

RESEARCH ARTICLE

Novel synaptobrevin-1 mutation causes fatal congenital myasthenic syndrome

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Abstract

Objective: To identify the molecular basis and elucidate the pathogenesis of a fatal congenital myasthenic syndrome. **Methods:** We performed clinical electrophysiology studies, exome and Sanger sequencing, and analyzed functional consequences of the identified mutation. **Results:** Clinical electrophysiology studies of the patient revealed several-fold potentiation of the evoked muscle action potential by high frequency nerve stimulation pointing to a presynaptic defect. Exome sequencing identified a homozygous c.340delA frameshift mutation in synaptobrevin 1 (SYB1), one of the three SNARE proteins essential for synaptic vesicle exocytosis. Analysis of both human spinal cord gray matter and normal human muscle revealed expression of the SYB1A and SYB1D isoforms, predicting expression of one or both isoforms in the motor nerve terminal. The identified mutation elongates the intravesicular C-terminus of the A isoform from 5 to 71, and of the D isoform from 4 to 31 residues. Transfection of either mutant isoform into bovine chromaffin cells markedly reduces depolarization-evoked exocytosis, and transfection of either mutant isoform into HEK cells significantly decreases expression of either mutant compared to wild type. **Interpretation:** The mutation is pathogenic because elongation of the intravesicular C-terminus of the A and D isoforms increases the energy required to move their C-terminus into the synaptic vesicle membrane, a key step for fusion of the synaptic vesicle with the presynaptic membrane, and because it is predicted to reduce expression of either isoform in the nerve terminal.

Introduction

In most congenital myasthenic syndromes (CMS) identified to date, the disease protein resides in the postsynaptic region.¹ The first identified presynaptic disease proteins were choline acetyl transferase in 2001² and agrin in 2009.³ Next generation sequencing methods in recent years facilitated discovery of mutations in other presynaptic proteins essential for neuromuscular transmission. These include synaptotagmin 2,⁴ SNAP25B (synaptosomal-associated protein 25B),⁵ Munc13-1,⁶ myosin 9A,⁷ the high-affinity choline transporter,⁸ and the vesicular acetylcholine (ACh) transporter.⁹ Here, we describe the clinical features and molecular pathogenesis of another presynaptic CMS caused by a homozygous frameshift mutation in synaptobrevin 1 (SYB1, also known as VAMPI; OMIM:

185880). SYB1 is one of the three core SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) components of the synaptic vesicle fusion machinery. Synaptobrevin 1 attached to the synaptic vesicles is a v-SNARE; syntaxin and SNAP25B anchored in the target presynaptic membrane are t-SNAREs.¹⁰

Synaptobrevin has two homologous-related genes, SYB1 and SYB2. SYB2 is expressed at central synapses¹¹ except for a small population of hippocampal neurons that express SYB1.¹² SYB1 is expressed in spinal cord motor neurons and in motor nerve terminals.¹¹ SYB1 has four alternatively spliced isoforms (A-D) with three to five different C-terminal amino acids. Isoform 1B localizes to mitochondrial membranes.¹³ In preliminary studies, we detected isoforms 1A and 1D but not isoform C in spinal

cord anterior gray column as well as skeletal muscle and assumed that either the A or D or both isoforms are expressed at the motor nerve terminal.

Materials and Methods

Human studies

All human studies were approved by the Institutional Review Board of the Mayo Clinic and by Ethics Committee for Human Research, Hospital das Clinicas of the Federal University of Parana of Brazil. The parents gave written informed consent forms to participate in the study.

Genetic analysis

Genomic DNA was isolated from the patient's and from parents' blood by standard methods. Sanger sequencing was performed with PCR primers to sequence exons and flanking noncoding regions. Exome sequencing was performed at the Mayo Medical Genome Facility Sequencing Core. Paired-end libraries were prepared following the manufacturer's protocol (Agilent) using genomic DNA. Whole exome capture was carried out using the protocol for Agilent's SureSelect Human All Exon 71 MB v4 kit. The coverage was above 60 \times in all samples. The identified putative variants were scrutinized with Ingenuity Variant Analysis software (Qiagen, Redwood City, CA). We focused on variants in genes that affect quantal release from motor nerve terminals.

Expression of wild-type and mutant synaptobrevin in HEK cells

We analyzed cDNA isolated from spinal cord anterior gray column of an accident victim and from normal control skeletal muscle and detected isoforms 1A and 1D in both tissues. We then amplified the cDNAs of SYB1A and SYB1D and cloned each into pmCherry-C1 vector (Clontech, Mountain View, CA) with the tags at the N-terminal position. The mutation was engineered into wild-type SYB1-pmCherry-C1 plasmids using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). The mutation was verified by Sanger sequencing of the plasmid. HEK293T cells were transfected with mCherry-tagged wild-type or mutant isoforms 1A or 1D, or with mCherry-tagged empty plasmid using the TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI). Cells were harvested after 48 h of transfection. The transfections were done in triplicate. Extracts of transfected cells were immunoblotted with anti-mCherry-tag antibody (rat IgG fraction, ThermoFisher Scientific,

Invitrogen, Carlsbad, CA) followed by alkaline phosphatase-labeled goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA), and GAPDH antibody (mouse IgG fraction EMD Millipore Corp., Danvers, MA) followed by alkaline phosphatase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) to control for loading. The blots were developed by the alkaline phosphatase method, and quantitated using NIH Image 1.63.

Amperometric assay of exocytosis from chromaffin cells

Adult bovine adrenal glands were obtained from a local slaughter house. Chromaffin cells were prepared by collagenase digestion of medullary tissue followed by further separation from debris and erythrocytes by centrifugation on a Percoll gradient (GE-Amersham Biosciences, Uppsala, Sweden) as described.¹⁴ Approximately 3×10^5 chromaffin cells were electroporated with 8 μ g m-Cherry-tagged plasmids of the A or D isoforms of wild-type or mutant human SYB1 in 100 μ L resuspension buffer with a Neon electroporator (Life Technologies, Carlsbad, CA). To examine the effects of the transfected mutant and wild-type SYB1A and 1D on depolarization-induced exocytosis in the absence of SYB2 and SYB3 endogenous to chromaffin cells, we silenced them with two different green fluorescent siRNAs targeted against SYB2 and two different green fluorescent siRNAs targeted against SYB3 (prepared by Dharmacon, Lafayette, CO). Each siRNA contained 4–6 mismatched nucleotides for the human SYB1 cDNA. The siRNAs were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 1 day prior to transfection with SYB1 as described.¹⁵ Preparation of carbon fiber electrodes, and amperometric recordings from the depolarized chromaffin cells were done as previously described.⁵

Statistics

The data displayed in this article were compared by the two-tailed Student *t*-test. A *P* < 0.05 was considered significant.

Results

Clinical data

A Brazilian girl, the fourth child of first-cousin unaffected parents, was markedly hypotonic and could not feed well in the neonatal period. At 6 months of age, she lacked head control and could not sit without support. At 2 years of age, she had severe generalized hypotonia and muscle atrophy, slight arm and leg movements (Fig. 1A), mildly

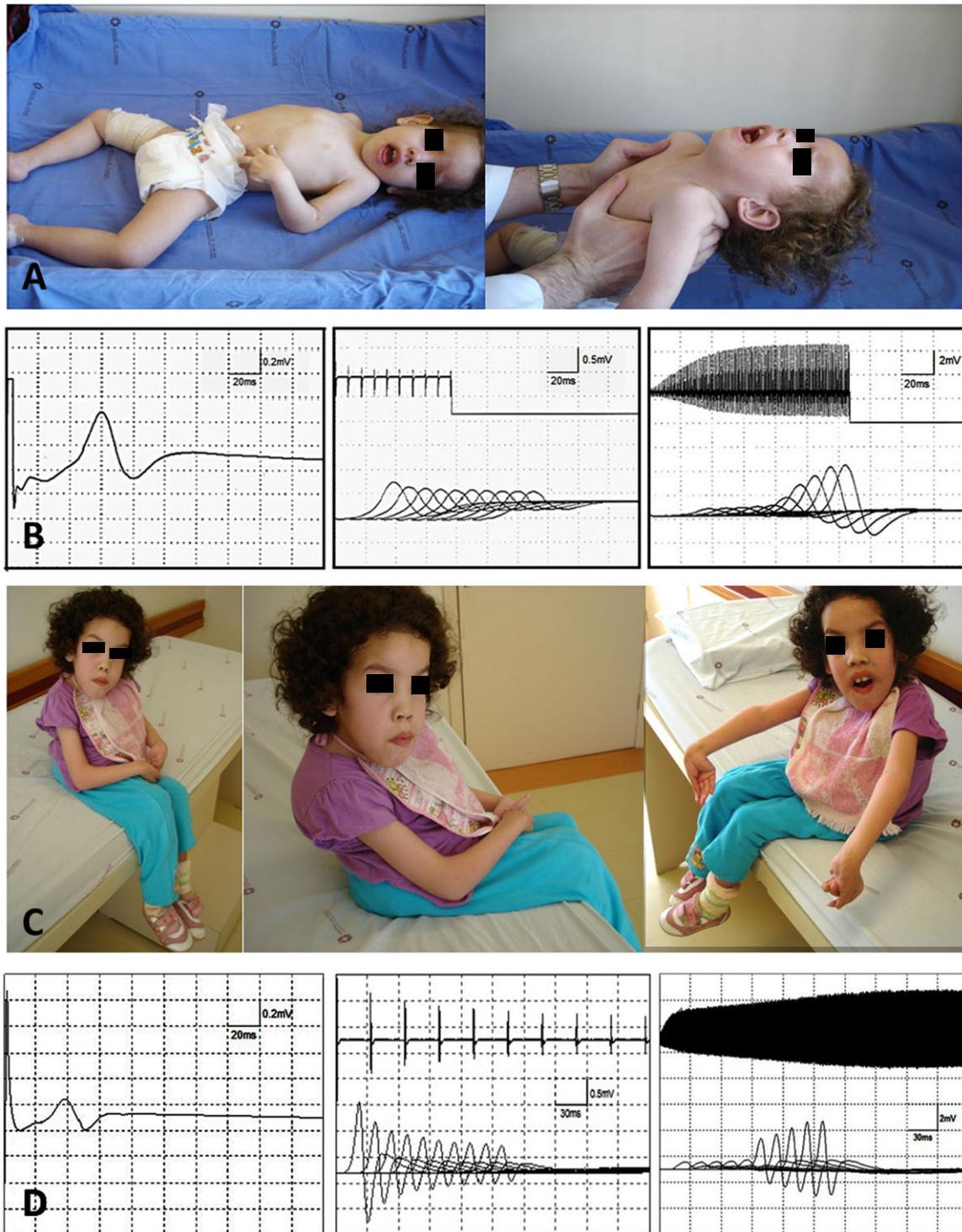


Figure 1. (A) Patient at age 2 years. Note dropped head, elongated face, severe weakness and atrophy of facial, bulbar, limb and axial muscles, lack of head control, and inability to sit unaided. (B) Electrophysiology study at age 2 years, tibial nerve stimulation: Left: Single supramaximal stimulus evokes a low-amplitude compound muscle action potential (CMAP). Center: Stimulation at 3 Hz elicits a decremental response with the fourth CMAP 25% smaller than the first CMAP. Right: 100 stimuli at 20 Hz increment the CMAP 8.8-fold. (C) Patient at age 8 years. Patient sits unaided and holds her head erect but cannot lift her arms, flex her wrists, or walk. Note kyphosis and lordosis, muscle atrophy, and short stature. (D) Electrophysiology study at age 8 years, tibial nerve stimulation. Left: Persistent low-amplitude CMAP. Center: Stimulation at 3 Hz elicits low-amplitude CMAPs with the fourth CMAP 60% smaller than the first CMAP. Right: 500 stimuli at 20 Hz increment the CMAP 6.5-fold.

restricted ocular ductions, and was areflexic. Her weakness worsened with respiratory infections but she had no episodes of dyspnea or apnea. Therapy with pyridostigmine slightly improved her arm and leg movements but she still could not feed well, support her head, or sit unaided. At 8 years of age, still receiving pyridostigmine, she was hypotonic and areflexic with severe facial, bulbar, limb and axial muscle weakness and atrophy, dropped head, kyphoscoliosis, and lordosis. She sat up unaided but could not walk (Fig. 1C); her speech was slurred but she was able to swallow. At age 14 years she remained diffusely hypotonic, had mildly limited ocular ductions with severe bulbar and limb but only mild facial muscle weakness, and remained areflexic. Shortly after her last visit she died of respiratory failure precipitated by a pulmonary infection. There was no history of similarly affected relatives.

Clinical electrophysiology studies at age 2 years revealed low-amplitude evoked compound muscle action potentials (CMAPs) on stimulation of the median, ulnar, deep peroneal, and posterior tibial nerves. Three-Hz stimulation of the same nerves evoked decremental responses, with the amplitude of the fourth evoked CMAP about 25% smaller than of the first evoked CMAP (Fig. 1B). Stimulation of the same nerves with 100 stimuli at 20 Hz increased the CMAP amplitude 5.2-, 3.8-, 2.3-, and 8.8-fold. The marked facilitation was like that observed in the Lambert-Eaton syndrome (Fig. 1B). Sensory nerve conduction velocities were normal.

Electrophysiology studies at the age of 8 years, gave similar results except the decremental response on 3-Hz stimulation had increased to 60% (Fig. 1D). Routine studies of blood, the serum CK level, and the brain MRI were normal. Biopsy specimens of the biceps brachii and quadriceps muscles showed only type 2 fiber atrophy.

Mutation analysis

Because the nerve stimulation studies pointed to a presynaptic defect of neuromuscular transmission, we focused on selected genes governing regulated fast synaptic vesicle exocytosis. Sanger sequencing of the *SYN1* (synapsin-1), *DNAJC5* (cysteine-string protein), *RAB3A* (Rab3A), *SYT1* and 2 (synaptotagmin 1 and 2), *STXBP1* (Munc18-1), *CPLX1* and *CPLX2* (complexin 1 and 2), and *SNAP25B* gave negative results. Next we performed whole exome sequencing using genomic DNA isolated from blood from the patient and her parents and analyzed the results with Ingenuity software to filter against common and benign variants. In the absence of similarly affected relatives, we searched for variants compatible with recessive as well as de novo inheritance that can affect quantal release from the motor nerve terminal. This revealed a frameshifting homozygous single-nucleotide deletion, c.340delA,

predicting to result in p.Ile114SerfrsTer72 for isoform A, and p.Ser114ValfsTer32 for isoform D, near the 3' end of *SYB1* in the patient, and a single heterozygous mutation in each parent. Sanger sequencing confirmed these findings and also identified a single heterozygous mutation in two of the patient's three siblings. According to ExAC browser (<http://exac.broadinstitute.org>), this mutation is present at heterozygosity in one of 11,574 Latino alleles but not in other ethnic groups.¹⁶

Neuronal synaptobrevins are anchored in synaptic vesicles by their C-terminal domains.^{17,18} The identified frameshift mutation elongates the intravesicular C-terminus of SYB1A (NM_014231.4; NP_055046.1) from 5 to 71 residues, and that of SYB1D (NM_199245.2; NP_954740.1) from 4 to 31 residues (see red bars in Fig. 2A). The heterozygous parents and siblings are unaffected.

Expression of mutant and wild-type SYB1A and SYB1D in HEK Cells

Immunoblot analysis of the A and D isoforms of wild-type and mutant SYB1 expressed in HEK293T cells revealed double bands (Fig. 2B), due to hydrolysis of the m-Cherry tag during preparation of the cell extract.¹⁹ The upper bands represent the fusion protein of intact m-Cherry and isoform A or D of wild-type or mutant SYB1. The lower bands represent the fusion protein of degraded m-cherry and isoform A or D of wild-type or mutant SYB1. For each lane, protein expression was estimated from the sum of both bands. Expression of the mutant A isoform was reduced to 15%, and that of the mutant D isoform to 68%, of the corresponding wild-type isoform.

Transfection of chromaffin cells with mutant SYB1A and SYB1D inhibits exocytosis of catecholamine-containing vesicles

Bovine chromaffin cells naturally express SYB2 and SYB3.²⁰ To examine the functional effect of mutant human SYB1A and D on synaptic transmission, we used amperometry to measure depolarization-evoked exocytosis of dense-core vesicles from chromaffin cells after forced expression with wild-type and mutant A and D isoforms.²¹ In this system, exocytosis of each vesicle is signaled by a single temporally resolved spike.^{22–24} The area of each spike is a measure of the charge generated by oxidation of the released catecholamine; the number of released molecules is obtained by dividing the generated charge by Faraday's constant.

Figure 3 and Table 1 show depolarization evoked exocytosis monitored by amperometry during the first minute after depolarization. Chromaffin cells transfected with wild-type SYB1A or D generate similar spikes (upper

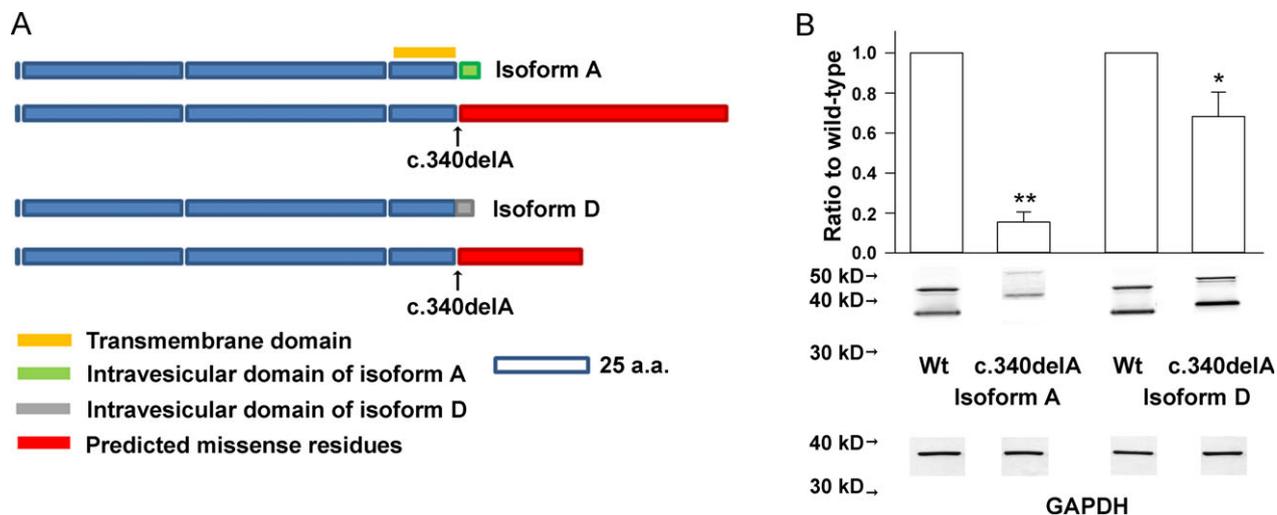


Figure 2. (A) The 340delA variant elongates the C-terminal vesicle domains of the A and D isoforms to 71 and 31 residues, respectively (red bars). Unique C-terminal amino acids are shown in green for isoform A and in gray for isoform D. Bar at the top indicates position of the transmembrane domains (aa 97-113) in yellow, the vesicle domain of isoform A (aa 114-118) in green, and the vesicle domain of isoform D (aa 114-117) in grey. (B) Immunoblots of wild-type and mutant SYB1 isoforms. Bars and vertical lines indicate mean and one standard error of three transfections. Expression levels were normalized for endogenous GAPDH expression. Each lane displays double bands due to hydrolysis of the m-Cherry tag during the preparatory procedure.¹⁹ The upper bands for lanes 1 to 4 represent intact m-cherry (~30 kD) fused with wild-type isoform A (13 kD), mutant isoform A (20 kD), wild-type isoform D (~13 kD), and mutant isoform D (~16 kD). The lower bands for lanes 1 to 4 represent the degraded m-Cherry (~24 kD) fused with wild-type isoform A (~13 kD), mutant isoform A (20 kD), wild-type isoform D (~13 kD), and mutant isoform D (~16 kD). For each lane, protein expression was estimated from the sum of both bands. Compared to the corresponding wild type, the mutant A isoform expresses at a lower level than the mutant D isoform. *P*: **<0.0001; *<0.05.

traces in panels A and B) and exocytose vesicles at rates similar to depolarized untransfected cells (open and closed circles, in panels C and D). In contrast, the mutant *SYB1A* or *SYB1D*-transfected cells generate smaller spikes (lower traces in panels A and B) and exocytose vesicles at a much slower rate (triangles, panels C and D), reducing the released catecholamine molecules to only 2% and 12% of that released by wild-type transfected cells, respectively (Table 1).

In additional experiments we also transfected chromaffin cells with wild-type and mutant *SYB1A* and *SYB1D* after silencing endogenous *SYB2* and *SYB3* expression. Depolarization of these cells showed findings similar to those obtained after forced transfection of the native chromaffin cells with *SYB1A* and *SYB1D* (Fig. 4).

Discussion

Our patient presented at birth with severe weakness, hypotonia, limited ocular ductions, and bulbar symptoms. The initial low amplitude of single evoked muscle action potentials and their marked potentiation by high frequency nerve stimulation (see Fig. 1B and D) pointed to a presynaptic defect like that seen in the Lambert-Eaton syndrome²⁵ and in the presynaptic CMS caused by defects in synaptotagmin 2,⁴ SNAP25B,⁵ and MUNC13-1.⁶

Synaptobrevin is the most abundant synaptic vesicle protein with 70 copies per vesicle, yet 1-3 copies are sufficient for exocytosis. A large vesicular pool of the protein is maintained by adaptor protein 180 which is crucial for synaptic vesicle reformation and efficient neurotransmission.²⁶ Synaptobrevin also interacts with synaptophysin in the synaptic vesicles with six synaptophysin molecules binding to synaptobrevin dimers to form a hexameric ring that directionally orients synaptobrevin toward the presynaptic membrane.²⁷ Exocytosed synaptobrevin and synaptophysin homo- or hetero-oligomerize into clusters which clears synaptobrevin from the active zone preventing short term depression.²⁸

The transmembrane domains of different synaptobrevin isoforms comprise 17 flexible amino acids which may function as catalysts for fusion of the vesicle with the presynaptic membrane.²⁹ The C-terminal intravesicular portion of wild-type *SYB1A* and *SYB1D* contains five and four amino acids, respectively.^{17,18} In our patient, C-terminals of both synaptobrevin isoforms 1A and 1D are markedly elongated. The addition of even two extra residues to the C-terminus of synaptobrevin 2 has been noted to inhibit exocytosis by increasing the energy required to move the C-terminal into the synaptic vesicle membrane which is a key step for vesicle fusion.³⁰⁻³² This finding, which likely also applies to the mutant A and D isoform of synaptobrevin 1, provides a plausible explanation for why

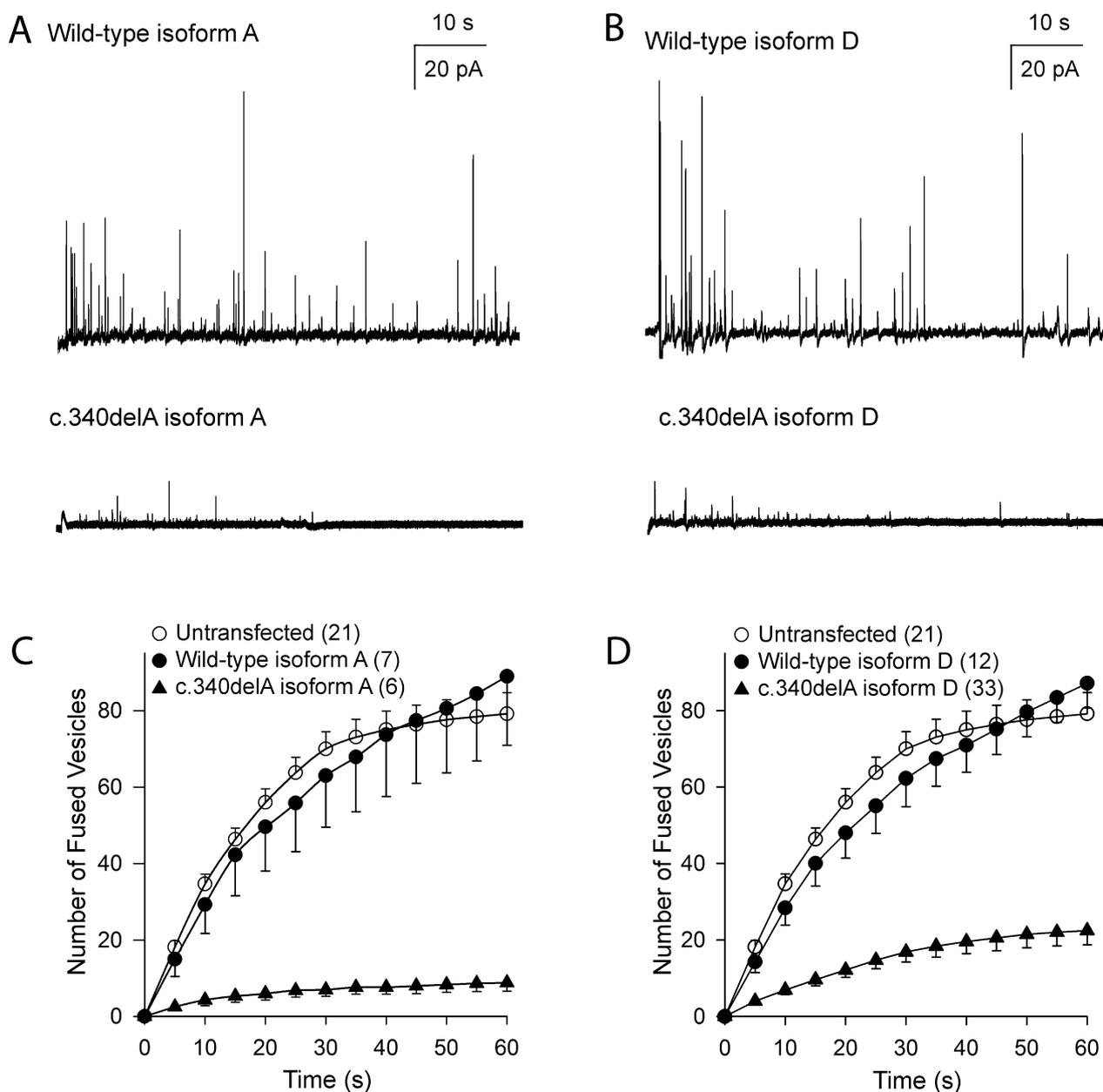


Figure 3. (A) and (B) Representative amperometric traces from depolarized chromaffin cells transfected with isoforms A and D of wild-type and mutant SYB1. Each spike represents a single exocytotic event. Cells transfected with mutant SYB1A or SYB1D generate fewer and lower amplitude spikes than the corresponding wild-type transfected cells. (C) and (D) Cumulative exocytotic events during the first minute after depolarization of cells transfected with wild-type or mutant isoform A (C) or isoform D (D). Numbers in parentheses indicate numbers of tested cells for each curve. For each group of cells, each point indicates the mean number of spikes over 5 sec. Vertical lines indicate one standard error. Cells transfected with either mutant Syb1A or D exocytose vesicles at a much lower rate than untransfected or wild-type-transfected cells.

expression of either mutant isoform severely depresses the exocytosis from depolarized chromaffin cells, and why the mutant A isoform with the longer intravesicular segment suppresses exocytosis to a greater extent than the mutant D isoform (see Figs. 2B and 3, Table 1).

A c.190G>T null mutation in *Syb1* had been observed in the lethal-wasting (*lew*) mouse mutant. The mutation

truncates nearly half the protein and disrupts all alternative splice forms of the gene. The homozygous animals show muscle wasting and no movement, and die before weaning.³³ Heterozygous animals are unaffected. In homozygous mutants the diaphragm EPs on postnatal day 14 are structurally normal but the miniature EP potential frequency and the EPP amplitude are reduced

Table 1. Amperometry studies of bovine chromaffin cells overexpressing A and D isoforms of wild-type and mutant synaptobrevin 1.

	Wild-type Isoform A	Mutant Isoform A	<i>P</i> *	Wild-type Isoform D	Mutant Isoform D	<i>P</i> *
Fusion events, min ⁻¹	89.0 ± 18.0 (7)	8.8 ± 2.2 (6)	<0.002	87.2 ± 6.5 (12)	22.4 ± 3.7 (33)	<0.001
Spike amplitude, pA	29.5 ± 2.0 (585)	12.2 ± 1.2 (33)	<0.05	30.2 ± 1.5 (1033)	8.9 ± 0.6 (696)	<0.001
Total charges detected, pC min ⁻¹	54.7 ± 12.9 (7)	1.2 ± 0.6 (6)	<0.003	46.2 ± 10.9 (12)	5.4 ± 1.10 (33)	<0.001
Total catecholamine molecules released ¹ , min ⁻¹	170.7 ± 40.3 E6 (7)	3.7 ± 1.8 E6 (6)	<0.003	144.2 ± 34.2 E6 (12)	16.8 ± 3.5 E6 (33)	<0.001

Values indicate mean ± SE. Numbers in parenthesis indicate number of cells except for spike amplitude where they indicate number of spikes.

*Determined by two-tailed *t*-test.

¹Derived by Faraday's law $Q = zFM/N_A$, where *Q* is charge in redox transfer; *z*, electrons transferred per molecule (2 for catecholamine); *F*, Faraday constant; *M*, number of reactant molecules; *N_A*, Avogadro's number.

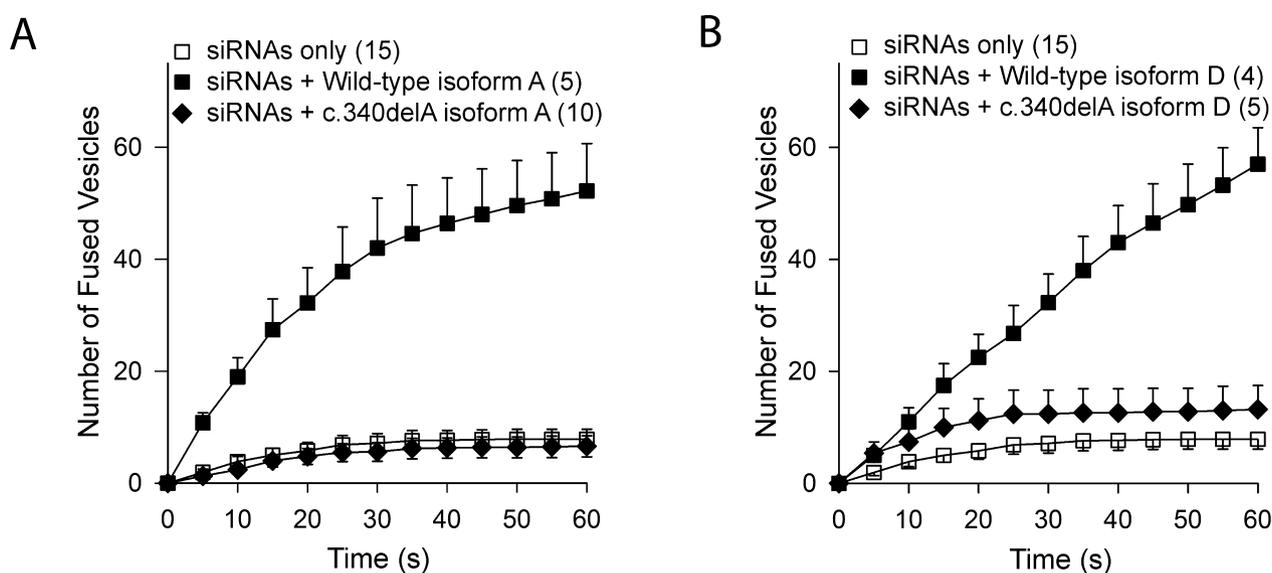


Figure 4. (A) Amperometry studies of bovine chromaffin cells cotransfected with siRNA and with wild-type or mutant isoform A. (B) Amperometry studies of bovine chromaffin cells cotransfected with siRNA and with wild-type or mutant isoform D. Numbers in parentheses indicate numbers of transfected cells for each curve. The graphs show cumulative exocytotic events during the first minute after depolarization. For each group of cells, each point indicates the mean number of spikes over 5 sec. Vertical lines indicate one SE. Cells transfected with either mutant Syb1A or Syb1D exocytose vesicles at a much lower rate than the corresponding wild-type-transfected cells.

to <40% of the control values, and stimulation at 30 Hz increases the EPP amplitude by 1.4-fold.¹¹

Interestingly, a dominant mutation in *SYB1* had been reported to result in hereditary spastic ataxia in Newfoundland families in Canada (MIM number 108600).³⁴ The symptoms included lower limb spasticity, ataxia, dysphagia, dysarthria, and eyelid ptosis presenting in the second decade of life. The mutation c.340+2T>G in *SYB1A* disrupts mRNA splicing by changing donor site resulting in loss of the isoform. The same mutation c.342T>G in *SYB1D* results in a missense amino acid change p.Ser114-Arg. The authors speculate that the haploinsufficiency of *SYB1A* isoform determines phenotype. However, our patient's parents have no neurologic symptoms, and each

carries a deletion mutation that elongates the *SYB1A* isoform and makes it nonfunctional. One plausible explanation for this discrepancy would be that in the Newfoundland families the missense mutation in *SYB1D* causes disease by the dominant effect.

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Conflict of Interest

None of the authors declare any conflict of interest.

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