Impaired Synaptic Development, Maintenance, and Neuromuscular Transmission in LRP4-Related Myasthenia

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**IMPORTANCE** Congenital myasthenic syndromes (CMS) are heterogeneous disorders. Defining the phenotypic features, genetic basis, and pathomechanisms of a CMS is relevant to prognosis, genetic counseling, and therapy.

**OBJECTIVES** To characterize clinical, structural, electrophysiologic, and genetic features of a CMS and to search for optimal therapy.

**DESIGN, SETTINGS, AND PARTICIPANTS** Two sisters with CMS affecting the limb-girdle muscles were investigated between 2012 and 2014 at an academic medical center by clinical observation, in vitro analysis of neuromuscular transmission, cytochemical and electron microscopy studies of the neuromuscular junction, exome sequencing, expression studies in HEK293 and COS7 cells, and for response to therapy, and they were compared with 15 historical control participants.

**MAIN OUTCOMES AND MEASURES** We identified the disease gene and mutation, confirmed pathogenicity of the mutation by expression studies, and instituted optimal pharmacotherapy.

**RESULTS** Quantitative analysis of single EP regions was done for all 15 control participants and microelectrode studies of neuromuscular transmission and α-bgt binding sites per EP was conducted for 13 control participants. Examination of the older sister’s intercostal muscle end plates (EPs) showed them to be abnormally small, with attenuated reactivities for the acetylcholine receptor and acetylcholinesterase. Most EPs had poorly differentiated or degenerate junctional folds, and some appeared denuded of nerve terminals. The amplitude of the EP potential (EPP), the miniature EPP, and the quantal content of the EPP were all markedly reduced. Exome sequencing identified a novel homozygous p.Glu1233Ala mutation in low-density lipoprotein receptor–related protein 4 (LRP4), a coreceptor for agrin to activate muscle-specific tyrosine kinase (MuSK), which is required for EP development and maintenance. Expression studies indicate that the mutation compromises the ability of LRP4 to bind to, phosphorylate, and activate MuSK. Treatment with albuterol sulfate improved the patient’s symptoms. A previously identified patient harboring 2 heterozygous mutations in LRP4 had structurally abnormal intercostal EPs but no identifiable defect of neuromuscular transmission at these EPs.

**CONCLUSIONS AND RELEVANCE** We identified a second CMS kinship harboring mutations in LRP4, identified the mechanisms that impair neuromuscular transmission, and mitigated the disease by appropriate therapy.

Published online June 8, 2015.

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The development and maintenance of the neuromuscular junction depends crucially on the agrin-MuSK-LRP4 signaling system. Low-density lipoprotein receptor-related protein 4 (LRP4) and muscle-specific tyrosine kinase (MuSK) are anchored in the postsynaptic membrane. Agrin, secreted into the synaptic space by the nerve terminal, binds to multiple sites on the extracellular domain of LRP4, which then binds to the extracellular domain of MuSK on the postsynaptic membrane. This results in phosphorylation and activation of MuSK and clustering of MuSK and LRP4.1-3 Activated MuSK, in concert with DOK7 and other postsynaptic proteins, acts on rapsyn to concentrate the acetylcholine receptor (AChR) in the postsynaptic membrane and promotes postsynaptic gene expression and differentiation. Clustered LRP4 also enhances presynaptic differentiation.3

Several reports have described congenital myasthenic syndromes (CMSs) caused by mutations in agrin4-6 and MuSK,7-11 but only 1 report has described mutations in LRP4.12 This was the case of a 14-year-old girl with moderately severe fatigable limb-girdle weakness, dysplastic synaptic contacts, borderline end-plate (EP) AChR deficiency, smaller-than-normal individual EP regions, but no demonstrable defect of neuromuscular transmission at intercostal muscle EPs. The patient’s weakness continued to progress, and by 24 years of age, she was barely able to walk. Herein we describe 2 young adult sisters with LRP4-related myasthenia caused by a novel homozygous LRP4 mutation. Intercostal muscle studies of the older sister revealed structurally and functionally abnormal EPs and EP AChR deficiency. Expression studies indicate that the mutant protein hinders LRP4 from binding to, activating, and phosphorylating MuSK.

Methods

Participants

All human studies were approved by the institutional review board of the Mayo Clinic in Rochester, Minnesota, and the 2 sisters (ie, patients) and 15 historical control participants provided written informed consent to participate in the studies. Intercostal muscle was obtained from the 15 historical control participants during chest surgery for unrelated diseases.

Genetic Analysis

Sanger sequencing for mutations in genes known to cause limb-girdle CMS (namely, RAPSN, DOK7, Gephyt1, and DPAGT1) gave negative results. Next, exome sequencing of genomic DNA from both siblings was performed at the Mayo Clinic. The identified putative variants were scrutinized with Ingenuity Variant Analysis software (Qiagen). Variants at intergenic and intronic sites, and genes not expressed in skeletal muscle or the spinal cord based on the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), were excluded. The identified mutation was confirmed by Sanger sequencing of the family. Nucleotides of LRP4 complementary DNA (cDNA) were numbered according to GeneBank accession number NM_002334.3.

To determine whether an identified nucleotide variant caused abnormal splicing, we isolated cDNA from the muscle specimens obtained from control participants and the 2 patients. To detect any alternative transcript, we amplified the cDNA from exons 25 to 29 and exons 26 to 28 with primers designed for the cDNA of the 2 patients and 2 control participants.

Structural Studies

Intercostal and serratus anterior muscle specimens were obtained from the older sister and from control participants without muscle disease undergoing thoracic surgery. Cryosections were used to colocalize the AChR and acetylcholinesterase as previously described.13 End plates were localized for electron microscopy14 and quantitatively analyzed15 by established methods. Peroxidase-labeled α-bungarotoxin was used for the ultrastructural localization of AChR.16 The number of AChRs per EP was measured with iodine 125-labeled α-bungarotoxin.17

In Vitro Electrophysiology Studies

Quantitative analysis of single EP regions was done for all 15 control participants and microelectrode studies of neuromuscular transmission and α-bgt binding sites per EP was conducted for 13 control participants. Intracellular microelectrode studies were performed on an intercostal muscle specimen obtained from the 34-year-old sister (patient 1). The amplitude of the miniature EP potential (MEPP) and the quantal content of the EPP (m) were determined as previously described.16-20

Plasmids

We used the following previously constructed plasmids: (1) full-length human LRP4 cDNA for the luciferase assay and cell surface binding assays; (2) mouse Musk cDNA for luciferase assay; (3) the extracellular domain of mouse Musk cDNA and a fraction (amino acids 1141-1937) of rat Agrn cDNA, both of which were fused to an myc-tag and alkaline phosphatase (MuSKect-mycAP and agrin-mycAP) for cell surface binding assay; and (4) human MUSK cDNA with a flag-tag at the N-terminal end for coimmunoprecipitation assay. Mutant LRP4 plasmid carrying p.Glu1233Ala was generated by the QuikChange Site-Directed Mutagenesis kit (Stratagene).12 The ATF2-luciferase (ATF2-luc) reporter14 and the phRL-TK Renilla luciferase vector (Promega) were used for the luciferase reporter assay.

Cell Cultures

HEK293 and COS7 cells were cultured in the Dulbecco Modified Eagle Medium supplemented with 10% fetal calf serum, and transfected with FuGENE 6 transfection reagent (Roche). The agrin-mycAP and MuSKect-mycAP proteins were produced as previously described.12 Recombinant rat C-terminal agrin (10 ng/mL; R&D Systems) was used for agrin treatment, except for the cell-binding assays.

Luciferase Assays

We used an ATF2-luc reporter to monitor MuSK activation. The basis for this approach is that agrin induces JNK activation in myotubes21 and that a previous report22 has demonstrated in-
teraction between JNK and ATF2. This suggested that report-

ers regulated by JNK might reflect MuSK activation. We there-
ore tested several JNK reporters and found that ATF2-luc

reporter responded to MuSK, LRP4, and agrin in a dose-

dependent manner.12

HEK293 cells were transfected with ATF2-luc and phRL-TK

along with the MUSK cDNA and the LRP4 cDNA. Cells were cul-
tured for 24 hours in a 96-well plate with or without 10 ng/mL

of agrin in the medium. Cells were lysed with the Passive Ly-
sis Buffer (Promega) and assayed for luciferase activity using

the Dual-Luciferase Reporter Assay System (Promega). Each

experiment was performed in triplicate.

Western Blotting

HEK293 cells transfected with MuSK and LRP4 plasmids were cul-
tured for 24 hours in the presence of 10 ng/mL of agrin, as

previously described.12 The primary antibodies were mouse

monoclonal anti-FLAG M2 (Sigma-Aldrich; F3165, dilution

1:4000), anti-β-actin (Santa Cruz Biotechnology; sc-47778, di-

lution 1:200), and the goat polyclonal anti-LRP4 (Abcam;

ab85697, dilution 1:1000). The secondary antibodies were goat

antimouse IgG (GE Healthcare; NA931V, dilution 1:6000) and

mouse anti-goat IgG (Santa Cruz Biotechnology; sc-2345, di-

lution 1:6000) conjugated to horseradish peroxidase.

Biotinylation Assay

HEK293 cells transfected with plasmids harboring wild-type

LRP4 or its Glu233Ala mutant were cultured for 48 hours. Cell

surface proteins were isolated by biotinylating the cell sur-

face proteins and precipitating the bound proteins with strep-
tavidin beads, as previously described.12

Results

Clinical Data

Two young adult sisters with CMS (patient 1 in her mid-30s and

patient 2 in her early 20s) who were born to nonconsanguin-

eous parents, were investigated. Their parents and other sib-

lings are unaffected.

Patient 1 was born after normal gestation and delivery.

months. Developmental milestones (sitting and walking) were

slightly delayed; after beginning to walk, she fell frequently. As

a young child, she had mild difficulty chewing and swallow-

ing. She never climbed steps or kept up with her peers in physi-

cal activities. Her weakness worsened around her menses. In

her teens, she could only walk a short distance with support and

became dependent on a wheelchair. On initial examination, she
could barely rise from the sitting position without support, and

her gait was waddling, hyperlordotic, and intoeing. Her weak-

ness was confined to the axial and limb muscles with selective

severe involvement of the dorsal forearm muscles. Her tendon

reflexes were hypoactive. Her vital capacity was reduced to 49%,

and the maximal inspiratory and expiratory pressures were re-
duced to 43% and 22% of normal, respectively. Repetitive nerve

stimulation at 2 Hz revealed a decremental response of 37% in

the trapezius and of 14% in facial muscles of the fourth com-
pared with the first evoked compound muscle action potential

(CMAP) (normal, <10%). Repetitive CMAPs, typical of the slow-

channel myasthenic syndrome or EP acetylcholinesterase
deficiency,23 were absent. Brief strenuous exercise did not ap-

preciably improve the decremental response or potentiate the

first evoked CMAP as in the CMS-caused synaptotagmin-2
deficiency,24 in some patients harboring mutations in agrin,4 or

in the Lambert-Eaton syndrome.23 Needle electromyographic

studies of multiple muscles revealed an increased proportion

of short-duration, polyphasic motor unit potentials without

spontaneous electrical activity, a common finding in myas-

thenic disorders owing to a variable proportion of muscle fi-

bers in motor units failing to generate an action potential.

After treatment with 4 mg of albuterol sulfate twice daily

for 1 week, patient 1 rose up from the sitting position 10 times

without support, and the electromyographic decrement in her

trapezius muscle decreased to 25%. One week later, she climbed

10 steps, walked 450 m (500 yd), and was able to groom her-

self independently. Additional treatment with pyridostig-

mine bromide or 3,4-diaminopyrididine made her weaker and

had to be discontinued.

Patient 2 appeared normal at birth and during infancy. She

walked at a developmentally appropriate age but never learned

to run or jump, found it difficult to climb steps, and could not

keep up with her peers in physical activities. By late adoles-

cence, her arm-elevation time was 22 seconds, and her gait was

lordotic and waddling; she required assistance to rise from a

sitting position and could climb only 20 steps before having to

rest. There was mild weakness of the cervical and proximal

arm muscles and mild to moderately severe weakness of the

hip girdle muscles. Her tendon reflexes were hypoactive. Res-

piratory function tests showed that her vital capacity was re-
duced to 68% and that her maximal inspiratory and expira-

tory pressures were reduced to 39% and 47% of normal,

respectively. After treatment with 4 mg of albuterol twice daily

for 2 weeks, her arm and cervical muscles were of normal

strength, her arm-elevation time was 60 seconds, and she could

climb 44 steps before having to rest. Additional treatment with

60 mg of pyridostigmine twice daily over the next 2 years nei-

ther improved nor worsened her condition. A needle electro-

myographic examination suggested a mild proximal myopa-

thy. After receiving albuterol for 2 years, repetitive stimulation

of multiple muscles revealed no significant decrement of the

evoked CMAP.

Structural Studies

Structural studies were performed in patient 1. Light micro-

scopy revealed markedly diminished EP size. In transverse fro-

zen sections reacted for acetylcholinesterase, the median

length of 34 EPs was 7.2 μm (Figure 1A, B, E, and F); the med-

ian length of 69 EPs of 3 control participants was 21.3 μm

(P < .001, determined by the use of the rank sum test)

(Figure 1C, D, G, and H). Paired fluorescence localization stud-

dies revealed reduced synaptic expression of acetylcholin-

esterase and of the AChR in the EPs of patient 1 (Figure 1E and

F) compared with the EPs in the 3 control participants

(Figure 1G and H).

Qualitative inspection of 15 EP regions revealed 7 postsyn-

aptic regions unoccupied or partially occupied by the nerve

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In transverse sections, the length of acetylcholinesterase (AChE)-reacted end-plate (EP) regions is markedly reduced in the EPs of patient 1 (A and B) compared with the EPs of control participants (C and D). Paired fluorescence studies reveal reduced expression of AChE (green) and acetylcholine receptors (AChRs) (red) in the EPs of patient 1 (E and F) compared with the EPs of control participants (G and H). Electron microscopy reveals simplified postsynaptic regions (I and J) and patchy expression of AChRs (black reaction product) at the EP (J). K, Electron microscopy also reveals a degenerating nerve terminal (NT) separated from a shallow postsynaptic region by a synaptic space containing coarse collagen fibrils; the region on the left shows a degenerate postsynaptic region displaying basal lamina remnants of preexisting folds (asterisk) and no NT. Electron microscopy also reveals a noninnervated postsynaptic region (L) and focal myofibrillar degeneration (M). Scale bars: A-D, 50 μm; E-H, 20 μm; I-L, 1 μm; M, 2 μm.
CharacteristicsofLRP4-RelatedMyasthenia

Original Investigation Research

The evoked EPPs were subthreshold to trigger action potentials and were recorded without curare. The mean values for the EPP amplitude, the quantal content of the EPP (m), and the amplitude of the MEPP derived from the quantal component of the EPP were reduced to 16%, 40%, and 43%, respectively, of the corresponding mean values for the control participants. The mean number of AChRs per EP, determined from the number of α-bungarotoxin binding sites, was decreased to 25% of the mean number for the control participants (Table 2).

**Table 2. Microelectrode Studies of Neuromuscular Transmission and α-bgt Binding Sites per EP**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control Participants (n = 13)</th>
<th>Older Sister</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP amplitude, mV</td>
<td>28.76 (1.98)</td>
<td>4.66 (0.74)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>MEPP amplitude, mV</td>
<td>1.00 (0.03)</td>
<td>0.43 (0.078)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Quantal content of EPP at 1 Hz (m)</td>
<td>26.9 (1.0)</td>
<td>10.8 (2.36)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>[125I]α-bgt binding sites per EP, No.</td>
<td>12.8 × 10^6 (0.8 × 10^6)</td>
<td>3.03 × 10^6</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviations: EP, end plate; [125I]α-bgt, iodine 125-labeled α-bungarotoxin; MEPP, miniature EP potential.

* More than 1 region can occur at a single end plate (EP).

**Mutation Analysis**

Whole-exome sequencing in both patients (confirmed by Sanger sequencing) revealed a novel homozygous p.Glu1233Ala (c.3698A>C) mutation in exon 26 (Figure 2). The mutated residue is positioned at the edge of the third β-propeller domain of LRP4 and close to the conserved YWTD motif important for β-sheet formation. The unaffected brother and the mother are heterozygous for the mutation. Glu1233 is highly conserved across vertebrates, but not in insects, and is not reported in the Exome Variant Database (Exome Variant Server, National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project, Seattle, Washington [http://evs.gs.washington.edu/EVS/]; January 2015), and it is predicted to be disease causing by MutationTaster but benign by Polymorphism Phenotyping v2. Interestingly, mutations in the central cavity of the third β-propeller domain of LRP4 were previously reported to impair Wnt signaling and cause bone disease, including Cenani-Lenz syndactyly syndrome and sclerosteosis-2.

Because the A>C variant is the penultimate nucleotide of exon 26 and because MutationTaster predicts it to alter splicing, we isolated cDNA from the patient’s intercostal and serratus anterior muscles and amplified the segment, including exon 26 by 2 different sets of primers. In both specimens, the mutant residue was homozygous, and there was no evidence for abnormal splicing.
Expression Studies
Expression studies show that Glu1233Ala inhibits the MuSK signaling pathway. During the formation of the neuromuscular junction, binding of agrin to LRP4 induces phosphorylation and activation of MuSK. Activated MuSK activates ATF2 downstream of JNK to induce clustering of AChRs. To investigate the effect of the Glu1233Ala mutation on this signaling pathway, we used a JNK-responsive ATF2-luc reporter that specifically monitors MuSK-dependent stimulation in transfected HEK293 cells. When LRP4 and MuSK are overexpressed, limited ATF2 activation occurs even in the absence of agrin. The addition of agrin to this system further enhances ATF2 activation by wild-type LRP4, whereas activation of ATF2 by mutant LRP4 is markedly attenuated. To validate this finding, the previously reported Glu1233Lys mutation was used in these experiments, and the currently identified Glu1233Ala mutation was found to be equivalently attenuated.

In another experiment using HEK293 cells, we examined the effects of wild- and mutant LRP4 on MuSK phosphorylation that occurs during the assembly of the agrin-LRP4-MuSK complex. Consistent with its effects on MuSK signaling activity, mutant LRP4 compromises agrin-enhanced MuSK phosphorylation (Figure 3C). Both experiments support the notion that Glu1233Ala in LRP4 compromises agrin-mediated activation of MuSK.

MuSK and agrin bind to different extracellular residues of LRP4. We therefore examined the binding of MuSK and agrin to mutant and wild-type LRP4 expressed on the surface of COS cells. We first confirmed that both wild-type and mutant LRP4 are similarly expressed on the plasma membrane by use of the biotinylation assay (Figure 3D). We then overlaid recombinant human MuSKect-AP and recombinant human neural agrin-AP on COS cells expressing LRP4. Measurement of alkaline phosphatase activity revealed that MuSK and agrin bind efficiently to wild-type LRP4 but not to mutant LRP4 (Figure 3E). Thus, all 3 experiments indicate that Glu1233Ala in LRP4 inhibits agrin-mediated upregulation of MuSK signaling.

Discussion
The 2 sisters described in this report harbor a homozygous p.Glu1233Ala mutation in the third propeller domain of LRP4 that binds to and activates MuSK. An intercostal muscle biopsy of the older sister revealed very small EPs with poorly developed postsynaptic regions, and postsynaptic regions that were degenerating or denuded of their nerve terminal. The amplitude of the EPP was reduced to 16% of normal owing to the combined decrease of the quantal content of the EPP and of the MEPP amplitude (Table 2). The decreased amplitude of the MEPP can be attributed to decreased input resistance of the EP owing to simplification of the postsynaptic membrane, as well as the attenuated expression of AChRs on the remaining junctional folds. The decreased quantal content of the EPP is adequately explained by the small size of individual EPs, and hence of the total number of synaptic vesicles available for release by nerve impulse. Thus, the observed structural and electrophysiologic abnormalities are consistent with an abrogated role played by LRP4 in the development and maintenance of the neuromuscular junction.
Conclusions

The first reported patient with LRP4-related myasthenia harbored 2 heterozygous mutations in LRP4, p.Glu1233Ala and p.Arg1277His. At 14 years of age, her synaptic contacts were dysplastic, the individual EP regions were smaller than normal, and the EP AChR content slightly reduced, but, paradoxically, in vitro electrophysiology studies of intercostal muscle EPs revealed no abnormality. Interestingly, expression studies of both mutant proteins in the first patient and of the single mutant protein in the 2 sisters in this study revealed impaired LRP4 binding to, activating, and phosphorylating MuSK. The reason for the much milder clinical, structural, and electrophysiologic abnormalities observed in the first identified patient with LRP4-related myasthenia compared with the patients described in this report remains unknown. We initially attributed the milder findings in the first reported patient to

Figure 3. Expression Studies

A Agrin-mediated activation of MuSK by LRP4 mutant Glu1233Ala

B Agrin-mediated activation of MuSK by LRP4 mutant Glu1233Lys

C Agrin-mediated phosphorylation of MuSK by LRP4 mutant Glu1233Ala

D Western blotting of membrane-bound and total LRP4

The lipoprotein receptor–related protein 4 (LRP4) mutants Glu1233Ala (A) and Glu1233Lys (B) inhibit agrin-mediated upregulation of muscle-specific tyrosine kinase (MuSK) signaling. Agrin-mediated upregulation of MuSK signaling in HEK293 cells is monitored by the ATF2-luciferase (ATF2-luc) reporter assay. Cells were transfected with the ATF2-luc and Renilla reporter plasmids, along with MuSK complementary DNA (cDNA) and wild-type (WT) or mutant LRP4, and then incubated with or without 10 ng/mL of agrin. Error bars indicate mean (SD) values (n=3) of relative luciferase activity (RLA) calculated by dividing the firefly luciferase activity by the Renilla luciferase activity, which is further normalized for activity without MuSK and LRP4.

C, Effect of mutant and WT LRP4 on MuSK phosphorylation in HEK293 cells transfected with Flag-MuSK and the indicated LRP4 cDNA with or without agrin (10 ng/mL). Phosphorylated MuSK was detected by immunoprecipitation of cell lysate by antiphosphotyrosine antibody (IP:p-Tyr) followed by immunoblotting with anti-FLAG antibody. Wild-type LRP4 phosphorylates MuSK, which is further enhanced by agrin. Mutant LRP4 abolished responsiveness to agrin. D, Western blotting detected membrane-bound LRP4 (memLRP4) and total LRP4. Membrane proteins were biotinylated and precipitated with streptavidin. β-Actin in each sample serves as a loading control. E, The LRP4 mutant impairs binding of LRP4 to MuSK and agrin in cell surface binding assays. COS7 cells were transfected with the WT or mutant LRP4 cDNA and treated with a concentrated conditioned medium containing either neural agrin-mycAP or MuSKect-mycAP, both of which expressed an alkaline phosphatase (ALP) fusion protein. Control cells were transfected with an empty vector. The error bars indicate mean (SD) of ALP activity of bound agrin-mycAP and MuSKect-mycAP in 3 independent wells. Mutant LRP4 reduces binding of MuSKect-mycAP and agrin-mycAP.
relative sparing of the intercostal muscles. Alternatively, the structural and physiologic abnormalities at the EPs in LRP4-related myasthenia could worsen age, consistent with the progressive clinical course in all 3 LRP4-deficient patients observed to date. The greater suppression of ATF2 activation by the Glu1233Ala mutation in LRP4 than by the previously reported Glu1233Lys mutation in LRP4 likely also contributes to the phenotypic differences between the first reported patient and the 2 patients described herein. Finally, single-nucleotide polymorphisms in modifier genes may also contribute to phenotypic differences. That both kinships harbor mutations at the edge of the third β-propeller domain and that 2 of the 3 identified mutations occur at codon 1233 suggest a hot spot for causing CMS.

ARTICLE INFORMATION

Accepted for Publication: April 6, 2015.

Published Online: June 8, 2015.

Author Contributions: Dr Engel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Selcen, Shen, Ohno, Engel.

Acquisition, analysis, or interpretation of data: All authors.

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Critical revision of the manuscript for important intellectual content: All authors.

Statistical analysis: Shen, Engel.

Obtained funding: Selcen, Ohkawara, Ohno, Engel.

Administrative, technical, or material support: Ohno, Engel.

Study supervision: Ohno, Engel.

Conflict of Interest Disclosures: None reported.

Funding/Support: This work was supported by National Institutes of Health grant NS26777 (Dr Engel), the Mayo Clinic Center for Individualized Medicine, and by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare of Japan (Dr Ohkawara and Ohno).

Role of Funder/Sponsor: The funding sources had no role in the design and conduct of the study; collection, management, analysis, or interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

REFERENCES


