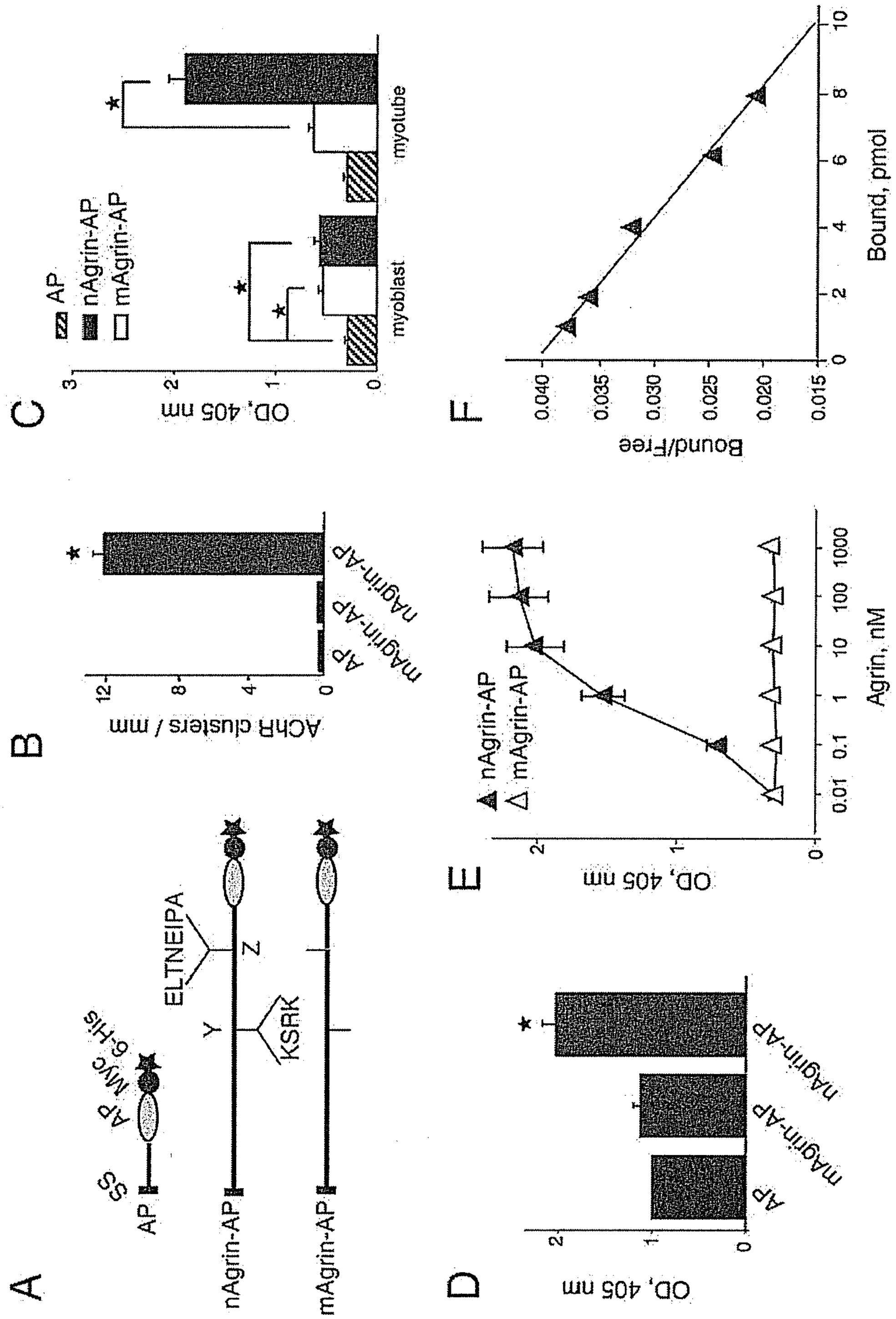
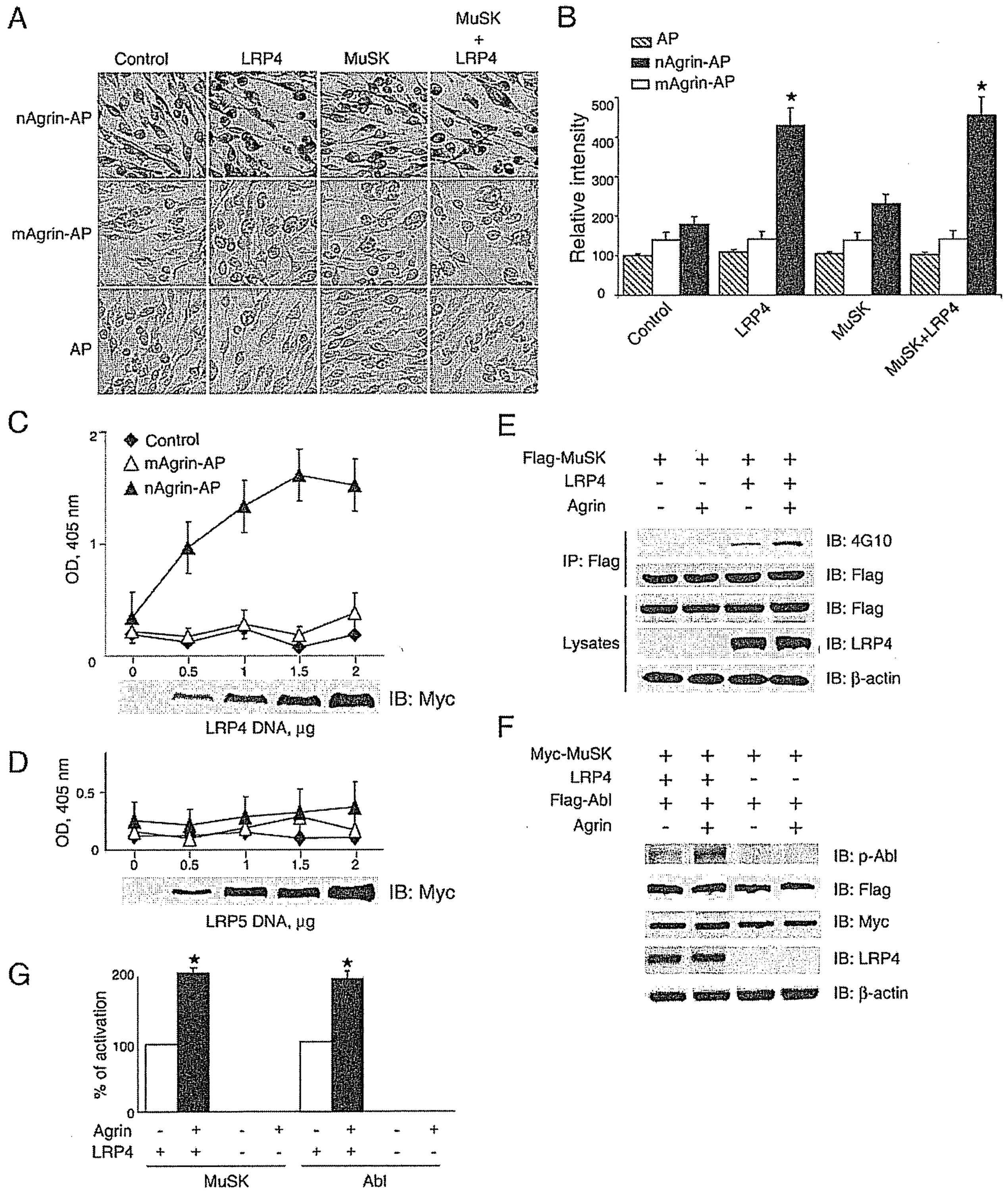


Figure 3

Figure 4



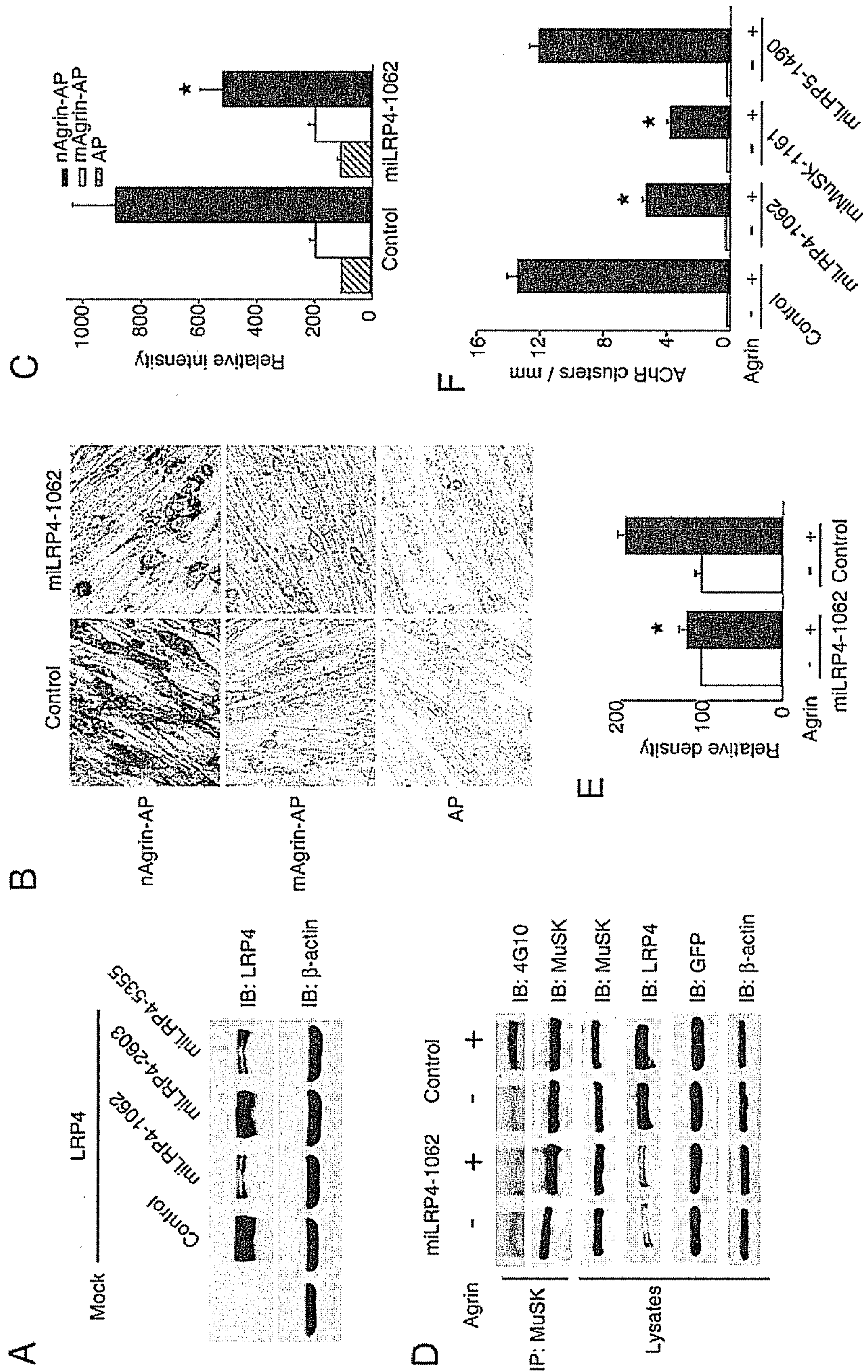


Figure 5

Figure 6

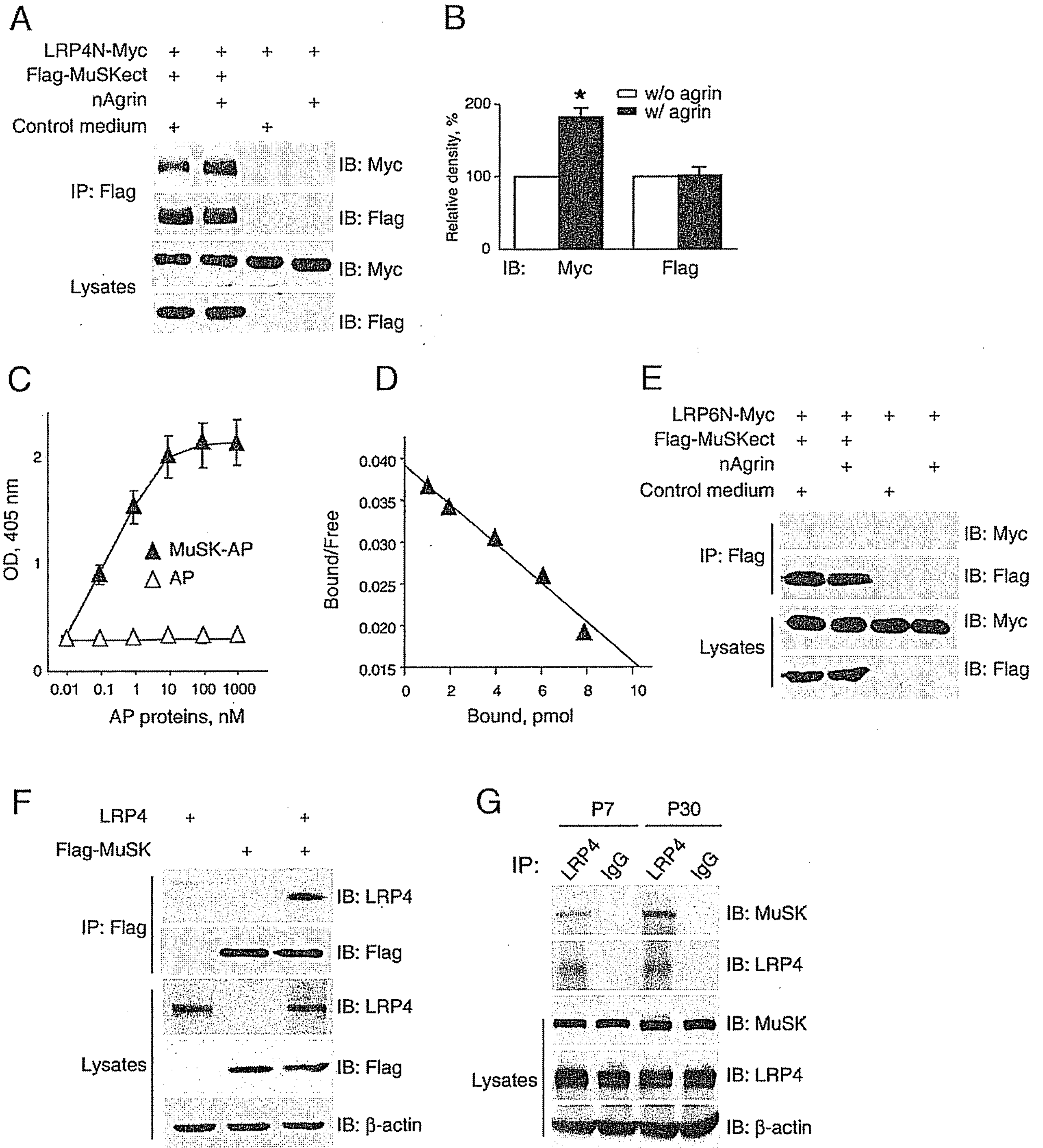


Figure 7

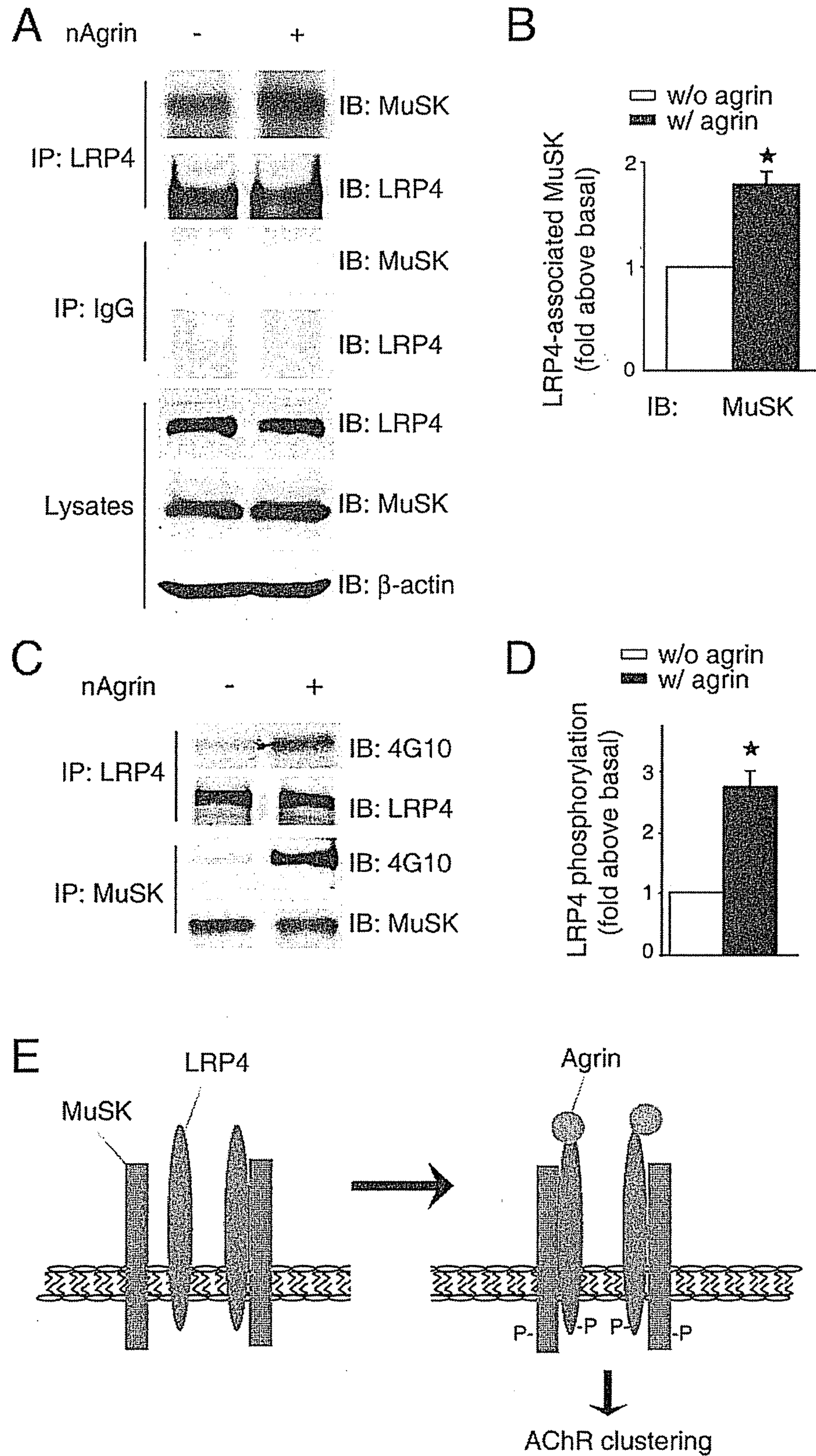


Figure 8

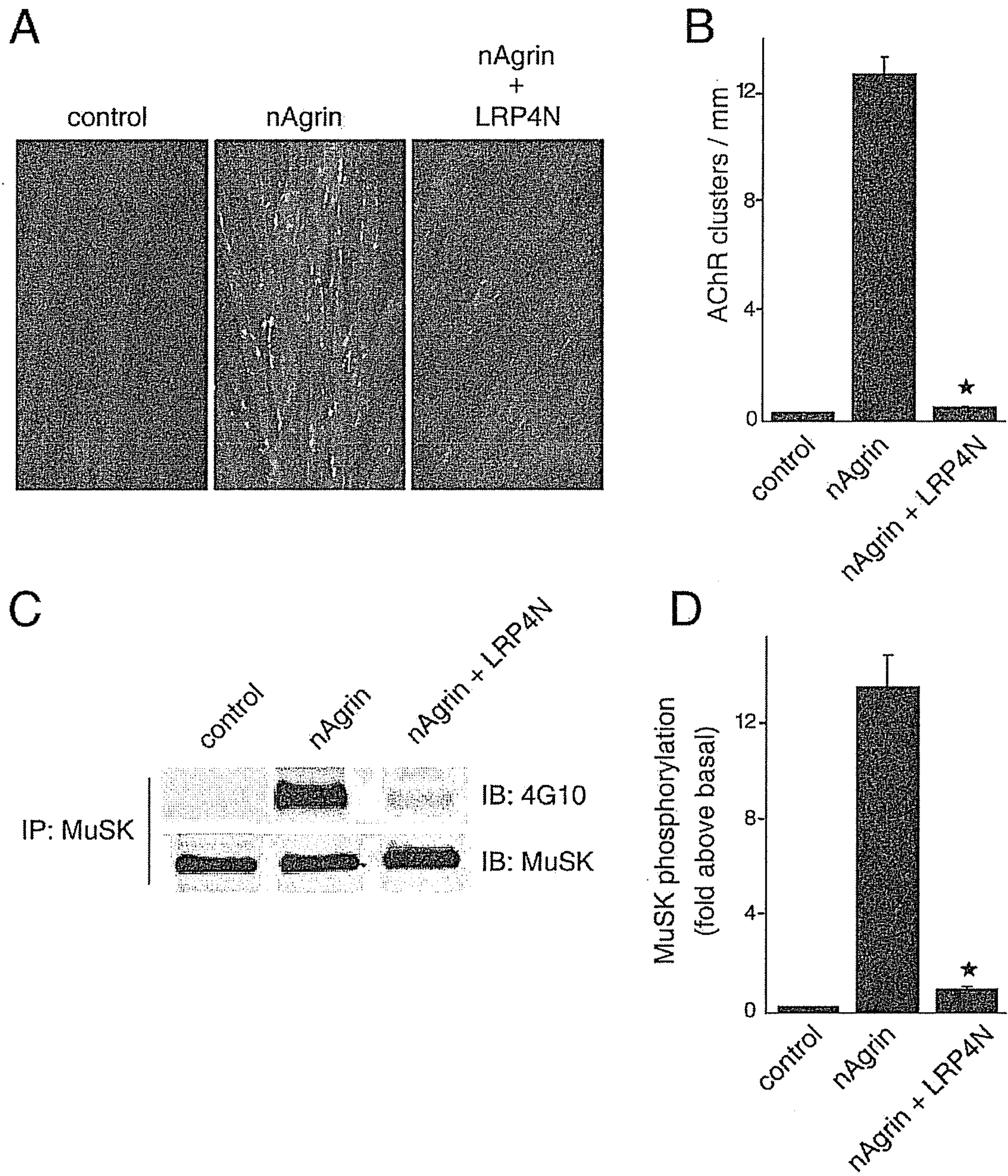


Figure 9

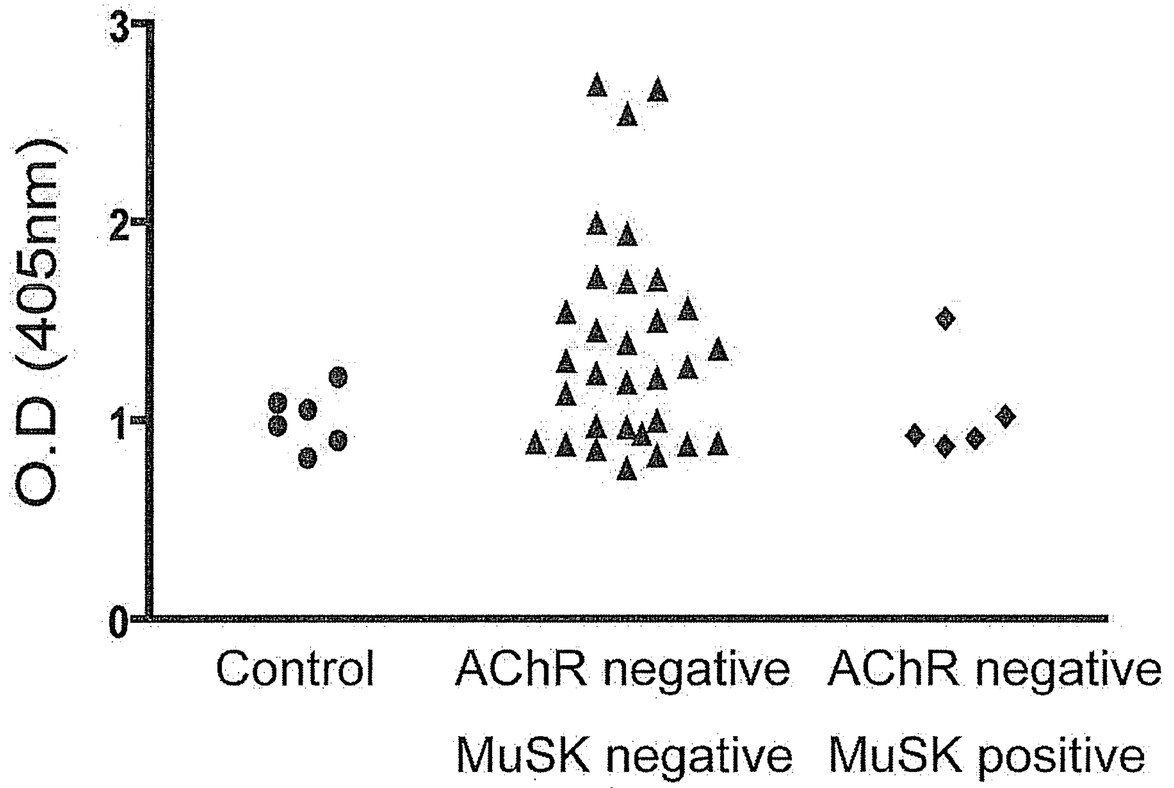


Figure 10

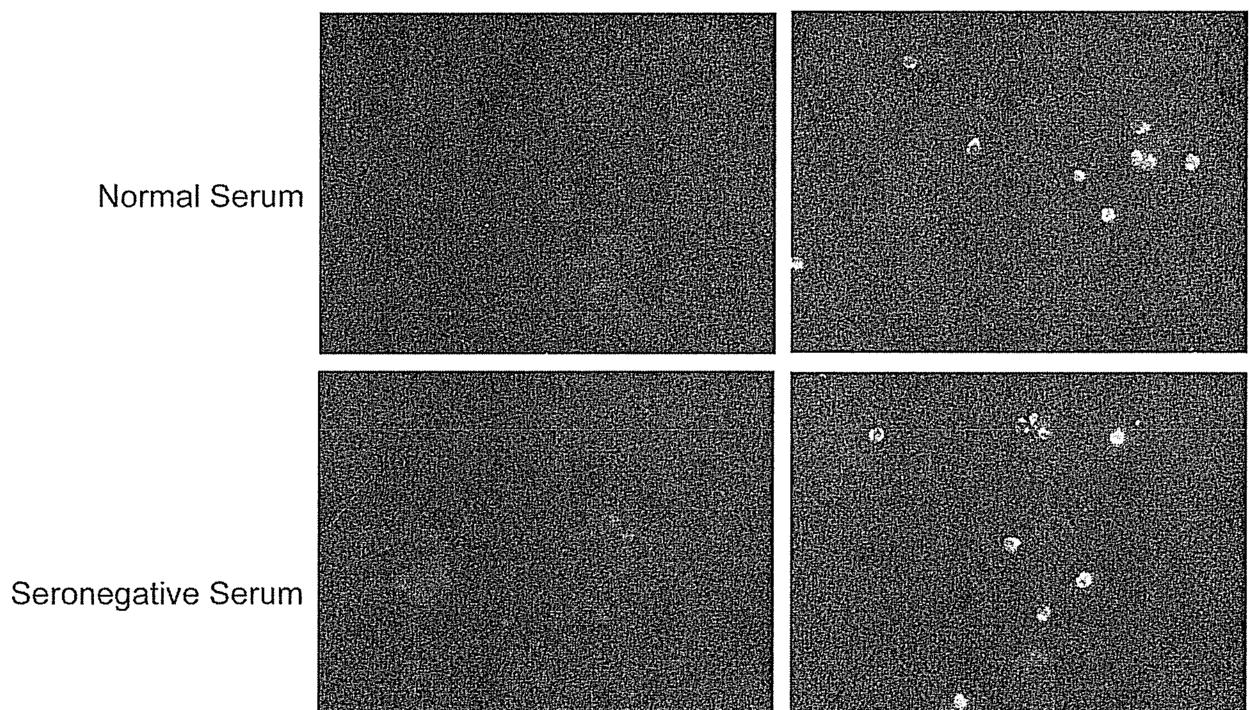


Figure 11-1

```
        10         20         30         40         50         60
MRRQWGALLL GALLCAHGLA SSPECACGRS HFTCAVSALG ECTCIPAQWQ CDGDNDCGDH

        70         80         90        100        110        120
SDEDGCILPT CSPLDFHCDN GKCIRRSWVC DGDNDCEDDS DEQDCPPREC EEDEFPCQNG

       130        140        150        160        170        180
YCIRSLWHCD GDNDCGDNSD EQCDMRKCSD KEFRCSDGSC IAEHWYCDGD TDCKDGSDEE

       190        200        210        220        230        240
NCPSAVPAPP CNLEEFQCAY GRCILDIYHC DGDDDCGDWS DESDCSSHQP CRSGEFMCDS

       250        260        270        280        290        300
GLCINAGWRC DGDADCDDQS DERNCTTSMC TAEQFRCHSG RCVRLSWRCD GEDDCADNSD

       310        320        330        340        350        360
EENCENTGSP QCALDQFLCW NGRCIGQRKL CNGVNDCGDN SDESPQQNCR PRTGEENCNV

       370        380        390        400        410        420
NNGGCAQKCQ MVRGAVQCTC HTGYRLTEDG HTCQDVNECA EEGYCSQGCT NSEGAFQCWC

       430        440        450        460        470        480
ETGYELRPDR RSCKALGPEP VLLFANRIDI RQVLPHRSEY TLLLNNLENA IALDFHHRRE

       490        500        510        520        530        540
LVFWSDVTLD RILRANLNGS NVEEVVSTGL ESPGGLAVDW VHDKLYWTDS GTSRIEVANL

       550        560        570        580        590        600
DGAHRKVLLW QNLEKPRAIA LHPMEGTIYW TDWGNTPRIE ASSMDGSGRR IIADTHLFWP

       610        620        630        640        650        660
NGLTIDYAGR RMYWVDAKHH VIERANLDGS HRKAVISQGL PHPFAITVFE DSLYWTDWHT
```

Figure 11-2

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       670        680        690        700        710        720
KSINSANKFT GKNQEIIRNK LHFPMDIHTL HPQRQPAGKN RCGDNNGGCT HLCLPSGQNY

       730        740        750        760        770        780
TCACPTGFRK ISSHACAQSL DKFLLFARRM DIRRISFDTE DLSDDVIPLA DVRSAVALDW

       790        800        810        820        830        840
DSRDDHVYWT DVSTDTISRA KWDGTGQEVV VDTSLESPAG LAIDWVTNKL YWTDAGTDRI

       850        860        870        880        890        900
EVANTDGSMR TVLIWENLDR PRDIVVEPMG GYMYWTDWGA SPKIERAGMD ASGRQVIISS

       910        920        930        940        950        960
NLTWPNGLAI DYGSQRLYWA DAGMKTIEFA GLDGSKRKVL IGSQLPHPFG LTLYGERIYW

       970        980        990       1000       1010       1020
TDWQTKSIQS ADRLTGLDRE TLQENLENLM DIHVFHRRRP PVSTPCAMEN GGCSHLCLRS

      1030       1040       1050       1060       1070       1080
PNPSGFSCTC PTGINLLSDG KTCSPGMNSF LIFARRIDIR MVSLDIPYFA DVVVPINITM

      1090       1100       1110       1120       1130       1140
KNTIAIGVDP QEGKVYWSDS TLHRISRANL DGSQHEDIIT TGLQTTDGLA VDAIGRKVYW

      1150       1160       1170       1180       1190       1200
TDTGTNRIEV GNLDGSMRKV LVWQNLDSPR AIVLYHEMGF MYWTDWGENA KLERSGMDGS

      1210       1220       1230       1240       1250       1260
DRAVLINNNL GWPNGLTVDK ASSQLLWADA HTERIEAADL NGANRHTLVS PVQHPYGLTL

      1270       1280       1290       1300       1310       1320
LDSYIYWTDW QTRSIHRADK GTGSNVILVR SNLPGLMDMQ AVDRAQPLGF NKCGSRNGGC
```

Figure 11-3

```
      1330       1340       1350       1360       1370       1380
SHLCLPRPSG FSCACPTGIQ LKGDGKTCDP SPETYLLFSS RGSIRRISLD TSDHTDVHVP

      1390       1400       1410       1420       1430       1440
VPELNNVISL DYDSVDGKVY YTDVFLDVIR RADLNGSNME TVIGRGLKTT DGLAVDWVAR

      1450       1460       1470       1480       1490       1500
NLYWTDTGRN TIEASRLDGS CRKVLINNSL DEPRAIAVFP RKGYLFWTDW GHIAKIERAN

      1510       1520       1530       1540       1550       1560
LDGSERKVLI NTDLGWPNGL TLDYDTRRIY WVDAHLDRIE SADLNGKLRQ VLVSHVSHPF

      1570       1580       1590       1600       1610       1620
ALTQQDRWIY WTDWQTKSIQ RVDKYSGRNK ETVLANVEGL MDIIVVSPQR QTGTNACGVN

      1630       1640       1650       1660       1670       1680
NGGCTHLCFA RASDFVCACP DEPDSRPCSL VPGLVPPAPR ATGMSEKSPV LPNTPPTTLY

      1690       1700       1710       1720       1730       1740
SSTTRTRTSL EEVEGRCSER DARLGLCARS NDAVPAAPGE GLHISYAIGG LLSILLILVV

      1750       1760       1770       1780       1790       1800
IAALMLYRHK KSKFTDPGMG NLTYSNPSYR TSTQEVKIEA IPKPAMYNQL CYKKEGGPDH

      1810       1820       1830       1840       1850       1860
NYTKEKIKIV EGICLLSGDD AEWDDLKQLR SSRGGLLRDH VCMKTDTVSI QASSGSLDDT

      1870       1880       1890       1900
ETEQLLQEEQ SECSSVHTAA TPERRGSLPD TGWKHERKLS SESQV
```

patsnap

专利名称(译)	检测和治疗LRP4相关的神经传递障碍		
公开(公告)号	EP2491389A4	公开(公告)日	2013-07-31
申请号	EP2010825651	申请日	2010-10-21
[标]申请(专利权)人(译)	格鲁吉亚健康科学大学研究所公司		
申请(专利权)人(译)	格鲁吉亚医学版大学研究所，INC.		
当前申请(专利权)人(译)	格鲁吉亚医学版大学研究所，INC.		
[标]发明人	MEI LIN XIONG WEN CHENG ZHANG BIN		
发明人	MEI, LIN XIONG, WEN-CHENG ZHANG, BIN		
IPC分类号	G01N33/53 G01N33/534 G01N33/15 C12Q1/68		
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外部链接	Espacenet		

摘要(译)

本发明包括检测神经传递或发育障碍的方法，包括但不限于重症肌无力，其对乙酰胆碱受体（AChR）和/或肌肉特异性酪氨酸激酶（MuSK）的自身抗体是血清阴性的，该方法包括检测结合LRP4或其表位的自身抗体。还包括治疗患有神经传递障碍的个体的方法，该方法包括在体液中检测与LRP4或其表位结合的各个自身抗体，并向患者施用有效量的免疫抑制剂和/或或其他适当的治疗方式。还包括与LRP4的自身抗体结合的抗体和用于检测神经传递或发育障碍的试剂盒。