

## Corrections

**CHEMISTRY, GENETICS.** For the article “Genomewide studies of histone deacetylase function in yeast” by Bradley E. Bernstein, Jeffrey K. Tong, and Stuart L. Schreiber, which appeared in number 25, December 5, 2000, of *Proc. Natl. Acad. Sci. USA* (**97**, 13708–13713; First Published November 28, 2000; 10.1073/pnas.250477697), the authors note the following corrections. As a result of an error at the proof stage, there is a shift in the references. Ref. nos. 9–16 and 31–34 in the text should be 10–17 and 30–33, respectively.

www.pnas.org/cgi/doi/10.1073/pnas.111142398

**ANTHROPOLOGY.** For the article “A highly variable segment of human subterminal 16p reveals a history of population growth for modern humans outside Africa” by Santos Alonso and John A. L. Armour, which appeared in number 3, January 30, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 864–869; First Published December 19, 2000; 10.1073/pnas.011244998), the authors note

**BIOCHEMISTRY.** For the article “Mapping the intrinsic curvature and flexibility along the DNA chain” by Giampaolo Zuccheri, Anita Scipioni, Valeria Cavaliere, Giuseppe Gargiulo, Pasquale De Santis, and Bruno Samori, which appeared in number 6, March 13, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 3074–3079; First Published February 27, 2001; 10.1073/pnas.051631198), the authors note the following correction. In the last part of the *Discussion*, the following DNA base steps were termed incorrectly: AT·TA should be AT·AT; TA·AT should be TA·TA; CG·GC should be CG·CG; and GC·CG should be GC·GC. The other sections and the figure legends are not affected.

www.pnas.org/cgi/doi/10.1073/pnas.111142498

the following corrections. Table 1 on page 865 was misaligned; therefore, a corrected table is printed below. In addition, the circle representing lineage e in Fig. 1*b* should be split into two sections to indicate equal representation of this haplotype in Pygmies and Kenyans.

www.pnas.org/cgi/doi/10.1073/pnas.111142598

**Table 1. Polymorphic positions**

Ancestor	ggga+cccgggcccgggcccccgacgggtaagctagggcggt
3P	a.....t.....t.....a.....a.....
1U	.a.....a.a.....a.....
1J	.c.....aa.....a.....
3B+4J	.c.....a.....a.....
1P	.....ca.t.....a.....a.....at.....
1P	.....a.a...t...a.....a.....
1J	.....a.....c.a.....
1U	.....a.....a.a...
2P	.....a.....a...c...
1K	.....a.....a...c...
13B+11J+14U+8K+2P	.....a.....a.....
1U	.....a.....a...t...
1B+2UK	.....a.....ca.....
1J	.....a.....t...a.....
1B+1K	.....a.....c.....a.....
1B+2J+4K+4P	.....a...t.....a.....
1P	.....a...t...a.....a.a...
2K	.....a...t...a.....a.....
1K	.....a...t.....a.....
1P+1K	.....a.....
1U	.....t...a.....ca.....
1B	.....t.....
1P	.....t.....t.....a.acg.....
3P	...-...t.....a.....
1P	...-...gt.....a.....
2K	...g.....a.....a.....

Dots represent the same state as in the ancestor sequence. + and – in polymorphism number 5 represent presence or absence of a 5-bp motif, respectively. Abbreviations: B, Basques; J, Japanese; K, Kenyans; P, Pygmies; and U, U.K.

**BIOCHEMISTRY.** For the article “Functional transitions in myosin: Formation of a critical salt-bridge and transmission of effect to the sensitive tryptophan” by Hirofumi Onishi, Shin-ichiro Kojima, Kazuo Katoh, Keigi Fujiwara, Hugo M. Martinez, and Manuel F. Morales, which appeared in number 12, June 9, 1998, of *Proc. Natl. Acad. Sci. USA* (**95**, 6653–6658), the authors note the following correction. Recently, it has been discovered that the heavy meromyosin (HMM) mutant described as E470R/R247E HMM was actually P548G HMM. Examination and subsequent experiments with authentic E470R/R247E HMM revealed that although its tryptophan fluorescence is increased upon addition of ADP or ATP, its intrinsic ATPase at all ATP concentrations examined, 0.5–4 mM, was far less than that of wild type. As before, it was not actin-activated. Therefore, our revised conclusions are: (i) our observations do not conflict with Rayment’s suggestion that at some stage preceding hydrolysis, bridge formation occurs; (ii) for fluorescence enhancement, the reversed dipole of the mutant is at least partly effective; (iii) although ATP binds as suggested by the partial tryptophan enhancement, the salt bridge does not form properly, so hydrolysis is therefore precluded; and (iv) we cannot deduce anything about actin activation because intrinsic ATPase is absent. It seems that in E470R and R247E HMMs, electrical repulsion precludes bridge formation, and therefore, hydrolysis.

[www.pnas.org/cgi/doi/10.1073/pnas.111149198](http://www.pnas.org/cgi/doi/10.1073/pnas.111149198)

**CELL BIOLOGY.** For the article “Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands” by Shila Jalali, Miguel A. del Pozo, Kuang-Den Chen, Hui Miao, Yi-Shuan Li, Martin A. Schwartz, John Y.-J. Shyy, and Shu Chien, which appeared in number 3, January 30, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 1042–1046; First Published January 23, 2001; 10.1073/pnas.031562998), the authors note the following correction. The first sentence on page 1045, right column, fourth paragraph, lines 1–3, reads, “Recent study (23) has indicated that vascular endothelial growth factor receptor (VEGF-R) may be involved in integrin/Shc association.” This sentence should be changed to read, “Recent study (23) indicated that vascular endothelial growth factor receptor (VEGF-R) may interact with integrin for VEGF signaling.”

[www.pnas.org/cgi/doi/10.1073/pnas.111142898](http://www.pnas.org/cgi/doi/10.1073/pnas.111142898)

**GENETICS.** For the article “Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10” by Hyoung Doo Shin, Cheryl Winkler, J. Claiborne Stephens, Jay Bream, Howard Young, James J. Goedert, Thomas R. O’Brien, David Vlahov, Susan Buchbinder, Janis Giorgi, Charles Rinaldo, Sharyne Donfield, Anne Willoughby, Stephen J. O’Brien, and Michael W. Smith, which appeared in number 26, December 19, 2000, of *Proc. Natl. Acad. Sci. USA* (**97**, 14467–14472), the authors note the following: “The discovery described by Shin *et al.* in this paper was the subject of U.S. patent application no. PCT/US00/09355 filed on behalf of the U.S. Department of Health and Human Services on April 9, 1999, and internationally on April 6, 2000. M. W. Smith, H. D. Shin, and S. J. O’Brien are listed as inventors on the patent.”

[www.pnas.org/cgi/doi/10.1073/pnas.101139898](http://www.pnas.org/cgi/doi/10.1073/pnas.101139898)

**MEDICAL SCIENCES.** For the article “Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis” by Lubor Borsig, Richard Wong, James Feramisco, David R. Nadeau, Nissi M. Varki, and Ajit Varki, which appeared in number 6, March 13, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 3352–3357), the authors note the following correction. On page 3355, the URL for NEARCOUNT software was incorrectly listed as <http://vis.fdsc.edu>. The correct URL is <http://vis.sdsc.edu>.

[www.pnas.org/cgi/doi/10.1073/pnas.111148898](http://www.pnas.org/cgi/doi/10.1073/pnas.111148898)

**MICROBIOLOGY.** For the article “Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens” by Kelly A. Brayton, Donald P. Knowles, Travis C. McGuire, and Guy H. Palmer, which appeared in number 7, March 27, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 4130–4135), the authors note the following correction. The correct address for Donald P. Knowles is Animal Disease Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Pullman, WA 99164-6630.

[www.pnas.org/cgi/doi/10.1073/pnas.111153498](http://www.pnas.org/cgi/doi/10.1073/pnas.111153498)

**SPECIAL FEATURE, MICROBIOLOGY.** For the article “Chains of magnetite crystals in the meteorite ALH84001: Evidence of biological origin” by E. Imre Friedmann, Jacek Wierzchos, Carmen Ascaso, and Michael Winklhofer, which appeared in number 5, February 27, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 2176–2181), the authors note the following correction. On page 2178, left column, 2nd line from the bottom, insert after “fractured”: “along existing microscopic cracks to expose carbonate globules.”

[www.pnas.org/cgi/doi/10.1073/pnas.091151998](http://www.pnas.org/cgi/doi/10.1073/pnas.091151998)

**NEUROBIOLOGY.** For the article “Dopamine D1/D5 receptor modulation of excitatory synaptic inputs to layer V prefrontal cortex neurons” by Jeremy K. Seamans, Daniel Durstewitz, Brian R. Christie, Charles F. Stevens, and Terrence J. Sejnowski, which appeared in number 1, January 2, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 301–306; First Published December 26, 2000; 10.1073/pnas.011518798), the authors note the following correction. Line 10 of the abstract should read: “With 20 Hz synaptic trains we found that the D1/D5 agonists increased the depolarization produced by summing NMDA excitatory postsynaptic potentials (EPSPs).” In addition, on line 3 of page 305, “signal” should be “single.”

[www.pnas.org/cgi/doi/10.1073/pnas.091115298](http://www.pnas.org/cgi/doi/10.1073/pnas.091115298)

**PHYSIOLOGY.** For the article “Choline acetyltransferase mutations cause myasthenic syndrome associated with episodic apnea in humans” by Kinji Ohno, Akira Tsujino, Joan M. Brengman, C. Michel Harper, Zeljko Bajzer, Bjarne Udd, Roger Beyring, Stephanie Robb, Fenella J. Kirkham, and Andrew G. Engel, which appeared in number 4, February 13, 2001, of *Proc. Natl. Acad. Sci. USA* (**98**, 2017–2022), the authors note the following correction. Dr. Xin-Ming Shen’s name and affiliation were inadvertently omitted from the list of authors. Dr. Shen’s affiliation is Department of Neurology and Neuromuscular Research Laboratory, Mayo Clinic, Rochester, MN 55905. The corrected list of authors is: Kinji Ohno, Akira Tsujino, Xin-Ming Shen, Joan M. Brengman, C. Michel Harper, Zeljko Bajzer, Bjarne Udd, Roger Beyring, Stephanie Robb, Fenella J. Kirkham, and Andrew G. Engel.

[www.pnas.org/cgi/doi/10.1073/pnas.101139998](http://www.pnas.org/cgi/doi/10.1073/pnas.101139998)

# Choline acetyltransferase mutations cause myasthenic syndrome associated with episodic apnea in humans

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Choline acetyltransferase (ChAT; EC 2.3.1.6) catalyzes the reversible synthesis of acetylcholine (ACh) from acetyl CoA and choline at cholinergic synapses. Mutations in genes encoding ChAT affecting motility exist in *Caenorhabditis elegans* and *Drosophila*, but no *CHAT* mutations have been observed in humans to date. Here we report that mutations in *CHAT* cause a congenital myasthenic syndrome associated with frequently fatal episodes of apnea (CMS-EA). Studies of the neuromuscular junction in this disease show a stimulation-dependent decrease of the amplitude of the miniature endplate potential and no deficiency of the ACh receptor. These findings point to a defect in ACh resynthesis or vesicular filling and to *CHAT* as one of the candidate genes. Direct sequencing of *CHAT* reveals 10 recessive mutations in five patients with CMS-EA. One mutation (523insCC) is a frameshifting null mutation. Three mutations (I305T, R420C, and E441K) markedly reduce ChAT expression in COS cells. Kinetic studies of nine bacterially expressed ChAT mutants demonstrate that one mutant (E441K) lacks catalytic activity, and eight mutants (L210P, P211A, I305T, R420C, R482G, S498L, V506L, and R560H) have significantly impaired catalytic efficiencies.

Congenital myasthenic syndromes (CMS) are heterogeneous disorders caused by postsynaptic, synaptic, and presynaptic defects. Recent studies identified CMS caused by mutations in the postsynaptic acetylcholine receptor (AChR) and in synaptic acetylcholinesterase (reviewed in ref. 1), but the molecular basis of presynaptic CMS has not been elucidated to date. One form of presynaptic CMS presents at birth or in the neonatal period with hypotonia, variable eyelid ptosis, severe bulbar weakness causing dysphagia, and respiratory insufficiency with cyanosis and apnea. If the infant survives, the symptoms improve, but the crises recur abruptly with infections, fever, excitement, vomiting, or overexertion. During crises, episodes of apnea can cause sudden death or anoxic brain injury. Between crises, patients may have only mild or no myasthenic symptoms. When weakness is absent, it can be induced readily by exercise (2–10). The crises can be prevented or mitigated by anticholinesterase drugs. In 1960, Greer and Schotland (2) described the clinical features of the disease, and in 1975 Conomy *et al.* (3) referred to it as “familial infantile myasthenia.” Because all CMS can be familial and because most CMS presents in infancy, the term “familial infantile myasthenia” has become a source of confusion (11). We therefore refer to this disease as CMS with episodic apnea (CMS-EA).

Previous studies of CMS-EA revealed no endplate AChR or acetylcholinesterase deficiency but suggested impaired resynthesis or vesicular packaging of ACh (9, 12). We here show that spontaneous mutations in *CHAT*, the gene encoding choline acetyltransferase (ChAT; EC 2.3.1.6), cause CMS-EA. In the course of this study, we also determine the genomic structure of 11 previously uncharacterized exons at the 3' end of *CHAT* and identify an S transcript in human spinal cord.

## Materials and Methods

**Clinical Data.** Five patients, now 26, 40, 5, 6, and 4 years old, were studied. Four were male and one was female. All had myasthenic

symptoms since birth or early infancy, negative tests for anti-AChR antibodies, and abrupt episodic crises of increased weakness, bulbar paralysis, and apnea precipitated by undue exertion, fever, or excitement. Electromyographic studies in the five patients between crises revealed no decremental response on 2-Hz stimulation of motor nerves, but such a response appeared after a conditioning train of 10-Hz stimuli for 5 min. Patient 2 had three affected siblings, and patient 4 had two; three of the five affected siblings died during febrile episodes, and one died suddenly without apparent cause. Previous studies of intercostal muscle specimens from patients 1 and 2 revealed no endplate AChR deficiency, a normal miniature endplate potential (MEPP) amplitude in rested muscle, and an abnormal decrease of the MEPP amplitude after 5 min stimulation at 10 Hz, suggesting a defect in ACh resynthesis or vesicular packaging (9, 12).

**Endplate Studies.** Intercostal muscle specimens were obtained from patients 3 and 4 and a control subject without muscle disease who was undergoing thoracic surgery. In patient 5, CMS-EA was suspected on clinical grounds. All human studies were in accordance with the guidelines of the Institutional Review Board of the Mayo Clinic.

The number of AChRs per endplate was measured with <sup>125</sup>I-labeled  $\alpha$ -bungarotoxin (13). MEPP and EPP recordings and estimates of the number of transmitter quanta released by nerve impulse were carried out as described (13). MEPP recordings were obtained before and within 5 min after 10-Hz stimulation for 5 min. EPP recordings were obtained from curarized muscle fibers before and within 1 min after 10-Hz stimulation for 5 min.

**Sequencing Procedures.** PCR-amplified fragments were purified by the QIAquick 8 PCR Purification kit (Qiagen, Chatsworth, PA). Plasmids were purified by the QIAprep Spin Miniprep kit (Qiagen, Chatsworth, CA). PCR products and plasmids were sequenced with an ABI 377 DNA sequencer (Applied Biosystems) by using fluorescently labeled dideoxy terminators.

**Analysis of Genomic Structure of *CHAT*.** The genomic structure of exons R to 7 of the human *CHAT* gene was reported previously (14–16). To determine the remaining genomic structure, we synthesized 34 cDNA primers based on the published cDNA

Abbreviations: CMS, congenital myasthenic syndrome; ACh, acetylcholine; AChR, ACh receptor; CMS-EA, CMS associated with episodic apnea; ChAT, choline acetyltransferase; EPP, endplate potential; MEPP, miniature EPP; RT, reverse transcription; AcCoA, acetyl CoA.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (*CHAT* genomic sequence accession nos. AF305894–AF305906; *CHAT* cDNA sequence accession nos. AF305907–AF305909).

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sequence (17) and amplified overlapping genomic DNA segments by using long-distance PCR.

**Mutation Analysis.** DNA was isolated from blood by using a QIAamp DNA Blood kit (Qiagen). By using the genomic sequence determined above, we synthesized 18 pairs of PCR primers to amplify and directly sequence 18 exons and their flanking intronic or untranslated regions. After identifying mutations in *CHAT*, we screened the patients' relatives for the observed mutations by restriction analysis. We used allele-specific PCR to screen for the identified missense mutations in 400 normal alleles.

**cDNA Cloning of Human *CHAT*.** We used nested reverse transcription (RT)-PCR to amplify the entire coding region of the M transcript of human *CHAT* (nucleotides 90–2425; GenBank accession no. AF305907) from human spinal cord cDNA (Quick-Clone cDNA, CLONTECH). We used spinal cord cDNA because ChAT is synthesized in anterior horn cells and reaches the nerve terminal by slow axonal transport (18, 19). The attempt to clone the M transcript unexpectedly amplified an S transcript. The RT-PCR products were cloned into a pGEM-T vector (Promega) and were sequenced to confirm absence of PCR artifacts.

**Construction of Expression Vectors.** Presence of *EcoRI* sites in both the untranslated region of exon S and pGEM-T enabled us to clone the *EcoRI*-digested S transcript into pRBG4, a cytomegalovirus-based expression vector (20). Naturally occurring or artificial *CHAT* mutations were introduced into *CHAT* cDNA in pRBG4 by using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The presence of desired mutations and the absence of unwanted mutations was confirmed by sequencing the entire insert.

We next amplified by PCR the coding region for 70-kDa ChAT (nucleotides 578–2470 of S transcript, GenBank accession no. AF305908) from wild-type and mutant *CHAT* cDNAs in pRBG4 and cloned them into the bacterial-expression vector pCRT7/NT (Invitrogen), which introduces six N-terminal histidine residues. We also cloned the coding region of the wild-type 74-kDa ChAT into pCRT7/NT and replaced the translational start site of 70-kDa ChAT with alanine to suppress the production of 70-kDa ChAT.

**Immunoblot Analysis of ChAT Expressed in COS Cells.** COS-7 cells were grown and transfected 1 day after spreading by the DEAE-dextran method (21) with 10  $\mu$ g of wild-type or mutant *CHAT* cDNA and 2  $\mu$ g of pSV- $\beta$ -galactosidase control vector (Promega) per 10-cm dish. COS cells were extracted 3 days after transfection with 900  $\mu$ l per dish of Reporter lysis buffer (Promega). Aliquots of each extract (10  $\mu$ l) were separated on 10% NuPage Bis-Tris gel (Invitrogen) and transferred electrophoretically to nitrocellulose membrane (*Trans-Blot*, Bio-Rad). The membrane was immunostained with affinity purified goat anti-human ChAT antibody (Chemicon International) and alkaline phosphatase-conjugated rabbit-anti-goat IgG (Southern Biotechnology Associates). Plate-to-plate variations of transfection efficiency were corrected for by measuring the  $\beta$ -galactosidase activities of transfected cells. The National Institutes of Health's IMAGE 1.61 software was used for densitometric analysis.

**Expression and Purification of Recombinant ChAT Expressed in Bacteria.** BL21(DE3)pLysS (Invitrogen) was transformed with pCRT7/NT-*CHAT* and grown overnight at 37°C in 2 ml LB medium containing 100  $\mu$ g/ml ampicillin and 34  $\mu$ g/ml chloramphenicol. The entire overnight culture was transferred to 100 ml LB medium containing 100  $\mu$ g/ml ampicillin, and incubation was continued at 25°C. When  $A_{600}$  of the culture reached 0.5 ( $\approx$ 7 h), isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 0.5 mM, and growth continued overnight at 25°C. The bacterial cell pellet from 50-ml culture medium was suspended in 4 ml lysis buffer containing 50 mM sodium phosphate (pH 7.4), 300

mM NaCl, 10 mM imidazole, 1 mM PMSF, 0.1 mM benzamidine, 1 mM MgSO<sub>4</sub>, and 11 units per ml DNase I and was disrupted by freezing and thawing three times. A mixture of the supernatant of the lysate and 1 ml Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) was shaken at 1,000 rpm at 4°C for 1 h. Next, Ni-NTA agarose was washed three times with 4 ml of 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, and 20 mM imidazole. ChAT peptide was eluted with 1 ml of 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, and 250 mM imidazole and diluted with 9 ml of 20 mM sodium phosphate (pH 7.4). The diluted eluate was loaded to 0.5-ml Amicon DyeMatrex blue B gels (Millipore), washed with 5 ml of 20 mM sodium phosphate (pH 7.4), and eluted with 1 ml of linear gradient of 0.2–2.0 M NaCl in 20 mM sodium phosphate (pH 7.4). Elution fractions of 0.2–0.8 M NaCl were combined and concentrated/desalted with Centricon YM-30 (Millipore) and resuspended in 300  $\mu$ l 20 mM sodium phosphate (pH 7.4). Protein concentrations were determined by densitometric assay of Coomassie G-250-stained SDS/PAGE against IgG-free BSA as a standard. We obtained 29–101  $\mu$ g of ChAT peptide from 50 ml culture medium.

#### Measurement of Enzyme Activity and Treatment of Kinetic Data.

ChAT activity was measured under steady-state conditions by the radioactive assay of Fonnum (22). Initial reaction velocities were determined at 37°C in a 40- $\mu$ l reaction mixture containing 5–1,200  $\mu$ M [<sup>14</sup>C]acetyl-CoA (AcCoA; NEN), 0.1–3.5 mM choline, 0.2–10 ng ChAT protein, 50 mM sodium phosphate (pH 7.4), 250 mM NaCl, 1 mM EDTA, and 0.5 mg/ml BSA.

The kinetic mechanism of the ChAT reaction follows a random Theorell-Chance mechanism in which a low but finite amount of ternary complex exists (23). Accordingly, the initial velocity of the reaction is defined by Eq. 1,

$$v = k_{cat}[\text{ChAT}][\text{AcCoA}][\text{Choline}] / (K_{ia}K_m^{\text{chol}} + K_m^{\text{AcCoA}}[\text{Choline}] + K_m^{\text{chol}}[\text{AcCoA}] + [\text{AcCoA}][\text{Choline}]) \quad [1]$$

where  $v$  is the observed velocity,  $k_{cat}$  is the turnover number ( $V_{max}/[\text{ChAT}]$ ),  $K_{ia}$  is the dissociation constant for the AcCoA-enzyme complex, and  $K_m^{\text{AcCoA}}$  and  $K_m^{\text{chol}}$  are the Michaelis-Menten constants. The kinetic constants for the equation were determined by weighted nonlinear regression using a SIGMAPLOT 5 computer program (SPSS, Chicago) and were confirmed by using the FIT function of MLAB (Civilized Software, Bethesda).

For the L210P mutant, for which the obtainable concentration of AcCoA is much lower than  $K_m^{\text{AcCoA}}$ , Eq. 1 reduces to Eq. 2:

$$v = (k_{cat}/K_m^{\text{AcCoA}})[\text{ChAT}][\text{AcCoA}][\text{Choline}] / (K_{ib} + [\text{Choline}]) \quad [2]$$

where  $K_{ib}$  is equivalent to  $K_{ia} \cdot K_m^{\text{chol}} / K_m^{\text{AcCoA}}$  in Eq. 1. Similarly, for the R560H mutant, for which the obtainable concentration of choline is much lower than  $K_m^{\text{chol}}$ , Eq. 1 reduces to Eq. 3:

$$v = (k_{cat}/K_m^{\text{chol}})[\text{ChAT}][\text{AcCoA}][\text{Choline}] / (K_{ia} + [\text{AcCoA}]) \quad [3]$$

The catalytic efficiencies ( $k_{cat}/K_m^{\text{AcCoA}}$  and  $k_{cat}/K_m^{\text{chol}}$ ) in Eqs. 2 and 3 were calculated by nonlinear regression using SIGMAPLOT 5 and were confirmed by MLAB.

## Results

**Endplate Studies in Patients 3 and 4.** The number of  $\alpha$ -bungarotoxin binding sites per endplate was  $6.02 \times 10^6$  in patient 3 (age 5 years) and  $6.13 \times 10^6$  in patient 4 (age 6 years). These values were lower than in 13 adult controls ( $12.82 \pm 2.84 \times 10^6$ , mean  $\pm$  SD) but were similar to that in a 6-year-old control ( $6.9 \times 10^6$ ). In both patients, the MEPP amplitude was slightly lower than

**Table 1. Endplate studies**

	Patient 3	Patient 4	Control*
MEPP amplitude before stimulation, mV <sup>†</sup>	0.77 ± 0.04 (n = 20)	0.88 ± 0.06 (n = 17)	1.22 ± 0.062 (n = 12)
MEPP amplitude after stimulation, mV <sup>†</sup>	0.38 ± 0.04 (n = 5)	0.41 ± 0.04 (n = 10)	1.09 ± 0.043 (n = 13)
EPP quantal content before stimulation <sup>‡</sup>	28.94 ± 2.44 (n = 18)	28.91 ± 2.9 (n = 10)	36.5 ± 3.9 (n = 7)
EPP quantal content after stimulation <sup>‡</sup>	ND	28 ± 3.1 (n = 10)	39.3 ± 6.09 (n = 7)

Values shown are means ± SEM. T = 30°C; n = number of fibers; ND, not determined.

\*For 164 unstimulated muscle fibers of 15 other controls, the MEPP amplitude was 1.00 ± 0.025 mV.

<sup>†</sup>Corrected to a resting potential of -80 mV and for a fiber diameter of 50 μm.

<sup>‡</sup>Recordings before and after 10-Hz stimulation were obtained from the same fibers of curarized muscle at 1 Hz with correction for resting membrane potential of -80 mV, nonlinear summation, and non-Poisson release.

normal, and the quantal content of EPP was normal in rested muscle. In both patients, 10-Hz stimulation for 5 min reduced the amplitude of the MEPP by 50% but had no effect on the quantal content of the EPP (Table 1). The stimulation-dependent decrease of the MEPP amplitude was consistent with insufficient resynthesis or vesicular packaging of ACh in response to increased functional demand. Candidate proteins for this type of defect would be the presynaptic high-affinity choline transporter (24, 25), ChAT, the vesicular ACh transporter (vAChT; ref. 26), or the vesicular proton pump (27). Direct sequencing of the *VACHT* in patients 1–4 revealed no mutations (data not shown). We next searched for mutations in *CHAT*.

**Genomic Structure and Mutation Analysis of *CHAT*.** Preparatory to sequencing *CHAT*, we wished to determine its genomic structure. *CHAT* is in the cholinergic gene locus at 10q11.2 that also comprises *VACHT* in the first intron of *CHAT* (26). The genomic structure of *CHAT* between exons R and 7 was reported previously (14–16); we therefore determined the genomic structure of the remaining 11 exons (Fig. 1). Comparison of *CHAT* with rat *Chat* (28) revealed size identity and structural homology of exons 6–17 and structural homology of exons R, 5, and 18.

Next, we sequenced 18 exons and their flanking intronic or untranslated regions directly by using patients' blood DNA and identified two heterozygous mutations in each patient (Table 2 and

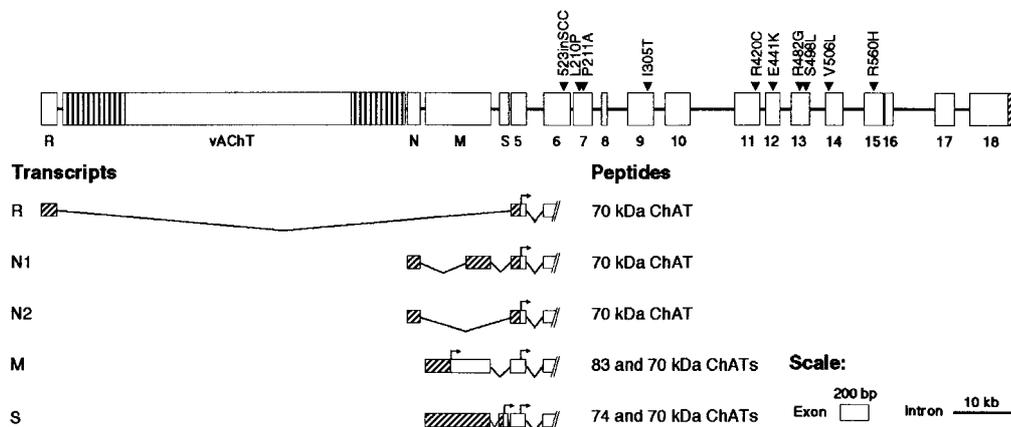
**Table 2. Identified *CHAT* mutations**

Patient	Nucleotide change*	Amino acid change*	Effects
1	523insCC 931C→G	Frameshift P211A	No expression Very high $K_m^{AcCoA}$
2	1371G→A 1516G→T	E441K V506L	Low expression, no catalytic activity High $K_m^{AcCoA}$
3	1444A→G 1679G→A	R482G R560H	High $K_m^{AcCoA}$ Very high $K_m^{AcCoA}$ and $K_m^{chol}$
4	629T→C 1493C→T	L210P S498L	Very high $K_m^{AcCoA}$ and $K_m^{chol}$ High $K_m^{AcCoA}$
5	914T→C 1258C→T	I305T R420C	Low expression, high $K_m^{AcCoA}$ Low expression, very high $K_m^{AcCoA}$ and $K_m^{chol}$

\*Nucleotide and codon numbers start from the translational start site of 83-kDa ChAT.

Figs. 1 and 2). Restriction analysis of DNA from relatives revealed that a living affected sibling harbors both mutations, the asymptomatic parents carry a single mutation, and other unaffected relatives carry either no or a single mutation (data not shown). Therefore, each mutation is heteroallelic and recessive. None of the nine missense mutations were detected in 400 normal alleles. All nine missense mutations alter residues conserved in mouse, rat, and pig (29–31; see Fig. 2). Patients 1 and 2 are also heterozygous for 358G/A (A120T), a polymorphism detected in 17/60 in normal alleles.

**Identification of an S Transcript in Human Spinal Cord.** For expression studies of wild-type and mutant ChATs, we cloned *CHAT* cDNA by RT-PCR. RT-PCR amplification with primers designed to amplify the M transcript from a human spinal cord cDNA library yielded a transcript (S transcript) with a 70-bp insertion (exon S) between exons M and 5 (Fig. 1). Sequencing of 14 plasmids cloned from four different RT-PCR reactions failed to reveal the M transcript. Expression of the S transcript in COS cells showed two distinct proteins (Fig. 3). Replacement of the initial ATG methionine codons with GCG alanine codons identified two translational start sites of the S transcript, yielding 74- and 70-kDa ChATs (Fig. 3). The kinetic properties of the purified recombinant 74-kDa ChAT expressed in *Escherichia coli* are identical essentially with those of 70-kDa ChAT (data not shown).



**Fig. 1.** Genomic structure and alternative transcripts of human *CHAT* and 10 identified mutations. *CHAT* and *VACHT* are encoded in the cholinergic gene locus (26). Four alternative *CHAT* transcripts (R, N1, N2, and M) have been reported (15, 17, 41). The S transcript is a moiety identified in the spinal cord. All transcripts encode a 70-kDa ChAT. M transcript encodes an additional 83-kDa ChAT and S transcript codes for an additional 74-kDa ChAT. Vertical shading indicates untranslated regions of *VACHT*. Diagonal shading show untranslated regions of *CHAT*. For M and S transcripts, diagonal shading indicates untranslated regions for yielding 83- and 74-kDa ChATs, respectively. Arrows indicate translational start sites. Exons and introns are drawn to indicated scale.

83-kDa MGLRTAKKRLGGGGKWKREEGGTRGRREVRFACFLQSGGRGDPGDVGGPAGNPGCSPH 60

83-kDa PRAATRPPPLPAHTFAHTPEWCGAASARAAEFRRAGPLHCIPAPGLTKTPILEKVPKMA 120

74-kDa MWPECRDEALSTV----- 38

70-kDa ----- 2

Pig M----T-P-- 12

Mouse M----P--P 12

Rat M----A-Q--P 12

523insCC

83-kDa AKTPSSEES GLFKLPVPLQQTLLATYLQCMRHLVSEEQFRKSQAIVQQFGAPGGLGETL 179

Pig --S---EP-----R--Q--P--R----- 72

Mouse VQAS-C--VLD-----Q--P-----KR----- 72

Rat V-A--W-ELD-----Q--P-----KR----- 71

L210P P211A

83-kDa QQKLLERQERTANWVSEYWLNDMYLNNRLALFVNSSPAVIFARQHFFGTDQLRFAASLI 239

Pig -----Q-----QD-N-----N- 132

Mouse -E-----QD-N----- 132

Rat -E-----QD-N-----C- 131

83-kDa SGVLSYKALLDSHS IPTDCAKGLSGQPLCMKQYVGLFSSYRLPGHTQDTLVAQNSSIMP 299

Pig -----I-----R--V-- 192

Mouse -----Q--W-----R-----K----- 192

Rat -----T--L--W-----R-----K----- 191

I305T

83-kDa EPEHVIVACCNQFFVLDVVINFRLRSEGDLFTQLRRIVKMASNEDERLPPIGLLTSDGRS 359

Pig -----R----- 252

Mouse ----- 252

Rat ----- 251

83-kDa EWAEARTVLVKDSTNRDSDMIERICICLVCLDAPGVELSDTHRALQLLHGGGYSKNGAN 419

Pig -----M-----N-----C----- 312

Mouse --X---L-----G--TGD-----C-L----- 312

Rat --X---L-----G--TG-----C-L----- 311

R420C E441K

83-kDa RWYDKSLQFVVGDRDGTGVVCEHSFFDGIIVLVQCTEHLHKMTQSSRKLIRADSVSELPA 479

Pig -----VK--K-MV----- 372

Mouse -----MTGNK--V-V----- 372

Rat -----MT-NK--V----- 371

R482G S498L V506L

83-kDa PRRLRWKCSPEIQGHLSAELKLRIVKNDLFIYKFDNYGKTFIKKQKSPDPAFIQVAL 539

Pig -----L-----Q-----T-----D-----Q----- 432

Mouse -----T-----Q-----G----- 432

Rat -----L-----T-----Y-----G----- 431

R560H

83-kDa QLAFYRLHRRLLVPTYESASIRRFQEGRVDNIRSATPEALAFVRAVTDHKAAPASEKLLL 599

Pig -----G-----H-----H--K--I--AS-M-D----- 492

Mouse --Y--YQ-----Q-M-----L-----Q----- 492

Rat --Y--YQ-----Q-M-----M-----Q----- 491

83-kDa LKDAIRAQTAYTVMAITGMAIDNHLALRELARAMCKELPEMFHDETYLMSNRFLVLSQ 659

Pig -----Q-----G-----EV-----T----- 552

Mouse -QR-Q--E-----DL--P-----I----- 552

Rat -QT-MQ-HKQ-----DL--P----- 551

83-kDa VPTTTFMFCYGPVVFVNGYACYNPQPETILFCISSFHSCKETSSKFAKAVEESLIDNR 719

Pig -----S-----G-----T-----F-E-K----- 612

Mouse -----M-----HA-A-T-----G-----VE--E--GA--V----- 612

Rat -----M-----N-----A-T-----VE--E--GA--V----- 611

83-kDa DLCSLLPPTESKPLATKERTRPSQGHOPN 749

Pig G---SQGMG-----V-----V----- 641

Mouse ---SRQ-AD---PTA--R-RG---AK-S 641

Rat ---SRQ-AD---P-P---RG---AK-S 640

Fig. 2. Alignment of the amino acid sequences of human 83-kDa ChAT and of single isoforms identified in pig, mouse, and rat ChAT. Divergent N-terminal ends of the human 74- and 70-kDa ChATs are shown also. Dashes indicate residues identical to the human 83-kDa ChAT. Gaps are inserted for alignment. The right column indicates codon numbers. Arrowheads indicate the 10 recessive mutations identified in five patients. The arrowhead under 523insCC points to the first residue substituted by the frameshift.

**Expression Studies in COS Cells.** To study the effects of the mutations at the protein level, we performed immunoblot analysis of wild-type and mutant ChAT proteins expressed in COS cells. Densitometric analysis of immunoblots showed no expression of the 523insCC mutant, and 30, 49, and 26% expression of wild-type for E441K, I305T, and R420C mutants, respectively (Fig. 4).

**Kinetic Analysis of ChAT Mutants Expressed in *E. coli*.** To determine whether the mutations affect the kinetic properties of ChAT, we

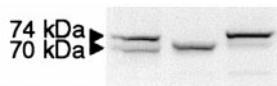


Fig. 3. Immunoblot identifying translational start sites. (Left) Wild type. A Met-to-Ala substitution at the putative translational start site of 74-kDa ChAT prevents expression of the 74-kDa ChAT (Center). A similar Met-to-Ala substitution of 70-kDa ChAT prevents expression of the 70-kDa ChAT (Right).

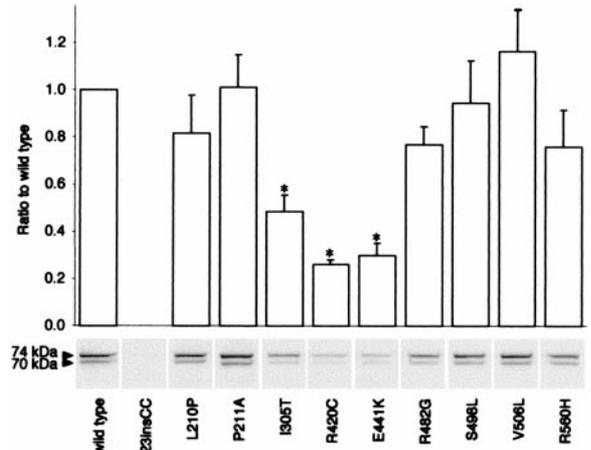


Fig. 4. Immunoblot demonstrating expression of wild-type and mutant ChATs in COS cells. Expression levels are normalized for cotransfected  $\beta$ -galactosidase activity and compared with that of wild-type. Bars indicate means and standard errors of three to four transfections. \* indicates significant difference from wild type ( $P < 0.01$ ). The 74- to 70-kDa ChAT ratios for the mutants were not significantly different from that of wild type.

expressed recombinant wild-type and mutant ChATs in *E. coli* using truncated S transcripts that yield only 70-kDa ChAT. SDS/PAGE of purified recombinant ChATs revealed a single band of 74 kDa comprising the 4-kDa hexahistidine tag and 70-kDa ChAT (Fig. 5). The 523insCC mutant did not express in *E. coli*. The E441K mutant showed no catalytic activity over a range of substrate concentrations. The eight remaining mutants all showed abnormal activation with AcCoA or choline or both, a catalytic efficiency for AcCoA that varied from 1–49% of wild type, and an overall catalytic efficiency that ranged 0.2–64% of wild type (Table 3 and Fig. 6).

## Discussion

In this study, we identify and functionally characterize loss-of-function mutations in *CHAT* as the cause of a highly fatal presynaptic myasthenic syndrome in humans. That *CHAT* became a candidate gene for CMS-EA was owing to *in vitro* electrophysiologic studies that pointed to a defect in ACh resynthesis or vesicular filling. Defects in the presynaptic high-affinity choline transporter (24, 25), the vesicular ACh transporter (26), or the vesicular proton pump (27) may have similar phenotypic consequences, but no mutations in humans of these proteins have been detected to date.

Previous studies identified recessive loss-of-function mutations of ChAT in invertebrates. Homozygous *cha-1* mutants of *C. elegans* have marked ChAT deficiency, small size, uncoordinated behavior, and resistance to cholinesterase inhibitors. In mutants retaining

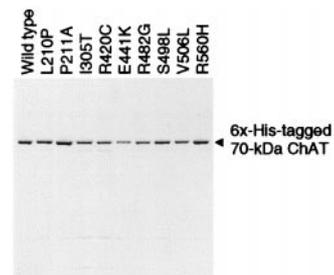


Fig. 5. SDS/PAGE of purified wild-type and mutant recombinant ChATs expressed in *E. coli* stained with Coomassie G-250.

**Table 3. Kinetic parameters of wild-type and mutant ChAT enzymes**

	$k_{cat}$ , s <sup>-1</sup>	$K_m^{AcCoA}$ , $\mu$ M	$K_m^{chol}$ , mM	$k_{cat}/K_m^{AcCoA*}$	$k_{cat}/(K_m^{AcCoA} \cdot K_m^{chol})^*$
wild type	99.6 $\pm$ 3.5	26.8 $\pm$ 1.9	0.478 $\pm$ 0.036	1.00	1.00
L210P	35.0 $\pm$ 2.2 <sup>†</sup>	n.d. <sup>‡</sup>	2.693 $\pm$ 0.280 <sup>†</sup>	0.09	0.02 <sup>†</sup>
P211A	418.8 $\pm$ 15.9	287.9 $\pm$ 28.6	0.514 $\pm$ 0.046	0.39	0.36
I305T	60.4 $\pm$ 3.4	70.6 $\pm$ 6.5	0.590 $\pm$ 0.064	0.23	0.19
R420C	176.8 $\pm$ 42.9	461.8 $\pm$ 134.4	1.018 $\pm$ 0.389	0.10	0.05
R482G	99.0 $\pm$ 6.9	91.7 $\pm$ 10.7	0.500 $\pm$ 0.067	0.29	0.28
S498L	88.6 $\pm$ 1.5	94.6 $\pm$ 7.2	0.418 $\pm$ 0.018	0.25	0.29
V506L	125.8 $\pm$ 7.4	68.4 $\pm$ 7.7	0.370 $\pm$ 0.053	0.49	0.64
R560H	34.1 $\pm$ 1.3 <sup>§</sup>	870.8 $\pm$ 62.4 <sup>§</sup>	n.d. <sup>¶</sup>	0.011 <sup>§</sup>	0.002 <sup>§</sup>

Values indicate estimate  $\pm$  standard error of estimate.

\*Catalytic efficiency ( $k_{cat}/K_m^{AcCoA}$ ) and the overall catalytic efficiency [ $k_{cat}/(K_m^{AcCoA} \cdot K_m^{chol})$ ] are normalized with respect to wild type. In wild type,  $k_{cat}/K_m^{AcCoA} = 3.72 \times 10^6 \text{ s}^{-1} \cdot \text{M}^{-1}$  and  $k_{cat}/(K_m^{AcCoA} \cdot K_m^{chol}) = 7.77 \times 10^9 \text{ s}^{-1} \cdot \text{M}^{-2}$ .

<sup>†</sup>Apparent values calculated at 116  $\mu$ M AcCoA.

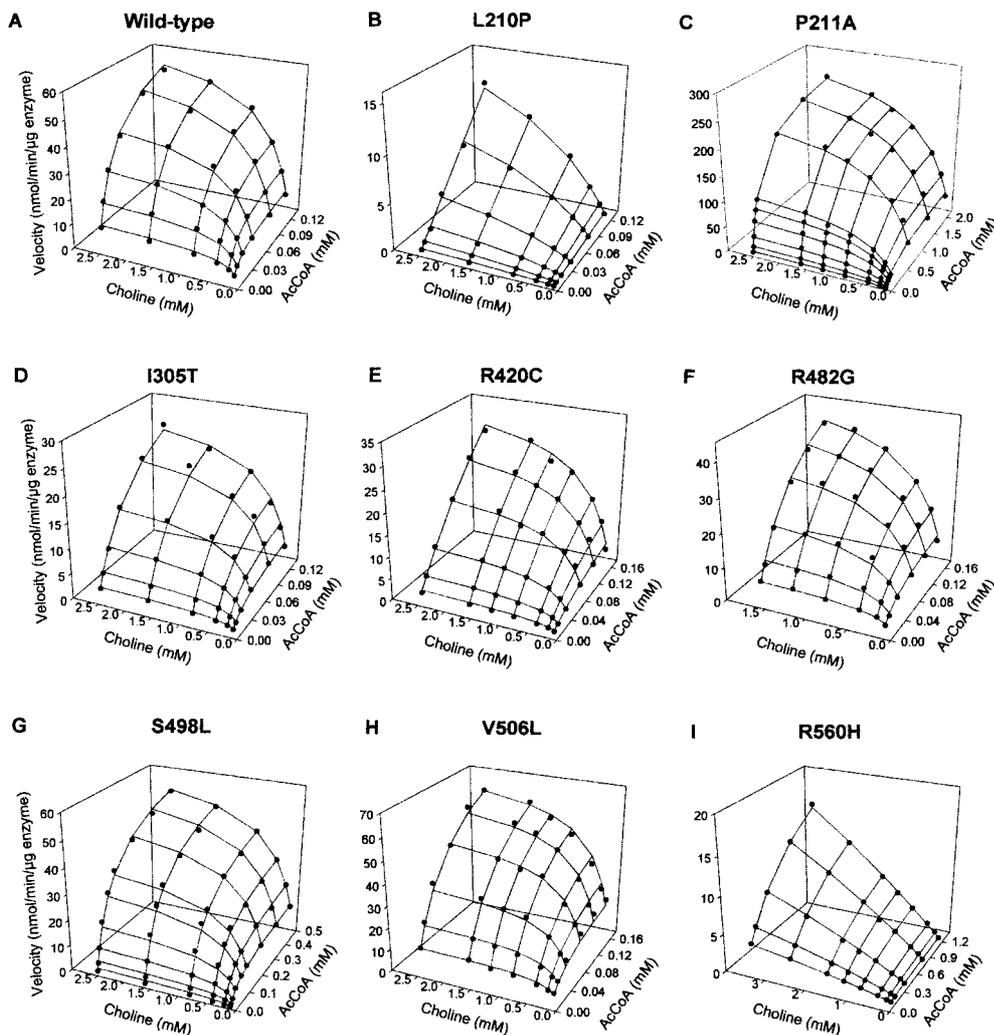
<sup>‡</sup>Not determined because  $K_m^{AcCoA}$  exceeds practical concentration range of AcCoA. Catalytic efficiency is calculated from Eq. 2 (see *Materials and Methods*).

<sup>§</sup>Apparent values are calculated at 3.5 mM choline.

<sup>¶</sup>Not determined because  $K_m^{chol}$  exceeds practical concentration range of choline. Catalytic efficiency is calculated from Eq. 3 (see *Materials and Methods*).

10% of wild-type enzyme activity, the residual enzyme has an altered  $K_m$  for both choline and AcCoA (32). *Drosophila Cha* mutants are temperature sensitive, becoming paralyzed at a restrictive temperature of 32°C (33). No spontaneous ChAT mutations of vertebrates have been identified, but studies of *Chat* knockout mice

were reported recently (34). Homozygous null mice lacking ChAT are stillborn and have underdeveloped muscles; heterozygous mice have 40–50% ChAT activity and seem normal. Similarly, in our series, heterozygous parents are unaffected even when they carry a nonfunctional allele such as 523insCC or E411K. Patient 2, how-



**Fig. 6.** Individually scaled kinetic landscapes of wild-type and mutant ChATs. Dots represent observed values. Meshes show curves fitted to Eq. 1, except for panels B and I in which the curves are fitted to Eqs. 2 and 3, respectively (see *Materials and Methods*).

ever, who is a compound heterozygote for 523insCC and V506L, is symptomatic, although the kinetics of the V506L mutant are the least abnormal among the catalytically active mutants (see Fig. 6H and Table 3). This observation implies that even a small decrease of ChAT activity below 50% can impair neuromuscular transmission when the functional demand for ACh resynthesis is increased.

**Effects of the CHAT Mutations.** The 523insCC mutation is frame-shifting and does not express in COS cells (see Fig. 4) or *E. coli*. The E441K protein expresses at 29% of wild type in COS cells, but the purified mutant is devoid of catalytic activity. Interestingly, the residue corresponding to the adjacent H442 in *Drosophila* is essential for affinity for AcCoA (35). Of the eight enzymatically active mutants, seven predominantly affect affinity for AcCoA and five have a minor or no effect on affinity for choline (see Table 3).

R420C and I305T, both in patient five, resulted in the most severe phenotype in our series. Protein expression of these mutants is 26 and 49% of wild type in COS cells (see Fig. 4). The  $K_m^{\text{AcCoA}}$  of the R420C mutant is 17-fold higher than wild type but carries a high standard error owing to incomplete saturation of the enzyme with up to 0.16 mM AcCoA (Table 2 and Fig. 6E). Interestingly, an artificial arginine-to-alanine mutation of a corresponding residue in rat (see Fig. 2) does not affect kinetics of the reverse ChAT reaction significantly (36). The  $K_m^{\text{AcCoA}}$  for I305T is 2.6-fold higher than that of wild type. The severe phenotypic consequences in patient five probably owe to combined effects of reduced expression and kinetic abnormalities of both mutants.

The L210P mutant exhibits no saturation over a practical range of AcCoA concentrations, indicating an extremely high  $K_m^{\text{AcCoA}}$  (Table 3 and Fig. 6B). An adjacent mutation, P211A, also results in a very high  $K_m^{\text{AcCoA}}$  (Table 3 and Fig. 6C). These findings implicate L210 and P211 as residues that participate in governing affinity for AcCoA.

The R560H mutation markedly reduces affinity for AcCoA and shows no saturation with 3.5 mM choline (Table 3 and Fig. 6F). A residue corresponding to human R560 in rat (see Fig. 2) interacts with 3' phosphate of CoA (36). R560 is also close to the VDN residues at 567–569; the corresponding residues in rat participate in determining affinity for choline (37).

For the three remaining mutants (R482G, S498L, and V506L),  $K_m^{\text{AcCoA}}$  is 3.4-, 3.5-, and 2.6-fold higher than that of wild type, respectively, and the catalytic efficiency for AcCoA ( $k_{\text{cat}}/$

$K_m^{\text{AcCoA}}$ ) is diminished to 29, 25, and 49% of wild type (Table 3 and Fig. 6 F–H).

**CHATs transcript.** The S transcript isolated from human spinal cord has two functional translational start sites that encode 74- and 70-kDa ChATs (Figs. 1 and 3). The 74-kDa ChAT differs from 70-kDa ChAT by 36 extra N-terminal residues that have no known functional significance. That the boundaries of exon S have high consensus values for splicing (38) and that the two translational start sites are used in COS cells consistently (Figs. 1 and 3) argue that the S transcript is not an RT-PCR artifact or immature mRNA. However, the level or site of expression of the 74-kDa ChAT in humans is not known. The S transcript also harbors the translational start site of 83-kDa ChAT (see Fig. 1). However, the 83-kDa start site within the S transcript is of doubtful significance, because after 112 codons it leads to a stop codon within exon S.

**Possible Reasons for Selective Vulnerability of the Neuromuscular Synapse.** That none of our CMS-EA patients had symptoms referable to the central or autonomic nervous system is of special interest, because it implies a larger safety margin for central and autonomic synapses than for neuromuscular synapses. Reasons for selective vulnerability of the neuromuscular synapse may be owing to differences in presynaptic levels of ChAT, choline, or AcCoA, rates of choline uptake, or rates of ACh release under conditions of increased neuronal impulse flow. Presently, there is no evidence that tissue-specific isoforms explain the selective vulnerability of neuromuscular transmission. Although there are five alternative CHAT transcripts (Fig. 1) with at least three different promoters in human (39), and CHAT expression may be regulated by different control mechanisms in brain, spinal cord, and autonomic ganglia, the observed mutations are in the shared coding region of the recognized ChAT isoforms. Moreover, the 83- and 70-kDa ChATs synthesize ACh at similar rates (40), and the kinetic properties of the 74- and 70-kDa ChATs are identical (see Results). Therefore, tissue specific expression of ChAT isoforms cannot explain the tissue-specific vulnerability, but tissue-specific vulnerability could be affected by promoters of differing strength producing different levels of ChAT expression in different tissues.

This work was supported by National Institutes of Health Grant NS6277 (to A.G.E.) and a Muscular Dystrophy Association grant (to A.G.E.).

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