Vesicular acetylcholine transporter defect underlies devastating congenital myasthenia syndrome

ABSTRACT

Objective: To identify the genetic basis of a recessive congenital neurologic syndrome characterized by severe hypotonia, arthrogryposis, and respiratory failure.

Methods: Identification of the responsible gene by exome sequencing and assessment of the effect of the mutation on protein stability in transfected rat neuronal-like PC12A123.7 cells.

Results: Two brothers from a nonconsanguineous Yemeni Jewish family manifested at birth with severe hypotonia and arthrogryposis. The older brother died of respiratory failure at 5 days of age. The proband, now 4.5 years old, has been mechanically ventilated since birth with virtually no milestones achievement. Whole exome sequencing revealed homozygosity of \( SLC18A3 \) \( c.1078G\rightarrow C, p.\text{Gly}360\text{Arg} \) in the affected brothers but not in other family members. \( SLC18A3 \) \( p.\text{Gly}360\text{Arg} \) is not reported in world populations but is present at a carrier frequency of 1:30 in healthy Yemeni Jews. \( SLC18A3 \) encodes the vesicular acetylcholine transporter (VAChT), which loads newly synthesized acetylcholine from the neuronal cytoplasm into synaptic vesicles. Mice that are VAChT-null have been shown to die at birth of respiratory failure. In human VAChT, residue 360 is located in a conserved region and substitution of arginine for glycine is predicted to disrupt proper protein folding and membrane embedding. Stable transfection of wild-type and mutant human VAChT into neuronal-like PC12A123.7 cells revealed similar mRNA levels, but undetectable levels of the mutant protein, suggesting post-translational degradation of mutant VAChT.

Conclusion: Loss of function of VAChT underlies severe arthrogryposis and respiratory failure. While most congenital myasthenic syndromes are caused by defects in postsynaptic proteins, VAChT deficiency is a presynaptic myasthenic syndrome.

GLOSSARY

ChAT = choline acetyltransferase; CMS = congenital myasthenic syndromes; VAChT = vesicular acetylcholine transporter.

Congenital myasthenic syndromes (CMS) are a heterogeneous group of genetic disorders characterized by early-onset weakness and fatigue of skeletal muscles with no involvement of the immune system. CMS are traditionally classified into 3 categories based on the location of the affected protein within the neuromuscular junction (figure 1).1-3 However, some proteins have both presynaptic and postsynaptic effects.4 Most CMS cases are due to defects in postsynaptic proteins, some 10% to defects in synaptic proteins, and fewer than 5% to defects in presynaptic proteins. Mutations in more than 20 genes that affect the structure and function of the neuromuscular junction have been identified so far1-3 (table 1). Presynaptic defects can affect the synthesis and release of acetylcholine from nerve terminals.1,5,6 Several genes were reported to underlie presynaptic CMS, including \( CHAT \), encoding choline acetyltransferase, which is critical for the synthesis of acetylcholine in neurons; \( MUNC13 \), \( SYT2 \), and \( SNAP25B \), which are required for priming the synaptic vesicles for exocytosis and calcium-evoked transmitter release;

*These authors contributed equally to this work.
and $GFPT1^{10}$ and $DPAGT1$, $^{11}$ which glycosylate nascent peptides. In this report, we describe a devastating CMS due to a defect in a different presynaptic protein and the underlying genetics.

**METHODS**

Standard protocol approvals, registrations, and patient consents. The study was approved by the Institutional Review Board of Shaare Zedek Medical Center and the Israel National Ethics Committee for Genetic Studies (protocol 20/10) and the University of Washington Institutional Review Board. Blood samples were obtained after written informed consent.

Genomic analyses. Genomic DNA was extracted from peripheral blood. Whole exome sequencing $^{12,13}$ and homology mapping $^{14}$ were performed as described previously. Mutation and haplotype analyses were performed using primers listed in table e-1 at Neurology.org.

Homology modeling. Homology models of human vesicular acetylcholine transporter (VAChT) structure were generated using Phyre2 in normal modeling mode. $^{15}$ The best homology model based on the YajR transporter of *Escherichia coli*, protein data bank (PDB) ID 3WDO, was chosen and embedded in a lipid bilayer that appears in the MemProtMD database. $^{16}$ The structural effects of the Gly360Arg mutation were assessed using PyMol (The PyMOL Molecular Graphics System, version 1.3, Schrödinger, LLC, New York, NY).

Gene expression via stable transfection of neuronal-like PC12 $^{24-27}$ cell lines. PC12 $^{24-27}$ cell culture, site-directed mutagenesis, stable transfection, selection of stable clones, and Western blot analysis were performed as previously described. $^{17}$
cDNA for VACHT was obtained from Invitrogen (Carlsbad, CA) and transfected into pcDNA 6.2/V5-destination vector using the LR Recombinase (Invitrogen). Mutations in VACHT were made using the Stratagene (San Diego, CA) QuickChange kit according to the manufacturer’s instructions. Plasmids were purified from XL1-Blue super-competent cells (Stratagene) using a commercial kit (Qiagen, Venlo, Netherlands). Mutant plasmids were verified by Sanger sequencing. Purified plasmids were transfected into PC12A123.7 cells with lipofectamine in antibiotic-free complete medium. Blastcidin (10 μg/mL) was added to each plate to select for stable transfectants, which required 2–3 weeks of selection. To evaluate transcript levels, mRNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO) from untransfected and transfected lines and reverse transcribed to cDNA with ImProm-II Reverse Transcriptase (Promega, Madison, WI) using random hexamers and RNase inhibitor (RNasin; Promega). Quantitative real-time PCR analysis for VACHT protein levels measured presented in 25 clonal lines were evaluated by Western blot using standard techniques with primary antibody goat anti-VACHT (sc-7717) and secondary antibody donkey anti-goat immunoglobulin G horseradish peroxidase (sc-2020), both from Santa Cruz Biotechnology (Dallas, TX). Western blot ladder was Magic Marker XP (Invitrogen; Thermo Fisher Scientific, Waltham, MA). Western blots were imaged with a GE (Cleveland, OH) Typhoon Trio scanner.

**RESULTS** Clinical features. We describe 2 affected siblings born to nonconsanguineous parents of Yemenite Jewish extraction (figure 2). Both siblings were born at term (gestational ages 37 and 40 weeks) after uneventful pregnancies with minimal follow-up. Appgar scores were 4/5 and 3/3 at 1 and 5 minutes after birth, respectively. Birthweights were at the 10th percentile (2.416 and 2.670 Kg) and head circumferences at the 25th centile (32 and 33 cm). Both affected siblings had retrognathia, severe axial and peripheral hypotonia with distal arthrogryposis in all extremities (lower worse than upper), bilateral dislocated hips, bilateral descended testes, microopenis, and marked hirsutism. They were extremely hypotonic and needed mechanical ventilation. The first child died of respiratory insufficiency at age 5 days. Karyotype was normal, and SMN exon 7 deletion was ruled out; thus spinal muscular atrophy was unlikely. Brain CT demonstrated delayed myelination with normal size and structure of ventricles and corpus callosum. A large patent ductus arteriosus, relatively small

### Table 1
Clinical characteristics and treatments for congenital myasthenic syndromes, including vesicular acetylcholine transporter (VACHT) deficiency described here

<table>
<thead>
<tr>
<th>Clinical syndrome</th>
<th>Relative frequency, %*</th>
<th>Main clinical characteristics</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Presynaptic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline acetyltransferase (ChAT) deficiency</td>
<td>&lt;5</td>
<td>Onset at birth or early infancy; episodic respiratory distress and apnea, triggered by infections; no ptosis or ophthalmoplegia</td>
<td>AChE inhibitors, 3,4-DAPb</td>
</tr>
<tr>
<td>VACHT deficiency</td>
<td>Rare</td>
<td>Onset at birth, severe arthrogryposis, severe generalized weakness, respiratory failure, no ptosis or ophthalmoplegia</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Synaptic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholinesterase (COLQ) deficiency</td>
<td>6-12</td>
<td>Onset at early infancy or later; progressive proximal weakness and poor feeding; chronic hypventilation and respiratory crises during childhood; ophthalmoplegia and ptosis in 50%–80%</td>
<td>Ephedrine, salbutamolc</td>
</tr>
<tr>
<td>Postsynaptic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine receptor deficiency</td>
<td>30-35</td>
<td>Onset at early infancy, poor feeding, ptosis, weakness; usually stable course, severe ophthalmoplegia develops during first year</td>
<td>AChE inhibitors, 3,4-DAP,b; Fludrinred</td>
</tr>
<tr>
<td>Rapsyn deficiency</td>
<td>15-20</td>
<td>Onset at birth, feeding and respiratory difficulties and generalized weakness improve after childhood; life-threatening crises occur during infancy and early childhood; ptosis, strabismus, no ophthalmoplegia</td>
<td>AChE inhibitors, 3,4-DAPb</td>
</tr>
<tr>
<td>Downstream of kinase-7 deficiency</td>
<td>10-20</td>
<td>Onset at early childhood, recurrent falls, slowly progressive course, facial and limb girdle weakness, ptosis, no ophthalmoplegia</td>
<td>Ephedrine, salbutamolc</td>
</tr>
<tr>
<td>Slow channel syndrome</td>
<td>10</td>
<td>Variable onset: birth to adulthood, weakness in limbs (more upper and distal) and neck, mild ptosis and ophthalmoplegia</td>
<td>Fluoxetine, quinidine^d</td>
</tr>
<tr>
<td>Fast channel syndrome</td>
<td>5</td>
<td>Onset at birth, mild arthrogryposis; severe generalized weakness, feeding and respiratory difficulties; severe life-threatening crises; severe ptosis and ophthalmoplegia</td>
<td>AChE inhibitors, 3, 4-DAPb; Ephedrine^e</td>
</tr>
<tr>
<td>Glycosylation defects*</td>
<td>5</td>
<td>Onset at early childhood, recurrent falls, limb-girdle weakness with stable course; mild ptosis, no ophthalmoplegia; tubular aggregates in muscle biopsy</td>
<td>AChE inhibitors, 3,4-DAP,b; Ephedrine^f</td>
</tr>
</tbody>
</table>

*Relative frequency is based on references 1, 3, and 5.

b3,4-Diaminopyridine (3,4-DAP) is a potassium channel blocker that prolongs presynaptic depolarization and exocytosis of synaptic vesicles.

cEphedrine and salbutamol stabilize postsynaptic structures through p2-adrenergic receptors.

dFluoxetine and quinidine are open channel blockers.

*Glycosylation defects due to mutations in **GFP**T1, DPAGT1, ALG2, and ALG14.
kidneys, and normal thymus, liver, spleen, and pancreas were visualized.

The second affected sibling, currently age 4.5 years, has been ventilated since birth. He has profound global developmental disability with extreme hypotonia. At age 2 months, he had severe necrotizing enterocolitis complicated by small intestine perforation. Serial head ultrasounds at the first 3 months of life were normal. At 3 months, brain atrophy was first noticed in head ultrasound and CT. Normal kidneys, thymus, liver, spleen, and pancreas were visualized. Brainstem evoked response audiometry revealed a hearing threshold of 60 and 80 dB in the right and left ears and prolonged absolute latencies and interpeak interval latencies for waves I to VI with prolonged interaural difference. His current neurologic examination is characterized by progressive microcephaly (33 cm at birth, 37 cm at 4 months, and 42 cm at 4 years [−7 SD]), horizontal nystagmus, and severe hypotonia with absent deep tendon reflexes and minimal voluntary movements in the upper limbs and eyes. He recognizes his caregivers, tracks, smiles socially, and enjoys company and music. His family wished to refrain from therapeutic trials or neurophysiologic studies.

Gene discovery. Whole exome sequencing of genomic DNA of the proband was carried out with median 105-fold coverage with >95% of targeted exons having >10 high-quality reads. DNA from the deceased child was not suitable for whole exome sequencing, although adequate for individually targeted PCR. In interpreting the variant profile of the proband, we considered the historical endogamy of the Yemeni Jewish community. Many generations of marrying within the community suggested the possibility of shared ancestry of the parents, despite their not being closely related, and thus the possibility of a critical homozygous allele. The proband was homozygous for 2 rare potentially damaging missense mutations. SLC18A3 c.1078G>A, p.Gly360Arg (NM_003055.2), at chr10:50,819,864 in a 2.3 MB region of homozygosity, was present as a heterozygote in 9 of 60,000 individuals from combined world populations (exac.broadinstitute.org). NECAB2 c.920G>A, p.R307H (NM_019065.2), at
chr16:84,031,880 in a 2.5 MB region of homozygosity was present as a heterozygote in 3 of 60,000 individuals. Both amino acid substitutions occurred at sites completely conserved in all sequenced vertebrates, and both were predicted to be damaging by in silico tools. DNA from the deceased affected child, both parents, and 3 unaffected children was genotyped. Each mutation was homozygous in both affected children, heterozygous in both parents, and either heterozygous or absent in the unaffected children (figure 2). Thus both variants were co-inherited with the myasthenia phenotype under a recessive model. Among unrelated healthy controls of Yemeni Jewish ancestry, SLC18A3 p.Gly360Arg was inherited in 4 of 166 participants (allele frequency 0.012), yielding an estimated frequency of homozygotes in this community of 1 in 7,000 newborns. NECAB2 p.R307H was inherited in 7 of the 108 controls (allele frequency 0.032), yielding an estimated frequency of homozygotes in this community of 1 in 1,000 newborns.

We decided to focus functional studies on the SLC18A3 mutation, because SLC18A3 encodes the vesicular acetylcholine transporter VACht, which is known to function in the same pathway as choline acetyltransferase (ChAT) (figure 1), mutations in which cause congenital myasthenia (table 1). Indeed, the SLC18A3 locus is entirely embedded in the first intron of CHAT. Furthermore, the VACht-null mouse dies shortly after birth due to respiratory failure. In contrast, the homozygous null mouse for NECAB2, which encodes a neuronal calcium binding protein, is healthy, with increased body weight as the only phenotypic feature.

In silico structural analysis of Gly360Arg mutation. The presynaptic vesicular transporter VACht is composed of 12 transmembrane α-helices that enable loading of cytoplasmic acetylcholine into the presynaptic vesicle, which under conditions of long-term high-level ACh demand can be the rate-limiting step in cholinergic neurotransmission. Gly360 is located in the beginning of the ninth transmembrane α-helix. It is embedded within the hydrophobic membrane near the cytoplasm. Its substitution by the hydrophilic and positively charged arginine disfavors embedding in the hydrophobic membrane. Gly360 is part of a postulated GXXG motif, G360-A361-L362-G363 (underlined), which is highly conserved in vertebrates. Mutation of Gly360 to arginine breaks the GXXG motif and is predicted to result in a partially misfolded protein that is degraded more readily.

Wild-type and mutant VACht protein levels in stably transfected rat neuronal cell lines. In order to experimentally evaluate VACht p.Gly360Arg, wild-type and mutant human VACht expression vectors were stably transfected into the rat pheochromocytoma cell line PC12, which contains multiple synaptic vesicles but lacks endogenous VACht.
expression. RT-PCR analysis of VACHT mRNA in stably transfected Gly360Arg mutant and WT clonal cell lines revealed comparable VACHT mRNA levels in all cell lines (figure e-1). However, Western blot analysis of the same colonies demonstrated undetectable protein levels in all the mutant VACHT lines, whereas the wild-type VACHT protein was clearly present (figure 3C). Taken together, these results suggest that the Gly360Arg mutant VACHT protein undergoes posttranslational degradation.

DISCUSSION

Myasthenia is caused by any flaw in the complex process of cholinergic neurotransmission at the neuromuscular junction. Most cases of myasthenia in adolescents and adults are caused by blockage of the postsynaptic acetylcholine receptors by circulating antibodies. In infants and young children, most cases of myasthenia result from an inherited defect in the neuromuscular junction (figure 1) and do not involve immune mechanisms. The 2 brothers described here have a severe form of congenital myasthenia syndrome, caused by a homozygous mutation in \( SLC18A3 \) leading to loss of function of the vesicular acetylcholine transporter VACHT.

Under normal conditions, acetylcholine is synthesized in the neuronal cytoplasm by ChAT and then packed into the presynaptic vesicles by VAChT prior to being released by exocytosis (figure 1). Synaptic vesicles contain very high concentrations of acetylcholine molecules that form a quantum to guarantee neuromuscular transmission. VACHT is a very slow transporter and likely to be the rate-limiting step in cholinergic neurotransmission.21,24 Both ChAT and VACHT are crucial for cholinergic neurotransmission and proper wiring of the neuromuscular junction during embryonic development. Absence of either ChAT or VACHT is lethal,25,26 and reduced expression of either ChAT or VACHT causes myasthenia and cognitive deficits.27

In our patients, the complete loss of function of VACHT apparently disrupts cholinergic neurotransmission, resulting in severe hypotonia and respiratory failure, similar to the phenotype of VACHT-null mice. The highly conserved glycine at residue 360 is located within the hydrophobic membrane bilayer and is likely to be essential for the tight packing of the transmembrane \( \alpha \)-helix. Its substitution by the positively charged, hydrophilic arginine is predicted to disrupt proper folding and membrane embedding, leading to rapid degradation, consistent with the absence of VACHT mutant protein in our experimental results. Acetylcholine is also an important neurotransmitter in the CNS, and reduced VACHT activity results in severe central as well as peripheral nervous system deficits.28–30 consistent with the phenotype of our patients. Animal studies demonstrated cognitive and behavioral deficits with only 40%–50% decrease in VACHT expression,27,31–33 suggesting that central cholinergic synapses are even more sensitive to decreased VACHT activity than peripheral synapses, where symptoms appear only after 70% decrease in VACHT expression.27

Notably, mutations in other genes reported to underlie CMS with presynaptic defects are also frequently associated with CNS manifestations. These include genes that encode presynaptic proteins that regulate vesicular exocytosis (MUNC13, SNAP25B) and proteins important for glycosylation, a more general cellular process (DPAGT1 and GFPT1). MUNC13 is required for docking and priming of synaptic vesicles in cholinergic neuromuscular synapses as well as in central glutamatergic synapses. Homozygous MUNC13 nonsense mutations result in severe global disability.7 SNAP25B is a SNARE protein essential for exocytosis of synaptic vesicles from nerve terminals. Global disability, milder than that reported in MUNC13 mutation homozygotes, was observed in a single case heterozygous for a SNAP25B missense mutation.9 SYT2 encodes synaptotagmin II, involved in calcium-evoked vesicular transmitter release, and heterozygotes for SYT2 mutations have even milder motor symptoms with no CNS manifestations.10 Hypoglycosylation of synaptic-specific proteins due to recessive mutations in DPAGT111 and GFPT110 can functionally impair both central and motor synapses and CNS symptoms were observed even in cases with relatively mild motor deficits. This is likely explained by the wider implications of glycosylation defects.

Finally, 2 patients with a relatively mild presynaptic CMS, from 2 different families, were recently reported to have recessive mutations in \( SLC18A3 \).34 The first patient, a 14-year-old boy, is a compound heterozygote for a genomic deletion including \( SLC18A3 \) and for \( SLC18A3 \) p.Gly186Ala. He presented with ptosis, ophthalmoplegia, and mild facial weakness, as well as mild cognitive deficits. The second patient, a 6-year-old girl, is homozygous for \( SLC18A3 \) p.Asp398His. She presented with hypotonia, feeding difficulties, apneic episodes, ptosis, and ophthalmoplegia. She walked for some months at 4 years of age but lost independent ambulation by age 5. No CNS symptoms were reported. Both patients had electrophysiologic findings suggestive of myasthenia and demonstrated moderate improvement in pyridostigmine treatment. This phenotype is substantially milder than the phenotype we report, which is essentially lethal without mechanical ventilation. We hypothesize that phenotypic variability may be explained by different alleles. We show that VACHT is rendered dysfunctional by the codon 360 substitution of arginine for glycine. The resulting clinical
presentation is of severe muscle weakness and cognitive defects. Knowledge of consequences for loss of function of this gene aids in genetic counseling, suggests possible causes of as-yet-unresolved cases of presynaptic CMS, and further our knowledge of the complex process of synaptic neurotransmission.

AUTHOR CONTRIBUTIONS
Adi Aran assisted in conceptualizing the study, identified and recruited patients and family members, examined the participants, collected DNA samples and clinical data, wrote the first draft, and approved the final manuscript version. Reeval Segel assisted in conceptualizing the study, identified and recruited patients and family members, examined the participants, collected DNA samples and clinical data, wrote the first draft together with A.A., and approved the final manuscript version. Kota Kaneshige and Scott Oliphant assisted in conceptualizing the study, performed all the cell culture experiments (site-directed mutagenesis, stable transfection, selection of stable clones, and Western blot analysis), and approved revision of the manuscript. Sukayem Galusiner performed genomic analysis and interpretation and wrote sections of the manuscript. Paul Renbaum assisted in conceptualizing the study, provided insights about the hypothesis, and directed genetic testing and analysis. Tamar Meiron and Abraham O. Samson performed the homology modeling. Anella Weinberg-Shukron, Sharon Zeligson, and Yair Hershkovitz carried most of the testing and analysis and interpreted results. Ming K. Lee carried out genomic analysis and interpreted results. Stanley M. Parsons assisted in conceptualizing the study, directed functional analysis, interpreted results, and revised the manuscript. Mary-Claire King directed genomic analysis, interpreted results, and critically revised the manuscript. Ephrat Levy-Lahad initiated and conceptualized the study, obtained funding, directed genetic testing, analysis, and interpretation of the results, and critically revised the manuscript drafts. Tom Walsh carried out genomic analysis and interpreted results and critically revised the manuscript drafts.

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