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**ORIGINAL ARTICLE**

Biallelic loss of function variants in *SYT2* cause a treatable congenital onset presynaptic myasthenic syndrome

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Abstract

Synaptotagmins are integral synaptic vesicle membrane proteins that function as calcium sensors and regulate neurotransmitter release at the presynaptic nerve terminal. Synaptotagmin-2 (*SYT2*), is the major isoform expressed at the neuromuscular

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junction. Recently, dominant missense variants in *SYT2* have been reported as a rare cause of distal motor neuropathy and myasthenic syndrome, manifesting with stable or slowly progressive distal weakness of variable severity along with presynaptic NMJ impairment. These variants are thought to have a dominant-negative effect on synaptic vesicle exocytosis, although the precise pathomechanism remains to be elucidated. Here we report seven patients of five families, with biallelic loss of function variants in *SYT2*, clinically manifesting with a remarkably consistent phenotype of severe congenital onset hypotonia and weakness, with variable degrees of respiratory involvement. Electrodiagnostic findings were consistent with a presynaptic congenital myasthenic syndrome (CMS) in some. Treatment with an acetylcholinesterase inhibitor pursued in three patients showed clinical improvement with increased strength and function. This series further establishes *SYT2* as a CMS-disease gene and expands its clinical and genetic spectrum to include recessive loss-of-function variants, manifesting as a severe congenital onset presynaptic CMS with potential treatment implications.

KEYWORDS

congenital myasthenic syndrome, neuromuscular junction, presynaptic CMS, synaptotagmins, *SYT2*

1 | BACKGROUND

Congenital myasthenic syndromes (CMS) are a clinical and genetically heterogeneous group of disorders characterized by impaired neuromuscular transmission (Rodriguez Cruz, Palace, & Beeson, 2018). Genetic confirmation of the CMS subtype can inform the use of available treatment options tailored to the underlying molecular mechanism; however, the considerable clinical and genetic heterogeneity of CMS contribute to delayed diagnosis. In addition, approximately 40% of patients with a CMS currently remain without a confirmed genetic diagnosis (Engel, Shen, Selcen, & Sine, 2015; Nicole et al., 2017). Variants in genes associated with presynaptic transmitter release are increasingly recognized as causes of presynaptic CMS (Engel, 2018).

Synaptotagmins, are a large family of integral membrane proteins that are essential for regulating Ca^{2+} mediated exocytosis (Hilbush & Morgan, 1994). Synaptotagmin-2 encoded by *SYT2* is the main isoform expressed at the presynaptic neuromuscular junction and functions as a calcium sensor for neurotransmission (Littleton, Stern, Perin, & Bellen, 1994; Mackler, Drummond, Loewen, Robinson, & Reist, 2002; Pang et al., 2006). *SYT2* is a synaptic vesicle protein that contains two calcium binding domains, C2A (AA 141–242) and C2B (AA 272–375), and regulates neurotransmitter release through mediating fast synaptic vesicle exocytosis (Littleton et al., 1994; Mackler et al., 2002; Pang et al., 2006). At this time, three dominantly acting missense variants impacting the *SYT2* C2B domain have been reported as a rare cause of distal motor neuropathy and myasthenic syndrome. Patients clinically manifest with relatively stable or slowly progressive distal weakness of variable severity with physiologic evidence of presynaptic NMJ impairment, responsive to 3,4

diaminopyridine treatment in some patients (Herrmann et al., 2014; Montes-Chinea et al., 2018; Whittaker et al., 2015). These heterozygous *SYT2* variants are thought to impair Ca^{2+} binding and disrupt synaptic transmission in a dominant-negative manner, however, their detailed mode of action remains to be fully explored. Recently, one patient with severe presynaptic CMS and denervation atrophy was reported to have biallelic variants in *SYT2* (Maselli, van der Linden, & Ferns, 2020).

In this study we report seven patients of five independent families with biallelic loss of function (lof) variants in *SYT2*, clinically manifesting with a remarkably consistent phenotype of severe congenital onset hypotonia and profound weakness, with minimal attainment of motor milestones and with variable degrees of respiratory involvement. Treatment with an acetylcholinesterase inhibitor in three patients showed clinical improvement. Taken together, our data introduce recessive loss of function variants in *SYT2* as an important mutational mechanism, establish the role of *SYT2* in neuromuscular disease, and expand its clinical spectrum to a severe congenital onset phenotype that is potentially treatable.

2 | METHODS

2.1 | Patient recruitment and sample collection

Patients presented with a history of congenital onset muscle weakness and underwent detailed clinical examination. DNA, tissues including muscle, and medical records were obtained based on standard procedures. For research studies, written informed consent and

age appropriate assent were obtained. This study was approved by the NIH, National Institute of Neurological Disorders and Stroke (NINDS), Institutional Review Board (Protocol 12-N-0095), the Yorkshire & The Humber–Leeds Bradford Research Ethics Committee (13/YH/0310). P3 was identified through GeneMatcher (Sobreira, Schiettecatte, Valle, & Hamosh, 2015).

2.2 | Genetic analysis

Trio whole exome sequencing was performed in all patients. Variants were confirmed by Sanger sequencing. For P1 exon-level oligo array comparative genomic hybridization (CGH) was performed for *SYT2*. Detailed methods are provided in Supporting Information 1.

2.3 | Motor assessments

Motor capacity and strength for Patient 1 (P1) were evaluated at baseline (visit 1) and at 13 months after treatment initiation (visit 2). Motor capacity was measured using the Motor Function Measure-32 (MFM-32), comprised of three domains (D1: standing and transfers; D2: axial and proximal motor function; and D3: distal motor function) with results expressed as percentages of total and domain scores. A higher score indicates better performance. Motor strength was quantitatively measured using a handheld dynamometer (MicroFET®2 [hogganhealth.net]) for knee, hip, and elbow strength with distal strength measured using grip and pinch dynamometers (MyoGrip and MyoPinch (Ateliers Laumonier, France) (Hogrel, 2015). Handheld dynamometry results were expressed as the percentage of the highest value compared to age/weight/sex-based norms (Beenakker, van der Hoeven, Fock, & Maurits, 2001), and distal strength results were expressed as the percentage of the highest value compared to age-based norms (Annoussamy et al., 2019). A single pediatric physical therapist completed all motor capacity and strength assessments at both visits using standard positions and instructions.

3 | RESULTS

3.1 | Clinical characteristics

Patient 1 (P1) is a 15-year-old Caucasian female born to consanguineous parents. Pregnancy was notable for reduced fetal movements. At birth she was noted to have hypotonia, abnormal wrist and ankle positioning due to joint laxity, a high-arched palate, and a weak cry. She was diagnosed with failure to thrive requiring nasogastric (NG) tube feeding which was subsequently converted to gastrostomy tube. Motor milestones were delayed. She was able to sit independently at the age of 2 years but has not attained the ability to roll, stand or walk. She has a history of structural cardiac disease with subaortic stenosis and mitral valve stenosis requiring surgery in the first year of life. Additionally, she has a baseline prolonged QT interval and a

history of eye deviation requiring corrective surgery. Progressive scoliosis was noted at an early age for which she underwent corrective surgery at age 11 years (Figure 1). Examination at age 15 years revealed significant axial weakness (Medical Research Council [MRC] grade 2/5) and proximal weakness (MRC grade 2–3/5) more than distal weakness (MRC grade 4–/5). Sensation appeared normal. Reflexes were absent throughout the bilateral upper and lower extremities; however, post-exercise facilitation of biceps reflex was noted bilaterally. Bilateral upper and lower facial weakness was noted. Extraocular movements were notable for slow saccades, and bilateral adduction and abduction restriction and supraduction limitations that were still present post corrective surgery. Cognition was normal. Forced vital capacity (FVC) was 40% predicted. Brain magnetic resonance imaging (MRI) at age 14 years revealed mild cerebral atrophy, including thinning of the corpus callosum (Figure 1f). Muscle ultrasound at age 14 years showed a mixed granular and streaky pattern of increased echogenicity with rare fasciculations (Figure 1g,h).

Treatment of pyridostigmine was initiated in P1 at age 14 years. Her family reported an improvement in her function over the three months following the start of pyridostigmine. Subsequently, she has continued to improve in some functional domains but at a slower pace. Between visit 1 and visit 2, 12 months after initiation of pyridostigmine, the patient improved in the relative strength of all muscle groups between 1.2 and 10.8%, except for the right elbow extensors which decreased by 1.7%. In particular, the patient improved in the relative strength of both grip and pinch between 1.8 and 7.8%. The total MFM score between visit 1 and visit 2 increased by seven points, from 26 to 33, out of a total of 96 possible points, with an increase of five points considered reaching minimal clinically important difference (MCID) (Vuillerot et al., 2012). D1 remained stable, while scores on D2 and D3 increased from visit 1 to visit 2. 3,4-Diaminopyridine (3,4-DAP) has been successfully used in some patients with CMS. However, given P1's cardiac disease and baseline prolonged QT interval, this medication was not initiated.

We subsequently identified six additional patients, four males and two females of four independent families. The clinical presentation is summarized in Table 1, with ages ranging from 25 months to 15 years. All of the parents reported no clinical symptoms, and family history was significant for consanguinity in three families (Figure 3a). All patients presented with a remarkably consistent phenotype of congenital onset severe hypotonia with significant generalized weakness noted at birth. Patients had attainment of only minimal motor milestones, with maximal motor milestones ranging from minimal head control in two patients, to independent sitting in four patients. At the time of the last examination, all patients were found to have significant generalized weakness. Slow and limited extraocular movements were seen in five patients. Pulmonary function ranged from normal to requiring full-time ventilation (P5). Similar to P1, the brain MRI performed in P6 also revealed a thin corpus callosum. Additionally, P6 had evidence of mild vermian hypoplasia. The brain MRI in P2 and P3 was normal. None of the patients had a history of seizures, cataracts, hearing loss or cognitive involvement. Unlike P1, cardiac involvement was not observed in P2–7. A trial of pyridostigmine was initiated in P4

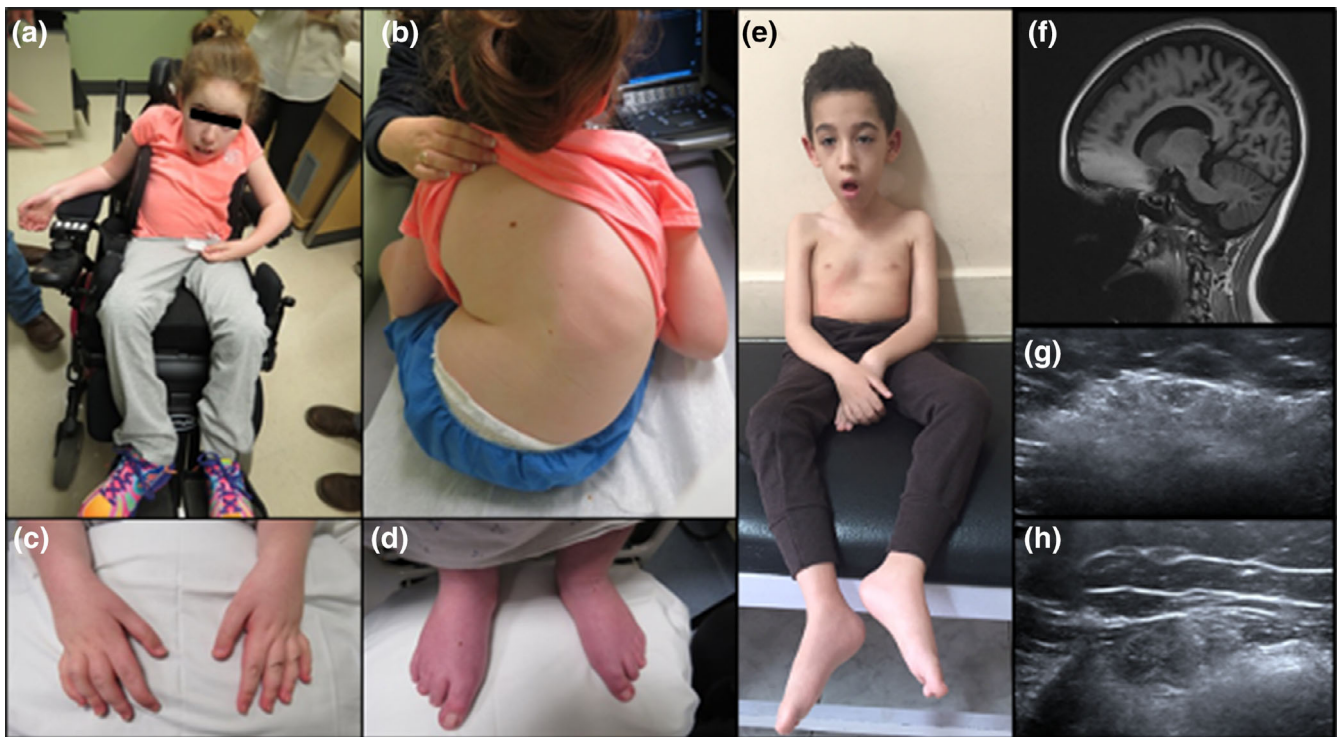


FIGURE 1 Clinical presentation (a) P1 with evidence of overall reduced muscle bulk, facial weakness, contractures of the elbows and fingers, (b) scoliosis, with (c) neuropathic looking hands and (d) feet. (e) P6 with evidence of reduced muscle bulk and severe facial weakness. (f) Brain MRI of P1 at age 14 years revealed thinning of the corpus callosum (exact midline section not available due to artifact related to dental braces). (g and h) Muscle ultrasound in P1 of the right deltoid (g) which is atrophic and with a moderate to severely increased echogenicity, and the right rectus femoris (h) which showed a mild to moderately increased echogenicity. In both muscles (g and h) the echogenicity had a mixed pattern (granular and streaky in qualitative appearance)

without evidence of a clinical response. Both siblings in Family 2 (P6 and P7) initiated neostigmine (an acetylcholinesterase inhibitor) and subsequently reported an increase in overall stamina and motor activity, and both patients were less susceptible to chest infections. On strength examination, P6 and P7 were noted to have increased head control and increased movement against gravity since initiation of neostigmine.

3.2 | Electrodiagnostic findings

A subset of patients had electrodiagnostic studies performed. These studies showed normal sensory responses. Motor nerve conduction studies universally revealed significantly reduced compound muscle action potential (CMAP) amplitudes and normal nerve conduction velocities. EMG showed reduced recruitment; however, motor unit action potential (MUAP) morphology varied with some patients having short duration, low amplitude MUAPs, while others having large amplitude and long duration units. Abnormal spontaneous activity, in the form of positive sharp waves and fibrillation potentials were present in some but not all patients. In those that had repetitive nerve stimulation (RNS) studies, low frequency (3–5 Hz) stimulation resulted in significant decremental responses pointing to myasthenic syndrome. Post-exercise

facilitation could not be reliably observed at the very low CMAP amplitudes.

3.3 | Histological characteristics

Muscle biopsies were performed in three patients (P1, P2 and P6). Common findings include variation in fiber size (Figure 2a) with occasional angular fibers. Gömöri trichome staining in P1 demonstrated occasional fibers with fusciphilic sarcoplasmic and perinuclear inclusions (Figure 2b) which were NADH positive (Figure 2c) suggestive of tubular aggregates. Non-specific esterase staining in P1 highlighted the tubular aggregates and appeared to show segmented NMJs. A rare angular, atrophic fiber is also noted (Figure 2d).

3.4 | Genetic findings

Trio whole exome sequencing for P1 was unrevealing. Subsequent analysis of the data for copy number variants identified an apparent homozygous loss of coverage of a portion of *SYT2* in P1 compared to controls, with each parent showing apparent heterozygosity for this deletion (Figure 3b). This was confirmed

TABLE 1 Clinical details of the SYT2 patients

	P1	P2	P3	P4	P5	P6	P7
Pathogenic SYT2 variant	Homozygous Exon 3-9 deletion	Homozygous c.927C>A; p.(Tyr309*)	Homozygous c.725dupA p.(Val243Glyfs*13)	Homozygous c.805G>T; p.(Glu269*)	Homozygous c.805G>T; p.(Glu269*)	Homozygous c.725dupA p.(Val243Glyfs*13)	Homozygous c.725dupA p.(Val243Glyfs*13)
Inheritance	Recessive	Recessive	Recessive	Recessive	Recessive	Recessive	Recessive
Consanguinity	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Sex/ethnicity	F/Caucasian	M/Turkish	M/Egyptian	M/Caucasian	F/Caucasian	M/Egyptian	F/Egyptian
History and progression							
Symptoms onset	Congenital: Reduced fetal movements; birth: Hypotonia, weak cry, failure to thrive	Birth: Hypotonia, weak cry, failure to thrive	Birth: Hypotonia	Birth: Hypotonia and tongue fasciculations	Birth: Hypotonia	Congenital: Reduced fetal movements; birth: Hypotonia	Congenital: Reduced fetal movements; birth: Hypotonia
Maximal motor development	Sat independently (2 years)	Minimal head control	Sat independently (34 months)	Head control	Not able to roll or sit	Sat independently	Sat independently
Loss of motor milestones	No	No	No	No	No	No	No
Fatigability	Family denies	Family denies	Family denies	Family denies	Family denies	Easily fatigability	Easily fatigability
Cognition	Learning difficulties	Mild cognitive delay, communication via computer interface	Normal	Normal	Normal	Normal	Normal
Exam findings							
Age at last examination	15 (years)	10 (years)	7 (years)	9 (years)	25 (months)	7 (years)	5 (years)
Muscle strength (MRC-grade)	Neck flex: 2;deltoids: 3; elbow flex: 4-; wrist flex: 4-; wrist ext: 2; LEs: Subgravity except for ankle dorsiflex and plantarflex; 4-; toe ext: 3; toe flex: 4-	Neck flex: 1; UEs: Subgravity except for wrist flex and ext; 3; LEs: Subgravity for toe ext and flex: 3	Neck flex: 3; deltoids: 2-; elbow flex 3-; wrist flex: 3-; wrist ext: 3; LEs: Subgravity except for toe ext: 3	Neck flex: 3; deltoids: 3; elbow flex: 3-4-; wrist flex 3, wrist ext 4; LEs: Subgravity	Forearm flex and finger ext: 3 (Exam limited due to young age)	Neck flex: 2; UEs: Subgravity; LEs: 2-3	Neck flex: 2/5, UEs and LEs: Subgravity
Deep tendon reflexes	Absent	Absent	Absent	Absent in UEs; minimal at knees	Absent	Absent	Absent
Sensory examination	Normal	-	-	-	Normal	Normal	Normal
Face	Bilateral upper and lower facial weakness; maintains mouth open	Bilateral upper and lower facial weakness; maintains mouth open	Bilateral upper and lower facial weakness; maintains mouth open	Bilateral upper and lower facial weakness; maintains mouth open	Bilateral upper and lower facial weakness; maintains mouth open	Bilateral upper and lower facial weakness; maintains mouth open	Bilateral upper and lower facial weakness; maintains mouth open

(Continues)

TABLE 1 (Continued)

	P1	P2	P3	P4	P5	P6	P7
Extraocular movements	Slow eye movements; slow saccades; adduction and abduction restriction; mild up-gaze limitation	—	Mild vertical limitation	Bell's phenomenon; vertical limitation; occasional divergent gaze; hypermetropia	Mild vertical limitation	Variable ptosis; slow eye movements; variable divergent gaze	Weak upgaze
Bulbar	Nasal and high-pitched voice; weak tongue, no tongue fasciculations	Nasal and quiet voice; weak tongue, no tongue fasciculations	No tongue fasciculations	Dysarthria	Soft cry; tongue fasciculations	Dysphagia; quiet voice	Quiet voice
Spine / joints	Severe scoliosis; mild contractures	Scoliosis; mild knee contractures	Joint laxity of shoulders, elbows, wrists and fingers	Mild scoliosis; hip flexor, knee and mild ankle contractures	None	Mild kyphosis; knee contractures, mild elbow contractures	Mild kyphosis; mild knee contractures
Review of systems							
Pulmonary	FVC 40% with 10% decrease in supine (14 years)	Intubated (9 years)	Recurrent chest infection	FVC: 50%	Ventilated full time via tracheostomy	—	—
Cardiac	Subaortic stenosis and mitral valve stenosis requiring surgery (1 year). Prolonged QT interval (14 years)	Normal echo, ECG (7 years)	Normal echo, ECG (7 years)	Normal echo, ECG (9 years)	Normal echo, ECG (2 years)	Normal echo, ECG (7 years)	Normal echo, ECG (5 years)
Gastrointestinal	Gastrostomy tube	Pylorus stenosis requiring surgery (2 months); gastrostomy tube (3 years)	—	Gastrostomy tube	Gastrostomy tube	Normal	Normal
Evaluations							
EMG/NCS	EMG: Myopathic with considerable connective tissue replacement of biceps muscle; NCS: Low motor responses with spared sensory responses (14 years)	NCS: Severe axonal polyneuropathy (6 years)	EMG: Neurogenic; NCS: Reduced amplitudes, normal velocity (10 months)	EMG: Neurogenic; NCS: Normal (6 months)	—	Myopathic	Myopathic

TABLE 1 (Continued)

	P1	P2	P3	P4	P5	P6	P7
Repetitive nerve stimulation	Decrement on low frequency stimulation (5 Hz). Post-exercise repair of decrement noted. CMAP did not significantly increase after either 15 s or 1 min exercise (14 years)	—	Decrement on stimulation	—	—	Decrement on stimulation	Decrement on stimulation
Muscle ultrasound	Mixed pattern of echogenicity with rare fasciculations (14 years)	—	Abnormal	—	—	—	—
Brain MRI	Mild cerebral atrophy, including thinning of the corpus callosum (14 years)	Brain MRI: Normal (neonatal)	Normal	—	—	Thin corpus callosum, mild vermian hypoplasia	—
Muscle histology (age)	Mixed neurogenic and myopathic pattern; few fibers with tubular aggregates (4 years)	Mixed neurogenic and myopathic pattern (2 years)	—	—	—	Mixed myopathic and neurogenic pattern; fiber size variation and angular fibers with grouping; occasional nuclear bags (2 years)	—

Abbreviations: ECG, electrocardiogram; EMG, electromyography; ext, extension; F, female; flex, flexion; Hz, hertz; LEs, lower extremities; m, male; MRC, Medical Research Council; NCS, nerve conduction studies; UEs, upper extremities.

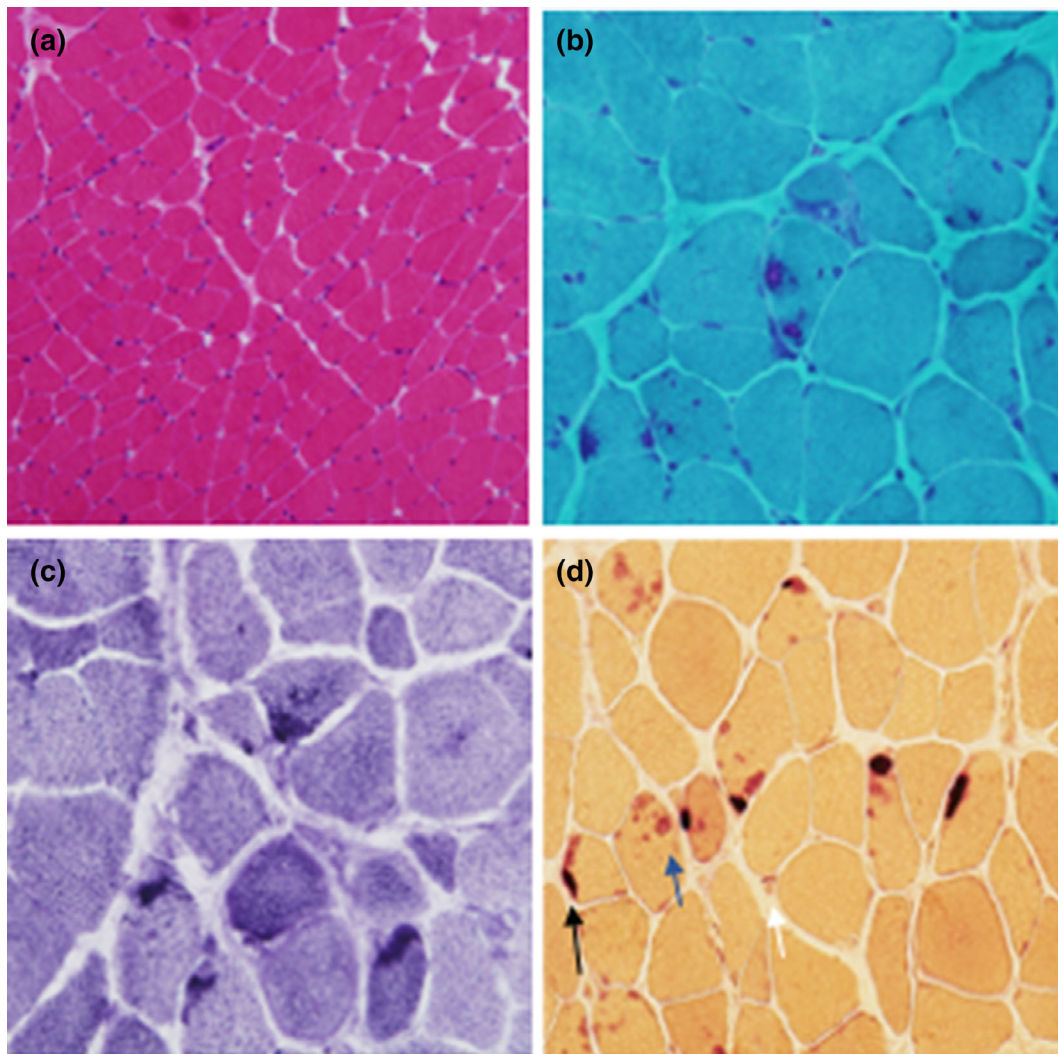


FIGURE 2 Quadriceps muscle biopsy of P1 age 4 years. (a) Variation in fiber size on hematoxylin and eosin (H&E) stain. (b) Inclusions suggestive of tubular aggregates on Gömöri trichome ($\times 60$). (c) Nicotinamide adenine dinucleotide (NADH) ($\times 60$) positive staining seen in the tubular aggregate-like inclusions. (d) Esterase highlighting the neuromuscular junction (black arrow), tubular aggregates (blue arrow), and an angulated atrophic fiber (white arrow)

through targeted array CGH analysis with exon-level resolution, which revealed a homozygous deletion including at least exons 3–9 of the *SYT2* gene in P1 ($\text{arr}[\text{GRCh37}] 1\text{q}32.1(202565663_202574633)\times 0$).

Using whole exome sequencing we identified three homozygous bi-allelic loss of function variants in *SYT2* (ENST00000367267 in six patients of four families: P2:c.[927C>A];[927C>A];p.[(Tyr309*)]; [(Tyr309*)], P3, P6 and P7: c.[725dup]; [725dup]; p. [(Val243Glyfs*13)]; [(Val243Glyfs*13)], P4 and P5: c.[805G>T]; [805G>T]; p. [(Glu269*)]; [(Glu269*)]. The *SYT2* loss of function variants are scattered throughout the gene and do not seem to cluster in a specific domain (Figure 3c). Variants are classified as pathogenic (Strong: PS3, PS4, Moderate: PM2, PM3, Supporting: PP3, PP4) based on the 2015 American College of Medical Genetics and Genomics-Association for Molecular Pathology (ACMG-AMP) guideline for variant interpretation (Richards et al., 2015).

4 | DISCUSSION

To date, three independent dominantly acting heterozygous variants in *SYT2* have been reported in five families as a rare cause of slowly progressive distal motor neuropathy and myasthenic syndrome (Herrmann et al., 2014; Montes-Chinea et al., 2018; Whittaker et al., 2015). Here we report a series of seven patients with recessive loss of function variants in *SYT2*, clinically manifesting with a severe presynaptic CMS. Overall, we establish the role of *SYT2* in neuromuscular disease and expand its mutational mechanisms to include biallelic loss of function variants and its clinical spectrum to include a severe congenital disorder with predominant NMJ dysfunction.

Clinically, *SYT2*-deficient patients presented at birth with severe hypotonia and profound weakness significantly interfering with the attainment of motor milestones. The additional findings of fasciculations (P1 and P5) and apparent neurogenic MUAPs on EMG may

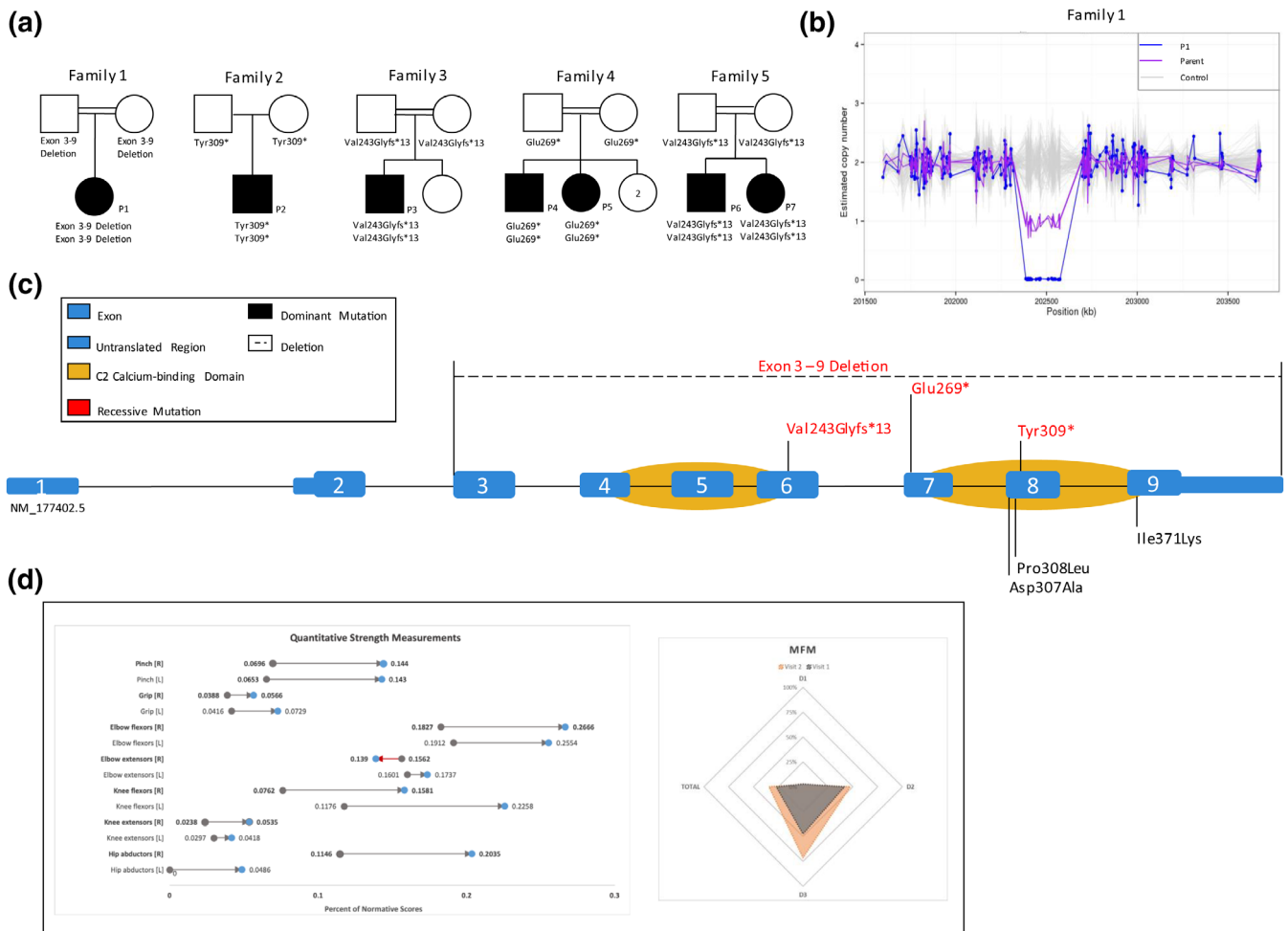


FIGURE 3 (a) Pedigrees of the patients with loss of function variants in *SYT2*. (b) Copy number variant analysis of the whole exome sequencing data identified an apparent homozygous loss of coverage of a portion of *SYT2* in P1 (blue) compared to control (gray), with each parent showing apparent heterozygosity for this deletion (purple). (c) New and reported human *SYT2* variants. The numbered, blue rectangles represent exons (RefSeq isoform NM_177402.5), while the orange ovals represent C2 calcium-binding protein domains. The slimmer blue rectangles represent untranslated region. The variants on the top half are reported in this series, while the variants on the bottom half have previously been reported. Variants in black represent dominant variants, while the ones in red represent recessive variants. The dotted line found on the top half of the figure represents a large deletion (Exons 3–9). (d) Normed quantitative strength measurements assessed using handheld dynamometry for P1 at visit 1 and visit 2 (left) and Radar graph representing MFM domain and total scores for P1 at visit 1 and visit 2, with the outer bounds of the chart representing 100% of total possible points in each domain (right). D1, standing and transfers; D2, axial and proximal motor function; and D3, distal motor function [Color figure can be viewed at wileyonlinelibrary.com]

phenotypically resemble lower motor neuropathy/neuronopathy (e.g., spinal muscular atrophy [SMA] or rarer causes of congenital onset SMA with respiratory failure such as those caused by variants in *IGHMBP2* and *LAS1L* [Butterfield et al., 2014; Grohmann et al., 2001]). The lack of fiber type grouping, a histologic hallmark of chronic neurogenic disease, as well as the overall mild changes on muscle biopsy compared to the profound level of weakness would not be consistent with a significant neuronopathy as the driver of the weakness, but would be more compatible with a dysfunctional NMJ. Additional histological clues are the tubular aggregates that were seen in P1's muscle biopsy, which have been reported in other forms of CMS. Namely CMS due to recessive variants in *GFPT1* or *DPAGT1* which encode enzymes in protein glycosylation pathways as well as tubular

aggregate myopathies caused by impaired intracellular Ca^{2+} handling (Bohm & Laporte, 2018; Chevessier et al., 2005). Post-exercise facilitation on RNS in P4 is consistent with this plausible pathophysiologic mechanism. Thus, we postulate that the profound muscle weakness seen in our *SYT2*-deficient patients is largely driven by significantly impaired presynaptic neuromuscular junction transmission, consistent with *SYT2*'s role, with an additional element of secondary axonal degeneration. Dedicated morphological analysis of the NMJ, the gold standard for evaluation of such disorders, was not available, however, and limited our ability to definitively characterize *SYT2*-related disease.

Brain MRI imaging was performed in four patients (P1, P2, P3 and P6). P1 and P6 had evidence of mild cerebral atrophy, including

thinning of the corpus, and P6 was also found to have a thin corpus callosum; the brain MRI in P2 and P3 was normal. Unfortunately, imaging was not available in the remaining patients. It remains unclear whether the brain findings are related to the SYT2-deficiency at this time, which however remains a possibility since presynaptic CMS as a whole are more likely to also include central nervous system manifestations. Thus, as additional patients are being recognized, this may very well be a more consistent finding of the SYT2-deficiency clinical spectrum. At this time, P1 is the only patient with cardiac involvement, it remains unclear whether this is related to the loss of SYT2, or whether there may be a second genetic etiology at play. It is also notable that arthrogyrosis, congenital brain malformations and epilepsy were not reported, which clinically distinguishes SYT2-deficiency from the various congenital motor neuropathies including pontocerebellar hypoplasias, and SMA with progressive myoclonic epilepsy (SMA-PME). Muscle fatigability, a hallmark of CMS, was difficult to assess in our SYT2-deficient cohort due to the severe weakness observed in all patients. Involvement of the ocular and facial muscles was seen in our patients. The profound congenital presentation is not unusual for CMS. More recently, patients with variants in presynaptic genes impacting neurotransmitter release were found to have more significant multisystemic involvement, including central nervous system. These proteins are often involved in SNARE-mediated vesicle fusion at the presynaptic nerve terminal with pathogenic variants resulting in a wide clinical spectrum ranging from a severe presynaptic CMS similar to SYT2-deficient patients, as seen in patients with pathogenic variants in *VAMP1*, to those with multisystemic involvement including cortical hyperexcitability, ataxia, and intellectual disability as seen in patients with pathogenic variants in *SNAP25B* (Rodríguez Cruz et al., 2018; Salpietro et al., 2017; Shen, Selcen, Brengman, & Engel, 2014).

Treatment options are available for selected CMS, depending on the individual molecular mechanism of NMJ dysfunction (Engel et al., 2015). Thus, an understanding of the underlying disease mechanism is imperative for a rational therapeutic choice to be made and to avoid inadvertent worsening of the condition. Given SYT2's role at the presynaptic NMJ, a trial of pyridostigmine was initiated in P1 following her genetic confirmatory diagnosis at age 14 years. P1 has a history of cardiac disease and baseline prolonged QT interval; therefore, a trial of 3,4-DAP was decided against, given the potential of cardiac side effects. The patient and her parents reported an increase in muscle strength and stamina, with an improvement in daily activities. At her follow up 12 months after starting pyridostigmine, motor improvements were observed with repeat manual muscle testing and quantified through various physical therapy measures, with an increase in total MFM score reaching MCID (Vuillerot et al., 2012). The initiation of therapeutic intervention in P1 was delayed given a lack of a confirmed genetic diagnosis, and earlier treatment may have resulted in a larger clinical response. A therapeutic response to an acetylcholinesterase was not a universal finding in SYT2-deficient patients, and in particular, P4 did not note any clinical improvement with a pyridostigmine trial.

SYT2 is part of the large synaptotagmin family, which is made up of synaptic vesicle membrane proteins that function as calcium

sensors and regulate neurotransmitter release at the presynaptic nerve terminal. SYT2 shares the highest sequence homology with SYT1, and they are often co-expressed with possible functional redundancy (Marqueze et al., 1995; Ullrich et al., 1994). In fact, overexpression of *Syt2* in cells from *Syt1* null mice was able to partially rescue the impaired exocytosis (Nagy et al., 2006). *Syt2* null mice were found to have moderate increase of *Syt1* in the spinal cord, which was not seen in the brain (Pang et al., 2006). Furthermore, developmental expression studies suggest that SYT1 is essential in pre- and early postnatal neuromuscular transmission, with an observed delayed expression and subsequent isoform switch to SYT2 (Berton, Iborra, Boudier, Seagar, & Marqueze, 1997; Kochubey, Babai, & Schneggenburger, 2016). Interestingly, de novo dominant variants in SYT1 were recently reported to cause a rare form of neurodevelopmental disorders (Baker et al., 2018). Unfortunately, access to patient tissue for further validation work is challenging as SYT2 is not expressed in human fibroblasts. Thus, we were unable to confirm a complete absence of SYT2 in our patients and to explore a potential compensatory upregulation of SYT1.

In our patients we see maximal manifestation of the phenotype at birth, without overt subsequent disease progression, indicating a possible phase of prenatal progression and a developmental role for SYT2 in humans. SYT2 deficiency in our patients may resemble the phenotype previously reported in *Syt2*-deficient mice, which were normal at birth but subsequently developed a rapid progressive motor dysfunction due to impaired synaptic transmission, with complete paralysis resulting in lethality at approximately 3 weeks of age (Pang et al., 2006). In addition, *Drosophila* synaptotagmin null mutants show early lethality. They have been reported to survive to adulthood, however, when placed on food, hence requiring limited movement, and they subsequently display motor defects with impaired synaptic transmission (Loewen, Mackler, & Reist, 2001).

Coincidentally, the SYT2-deficient mouse model was generated by replacing Exon 2 through 7 with a knock-in LacZ sequence, creating a null allele (Pang et al., 2006), which has a remarkable resemblance to P1 genetically (homozygous exon 3–9 deletion) and phenotypically. In this context it is notable that in humans SYT2 also does not seem very tolerant of loss of function variants. Only five null alleles are reported in gnomAD, all in heterozygous state only (probability of being loss-of-function intolerant (pLI) score of 0.98 and observed/expected (oe) score of 0.06). However, haploinsufficiency of SYT2 does not appear to cause disease, as the heterozygous carrier parents in this cohort did not report any symptoms. While the exact pathogenic mechanism of the previously reported dominant SYT2 missense variants remains largely unknown, they all impact the SYT2 calcium binding domains and are thought to impair Ca^{2+} binding in a dominant-negative manner, resulting in a loss of synaptic transmission beyond the haploinsufficiency state.

While the majority of SYT2 loss of function variants reported here can be accurately identified through standard next generation-based sequencing platforms, large deletions are typically missed. The partial deletion of SYT2 (Exon 3–9) identified in P1 was initially missed on whole exome sequencing but was

subsequently identified through research-based reanalysis of the WES data for copy number variants. Therefore, clinical recognition of this newly described SYT2-deficient phenotype is essential in facilitating appropriate diagnostic testing. Our series further establishes SYT2 as a CMS disease gene to now also include recessively acting loss of function variants, and expands its clinical spectrum to include severe congenital onset presynaptic CMS. Although not universal, some patients may benefit from CMS therapies directed toward improving the function of the presynaptic neuromuscular junction.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests to report.

AUTHOR CONTRIBUTIONS

Sandra Donkervoort, Payam Mohassel, Lucia Laugwitz, Rita Horvath, Tobias B. Haack, Carsten G. Bönnemann: Contributed to the concept, design, data collection, and manuscript writing. Maha S. Zaki, Corien C. Verschuuren-Bemelmans, Veronka Horber, Annemarie JM. Fock, Minal S. Jain, Victoria Biancavilla, Grace McMacken, Matthew Nalls, Nicol C. Voermans, Hasnaa M Elbendary, Molly Snyder, Chunyu Cai, Tanya Lehky, Susan T. Iannaccone, A. Reghan Foley: Contributed patient identification and characterization. Katherine R. Chao, Lucia Laugwitz, Erik-Jan Kamsteeg, Reza Maroofian, Riley M. McCarty, Valentina Stanley, Joseph Gleeson, Hanns Lochmüller, Henry Houlden, Tobias B. Haack: Contributed molecular data collection and interpretation. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings will be available in dbGAP and ClinVAR.

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