Agrin mutations lead to a congenital myasthenic syndrome with distal muscle weakness and atrophy

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Congenital myasthenic syndromes are a clinically and genetically heterogeneous group of rare diseases resulting from impaired neuromuscular transmission. Their clinical hallmark is fatigable muscle weakness associated with a decremental muscle response to repetitive nerve stimulation and frequently related to postsynaptic defects. Distal myopathies form another clinically and genetically heterogeneous group of primary muscle disorders where weakness and atrophy are restricted to distal muscles, at least initially. In both congenital myasthenic syndromes and distal myopathies, a significant number of patients remain genetically undiagnosed. Here, we report five patients from three unrelated families with a strikingly homogenous clinical entity combining congenital myasthenia with distal muscle weakness and atrophy reminiscent of a distal myopathy. MRI and
neurophysiological studies were compatible with mild myopathy restricted to distal limb muscles, but decrement (up to 72%) in response to 3 Hz repetitive nerve stimulation pointed towards a neuromuscular transmission defect. Post-exercise increment (up to 285%) was observed in the distal limb muscles in all cases suggesting presynaptic congenital myasthenic syndrome. Immunofluorescence and ultrastructural analyses of muscle end-plate regions showed synaptic remodelling with denervation–reinnervation events. We performed whole-exome sequencing in two kinships and Sanger sequencing in one isolated case and identified five new recessive mutations in the gene encoding agrin. This synaptic proteoglycan with critical function at the neuromuscular junction was previously found mutated in more typical forms of congenital myasthenic syndrome. In our patients, we found two missense mutations residing in the N-terminal agrin domain, which reduced acetylcholine receptors clustering activity of agrin in vitro. Our findings expand the spectrum of congenital myasthenic syndromes due to agrin mutations and show an unexpected correlation between the mutated gene and the associated phenotype. This provides a good rationale for examining patients with apparent distal myopathy for a neuromuscular transmission disorder and agrin mutations.

Keywords: agrin; congenital myasthenic syndrome; distal myopathy; neuromuscular junction; presynaptic
Abbreviations: AChR = acetylcholine receptor; NtA = N-terminal agrin

Introduction

Agrin is an extracellular matrix heparan sulphate proteoglycan with a critical role in the development and maintenance of the neuromuscular junction (McMahan, 1990; Bezakova and Ruegg, 2003; Singhal and Martin, 2011). This proteoglycan is a key organizer of the postsynaptic membrane through stabilization and maintenance of postsynaptic acetylcholine receptors (AChRs) formed on muscle fibres (McMahan, 1990; McMahan et al., 1992). A neural isoform of agrin, generated by alternative splicing, is secreted from the nerve terminal into the synaptic basement membrane and binds through its C-terminus a postsynaptic transmembrane receptor (LRP4), which activates the muscle tyrosine kinase MuSK to induce the stabilization of aneurally-formed AChR clusters and the establishment of new clusters (Kim et al., 2008). Agrin also interacts with α-dystroglycan and laminins present in the basement membrane through its C-terminal LG2 and N-terminal agrin (NtA) domains, respectively, but the functional importance of these binding interactions for neuromuscular junction is unknown (Yamada et al., 1996; Gesemann et al., 1998; Burgess et al., 2000; Stetefeld et al., 2001). Bearing in mind its critical role in the neuromuscular junction, recessive missense mutations in the AGRN gene were shown to lead to congenital myasthenic syndromes, a heterogeneous group of rare diseases with defective neuromuscular transmission (Huze et al., 2009; Maselli et al., 2012). The patients presented with ptosis and global fatigable limb weakness. One of the patients had a severe phenotype whereas the other case had fairly mild disease. Whether this phenotypic difference is due to the presence of one non-functional allele (p.Gln353X) in the more severe case or to other factors remains unclear.

Distal weakness is rare in congenital myasthenic syndromes and may point to another condition such as peripheral neuropathy or distal myopathy. The latter is a clinically and genetically heterogeneous group of diseases, often classified depending on the inheritance pattern, age of onset, pattern of muscle involved, evidence of cardiorespiratory insufficiency and histological muscle features (Mastaglia et al., 2005). More than 20 distinct distal myopathy entities are currently recognized, many of them remaining without gene characterization (Udd, 2012). Hereby, we describe five patients from three unrelated families with distal muscle weakness and atrophy resembling distal myopathy, but with electrophysiological features and muscle histology more consistent with congenital myasthenic syndrome, and demonstrate that this newly recognized entity is associated with missense mutations in agrin.

Materials and methods

Patients

Family 1 was selected from the Munich congenital myasthenic syndromes diagnostic database whereas Families 2 and 3 were recruited by the French congenital myasthenic syndromes network. Appropriate consent for research was obtained from each patient.

EMG, single fibre EMG, and repetitive nerve stimulation (3 Hz) were carried out in selected muscles using standardized protocols (Bauché et al., 2013). Patients 1 and 2 were investigated on a Keypoint machine (Medtronic) by S.L., E.S. and T.T, and Patients 3–5 on a Viking IV machine (Nicolet) by E.F. Motor conduction velocity studies were performed in the median, ulnar, tibial and peroneal nerve. Sensory neurographic studies were done in the median, ulnar, radial and sural nerves. Compound muscle action potentials were recorded at rest and after exercise. A pathological decrement was considered when the amplitude and surface were decreased by 5%, and an increment when they were increased by 20%. A possible presynaptic origin was considered for an increment >60% (Oh et al., 2005). Single fibre EMG was done on Patients 1 and 2 by S.L. and T.T using concentric needle. The filter setting was LF 1000 Hz, and HF 200 kHz. Criteria for abnormal jitter were 55 μs as the limit value.

Muscle biopsy

Muscle biopsies were obtained for Patient 2 at the age of 33 years (vastus lateralis), for Patient 3 at 36 years, for Patient 4 at 18 years and for Patient 5 at 31 and 34 years (deltoid muscles for the Patients...
Exome sequencing and data analysis

Blood exome DNA was isolated using the Wizard® Genomic DNA purification kit (Promega) for Family 1 and standard phenol–chloroform procedures for Families 2 and 3. DNA library preparation and whole-exome sequencing were outsourced to EuroFins MWG Operon (Germany) for Patients 1 and 2 and to IntegraGen (France) for Patient 3 and his two parents. Sequencing was achieved using Agilent SureSelect All Exon V5 50 Mb capture and Illumina HiSeq2000 platforms. Raw data were aligned to the human reference genome (hg19). The alignments were optimized for indel calling using indelalign (version 1.0.12) (Albers et al., 2011). Variants were called using SAMtools (0.1.16) and annotated using ANNOVAR (Wang et al., 2010). The filtering algorithm was applied to exclude non-coding, frequent variants (mean allele frequency >1%) found in public databases of single nucleotide polymorphisms (dbSNP, the Exome Variant Server, the 1000 Genomes Project) and in an in-house list of variants from unrelated controls. We applied the recessive model looking for heterozygous or homozygous changes shared between the two patients (Kinship 1) or inherited from both parents (Kinship 2). Variants were visualized using the UCSC Genome Browser and were assessed for conservation across species and pathogenicity using MutationTaster (http://www.mutationtaster.org) (Kent et al., 2002). PCR amplification on genomic DNA and Sanger sequencing verified the variant segregation with the disease. Sanger sequencing was performed using bi-directional fluorescent sequencing on an ABI 3730 XL with BigDye® Version 3.1 chemistry (Life Technologies). The GenBank reference number NM_198576.3 (NP_940978.2) was used as reference. Copy number variant analysis of Patients 1 and 2 was carried out using the ExomeDepth software (Plagnol et al., 2012). The read depth per exon was compared with a pool of reference exomes from the same capture targets and the same sequence machine run. Any copy number variation present in the test sample was detected as a deviation from the 1:1 ratio depth (0.5 = hemizygous deletion; 0.0 = complete deletion; 1.5 = duplication of one exonic copy). A confidence ‘BayesFactor’ score >20 was predictive of copy number variation. The copy number variation predictions were combined and compared with an in-house control list (191 exomes). We also excluded that the predicted copy number variation overlapped by at least 50% with a set of ‘common’ copy number variations from 40 controls (Conrad et al., 2010). Copy number variations were confirmed by conventional CGH array using Cytochip v1.0 180K (BlueGnome).

Production of wild-type and mutated agrin recombinant proteins

We used a construct encoding a mouse full-length agrin cloned into the pCEP-Pu vector kindly provided by Prof. Markus Ruegg (University of Basel, Switzerland) (Kohfeldt et al., 1997). This encodes for the full-length NTa isoform with the 4- and 8-amino acid inserts at the A/y and B/z sites corresponding to the neural isoform of mouse agrin with the signal peptide BM40 in N-terminus and a his(6) tag in C-terminus. Site-directed mutagenesis was performed by Mutagenex Inc. (Piscataway) to introduce the missense mutation found in Kinships 1 (c.226G>A, p.Gly76Ser) or 2 (c.314A>T, p.Asn105Ile).

In vitro acetylcholine receptor clustering assay

C2C12 cells were cultured to obtain myotubes as previously described (Huze et al., 2009). After 5 days of differentiation, culture media conditioned by 293 EBNA cells that were transiently transfected with the wild-type or the mutant agrin constructs, were added for 22 h at 37°C. Cells were fixed for 30 min with 4% paraformaldehyde. They were incubated with 1 mM α-bungarotoxin conjugated to Alexa Fluor® 594 (Life Technologies) for 1 h. Plates were mounted in Vectashield mounting medium with DAPI (Vector Laboratories) and observed with a ×40 objective lens under an Olympus IX70 inverted microscope (Olympus Europa) linked to a CCD camera (Princeton Cool SNAP Fx). The number of AChR clusters was counted manually from 10 fields per dish and data were expressed relative to the number of myotubes per field. The experiment was repeated twice.

Results

Clinical features of patients with congenital myasthenic syndrome and distal muscle weakness and atrophy

Kinship 1

Kinship 1 is a non-consanguineous family from Norway where both parents and the non-identical twin brother of the female patient are unaffected. The two affected siblings share a similar phenotype, were the product of a normal pregnancy and birth, and reported normal motor milestone and no autonomic symptoms. Patient 1 is a 45-year-old female who presented at the age of 15 years with weakness and thinning of her foot muscles with a tendency to trip. Three years later, she noted similar symptoms in her hands with marked thinning of her thenar eminence (Fig. 1A). The course of her disease was slow steady progression over years. She had no ptosis, diplopia, bulbar or facial weakness. Mild proximal limb weakness with a subtle waddling gait was reported several years into her illness. Neurological assessment at age 43 years revealed normal cranial nerves. She had mild weakness of her shoulder girdle muscles with preserved elbow and wrist flexion and extension. She had marked atrophy of her thenar and dorsal interossei muscles. Her hypothenar muscles and abductor digit minimi were spared. Hip flexion was marginally reduced with some suggestion of a waddling gait. Knee extension, ankle dorsiflexion and extensor hallucis longus were weak but extension of the four lateral toes, and foot evasion were markedly more affected (Fig. 1A). A trial with pyridostigmine was unhelpful.
Patient 2 is a 43-year-old male, who presented at the age of 15 years with muscle weakness and wasting of his feet, no longer being able to run and a tendency to fall. A few years later, he noted similar difficulties in both hands. He had no ocular, bulbar or facial involvement. The course of his disease was slowly progressive with no suggestion of fluctuations and little deterioration during the past 5–10 years. Neurological assessment showed normal cranial nerves. He had pronounced atrophy of the thenar and dorsal interossei muscles with preservation of the hypothenar muscles. Thumb abduction was weak. Proximal muscles were preserved in all four limbs. In the legs, knee extension was marginally weak, whereas ankle dorsiflexion and plantar flexion were significantly impaired. He was unable to heel or toe walk. Treatment with pyridostigmine showed no improvement. The patient declined further therapeutic trials given his mild symptoms. MRI of the lower limbs done 27 years after onset of disease, showed bilateral atrophy and fat infiltration of semitendinosus muscles (Fig. 1B). There was asymmetrical fat infiltration in medial gastrocnemius bilaterally, more marked on the right, and central fat infiltration only visible in the right medial soleus muscle.

The following investigations were normal: ECG, transthoracic echocardiogram, creatine kinase levels, anti-AChR and anti-MuSK antibodies. Histochemical analysis of the muscle biopsy (vastus lateralis) done for Patient 2 at the age of 33 years showed chronic myopathic changes with groups of atrophic and surrounding hypertrophic muscles fibres and selective type II atrophy (data not shown). No abnormalities of sarcolemma were detected when immunolabelling dystrophin, sarcoglycan, laminin α2, telethonin, caveolin 3, emerin, lamin A/C, calpain and dysferlin.

Kinship 2

Kinship 2 is a non-consanguineous French family with two patients where the parents and three siblings are unaffected. The two patients were the product of a normal pregnancy and birth and reported normal motor milestone.

Patient 3 is a 36-year-old male who presented with apnoeic episodes at the age of 2 years. No more respiratory episodes occurred during childhood, but exercise induced breathlessness since the age of 12 years and a persistent snoring was noted throughout childhood. The patient reported fatigability, muscle wasting and feet weakness after the age of 7 years requiring ankle foot orthotics at the age of 13 years. Disease worsened progressively and both hands became involved from the age of 19 years. At 30 years of age, he was admitted to intensive care for respiratory failure and has been on non-invasive ventilation ever since. While the course was mainly progressive, rapid exacerbations without recovery were reported at the age of 14 years, 19 years, and 32 years. At 32 years, weakness increased rapidly in hands and lower limbs with proximal involvement: the maximal walking distance on even ground was reduced to 200 m, and a walking aid became necessary. Daily fluctuations were present. No oculobulbar symptoms were present throughout the disease course. Examination at the age of 33 years revealed a severe distal weakness of all four extremities (Supplementary Video 1). A marked atrophy was found in hands and lower legs (Fig. 1A). Wrist, thumb and finger extensors were the most affected, right side more than left and third and fourth fingers more than the others (MRC grade 1/5), thumb-index pinch (grade 2–5/5). Wrist and finger flexors were much stronger (grade 4), biceps, triceps brachialis and scapular girdle were tested MRC grade 5, 5, 4 + , respectively with mild scapular winging; 90° arm antepulsion was normal (sustained for 2.5 min). Force in the posterior and anterior compartments of the lower legs was evaluated MRC grade 1/5, with foot drop and the inability to walk on heels or tiptoes (Supplementary Video 1). Knee flexors were tested MRC grade 4/5, other pelvic-femoral muscles were normal. Cervical, trunk,
facial and oculobulbar muscles (flexors and extensors) were normal. Scoliosis and joint contractures were not found. Vital capacity was 58% of the predicted value in the sitting position.

Pyridostigmine and 3, 4-diaminopyridine, administered at the ages of 25 and 29 years, respectively, were ineffective. Ephedrine initiated at the age of 33 years with increasing dose from 45 mg to 135 mg daily, provided a clear benefit quoted by the patient at 70% (100% being the 'normal functional capacities' of his own) that was immediate and remained stable for 3 years. He recovered walking ability from 50 m with a stick to 200 m in his 20s, and no longer required splints and walking aids. However, distal legs and finger extensors did not show significant gain of strength. Vital capacity increased by 20% (79% in sitting position), but overnight nasal ventilation was still necessary because of the persistent diaphragmatic involvement. The following ancillary investigations were normal: ECG, transthoracic echocardiogram, creatine kinase levels, and anti-AChR and anti-MuSK antibodies. Histological analyses of deltoid muscle biopsy, done at the age of 36 years to analyse synaptic area of one muscle not strongly affected by atrophy and weakness, revealed unspecific findings including small variability of myofibre size, and discrete predominance of type 1 myofibre with type 2 atrophy (Supplementary Fig. 1).

Patient 4 is the 27-year-old sister of Patient 3 and was first seen by us at the age of 23 years. A stridor was noted at birth without any other symptoms. She presented with fatigability and dyspnoea from the age of 7 years. At the age of 11 years, she began to stumble due to feet weakness. The predominantly distal, lower limb weakness and atrophy worsened progressively from the age of 15 years until now, with walking difficulties. Distal upper limbs are affected from the age of 16 years. Mild facial involvement was noted from the age of 18 years with difficulties in whistling. Fatigability was most marked in the evening and worsened with hot weather conditions. Unlike her brother, no daytime fluctuations but mild few days-long exacerbations were noted. Neurological assessment at the age of 23 years revealed marked symmetrical distal muscle wasting and weakness of posterior and anterior compartments in the lower legs with foot drop and inability to stand on tiptoes or heels, feet flexors and extensors (Supplementary Video 1). Toe extensors were very weak with a MRC grade 1+ for left peroneus to 2+ for toe extensors. Finger extensors and pinch grip (thumb-index finger) were weak (MRC grade 3). Proximal upper limbs were spared. Atrophy was marked for the first interosseous space, the thenar, and anterior and posterior legs. There was moderate facial weakness affecting mouth and orbicularis oculi, and mild swallowing impairment. There was a mild difficulty rising from a chair. Manual muscle testing revealed the following: tibialis anterior MRC grade 3+ for left peroneus longus, 4/5, triceps suralis 3−/5, ilopsoas 4+/5, quadriceps 4/5. Finger extensors were moderately affected (grade 3+/5), but other upper limbs and axial muscles were normal. There was no bulbar involvement, no ptosis but a minor limitation of ocular motility.

Creatine kinase levels were normal. Deltoid muscle biopsies performed at the age of 31 and 34 years showed small groups of atrophied fibres and moderate grouping of myofibre types (Supplementary Fig. 1). MRI revealed bilateral fatty degeneration of the posterior compartment (soleus and gastrocnemius) of the lower legs with sparing of the right gastrocnemius lateralis (Fig. 1B). Salbutamol (three tablets of 2 mg per day) introduced at the age of 34 years was partly beneficial with an improvement equaling 40% according to the patient’s own estimate, especially for climbing stairs.

**Electrophysiological and morphological evidence for diagnosis of congenital myasthenic syndrome**

All patients were examined by EMG. Motor conduction velocities and motor and sensory nerve distal latencies were within the normal range for all. Concentric needle EMG in Patient 1 revealed myopathic changes with short moderately polyphasic motor units with fibrillation potentials and positive sharp waves in most muscle groups, most marked in abductor pollicis brevis (Supplementary Fig. 2A). The deltoid and vastus lateralis muscles were the least affected. Repetitive nerve stimulation at 3 Hz showed decrement in all four limbs that was most pronounced in the distal muscles (Fig. 2A, Table 1 and Supplementary Fig. 2B). Minimal decrement was found in abductor digiti minimi and no decrement was detected in the facial muscles. The first-evoked compound muscle action potential was reduced in amplitude in some muscles (Fig. 2A). In most muscles, increment was seen after 10-s activation, most marked in tibialis anterior. In other muscles such as
abductor pollicis brevis, the amplitude of the motor response was reduced after 10-s activation followed by increment (+58%) after 20-s sustained APB muscle contraction, and decrement with marked increment after 20-s sustained muscle contraction (500%) in tibialis anterior (TA). Decrement with no increment after sustained muscle contraction for 20 s was seen for abductor digiti minimi (ADM). Similar findings were detected in Patient 2 (Table 1 and Supplementary Fig. 2B).

As for Patients 1 and 2, motor nerve conduction velocities and distal latencies were within the normal range in Patients 3 and 4, and EMG revealed myopathic changes in distal lower limbs. Repetitive nerve stimulation showed a decrement in many muscle groups, most marked in the distal muscles (Fig. 2B and Table 1). Post-exercise increment (up to 285% in tibialis anterior, Patient 4) was observed. Similar EMG features were observed in Patient 5 with EMG myopathic pattern of the lower limbs without spontaneous activity at rest, compound muscle action potential decrement most marked in the distal muscles in response to repetitive nerve stimulation, and post-exercise increment (Table 1).

### Table 1  Compound muscle action potential amplitude and variations in one proximal and two distal muscles in response to nerve stimulation and exercise in five patients with congenital myasthenic syndrome with distal muscle weakness and atrophy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Anconeus (3 Hz)</th>
<th>TA (mV) at rest</th>
<th>TA (3 Hz)</th>
<th>TA (post-exercise)</th>
<th>EDB (mV) at rest</th>
<th>EDB (3 Hz)</th>
<th>EDB (post-exercise)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−8%</td>
<td>0.9</td>
<td>−33%</td>
<td>+133%</td>
<td>1.6</td>
<td>−27%</td>
<td>+23%</td>
</tr>
<tr>
<td>2</td>
<td>−16%</td>
<td>0.3</td>
<td>−67%</td>
<td>+267%</td>
<td>2.8</td>
<td>−20%</td>
<td>+25%</td>
</tr>
<tr>
<td>3</td>
<td>−29%</td>
<td>1.3</td>
<td>−55%</td>
<td>+87%</td>
<td>0.9</td>
<td>−72%</td>
<td>+126%</td>
</tr>
<tr>
<td>4</td>
<td>−10%</td>
<td>0.1</td>
<td>−53%</td>
<td>+285%</td>
<td>4.7</td>
<td>−22%</td>
<td>+32%</td>
</tr>
<tr>
<td>5</td>
<td>−9%</td>
<td>1.5</td>
<td>−22%</td>
<td>+67%</td>
<td>1.8</td>
<td>−33%</td>
<td>+47%</td>
</tr>
</tbody>
</table>

TA = tibialis anterior; EDB = extensor digitorum brevis.
Deltoid muscle biopsies were used to analyse neuromuscular junctions for Patients 3 and 5. We chose this proximal muscle as it was less affected by muscle atrophy as compared to distal muscles, and expected to be free of synaptic damages secondary to muscle atrophy. Fluorescent staining of neuromuscular junctions on whole-mount preparations showed pre- and postsynaptic alterations in both patients (Fig. 3A). Normal fork-shaped nerve terminals and well-defined postsynaptic AChR organization were not observed in the patients’ samples (33 neuromuscular junctions for Patient 3 and 43 neuromuscular junctions for Patient 5). All the neuromuscular junctions showed fragmented and sometimes dispersed postsynaptic gutters with evanescent borders of AChR staining resulting in a postsynaptic streaky staining pattern. This peculiar staining was more pronounced in Patient 3. Neurofilament staining was totally (full neuromuscular junction) or partially (some postsynaptic gutters only) absent for most of the neuromuscular junctions (6/8 for Patient 3 and 8/22 for Patient 5). Other neuromuscular junctions showed strong arborization of terminal axons with nodal, terminal or en passant nerve sprouting innervating isolated clusters of AChR. Finally, S100 immunostaining revealed hypertrophic or phantom-like terminal Schwann cells covering synaptic gutters, which may indicate active denervation-reinnervation processes (Fig. 3B). Ultrastructural analyses of neuromuscular junction (n = 3) done for Patient 5 confirmed the occurrence of denervation processes with secondary synaptic folds not recovered by nerve terminals or terminal Schwann cells (Fig. 3C). When present, terminal nerves looked normal with numerous mitochondria, synaptic vesicles, and active zones facing the top of the postsynaptic crests. The depth and the number of secondary synaptic folds by synaptic gutters appeared to be reduced. These changes were not specific among those observed in other forms of congenital myasthenic syndrome. As they were observed in a proximal muscle that was not strongly affected, they may indicate a primary pathology of the neuromuscular junction rather than secondary changes due to muscle damage and atrophy.

Identification of agrin mutations

Kinships 1 and 2 had been excluded for the most frequent congenital myasthenic syndromes genes and were subjected to whole exome sequencing analysis. Based on the pattern of inheritance, we sequenced the two patients of Kinship 1 to focus on common genes with homozygous or two heterozygous variations, and Patient 3 and his two parents to focus on genes with homozygous or two heterozygous variations inherited from each parent. Restricting our search to coding and rare variants predicted to be deleterious shortlisted three variants in three genes for Kinship 1 and 18 variants in seven genes for Kinship 2 (Supplementary Table 1). We found no relevant variants in any of the known inherited peripheral neuropathy or distal myopathy genes in the two families. Agrin was the sole candidate gene common to the two kinships. For Kinship 1, the agrin variant was a novel seemingly homozygous transversion in exon 2 leading to the substitution of a conserved hydrophobic glycine to a polar serine in the N-terminal domain (c.226G>A; p.Gly76Ser). Patient 3 of Kinship 2 was heterozygous for one transversion (c.314A>T; p.Asn105Ile) in exon 2 on the maternal allele and one nucleotide duplication (c.1362dupC; p.Ser455Glnfs*8) in exon 7 on the paternal allele.

Sanger sequencing in Kinship 2 showed complete segregation of the two agrin variants with the disease (Fig. 4A). In Kinship 1, the mother was found to be heterozygous whereas the father and the healthy brother were homozygous for c.226G. The seemingly homozygous state of the father could not account for the homozygous state of both offspring for the mutant c.226A allele. Non-paternity was excluded based on haplotype analysis and exome data. We then run the ExomeDepth analysis algorithm and identified a ~0.48 Mb hemizygous deletion encompassing 236 exons in Patients 1 and 2, which included the entire agrin gene (Supplementary Table 2). This result was confirmed by CGH array done on Patient 1 that showed a 461 kb hemizygous deletion on chromosome 1 encompassing 22 genes (1p36.33, position 0.746,649–1,207,683 Mb). To summarize, Patients 1 and 2 are compound heterozygous for one missense mutation (p.Gly76Ser) and one large deletion encompassing the entire AGRN gene, suggesting that all the produced agrin carries the missense mutation.

Based on these results, we screened the AGRN gene in Patient 5 by Sanger sequencing and found two homozygous missense variants. One was located in exon 12 (c.2234C>T; p.Ala745Val) and was predicted to be a benign polymorphism by MutationTaster. The second variant was located in exon 32 (c.5611G>A) and led to the missense p.Gly1871Arg substitution in the C-terminal LG3 domain. The Gly1871 residue is well conserved in species and its substitution by an Arg residue is predicted to be disease-causing (Fig. 4B). We found it to segregate with the disease in the family of Patient 5 and then considered it as the disease-causing mutation.

Functional effect of the N-terminal agrin domain missense mutations on agrin function

The two NTA mutant agrins (p.Gly76Ser and p.Asn105Ile) are the first mutations ever described in this domain to be associated with a phenotype of congenital myasthenia (Fig. 4C). The implication of this domain in the formation of neuromuscular junction is not well known, and we focused our functional analyses on these two mutations to further investigate their effect. Staining transversal muscle sections containing neuromuscular junctions for Patients 2 and 3 revealed an apparent normal staining of synaptic agrin at the neuromuscular junctions compared to the control, suggesting that these mutations do not lead to a drastic reduction of agrin deposition into the synaptic basement membrane (Fig. 5A). Immunostaining of laminin β2 and LRP4, two synaptic binding partners of agrin, did not reveal any difference compared to the control (Supplementary Fig. 3).

The main known function of agrin at the neuromuscular junction is to stabilize postsynaptic clusters of AChR, and agrin mutations associated with congenital myasthenic syndromes alter this function (Huze et al., 2009; Maselli et al., 2012). We therefore assessed the in vitro ability of the two NTA mutant agrins to aggregate AChR in C2C12 myotubes. We treated myotube cultures with conditioned media from 293 EBNA cells transiently transfected with the wild-type or the NTA mutant agrin constructs,
and then counted the number of AChR clusters. The number of AChR clusters formed along the C2C12 myotubes were reduced for the two NtA mutant-containing media compared to the wild-type, indicating that the two mutations decrease the ability of agrin to cluster AChR in vitro as expected from loss of function mutations (Fig. 5B and C).

**Discussion**

Congenital myasthenic syndromes are inherited disorders with compromised neuromuscular transmission that are highly heterogeneous at the clinical and genetic levels with 19 genes identified to date (Hantai et al., 2013; Ohkawara et al., 2014).
Agrin mutations have been previously reported in two unrelated kinships with an unspecific form of congenital myasthenia displaying decrement but no increment at EMG (Huze et al., 2009; Maselli et al., 2012). By contrast, all the patients described in our report share a remarkable and homogenous clinical phenotype (Table 2). The common features include (i) marked distal muscle weakness and wasting affecting first the lower and later upper limbs; (ii) equal or greater lower limb involvement compared to the upper limbs with a fatty degeneration of the posterior leg compartment on muscle MRI; (iii) sparing of axial and oculobulbar muscles; (iv) slowly progressive course; (v) lack of efficacy of acetylcholinesterase inhibitors and beneficial effect of \( \beta \)2 adrenergic receptor agonists (ephedrine or salbutamol); and (vi) neurotransmission defect always present, predominating in distal muscles, with post-exercise increment. Nevertheless, some interindividual differences are noted: (i) the age at onset ranged from birth to early adulthood; (ii) fluctuations were more marked in Patient 3; (iii) severe respiratory involvement requiring intermittent ventilation was only observed in Patient 3; and (iv) mild facial weakness occurred in Patient 4, but not in other patients. An initial diagnosis of distal myopathy was suspected in all patients due to distal involvement of limbs, patchy myopathic features at EMG, and abnormal muscle MRI. Among the group of distal myopathy, three genetic entities share some phenotypic similarities: (i) MYH7-related distal myopathy, also called ‘Laing myopathy’, but this is a form that has an autosomal dominant inheritance and shows predominantly anterior compartment involvement (Laing et al., 1995); (ii) nebulin-related myopathies are recessively inherited, but differ by a selective and exclusive involvement of the leg anterior compartment (Wallgren-Pettersson et al., 2007); and (iii) dynamin 2-related distal myopathy affects the posterior compartment of legs, but shows dominant inheritance and is characterized by central nuclei in the muscle biopsy (Böhm et al., 2012). None of the related genes was found mutated by whole exome analyses in Kinships 1 and 2. Moreover, respiratory involvement, rapid worsening and fluctuations are atypical features in distal myopathy and are more suggestive of myasthenia. A degree of distal muscle weakness is not unusual in congenital myasthenia, but proximal and/or oculo-bulbar weakness often predominates (Abicht et al., 2012a, b). One rare congenital...
myasthenic syndrome entity with distal upper limb involvement is the slow channel form due to mutations in AChR-encoding genes, but these patients often present with neck extensor weakness, oculo-motor involvement, characteristic neurophysiology with double motor response to single nerve stimulation. These features were not seen in the patients reported here. Our report suggests that assessment of neuromuscular transmission including repetitive nerve stimulation and response to exercise should be carried out in patients with genetically unidentified, recessively inherited distal myopathy to exclude the diagnosis of congenital myasthenic syndrome with distal muscle weakness and atrophy.

We observed a clear relationship between congenital myasthenic syndrome with distal muscle weakness and atrophy and agrin mutations. The five patients harboured at least one missense agrin mutation that segregated with the disease. Two of the three missense mutations were located in the N-terminal region of agrin, which has never been reported to be mutated. We had initially hypothesized that a specific relationship between the NtA location of the mutation and the associated phenotype may exist, but the
Table 2 Clinical summary for the five patients suffering from congenital myasthenic syndrome with distal muscle weakness and atrophy

| Patient | Age (years), gender | Age at onset(course) | Symptoms/course | Fluctuation (daily/long- term exacerbation/other) | Signs | Proximal | Distal | Fatigue | Respiratory | Other features | Response to treatment | Creatine kinase levels | EMG pattern |
|---------|---------------------|---------------------|------------------|-----------------------------------------------|-------|----------|--------|---------|------------|----------------|---------------------|-----------------------|----------------------|-------------|
| 1       | 45, F               | 15                 | Weakness in feet, trips, muscle wasting, hand weakness and wasting. 3 years later, proximal weakness. Few years later, waddling gait. | No | –/−/−/− | UL+ | LL+ | UL+ | LL++ | – | – | Atrophy of thenar and interossei muscles. | Pyridostigmine – | N | Myopathic changes with fibrillation potentials and positive sharp waves (distal). Decrement (distal). Post exercise increment in some muscle groups. |
| 2       | 43, M               | 15                 | Weakness in feet, trips, difficulties running, hand wasting and weakness few years later. | No | –/−/−/− | UL− | LL− | UL+ | LL++ | – | – | Atrophy of thenar and interossei muscles. | Pyridostigmine – | N | Myopathic changes with fibrillation potentials and positive sharp waves (distal). Decrement (distal). Post exercise increment in some muscle groups. |
| 3       | 32, M               | 2                  | Episodic apnoea; Weakness in feet (7 y) and hands (23 y); Respiratory failure (30 y); Progressive worsening. | –/+/− | –/−/−/− | UL− | LL− | UL+ | LL++ | + | + NIV | Atrophy of thenar and interossei muscles, legs. AChE inhibitors –; 3,4diaminopyridine –; Ephedrine +70% (proximal muscles). | | |

(continued)
| Patient | Age (years), gender | Age at onset (years) | Symptoms/course | Fluctuation (daily/long term exacerbation/other) | Signs | Proximal | Distal | Fatigue | Respiratory | Other features | Response to treatment | Creatine kinase levels | EMG pattern |
|---------|-------------------|-------------------|-----------------|-----------------|-------|-----------|--------|---------|------------|--------------|-----------------|-------------------|------------------|-------------|
| 4       | 23, F             | Birth             | Isolated stridor at birth; Exercise fatigability (7 y); Stumble due to feet weakness (11 y); Distal upper limb weakness and atrophy (16 y); Progressive worsening | −/+ hotspot atmosphere worsening | −/+ mild swallowing/ − | UL − LL − (except right knee flexor) | UL + + LL + + | + | − | Atrophy of thenar and interossei muscles, legs | AChE inhibitors −; 3,4 diaminopyridine −; Ephedrine +70% (proximal muscles). | N | Myopathic pattern (distal) Decrement, post exercise increment (distal) |
| 5       | 25, M             | 5                 | Exercise fatigability (5 y); Distal lower limb weakness worsening since the age of 20 y; Successive periods of rapid worsening without regression (step pattern) | −/+ − | Mild questionable/ −/ − | UL − LL − | UL + LL + + | + | − | No hand atrophy, lower limbs atrophy | Salbutamol + 40% (distal muscles) | N | Myopathic pattern with reduced motor unit potential (distal) Decrement, post-exercise increment (distal) |

N = normal values; UL = upper limbs; LL = lower limbs; NIV = non-invasive ventilation.
identification of the LG3 mutation in Patient 5 makes this hypothesis less likely. Little is known about the function of the NTA domain in neuromuscular junction formation. This 135 amino-acid domain is present in the 150 amino acids-long (LN) secreted variant of agrin, and mediates the agrin binding to basement membrane by interacting with laminin γ1 (Kammerer et al., 1999). The X-ray structure of the chicken NTA domain revealed a β-barrel fold flanked by α-helices at both termini with a high content of charged amino acids (Stetefeld et al., 2001). The two missense mutations reported here do not modify the amino acid charge, nor do they lie in residues known to be critical for laminin binding interaction, but we cannot exclude a subtle effect on the ability of agrin to bind laminin γ1 in the dense basement membrane network in vivo. Another possible functional effect of the NTA mutations is protein misfolding, leading ultimately to a reduced amount of agrin in the synaptic basement membrane. Our current data are not in favour of such effect as we did not detect reduced immunostaining of agrin on muscle biopsies. Finally, our AChR binding assays are in favour of a loss-of-function effect of the NTA mutations on the AChR clustering activity of agrin, as did one previously described C-terminal mutation (Maselli et al., 2012). However, this effect appears to be subtle, especially for the p.Asn105Ile mutation, suggesting that these mutations may modify other agrin functions.

Muscle atrophy may result from either a neurogenic or myogenic process. We speculate that distal muscle weakness and atrophy associated with agrin mutations may be a consequence of primary synaptic dysfunction rather than muscle damage. If proximal muscle biopsy in Patient 2 (vastus lateralis) showed mild myopathic changes, those done in deltoid in Patients 3, 4 and 5 showed isolated angular fibres and fibre types grouping usually considered to be of neurogenic origin. Morphology of neuromuscular junctions in the clinically not affected deltoid muscle of two unrelated patients supports active denervation-reinnervation events with partial denervation of synaptic gutters, nerve sprouting, and Schwann cell extension. Furthermore, the muscles most clinically affected by weakness and atrophy were those with higher decrement and increment at EMG in all patients. Such a specific muscle involvement was observed in one mouse model of congenital myasthenic syndrome. Homozygous agrin knockout mice are not viable regardless of the mutated protein domain (Gautam et al., 1996; Burgess et al., 2000). A viable mouse model resulting from a homozygous missense mutation (p.Phe1061Ser) in the SEA domain of agrin was recently shown to accurately replicate human congenital myasthenic syndrome (Bogdanik and Burgess, 2011). This missense mutation caused partial loss of function with reduced glycosylation, reduced proteolytic cleavage and intracellular retention of mutant agrin. As depicted in the patients described here, mutant mice displayed neuromuscular junction denervation with late muscle atrophy and a disease severity that varied between muscles. This mouse model could therefore be useful to determine the factors involved in the selective distal muscle involvement in our cohort of congenital myasthenic syndrome with distal muscle atrophy and weakness due to agrin mutations.

In addition to the commonly seen synaptic structural changes resulting from denervation-reinnervation events, we observed a more unusual, although not specific, streaky pattern of postsynaptic AChR. This streaky pattern may have resulted from secondary synaptic folds not recovered by a terminal nerve or Schwann cells as observed at electron microscopy, pointing towards a presynaptic defect. This is in accordance with the marked increment seen in several muscles after exercise, which is compatible with presynaptic dysfunction. It is worth noting that increment is only observed in the choline acetyltransferase-deficient congenital myasthenia forms that accounts for ~10% of all congenital myasthenia, and in the auto-immune Lambert-Eaton syndrome targeting the presynaptic Ca2,1 calcium channel. Presynaptic defects were detected by in vitro microelectrodes studies of neuromuscular transmission in one patient with one nonsense mutation in the N-terminal domain and one missense mutation in the LG2 domain of agrin (Maselli et al., 2012). However, post-exercise increment at EMG was not reported in this patient. In our experience with six patients harbouring agrin mutations (two published, three in this report, and one unpublished), only those reported here displayed post-exercise increment at EMG as well as distal muscle atrophy. The genotype-phenotype correlation between agrin mutations, post-exercise increment suggesting facilitation of acetylcholine release by the nerve terminal and distal muscle atrophy is currently unexplained. Time-induced deletion has shown that whole agrin is required for the postsynaptic maintenance in adult mice, and that the postsynaptic alterations progressively lead to presynaptic changes (Samuel et al., 2012). The missense mutations identified here may point towards domains critical for presynaptic function of agrin. Loss of interaction between laminin β2 and Ca,2.1 results in a disassembly of the presynaptic active zone similar to that seen in the autoimmune Lambert–Eaton myasthenia (Nishimune et al., 2004). Through its binding interaction with laminins (NtA domain mutated in Kinships 1 and 2), agrin may participate to the presynaptic membrane specializations within the active zone and ensure their alignment with postsynaptic specializations. However, we did not identify abnormalities when immunostaining laminin β2 in muscle biopsies from the patients investigated here, and the active zone location and the amount of synaptic vesicles in nerve terminals appeared normal at the ultrastructural level. A direct effect of agrin on the presynaptic element can also be evoked because agrin affects axonal growth, differentiation and adhesion in vitro (Campaña et al., 1997). This effect may be a result of the interaction of agrin with LRP4 through its LG3 domain, which is mutated in Patient 5. This transmembrane receptor of agrin was recently shown to have a critical role in presynaptic formation (Wu et al., 2012; Yumoto et al., 2012). In conclusion, our report of this peculiar form of congenital myasthenic syndrome may reveal novel and critical roles for agrin with respect to the pre- and postsynaptic compartments, which deserve further functional studies.

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Supplementary material

Supplementary material is available at Brain online.

References


