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LG2 Agrin Mutation Causing Severe Congenital Myasthenic Syndrome Mimics Functional Characteristics of Non-neural (z-) Agrin

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Abstract

We describe a severe form of congenital myasthenic syndrome (CMS) caused by two heteroallelic mutations: a nonsense and a missense mutation in the gene encoding agrin (*AGRN*). The identified mutations, *Q353X* and *V1727F*, are located at the N-terminal and at the second laminin G-like (LG2) domain of agrin respectively. A motor-point muscle biopsy demonstrated severe disruption of the architecture of the neuromuscular junction (NMJ), including: dispersion and fragmentation of endplate areas with normal expression of acetylcholinesterase; simplification of postsynaptic membranes; pronounced reduction of the axon terminal size; widening of the primary synaptic cleft; and, collection of membranous debris material in the primary synaptic cleft and in the

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Ethical Standards

This study was approved by the institutional review board of the University of California, Davis, in accordance with National Institute of Health guidelines. The patient and his relatives were informed of their rights and the details of the research, and all signed an informed consent form.

Conflict of Interest

The authors declare that they have no conflict of interest.

subsynaptic cytoplasm. Expression studies in heterologous cells revealed that the *Q353X* mutation abolished expression of full-length agrin. Moreover, the *V1727F* mutation decreased agrin-induced clustering of the AChR in cultured C2 muscle cells by >100-fold, and phosphorylation of the MuSK receptor and AChR beta subunit by ~10-fold. Surprisingly, the *V1727F* mutant also displayed increased binding to α -dystroglycan but decreased binding to a neural (z+) agrin-specific antibody. Our findings demonstrate that agrin mutations can associate with a severe form of CMS and cause profound distortion of the architecture and function of the NMJ. The impaired ability of *V1727F* agrin to activate MuSK and clusters AChRs, together with its increased affinity to α -dystroglycan, mimics non-neural (z-) agrin and are important determinants of the pathogenesis of the disease.

Keywords

neuromuscular junction; myasthenia; synaptogenesis; genetic mutation; agrin

INTRODUCTION

Agrin is a large and ubiquitous proteoglycan that occurs in multiple isoforms generated by alternative RNA splicing, with diverse functions in different tissues. Agrin was originally identified as an essential regulator of neuromuscular synapse formation (Ngo et al. 2007; Sanes and Lichtman 2001), and also may contribute to neuronal synapse formation in the peripheral and central nervous systems (Gingras et al. 2002, 2007; Martin et al. 2005; Ksiazek et al. 2007; Matsumoto-Miyai et al. 2009). At the developing neuromuscular junction (NMJ), motoneuron-derived agrin is secreted into the synaptic basal lamina, and promotes synaptic differentiation by signaling through a receptor complex consisting of muscle specific receptor tyrosine kinase (MuSK) and low density lipoprotein receptor-related protein 4 (Lrp4) (Glass et al. 1996; Kim et al. 2008; Zhang et al. 2008). This activity is mediated by the carboxyl-terminal domains of agrin, and limited to neural-specific isoforms that contain inserts at the z splice site (z+ agrin). Indeed, mice deficient in all agrin forms or just z+ isoforms both die at birth due to a lack of functional NMJs with aligned nerve terminals and postsynaptic acetylcholine receptor clusters (Gautam et al. 1996; Burgess et al. 1999). Surprisingly, however, acetylcholine receptor clusters do form transiently in the central region of agrin-deficient or aneural embryonic muscle (Lin et al. 2001; Yang et al. 2001) and numerous nerve-associated clusters of AChR persist in ChAT/agrin double knockout mice where synaptic transmission is abolished (Lin et al. 2005; Misgeld et al. 2005). Thus, it is thought that agrin does not initiate synaptogenesis, but rather, that it stabilizes and promotes pre- and postsynaptic differentiation of nascent neuromuscular synapses, counteracting the dispersing effects of the transmitter, acetylcholine.

Congenital myasthenic syndromes (CMS) are a diverse group of genetic disorders characterized by failure of neuromuscular transmission, weakness and fatigability (Hantai et al. 2004). CMS are classified according with the location of the protein encoded by the causative defective gene in presynaptic, synaptic basal lamina-associated and postsynaptic types (Hantai et al. 2004). Detailed microelectrode studies performed in two forms of

synaptic basal-lamina associated CMS, due to mutations in *COLQ* and *LAMB2*, demonstrated profound impairment of neuromuscular transmission with combined pre- and postsynaptic failure (Engel et al. 1977; Maselli et al. 2009). Electron microscopy studies of the NMJ conducted in these human forms of CMS and in their animal models showed a pattern characterized by abnormally small nerve terminals, abnormal encasement of the nerve endings by Schwann cells, and moderate focal simplification of postsynaptic folds. Some of these findings were also observed in a previously reported case of a moderately severe CMS due to a homozygous *AGRN* mutation (*G1709R*), which appears to destabilize NMJs without impairing MuSK activation or receptor clustering (Huze et al. 2009).

Here, we describe a severe form of CMS resulting from a nonsense (*Q353X*) and a missense (*V1727F*) mutation in *AGRN*. We find that the *V1727F* mutation located in the second laminin-G-like domain (LG2) causes a striking reduction in agrin's receptor clustering activity, which likely explains the association of this mutation with a serious form of CMS.

MATERIALS AND METHODS

Muscle biopsy

A motor point biopsy of the right deltoid muscle was performed under local anesthesia as described elsewhere (Slater et al. 2006). Under a dissecting microscope the specimen was divided into multiple muscle bundles. Several of these bundles were frozen by rapid immersion into isopentane super-cooled with liquid nitrogen. Additional muscle bundles were fixed in glutaraldehyde, teased and subsequently stained for AChE in tissue culture wells using the Karnovsky method (Karnovsky 1964).

Electron microscopy and morphometric analysis of the NMJ—The ultrastructure of the NMJ and the morphometric analysis of the NMJ were performed as previously described (Anderson et al. 2008).

Immunohistochemical analysis—Frozen cryostat tissue sections of 8 μ m thickness were fixed on ice with cold acetone for 10 min as previously described (Maselli et al. 2010). The tissue was incubated overnight at 4°C with a goat polyclonal IgG antibody directed against the c-terminus of agrin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The next day, the tissue was labeled for 1.5 h at RT with a donkey anti-goat IgG FITC secondary antibody (1:200; Santa Cruz Biotechnology) and a rhodamine-conjugated α -BGT counterstain (125 nm; Sigma, St. Louis, MO, USA). The slides were washed and mounted with ProLong Gold Anti-Fade reagent (Invitrogen). The slides were visualized using a Nikon E-600 fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA). Surface extension and intensity of fluorescence were quantified using the imageJ software. Fluorescence intensity was corrected for background intensity and reported in arbitrary units.

Mutational analysis

DNA amplification and sequencing—DNA was extracted from patient blood using the QIAmp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). PCR products were sequenced

on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). We assembled and aligned sequences against the reference sequence downloaded from Genbank (NC_000001.10 GI:224589800; NM_198576.2) using BioEdit software. The patient's unaffected mother, paternal uncle and sisters were genotyped as well. This study was approved by the institutional review board of the University of California, Davis. The patient and his relatives were informed of their rights and the details of the research, and all signed an informed consent form.

Restriction digest analysis—Digestion of the Agrin *Q353X* and *V1727F* amplicons was performed using *Cac8I* and *SexAI* restriction enzymes respectively (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol. The cleaved product was run on a 15% TBE acrylamide gel and stained with EtBr for 45 min. The gel was analyzed using an ImageMaster VDS Imager (Molecular Dynamics).

Molecular modeling—Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081). The position of the *V1727F* mutation was modeled on the crystal structure of the LG2 domain of mouse agrin (Sampathkumar, P et al, unpublished; DOI: 10.2210/pdb3pve/pdb).

Functional analysis

Mammalian expression vectors—For expression studies in heterologous cells, we used a pCMV vector encoding full-length rat agrin(y4,z8) (Ferns et al. 1992) or pFlag-CMV1-vector encoding the C-terminal half of agrin(y4,z8); this is a Flag- and His6-tagged equivalent of C-Ag (Ferns et al. 1993) which we denote as T1-Ag. We performed site-directed mutagenesis on the WT construct to generate the agrin mutants as previously described (Maselli et al. 2010).

Expression of full-length agrin—The WT and *Q353X* agrin constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Subsequent protein extraction from cells was performed using RIPA buffer (Sigma) and Protease Inhibitor Cocktail (Sigma) added to the WCL to minimize protein degradation. Protein samples were harvested after 24 h and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The protein samples were diluted with sample application buffer (1.0 mL of 0.5 M Tris-HCl [pH 6.8], 1.9 g ultra pure, and 10% SDS), separated on a 4–20% SDS-PAGE gradient gel (Bio-Rad), and electroblotted as previously described (Arredondo et al. 2008). The primary goat anti-agrin antibody (Santa Cruz Biotechnology) was diluted 1:100 in LI-COR Odyssey Blocking Buffer and incubated overnight at 4 C. The secondary donkey anti-goat antibody, IRDye800cw (LI-COR Biosciences, Lincoln, NE), was diluted 1:5,000. Protein expression was quantitated using the Odyssey Imaging System (LI-COR).

Expression of soluble agrin—To generate soluble agrin, we transfected COS cells with T1-Ag constructs and determined the concentration of soluble agrin in the conditioned media by immunoblotting with anti-agrin antibody and comparing the signal with a known

amount of purified agrin (Ferns et al. 1993). Agrin-conditioned media was typically diluted ~1:1000 for experiments (ie. to 50pM), and has the advantage that it retains higher biological activity than purified agrin. To control for the presence of other proteins, we used matched dilutions of media from non-transfected cells.

To assay AChR clustering activity, we treated C2 myotube cultures with agrin for 16 hrs, labeled the AChR with AF594-BGT (Invitrogen), and counted the number of AChR clusters in random fields as described previously (Ferns et al. 1993).

To assay MuSK and AChR phosphorylation, we treated C2 myotubes with agrin for 1 hr, isolated MuSK and the AChR from cell extracts, and immunoblotted with anti-phosphotyrosine antibodies as described (Jacobson et al. 1998). After incubation with HRP-conjugated secondary antibody, bound antibody was visualized by chemiluminescence (ECL Plus; GE Healthcare), and digital images acquired using a Fujifilm LAS-400 and analyzed using MultiGuage v3.1 software. The membranes were reprobed with anti-MuSK and anti- α -subunit (mAb210) antibodies, respectively, to confirm equal loading.

For agrin binding to α -DG, we solubilized Torpedo synaptic membranes in HBS buffer (pH 7.3, 20 mM Hepes, 150 mM NaCl, protease inhibitors) containing 1% CHAPS and incubated with T1-Ag bound to Ni beads (Talon metal affinity resin; Clontech) in HBS buffer with 2 mM Ca and 5 mM imidazole. After washing, α -DG pulled down by the agrin beads was detected by immunoblotting with mAb IIH6 (Santa Cruz Biotechnology). To immunoprecipitate agrin, we incubated duplicate samples of WT or *V1727F* agrin-conditioned media with rabbit polyclonal antibody that recognizes all isoforms of agrin (Sugiyama et al. 1994), or monoclonal antibody mAb86 (Hoch et al. 1994) that recognizes only z+ agrin (Agr-520; Stressgen Biotechnologies).

RESULTS

Clinical data

Case report—Our patient is a 39-year-old man who was born full-term to non-consanguineous parents. During early childhood he was noted to have bilateral ptosis and to fatigue easily on persistent motor activities. Throughout his childhood and adolescence he has had several episodes of respiratory insufficiency, which have required tracheotomy. His cognitive development was normal and he has never experienced symptoms of autonomic dysfunction. An older brother, who had similar symptoms died during childhood, but two sisters and both parents were not affected. The diagnosis of CMS was made on him based on the history, electrodiagnostic studies and negative serum antibodies against AChR. Serum antibodies against MuSK were not tested. At the age of 24 years he had an intercostals muscle biopsy with in-vitro microelectrode studies of endplate physiology, which showed features consistent with presynaptic failure of neuromuscular transmission. A recent neurologic examination revealed normal cognition, bilateral ptosis—left more than right—normal pupillary function and relatively intact external ocular movements, except for mild weakness of the left inferior oblique and left lateral rectus muscles. He had bilateral facial weakness, high arched palate and intact elevation of soft palate. He had moderate weakness of proximal and distal muscles of upper extremities and mild weakness of proximal muscles

of the lower extremities with preserved deep tendon reflexes. Repetitive stimulation of the right spinal accessory nerve at 2 Hz revealed a 32% decrement of the amplitude of the compound muscle action potential (CMAP). Finally, he currently requires continuous respiratory support with bi-level positive airway pressure for large parts of the day and responds moderately to pyridostigmine bromide, but not to 3,4-diaminopyridine or ephedrine.

Muscle biopsy

To determine the nature of the CMS in this patient, we performed a motor-point biopsy of the right deltoid muscle.

Light microscopy—Except for the presence of occasional small angular fibers and type I fiber predominance there were no other significant histological abnormalities.

Electron microscopy of the NMJ—There was marked variability of junctional ultrastructure. Occasional junctions showed relatively normal ultrastructure, but most junctions showed simplification and distortion of the postsynaptic membranes. The presynaptic axon terminals were extremely small at all junctions and were partially encased by the Schwann cell. In addition, markedly widened primary synaptic clefts were filled with ECM material having the density of basal lamina but also containing globular debris of higher electron density than basal lamina. At many NMJs membranous debris in subsynaptic areas were also found (Fig 1).

Morphometric analysis—In comparison to control anconeus muscles, the patient showed a reduction in the endplate index (EI, length of the postsynaptic membrane/length of the presynaptic membrane) without significant reduction in the mean number of secondary clefts per micron of primary cleft (Table 1). The average axon terminal area was markedly diminished, but the average number of synaptic vesicles per area of nerve terminal was actually increased. Finally, relative to the controls, the widths of the primary and secondary synaptic clefts were notably increased.

Acetylcholinesterase (AChE) reaction in teased muscle bundles—The AChE reaction revealed that in comparison with two controls the endplate areas in the patient were markedly reduced ($54.5 \pm 28.0 \mu\text{m}^2$, $n = 23$ vs. $247.2 \pm 112.5 \mu\text{m}^2$, $n = 59$; $P < 0.001$). There was also dispersion and hypersegmentation of the endplate along individual fibers with occasional fibers showing chains of small junctional segments extended over several hundreds of microns (Fig 2).

Immunohistochemical analysis—Endplates were labeled with rhodamine-tagged α -bungarotoxin (BGT) and the expression of agrin at the endplate was visualized using a primary antibody directed against the C-terminal of human agrin in muscle sections of 8 μm thickness (Fig 2). In comparison with a control muscle, the patient showed a marked reduction in the mean surface expression of α -BGT per endplate (28.54 ± 14.15 , $n = 50$ versus 80.52 ± 37.30 , $n = 58$; $P < 0.001$). In addition, agrin was still present at patient NMJs,

although its staining intensity was moderately reduced relative to controls (23.16 ± 17.86 , $n = 85$ versus 43.94 ± 28.70 , $n = 78$; $P < 0.001$).

Mutational analysis

DNA sequencing—We amplified and sequenced all the coding and flanking intronic regions of the genes encoding the subunits of the acetylcholine receptor, rapsyn, MuSK, the choline-acetyltransferase gene (*CHAT*) and exons 5 and 7 of the *Dok-7* gene. We found no abnormalities in these genes, except for a heterozygous *C*→*T* transition at position 14,384 in *CHAT* in reference to the isoform 1 encoded by transcript variant 1. This single nucleotide transition, which is not reported in the single nucleotide polymorphism (SNP) data base, predicts a V346M change in exon 5, which in itself cannot account for the myasthenic syndrome of the patient. We then amplified and sequenced all the coding regions of *AGRN* and found two novel heteroallelic mutations, a nonsense mutation *Q353X* in exon 6, and a missense mutation *V1727F* in exon 30. Valine is a conserved amino acid in rodents and chicks, and *V1727F* was not detected in the DNA from 100 controls, or in the 1000 Genomes database.

Restriction digest—*Q353X* causes a loss of a restriction site for *Cac8I*. Treatment of the 198 base pair (bp) amplicon spanning *AGRN* exon 6 with *Cac8I*, which in the wild-type (WT) DNA results in a 96; 53 and 49 bp bands, in mutant DNA containing *Q353X* results in an indigested 102 bp band (53 + 49 bp). The presence of the 102 and 96 bp bands in the patient, one sister and the mother (Fig 3) along with the 53 and 49 bp bands (not shown in the figure) is consistent with heterozygosity for the *Q353X* mutation. By contrast, *V1727F* causes a gain of a restriction site for *SexAI*. Thus, treatment of the 267 bp amplicon containing *V1727F* in exon 30 results in an extra 190 bp band. The presence of the 267 and 190 bp bands in the patient, one sister and a paternal uncle (Fig 3) is consistent with heterogeneity for the *V1727F* mutation. In summary, the restriction digest assay showed that the patient is compound heterozygous for two *AGRN* mutations, whereas other non-affected members of his family are heterozygous for either one of the mutations but not for both.

Position of the mutations—As shown in Fig 4a, the *Q353X* mutation lies in the 4th Follistatin-like repeat of agrin, predicting a highly truncated and inactive protein. The *V1727F* mutation lies in the 2nd laminin-G-like (LG2) domain, preceding the LG3 and EGF4 domains that make up the minimal agrin fragment capable of activating MuSK and inducing AChR clustering. Based on known structures of the mouse agrin LG2 (unpublished; 10.2210/pdb3pve/pdb) and chicken agrin LG3 domains (Stetefeld et al. 2004), the *V1727F* mutation lies within a beta sheet that forms part of a beta jellyroll fold (Figure 4b). As the side-chains of V1727 face inward, substitution by a bulkier phenylalanine residue could disrupt the packing of the beta strands and alter the LG2 domain structure.

Functional analysis

Next, we analyzed the functional effects of the two mutations. We introduced the nonsense mutation *Q353X* into full-length agrin and confirmed that the expressed protein lacked the C-terminal half of agrin, which is required for its synaptogenic activity (Fig S1). Second, we

introduced the *V1727F* mutation into a C-terminal agrin construct (T1-Ag4,8) that is expressed by transfected COS cells as a secreted, soluble protein; this construct is used routinely in assays for MuSK activation and AChR clustering (Ferns et al. 1993; Jacobson et al. 1998). We determined the concentration of the recombinant, WT and *V1727F* agrin by immunoblotting the conditioned media with anti-agrin antibody and comparing the signal with a known amount of purified agrin (Ferns et al. 1993)(see later Figs).

To assay AChR clustering activity, we treated C2 myotube cultures with agrin for 16 hrs and then labeled the AChR with AF594-BGT. Wild type agrin at a concentration of 50 pM induced an ~6-fold increase in the number of AChR clusters (Fig 5a,b). By contrast, *V1727F* agrin was inactive at 50 pM and induced only occasional microclusters at 500 pM. At 100-fold higher concentration (50 nM), *V1727F* agrin induced an ~3-fold increase in AChR clusters, although these clusters tended to be less dense than those induced by WT agrin. Thus, the *V1727F* mutation decreases agrin's AChR clustering activity by >100-fold.

To define the molecular basis for this defect we assayed the ability of *V1727F* agrin to activate MuSK. To do this, we treated C2 myotubes with WT and mutant agrin for 1 hr, and then immunoprecipitated MuSK from cell extracts and immunoblotted with anti-phosphotyrosine antibodies (Fig 6). Interestingly, *V1727F* agrin induced lower levels of MuSK phosphorylation than WT agrin; 50% of WT levels at 50 pM but equivalent levels when used at 10-fold higher concentration (500 pM) (Fig 6a,b). A similar result was obtained when we assayed AChR β subunit phosphorylation, a signaling event that occurs downstream of MuSK activation. Compared to WT agrin (50 pM), *V1727F* agrin induced lower levels of β subunit phosphorylation at 50 pM but similar levels at 500 pM (Fig 6c). Together, these findings indicate that the agrin *V1727F* mutation impairs MuSK activation and downstream signaling by ~10-fold.

The *V1727F* mutation could also disrupt other agrin interactions that contribute to AChR clustering. One such possibility is agrin binding to α -dystroglycan (α -DG), which involves the LG2 domain and helps stabilize clusters at a step downstream of MuSK activation. To test this, we incubated WT or mutant agrin immobilized on beads with solubilized Torpedo synaptic membranes (TSM), and then immunoblotted the isolates with anti- α -DG antibody (IIH6) (Fig 7). Surprisingly, this revealed that *V1727F* agrin pulled down significantly more α -DG than WT agrin.

Together, our results show that *V1727F* agrin has decreased AChR clustering activity and increased α -DG binding compared to WT agrin. As these properties mimic the functional characteristics of non-neural (z-) agrin (Ferns et al. 1993; Gesemann et al. 1996), we tested whether the mutation might disrupt the conformation of neural (z+) agrin, rendering it more like z- agrin. To test this, we immunoprecipitated WT and mutant agrin from duplicate samples using pan-agrin antibody or neural-specific agrin antibody (Hoch et al. 1994) that recognizes a conformational change induced by splice inserts at the z site (Fig 8). As expected, WT z+ agrin (4,8) was immunoprecipitated efficiently by the neural as well as pan

Fig S1. Expression of agrin in HEK 293 cells. Full length WT agrin or full-length agrin-Q353X were transfected into HEK 293 cells and agrin expression was evaluated by incubating the whole cell lysate (WCL) with an anti-agrin antibody. The western blot analysis detects full-length agrin (~200 kDa) in cells transfected with WT but not the Q353X mutant. β -actin was used as an internal control.

agrin antibodies (neural/pan ~ 45%), whereas WT z– agrin (0,0) was only immunoprecipitated by the pan antibody (neural/pan ~0%). By contrast, *V1727F* agrin was immunoprecipitated poorly by the neural cf. pan agrin antibody (neural/pan ~12%), consistent with the mutation altering the conformation of neural agrin. We also noted a slight change in the mobility of *V1727F* agrin on SDS-Page gels compared to WT agrin (Fig 8a). This could reflect either a change in conformation or altered glycosylation of the protein, which feasibly could also contribute to its loss of synaptogenic activity.

DISCUSSION

We describe here a severe form of CMS caused by two heteroallelic mutations in *AGRN*. The analysis of the patient's pedigree revealed that unaffected family members were heterozygous for either one of the mutations and that the patient, and presumably his deceased brother, was compound heterozygous for both mutations. Thus, the disease is transmitted following an autosomal recessive pattern and since *Q353X* is a null allele, the phenotype is defined by *V1727F*. In contrast to previously reported cases of CMS due to homozygous *AGRN* mutations in human (Huze et al. 2009) and mouse (Bogdanik and Burgess 2011), our functional analysis shows that the *V1727F* mutation significantly reduces AChR clustering activity, in large part by impairing MuSK activation and downstream signaling.

The clinical features of our patient, including facial and limb weakness, moderate extraocular muscle involvement and chronic respiratory failure resemble those encountered in other CMS types resulting from mutations in genes encoding proteins of the ECM (Engel et al. 1977; Maselli et al. 2009). Yet, in contrast with most patients with CMS due to *COLQ* mutation, the electromyogram in our patient did not show repetitive CMAP in response to a single nerve stimulation to suggest underlying deficiency of AChE. Indeed, the histochemistry studies performed in the patient showed robust expression of AChE, which in that respect differs from the significantly reduced expression of AChE in muscles of agrin-deficient mutant mice (Gautam et al. 1996). Because our patient has ocular and respiratory involvement his clinical presentation does not strictly fit the phenotype of limb-girdle myasthenia (Engel 2006). Interestingly, his clinical presentation also differs from that of patients with CMS due to *DOK7* and *MUSK* mutations in that, in spite of respiratory failure and severe weakness of muscles of the upper extremities, he has only moderate weakness of proximal muscles of the lower extremities, which tends to be significant in patients with *DOK7* and *MUSK* mutations (Muller et al. 2007; Palace et al. 2007; Chevessier et al. 2004; Maselli et al. 2010).

Analysis of the NMJs of our patient revealed profound structural abnormalities, some of which resemble those previously reported in agrin-deficient mice (Gautam et al. 1996), or homozygous *AGRN* mutations in human (Huze et al. 2009) and mouse (Bogdanik and Burgess 2011). These include: (i) small-sized endplates, with marked reduction of AChR density and poor development of postsynaptic folds; (ii) fragmentation of endplates, with small junctional segments scattered over long distances in the myofibers; and (iii) severely reduced nerve terminal size, with decreased apposition with postsynaptic AChR clusters. Our results are also consistent with the remodeling of endplates and nerve sprouting

observed in these studies. Thus, as for the other agrin mutations, the *V1727F* mutation impairs agrin's ability to stabilize the NMJ and promote maturation of both the nerve terminal and postsynaptic apparatus.

Other abnormalities of the NMJ, including the pronounced reduction of the axon terminal size, the partial encasement of the nerve endings by Schwann cells, the prominent reduction of the area of apposition between the nerve terminal and the postsynaptic membrane, and the increased density of synaptic vesicles in the nerve terminals resemble the ultrastructural changes of the NMJ observed in patients with congenital deficiency of endplate AChE. Unexpectedly, we also found membranous debris in subsynaptic areas, which were reminiscent of the endplate myopathy seen in patients with deficiency of AChE and the slow channel syndrome due to cationic overload and activation of lytic enzymes (Vohra et al. 2004). This finding is surprising because our patient neither had overall deficiency of AChE nor had any mutation in the AChR subunit genes predicting slow ion-channel kinetics. A hypothetical explanation for the subsarcolemmal degeneration is that due to the profound remodeling of the NMJ there is a mismatching between presynaptic areas with highly concentrated synaptic vesicles and underdeveloped apposing postsynaptic membranes leading to focal ionic overflow and endplate damage. This kind of mismatch between pre- and postsynaptic structures has been observed at autonomic synapses in agrin-deficient embryos (Gingras et al. 2002), and at NMJs in mice treated with anti-MuSK antisera (Cole et al. 2008). In the patient this process would be even further accentuated by the intake of anticholinesterase medication. Together, these findings demonstrate severe pre- and postsynaptic defects at the NMJ in our patient, which likely both contribute to the impaired neuromuscular transmission.

The observed pathology at the NMJ is likely due to the drastically reduced ability of *V1727F* agrin to activate MuSK and induce AChR clustering. This contrasts with the reported human and mouse agrin mutations (Huze et al. 2009; Bogdanik and Burgess 2011), which destabilize the NMJ without affecting MuSK receptor activation. The severity of this defect for *V1727F* agrin is somewhat surprising because the mutation is located in the LG2 rather than LG3 domain, which is the minimal fragment of agrin sufficient to aggregate AChRs (Gesemann et al. 1995) and the proposed site of interaction with the LRP4/MuSK receptor complex (Fig 4). However, several earlier studies have hinted at a role for the LG2 domain in agrin's synaptogenic action. First, the 21 kDa LG3 agrin fragment induces AChR clustering with several hundred-fold lower potency than larger constructs that include the LG2 domain (Gesemann et al. 1995; Hoch et al. 1994). Second, several monoclonal antibodies that were found to block agrin-induced AChR clustering bind sites in the EGF3 and LG2 domains (Hoch et al. 1994). Similarly, heparin also blocks agrin-induced receptor clustering and MuSK activation and binds a site in the LG2 domain regulated by the y4 splice insert (Gesemann et al. 1996; Jacobson et al. 1998). Together, these findings highlight a critical role for the LG2 domain in agrin's synapse-organizing activity.

Modeling of the *V1727F* mutation on known structures of the chicken agrin LG3 (Stetefeld et al. 2004) and mouse LG2 domains (unpublished; 10.2210/pdb3pve/pdb) shows that the mutation lies in a beta strand, located within a beta jellyroll fold formed by two beta sheets (Fig 4). Potentially, substitution of valine by a bulkier phenylalanine residue may disrupt the

packing of the beta strands, thereby altering the LG2 domain structure. This is consistent with the observation that the probability of a mutation to be pathogenic increases with a decrease in the solvent accessibility of the site (Vitkup et al. 2003). How such changes in the LG2 domain affect agrin's interactions, and particularly, impair its ability to activate the LRP4/MuSK receptor is unclear. One possibility is that the LG2 domain contributes directly to the interaction with the LRP4/MuSK receptor, binding cooperatively or non-cooperatively with the LG3 domain. Alternatively, the LG2 domain might be involved in intramolecular interactions that affect agrin's structure. In this case, the mutation could produce conformational changes that indirectly affect the binding of the LG3 domain to LRP4/MuSK. Moreover, it could also account for the decreased binding of a neural (z+) specific agrin antibody that recognizes a conformational change induced by z splice inserts (Hoch et al. 1994), and the unexpected increase in affinity for α -DG, similar to that of non-neural (z-) agrin (Gesemann et al. 1996). Thus, the mutation may alter several properties of z+ agrin, giving it functional characteristics that are intermediary between z+ and z- agrin.

In summary, we identify a spontaneous agrin mutation that significantly reduces the ability of z+ agrin to activate MuSK and induce AChR clustering. This results in a severe CMS in the patient, with both pre- and post-synaptic defects at the neuromuscular junction. Intriguingly, as the patient exhibited no cognitive or autonomic deficits, agrin's proposed function at inter-neuronal synapses may be less critical or mediated by receptors other than MuSK (Burgess et al. 2002; Hilgenberg et al. 2006; Ksiazek et al. 2007; Matsumoto-Miyai et al. 2009).

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Fig 1.

Electron microscopy of the NMJ. (A) A NMJ from a control muscle showing normal nerve terminal (asterisk), postsynaptic folds, primary synaptic cleft (white arrows) and secondary synaptic clefts (black arrows). (B) A NMJ from the patient showing a small nerve terminal (asterisk) partially encased by a Schwann cell, simplified postsynaptic folds and increased diameter of the primary (white arrows) and secondary synaptic clefts (black arrows). (C) A NMJ from the patient showing similar findings as in B plus membranous debris in subsynaptic area (black arrow heads). (D) A NMJ from the patient showing comparable

findings as in B and C as well as more pronounced encasement of the nerve terminal by the Schwann cell (white arrow heads) which severely reduces the interface between the nerve terminal and the postsynaptic membranes. Calibration mark represents 1 μm in A and C, 0.75 μm in B, and 0.6 μm in D.

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Fig 2.

Immunohistochemistry analysis of the NMJ. (A,B) AChE reaction in teased longitudinal muscle fiber bundles showing an example of a small fragmented endplate in the patient (A) and normal endplates in a control muscle (B). (C) Transverse muscle section from the patient stained with tetramethyl-rhodamine conjugated- α -BGT showing a small fragmented endplate with reduced expression of AChRs. (D) Similar patient endplate as in C treated with a goat anti-agrin antibody and a donkey anti-goat FITC secondary antibody demonstrating mild reduced expression of agrin. (E,F) Multiple endplates from a control

muscle showing normal expression of AChRs (**E**) and agrin (**F**). Calibration mark represents 18 μm in A, C and D, and 25 μm in B, E and F.

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Fig 3.

Pedigree of the family and the results of the restriction analysis. Since *Q353X* results in a loss of a restriction site for *Cac8I*, digestion of exon 6 with *Cac8I* results in an undigested 102 band in the patient, in one of his sisters and mother. In contrast, *V1727F* causes a gain of a restriction site for *SexAI*. Thus, digestion of exon 31 with *SexAI* results in an extra 190 band and a 77 band (not shown in the figure) in the patient, his other sister and his paternal uncle. DNA from the deceased brother was not available, thus the presumptive compound heterozygous status could not be confirmed.

Fig 4.

Structure of agrin and position of the mutations. **(A)** Schematic showing agrin's domain structure, alternative RNA splice sites and protein interactions. The *Q353X* mutation introduces a premature stop codon in the third follistatin-like domain (FS). The *V1727F* mutation lies in the second laminin globular domain (LG2), which interacts with α -DG, HSPGs, and integrins. It is also located close to the y splice site, which regulates binding to HSPGs, and to the z splice site, which regulates binding to the MuSK receptor complex. **(B)** Ribbon representation of the mouse LG2 structure (10.2210/pdb3pve/pdb), which has 88% identity with the human sequence. Like the LG3 domain it consists of 2 beta sheets (orange strands) that form a beta jellyroll fold. The position of the *V1727F* mutation is highlighted in red and shows the probable inward orientation of the bulky phenylalanine sidechain. The y splice site is represented in green.

Fig 5.

Assay of receptor clustering activity. **(A)** C2 myotubes were treated with WT or *V1727F* agrin for 16 hrs and stained for the AChR with AF594-BGT. WT agrin induced numerous clusters at 50 pM, whereas *V1727F* agrin induced clusters only at significantly higher concentrations (5 nM). **(B)** The number of clusters per field induced by different concentrations of WT and mutant agrin. WT agrin increased cluster number ~6-fold at 50 pM ($p < 0.0001$, t-test, $n = 5$ exps), whereas *V1727F* agrin increased cluster number only 3-

fold at 5000 pM ($p < 0.0001$). Thus, *V1727F* agrin has dramatically impaired receptor clustering activity.

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Fig 6.

Assay of MuSK activation. C2 myotubes were treated with WT and *V1727F* agrin for 1 hr and then MuSK (A,B) or AChR (C) was immunoprecipitated from cell extracts and immunoblotted with anti-phosphotyrosine antibodies. (A,B) WT agrin induced robust MuSK phosphorylation at a concentration of 50 pM. Mutant agrin induced lower (~50%) but significant levels of MuSK phosphorylation at 50 pM ($p < 0.05$, t-test, $n = 3$ expts), and similar levels at 500 pM. Re-probing with anti-MuSK antibody confirmed that similar amounts of MuSK were present in each of the isolates. (C) Mutant agrin also induced slightly lower levels of AChR β -subunit phosphorylation compared to WT agrin. Re-probing with anti- α subunit antibody (mAb210) showed that equal amounts of receptor were present in each sample. Thus, *V1727F* agrin has a moderately impaired ability to activate MuSK and its downstream signaling pathway.

Fig 7.

Assay of α -DG binding. Torpedo synaptic membranes were solubilized with CHAPS and incubated with WT and *V1727F* agrin immobilized on beads. α -DG pulled down by the agrin beads was then detected by immunoblotting with mAb IIH6, and the blot was re-probed with anti-agrin antibody to confirm the presence of similar amounts of WT and mutant agrin. Significantly more α -DG bound to *V1727F* agrin than to WT agrin.

Fig 8.

Assay of neural-specific versus pan-agrin antibody binding. **(A)** Agrin was immunoprecipitated from duplicate samples using mAb86 that recognizes only z+ isoforms of agrin, and a rabbit polyclonal that recognizes all isoforms of agrin. Immunoblotting with pan-agrin antibody confirmed that WT z+ (4,8) but not z- (0,0) agrin was immunoprecipitated by mAb86. In contrast, *V1727F* 4,8 agrin was immunoprecipitated much less efficiently by mAb86. **(B)** Quantification showing the percentage of agrin immunoprecipitated by mAb86 versus pan agrin antibody. The neural agrin antibody immunoprecipitated significantly less *V1727F* agrin (12%) compared to WT agrin (45%) ($p < 0.02$, t-test, $n = 3$), suggesting that it has decreased affinity for the agrin mutant.

Table 1**Morphometric Data**

	Patient	Controls
EI [*]	5.59 ± 0.54 ^{a†} (<i>n</i> =14)	11.71 ± 2.36 (<i>n</i> =12)
Secondary clefts per primary cleft length	1.54 ± 0.21 (<i>n</i> =13)	1.79 ± 0.14 (<i>n</i> =12)
Nerve terminal area (μm ²)	2.17 ± 0.58 ^b (<i>n</i> =10)	7.34 ± 0.93 (<i>n</i> =12)
Number of synaptic vesicles/μm ²	52.21 ± 13.45 ^a (<i>n</i> =9)	16.77 ± 2.77 (<i>n</i> =12)
Cleft width (μm)	0.11 ± 0.01 ^a (<i>n</i> =9)	0.07 ± 0.004 (<i>n</i> =12)

^{*} EI, endplate index (postsynaptic membrane length/presynaptic membrane length).

[†] Values reported as mean ± SEM

^a $P < 0.05$;

^b $P < 0.001$.