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The Spectrum of Congenital Myasthenic Syndromes

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Abstract

The past decade saw remarkable advances in defining the molecular and genetic basis of the congenital myasthenic syndromes. These advances would not have been possible without antecedent clinical observations, electrophysiologic analysis, and careful morphologic studies that pointed to candidate genes or proteins. For example, a kinetic abnormality of the acetylcholine receptor (AChR) detected at the single channel level pointed to a kinetic mutation in an AChR subunit; endplate AChR deficiency suggested mutations residing in an AChR subunit or in rapsyn; absence of acetylcholinesterase (AChE) from the endplate predicted mutations in the catalytic or collagen-tailed subunit of this enzyme; and a history of abrupt episodes of apnea associated with a stimulation dependent decrease of endplate potentials and currents implicated proteins concerned with ACh resynthesis or vesicular filling. Discovery of mutations in endplate-specific proteins also prompted expression studies that afforded proof of pathogenicity, provided clues for rational therapy, lead to precise structure function correlations, and highlighted functionally significant residues or molecular domains that previous systematic mutagenesis studies had failed to detect. An overview of the spectrum of the congenital myasthenic syndromes suggests that most are caused by mutations in AChR subunits, and particularly in the ε subunit. Future studies will likely uncover new types of CMS that reside in molecules governing quantal release, organization of the synaptic basal lamina, and expression and aggregation of AChR on the postsynaptic junctional folds.

Index Entries: Congenital myasthenic syndromes (CMS); neuromuscular junction; acetylcholine receptor (AChR); acetylcholinesterase (AChE); choline acetyltransferase (ChAT); rapsyn; patch-clamp recordings.

Introduction

The congenital myasthenic syndromes (CMS) represent a heterogeneous group of genetically determined disorders in which the safety margin of neuromuscular transmission is compromised by one or more specific mechanisms.

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Table 1
Investigation of Congenital Myasthenic Syndromes

Clinical Data
- History, examination, response to AChE inhibitor
- EMG: conventional needle EMG, repetitive stimulation, SFEMG
- Serologic tests to exclude presence of anti-AChR and anti-calcium channel antibodies

Morphologic Studies
- Routine histochemical studies
- Cytochemical and immunocytochemical localization of AChE, AChR, agrin, β_2-laminin, utrophin, and rapsyn at the EP
- Estimate of the size shape, and configuration of AChE-reactive EPs or EP regions on teased muscle fibers
- Quantitative electron microscopy; electron cytochemistry

Endplate-Specific ^{125}I-α-Bungarotoxin Binding Sites
In Vitro Electrophysiology Studies
- Conventional microelectrode studies: MEPP, MEPC, evoked quantal release *(m, n, p)*
- Single-channel patch-clamp recordings: channel types and kinetics

Molecular Genetic Studies
- Mutation analysis (if candidate gene or protein identified)
- Linkage analysis (if no candidate gene or protein recognized)
- Expression studies (if mutation identified)

Abbreviations: AChE = acetylcholinesterase; AChR = acetylcholine receptor; α-bgt = α-bungarotoxin; EP = endplate; EMG = electromyography; MEEP = miniature endplate potential; MEPC = miniature endplate current; *m* = number of ACh quanta released by nerve impulse; *n* = number of readily releasable ACh quanta; *p* = probability of quantal release; SFEMG = single fiber EMG.

Generic identification of a CMS is often possible on clinical grounds on the basis of myasthenic symptoms since birth or early childhood, a typical pattern of the distribution of weakness with involvement of the cranial muscles, history of similarly affected relatives, decremental electromyographic (EMG) response of the compound muscle fiber action potential on low-frequency (2–3 Hz) stimulation, and negative tests for antibodies against the acetylcholine receptor (AChR) and P/Q type calcium channels. Some CMS, however, are sporadic or present in later life, a decremental EMG response may not be present in all muscles or at all times, and the weakness may be restricted in distribution and not involve cranial muscles. A clear understanding of the cause of a CMS and of the underlying pathogenic mechanisms requires special studies (*see* Table 1). If these tests point to a defect in a candidate gene or protein, then molecular genetic analysis becomes feasible. If a mutation is discovered in the candidate gene, then expression studies with the genetically engineered mutant molecule can be used to confirm pathogenicity and to analyze the properties of the mutant molecule.

On the basis of their studies of 146 CMS kinships at the Mayo Clinic, the authors classify the presently recognized CMS into three major categories: presynaptic, synaptic, and postsynaptic (*see* Table 2). This classification is useful, but it is still tentative because additional types of CMS may exist, and because in incompletely studied disorders, not listed in Table 1, as for example, the limb-girdle CMS, or the CMS associated with facial malformation in Iranian Jews, the site of the defect is not known *(1)*. Table 2 indicates that about 10% of CMS cases are presynaptic, 15% are caused by acetylcholinesterase (AChE) deficiency, and 75% are

Table 2
Site-of-Defect Based Classification of the CMS*

Site	Index cases
Presynaptic defects	
Choline acetyltransferase deficiency**	6
Paucity of synaptic vesicles and reduced quantal release	1
Lambert-Eaton syndrome like	1
Other presynaptic defects	4
Synaptic defects	
Endplate AChE deficiency**	24
Postsynaptic defects	
Kinetic abnormality of AChR with/without AChR deficiency**	37
AChR deficiency with/without minor kinetic abnormality**	67
Rapsyn deficiency**	5
Plectin deficiency	1
Total	**146**

* Classification based on cohort of CMS patients investigated at the Mayo Clinic between 1988 and 2001.

** Genetic defects identified.

postsynaptic. The representation of the different types of CMS, however, could be affected by embryonic or perinatal lethality, by reproductive capacity, or other factors. For example, the high frequency of the postsynaptic CMS is due to the high frequency of low-expressor or null mutations in the AChR ε subunit. A likely explanation for this is that expression of the fetal-type AChR γ subunit can partially compensate for a defect in the ε subunit, and thus rescue the phenotype, whereas a similar mechanism is not available for other types of CMS.

Presynaptic CMS

Four presynaptic CMS have been described to date: (i) CMS associated with episodic apnea (CMS-EA) caused by defects in choline acetyltransferase (ChAT) *(2);* (ii) a CMS with paucity of synaptic vesicles and reduced quantal release *(3);* (iii) a CMS resembling the Lambert-Eaton syndrome *(4);* and (iv) a CMS with reduced quantal release due to an undefined mechanism *(5).* The pathogenic mechanisms operating in syndromes (iii) and (iv) are still unclear; therefore only syndromes (i) and (ii) will be considered here.

Endplate Choline Acetyltransferase (ChAT) Deficiency (CMS with Episodic Apnea)

The distinguishing clinical feature is *sudden* episodes of severe dyspnea and bulbar weakness leading to apnea precipitated by infections, fever, or excitement. In some patients the disease presents at birth with hypotonia and severe bulbar and respiratory weakness requiring ventilatory support that gradually improves, but is followed by apneic attacks and bulbar paralysis in later life. Other patients are normal at birth and develop myasthenic symptoms and apneic attacks during infancy or childhood *(1).* Morphologic studies reveal no AChR deficiency and the postsynaptic region displays no structural abnormality. The synaptic vesicles are smaller than normal in rested muscle and increase or do not change in size after stimulation *(6).* Between attacks, a decremental EMG response is absent in rested muscles, but appears after a conditioning train of 10 Hz stimuli for 5 min. The synaptic response to ACh, reflected by the amplitude of the miniature endplate potential (MEPP) and EP potential (EPP), is normal in rested muscle but decreases abnormally during 10 Hz stimulation for 5 min and then recovers slowly over the next 10–15 min (*see* Fig. 1A) while the quantal content of the EPP is essentially unaltered *(6,7).*

That the MEPP and EPP amplitudes decline abnormally when neuronal impulse flow is increased and then recover slowly points to a defect in resynthesis or vesicular packaging of ACh and implicates four candidate genes: the presynaptic high-affinity choline transporter *(8,9),* ChAT *(10),* the vesicular ACh transporter (VAChT) *(11),* and the vesicular proton pump *(12)* (*see* Fig. 2). Mutation analysis in five CMS-EA patients uncovered no mutations in *VACHT* but revealed 10 recessive mutations in *CHAT (2)* (*see* Fig. 1B). One mutation (523insCC) was a null mutation; three others (I305T, R420C,

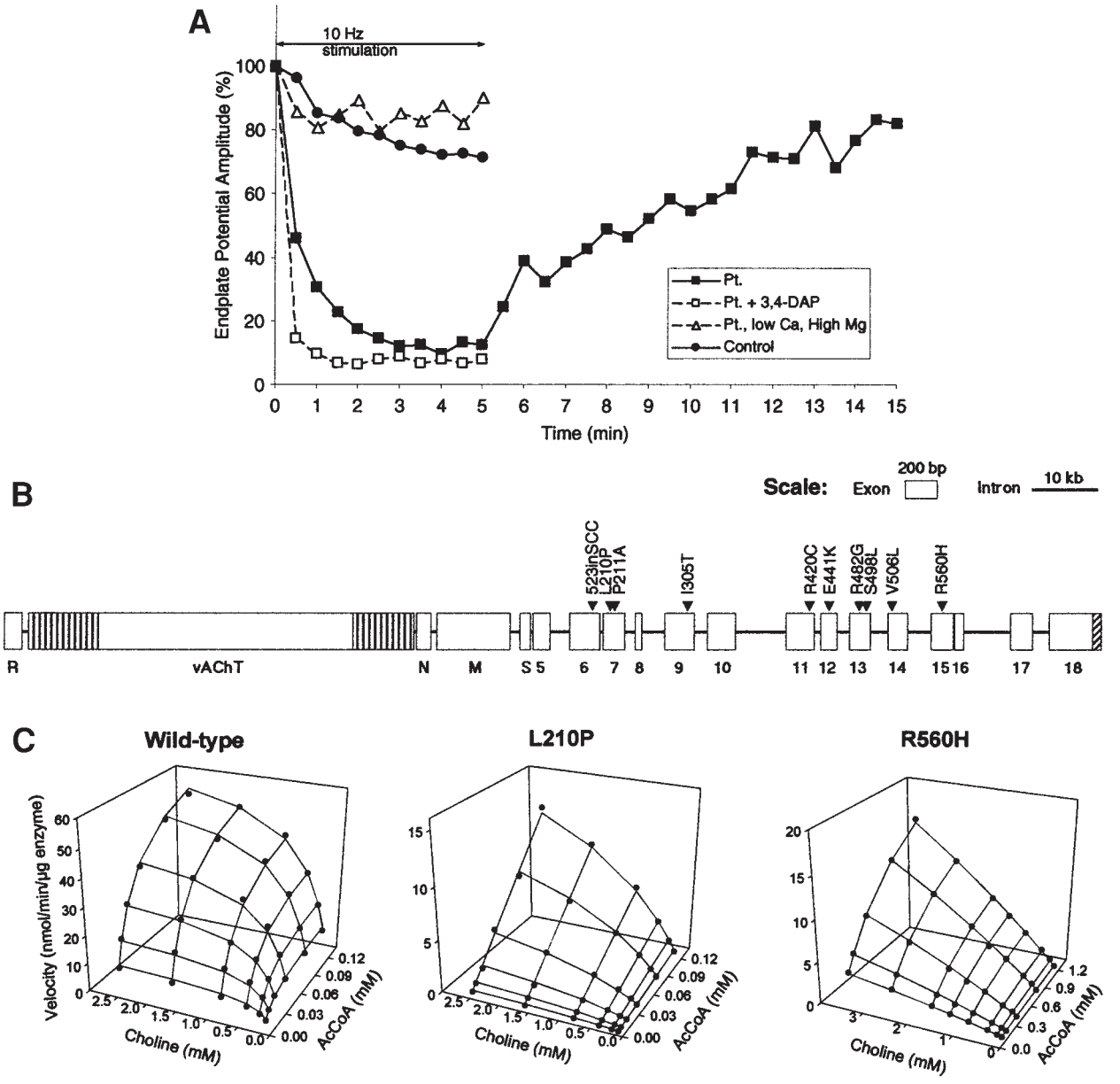


Fig. 1. CMS caused by mutations in choline acetyltransferase. **(A)** Ten Hz stimulation for 5 min results in a rapid abnormal decline of the endplate potential which then recovers slowly over ~10 min. 3,4-Diaminopyridine (3,4-DAP), which accelerates ACh release, enhances the defect, whereas a low CA^{2+}-high Mg^{2+} solution, which reduces ACh release, prevents the abnormal decline of the endplate potential. **(B)** Genomic structure of *CHAT* and identified mutations. The gene encoding the vesicular ACh transporter *VACHT* is located in the first *CHAT* intron. **(C)** Individually scaled kinetic landscapes of wild-type and of the L210P and R560H mutants of ChAT. The L210P mutant shows no saturation over a practical range of acetyl-CoA (AcCoA) concentrations, indicating an extremely hgih K_m for AcCoA. Similarly, the R560H mutant does not saturate with increasing concentrations of choline, indicating a very high K_m for choline. Figures 1 (B) and (C) are reproduced from ref. *(2)* by permission.

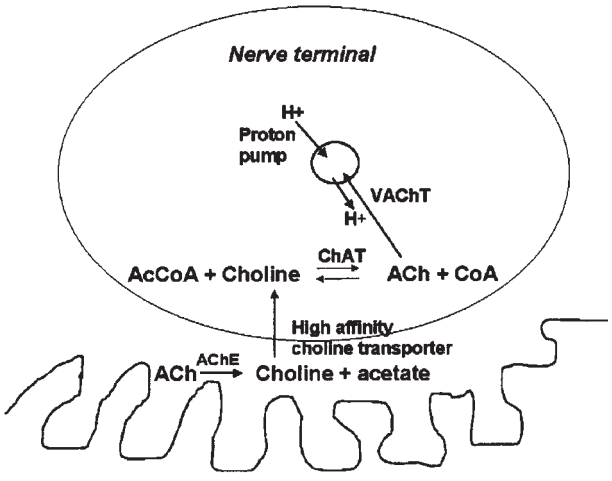


Fig. 2. Scheme of ACh resynthesis at the endplate. After ACh is hydrolyzed by AChE in the synaptic space, choline is transported into the nerve terminal by a high affinity choline transporter. ACh is resynthesized from choline and AcCoA by choline acetyltransferase (ChAT) and is then transported into the synaptic vesicle by the vesicular ACh transporter (VAChT) in exchange for protons delivered to the synaptic vesicle by a proton pump.

and E441K) markedly reduced ChAT expression in COS cells. Kinetic studies of nine bacterially-expressed and purified missense mutants revealed that one (E441K) lacked catalytic activity, and eight (L210P, P211A, I305T, R420C, R482G, S498L, V506L, R560H) had significantly impaired catalytic efficiencies (*see* Table 3 and Fig. 1C).

None of the observed CMS-EA patients had central or autonomic nervous system symptoms indicating that the neuromuscular synapse is selectively vulnerable to *CHAT* mutations. Presently there is no evidence that tissue-specific isoforms of ChAT explain this selective vulnerability. Although there are five alternative *CHAT* transcripts with at least three different promoters in human *(13),* the observed mutations are in the shared coding region of the recognized ChAT isoforms. The selective neuromuscular involvement may be owing to differences in presynaptic levels of ChAT, choline, or acetyl-CoA, rates of choline uptake, or rates of ACh release under conditions of increased neuronal impulse flow.

Paucity of Synaptic Vesicles and Reduced Quantal Release

This is a rare disorder. A presynaptic defect is indicated by a decrease to ~20% of the normal number of ACh quanta *(m)* released by nerve impulse. The decrease in *m* is due to a decrease in the number of readily releasable quanta *(n),* which is associated with a decrease in the numerical density of synaptic vesicles to ~20% of normal in unstimulated nerve terminals *(3).*

Synaptic vesicle precursors associated with different sets of synaptic vesicle proteins are produced in the perikaryon of the anterior horn cell and are carried distally along motor axons to the nerve terminal by kinesin-like motors *(14–17).* Mature vesicles containing a full complement of vesicular proteins are assembled in the nerve terminal *(17)* and are then packed with ACh. After ACh has been released by exocytosis, the vesicle membranes are recycled and then repacked with ACh *(18).* In the present syndrome, the reduction in synaptic vesicle density could arise from (i) a defect in the formation of synaptic vesicle precursors in the anterior horn cell, (ii) a defect in the axonal transport of one or more species of precursor vesicles, (iii) impaired assembly of the mature synaptic vesicles from their precursors, or (iv) impaired recycling of the synaptic vesicles in the nerve terminal. That synaptic vesicle density is reduced even in unstimulated nerve terminals argues against a defect in vesicle recycling.

Synaptic CMS

Endplate Acetylcholinesterase (AChE) Deficiency

This CMS is caused by the absence of AChE from the synaptic space *(19).* In most patients the disease presents in the neonatal period and is

Table 3
Kinetic Parameters of Wild-Type and Mutant ChAT Enzymes

k_{cat}, s^{-1}	K_m^{AcCoA}, μ*M*	K_m^{chol}, m*M*	k_{cat}/K_m^{AcCoA}*	k_{cat}/K_m^{AcCoA}*	$k_{cat}/(K_m^{AcCoA} \cdot K_m^{chol})$*
Wild-type	99.6 ± 3.5	26.8 ± 1.9	0.478 ± 0.036	1.0	1.00
L210P	35.0 ± 2.2†	n.d.‡	2.693 ± 0.280†	0.09	0.02†
P211A	418.8 ± 15.9	287.9 ± 28.6	0.514 ± 0.046	0.39	0.36
I305T	60.4 ± 3.4	70.6 ± 6.5	0.590 ± 0.064	0.23	0.19
R420C	176.8 ± 42.9	461.8 ± 134.4	1.018 ± 0.389	0.10	0.05
R482G	99.0 ± 6.9	91.7 ± 10.7	0.500 ± 0.067	0.29	0.28
S498L	88.6 ± 1.5	94.6 ± 7.2	0.418 ± 0.018	0.25	0.29
V506L	125.8 ± 7.4	68.4 ± 7.7	0.370 ± 0.053	0.49	0.64
R560H	34.1 ± 1.3§	870.8 ± 62.4§	n.d.¶	0.011§	0.002§

Values indicate estimate ± 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, $k_{cat}/K_m^{AcCoA} = 3.72 \times 10^6\ s^{-1}\ M^{-1}$ and $k_{cat}/(K_m^{AcCoA} \cdot K_m^{chol}) = 7.77 \times 10^9\ s^{-1}\ M^{-2}$.

† Apparent values calculated at 116 μ*M* AcCoA.

‡ Not determined because K_m^{AcCoA} exceeds practical concentration range of AcCoA. Catalytic efficiency was calculated from Equation 3 in ref. *(2)*.

§ Apparent values are calculated at 3.5 m*M* choline.

¶ Not determined because K_m^{chol} exceeds practical concentration range of choline. Catalytic efficiency is calculated from Equation 3 in ref. *(2)*. Reproduced with permission from ref. *(2)*.

highly disabling, but in some patients the onset is delayed and the disability is less marked.

The EP species of AChE is a heteromeric asymmetric enzyme composed of 1, 2, or 3 homotetramers of globular catalytic subunits ($AChE_T$) attached to a triple-stranded collagenic tail (ColQ) (*see* Fig. 3, right). ColQ has an N-terminal proline-rich region attachment domain (PRAD), a collagenic central domain, and a C-terminal region enriched in charged residues and cysteines (*see* Fig. 3, left). Each ColQ strand can bind an $AChE_T$ tetramer to its PRAD giving rise to A_4, A_8, and A_{12} species of asymmetric AChE *(20)*. Two groups of charged residues in the collagen domain (heparan sulfate proteoglycan binding domains, or HSPBD) *(21)* plus other residues in the C-terminal region *(22,23)* assure that the asymmetric enzyme is inserted into the synaptic basal lamina. The C-terminal region is also required for initiating the triple helical assembly of ColQ that proceeds from a C- to an N-terminal direction in a zipper-like manner *(24)*.

In 1998, human *COLQ* cDNA was cloned *(25,26)*, the genomic structure of *COLQ* determined *(25)*, and the molecular basis of EP AChE deficiency traced to recessive mutations in *COLQ (25,26)*. Twenty-two *COLQ* mutations in 23 kinships have been identified to date *(22,25–28)* (*see* Fig. 3, right). The mutations are of three major types *(22)*: (i) PRAD mutations prevent attachment of $AChE_T$ to ColQ; (ii) collagen domain mutations produce a short, single-stranded ColQ that binds a single $AChE_T$ tetramer and is insertion incompetent; (iii) C-terminal mutations hinder the triple helical assembly of the collagen domain, or produce an asymmetric species of AChE that is insertion incompetent, or both.

It is interesting that all EP AChE mutations identified to date reside in the collagenic tail subunit of the enzyme and prevent AChE expression at the EP. Low-expressor or null mutations in the catalytic subunit of AChE might also exist but would likely be lethal owing to the role of the catalytic subunit in the

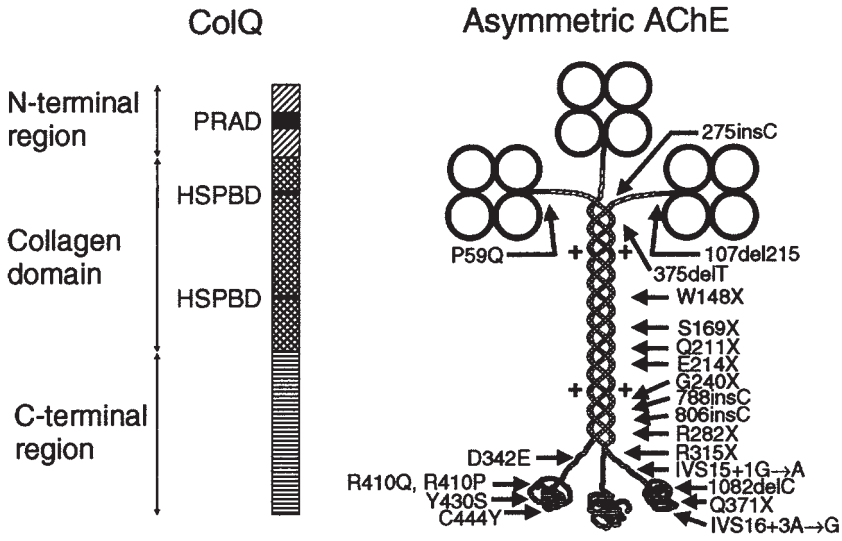


Fig. 3. (Left)-Schematic diagram showing domains of a ColQ strand. (Right)-Components of the A_{12} species of asymmetric AChE and 22 identified ColQ mutations. AChE = acetylcholinesterase; HSPBD = heparansulfate proteoglycan binding domain; PRAD = proline-rich attachment domain.

autonomic and central nervous system. Nonlethal kinetic mutations of the catalytic subunit could exist and affect transmission at both muscarinic and nicotinic synapses, but none has been identified to date.

Postsynaptic CMS

Overview of AChR Structure

Most postsynaptic CMS stem from a deficiency or kinetic abnormality of AChR. Therefore the authors begin with a brief review of the structure of the muscle type of AChR. Muscle AChR is a transmembrane macromolecule composed of five homologous subunits: two of α, one of β and δ, and one of ε in adult AChR, or one of γ instead of ε in fetal AChR. The genes coding for α, δ, and γ are at different loci on chromosome 2q, and those coding for β and ε are at different loci on chromosome 17p. The subunits are highly homologous, have similar secondary structures, fold similarly, and are organized like barrel staves around a central cation channel. Each subunit has an N-terminal extracellular domain that comprises ~50% of the primary sequence, four transmembrane domains (TMD1–TMD4), and a small C-terminal extracellular domain.

The two ACh binding sites are formed at interfaces between subunits: α–γ and α–δ in embryonic and denervated muscle, and α–ε and α–δ in the adult. Residues from both α and non-α subunits contribute profoundly to binding of ACh and competitive antagonists, indicating that the ACh binding sites are formed at interfaces between subunits *(29–31).* Mutagenesis, combined with ligands that select between the two binding sites, revealed seven linearly distinct loops among α and non-α subunits, designated loops A–G, that contribute to each site. Site-directed labeling studies physically localized all but one of the seven loops to the binding site, further demonstrating the presence of multiple loops *(32–35).* The recent x-ray structure of the snail glial acetylcholine binding protein (AChBP) confirmed that residues in all seven loops are present at the ligand binding site *(36)* including αY93 in loop A,

Table 4
Kinetic Abnormalities of AChR

	Slow-channel syndromes	Fast-channel syndromes
Endplate currents	Slow decay	Fast decay
Channel opening events	Prolonged	Brief
Open states	Stabilized	Destabilized
Closed states	Destabilized	Stabilized
Mechanisms[a]	Increased affinity Increased β Decreased α	Decreased affinity Decreased β Increased α Mode-switching kinetics
Pathology	Endplate myopathy from cationic overloading	No anatomic footprint
Response to therapy	Long-lived open channel blockade of AChR with quinidine or fluoxetine	3,4-Diaminopyridine and AChR inhibitors

β = channel opening rate; α = channel closing rate.

[a] Different combinations of mechanisms operate in the individual slow- and fast-channel syndromes.

αW149 in loop B, αY190 and αY198 in loop C, εK34 in loop D, εW55 in loop E, εL119 and εP121 in loop F, and εD175 in loop G. Each of these residues is highly conserved and may constitute minimal structures necessary for ACh binding. Additional residues at the periphery of the site have been identified by mutagenesis and confirmed by AChBP *(36).* These include αG153 in loop B, αS187, αV188 and αT189 in loop C, εK34 in loop D, εD59 in loop E, εL109, εY111, εS115 and εT117 in loop F, and εE177 in loop G. These peripheral residues may constitute structrues specialized for binding ACh at concentrations found at the motor synapse, or for releasing bound ACh with sufficient speed to terminate the response.

Unwin and colleagues recently resolved electron density in two-dimensional crystals of Torpedo AChR to a resolution of 4.6 Å *(37).* At the level of the membrane, the electron density reveals five rods typical of α-helices that twist upon activation to allow flow of permeant ions *(38).* These rods correspond to TMD2, with possible contributions from TMD1, based on substituted cysteine accessibility mutagenesis, and functional measurements. The collective studies indicate that the extracellular half of each TMD2 forms and α-helix, the middle three residues form an extended structure, and the remainder is an α helix followed by a β-strand that forms the channel gate and ion selectivity filter near the cytoplasmic limit of TMD2 *(29,39,40).* Electron densities corresponding to TMD3 and TMD4 have not been assigned, but the long cytoplasmic domain between them likely contributes to a fenestrated basket-like structure extending into the cytoplasm *(37).* This serves as an attachment site for cytoskeletal elements, bears phosphorylatable residues that may be important for desensitization and, in case of the ε subunit, stabilizes the gating mechanism *(41).*

CMS mutations have been found in all AChR subunits and in several domains of the subunits, including the major extracellular domain contributing to the ACh binding site, TMDs 1 through 3, and the long cytoplasmic domain between TMDs 3 and 4. Despite this diversity of targets, kinetic consequences of CMS mutations fall into two broad categories: increasing response to ACh in slow channel syndromes or decreasing response in fast channel syndromes. Table 4 lists reciprocal features of the slow- and fast-channel syndromes, and Fig. 4 B and C illustrate the abnormal single-channel and miniature EP currents in the two diseases.

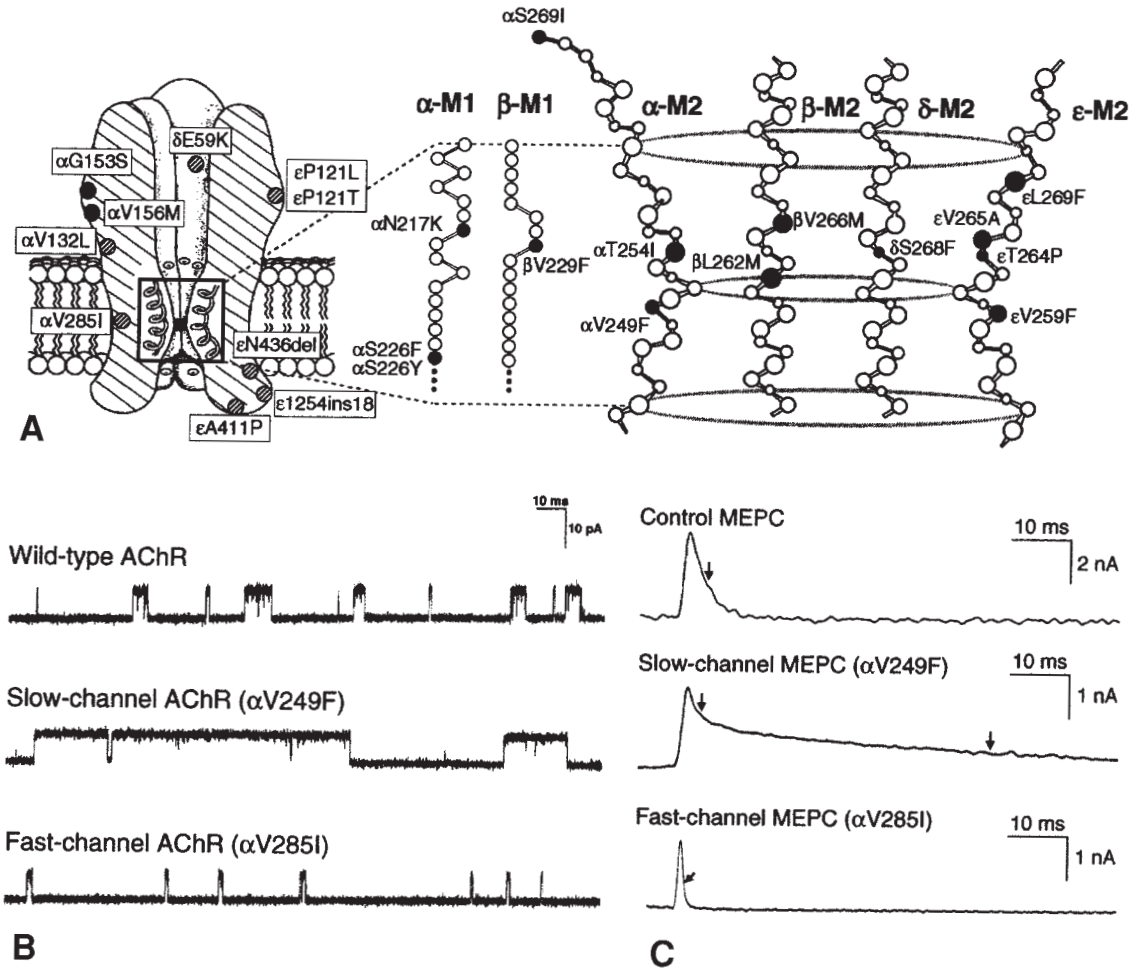


Fig. 4. **(A)** Schematic diagram of slow-channel (solid circles) and fast-channel (shaded circles) mutations. The drawing on the left shows a section through the acetylcholine receptor indicating approximate position of mutations that are not in transmembrane domains of the receptor. In the drawing on the right, dotted lines delimit transmembrane domains. Slow-channel mutations appear in the TMD2 domains of the α, β, δ, and ε subunits, and in the TMD1 domain of the α subunit. The αS2691 slow-channel mutation above the dotted line is in the extracellular TMD2/TMD3 linker. **(B)** Examples of single channel currents from wild-type, slow-channel (αV249F), and fast-channel (αV285I) AChRs expressed in human embryonic kidney (HEK) fibroblasts. **(C)** Miniature endplate currents (MEPC) recorded from endplates of a control subject, a patient harboring the αV249F slow-channel mutation, and a patient harboring the αV285I fast-channel mutation. Arrows indicate decay time constants. The slow-channel MEPC decays biexponentially due to expression of both wild-type and mutant AChRs at the EP, with one decay time constant that is normal and one that is markedly prolonged.

Slow-Channel Syndromes

Sixteen slow-channel mutations, all dominant and causing gain-of-function, have been reported to date *(42–51)* (solid symbols in Fig. 4A). The different mutations occur in different AChR subunits and in different functional domains of the subunits. The clinical consequences vary. In general, the disease is progressive owing to structural damage to the EP,

but mutations in the TMDs have more severe clinical consequences than those in the extracellular domain. Patch-clamp studies at the EP, mutation analysis, and expression studies in human embryonic kidney (HEK) cells indicate that the αG153S mutation near extracellular ACh binding site *(43)* (*also see* below) and the αN217K mutation in the N-terminal part of TMD1 *(45)* act mainly by enhancing affinity for ACh. This slows dissociation of ACh from the binding site and results in repeated channel reopenings during each receptor occupancy. αS226Y as well as αS226F in TMD1 enhance both affinity and gating efficiency *(50).* Mutations in TMD2 that lines the channel pore, such as βV266M, εL269F, εT264P and αV249F, as well as αS269I in the extracellular TMD2/TMD3 linker, act mainly by enhancing gating efficiency (channel opening rate β/channel closing rate α) *(42,44,46,52)* (*also see* below). Variable increases in steady-state affinity for ACh and concomitant increases in extent of desensitization are also observed with αV249F *(46),* εL269F *(44),* and εT264P *(42).*

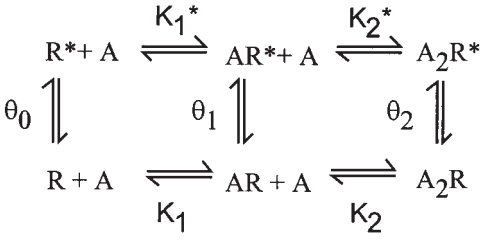
Other slow-channel mutations, namely αV156M near the ACh binding site *(48),* δS268F *(51),* βL262M *(47),* αT254I *(48),* and εV265A *(49)* in TMD2, and βV229F in TMD1 *(51)* have also been reported, but without detailed analysis of their effects on the kinetics of channel activation.

Functional Consequences of Mutations in TMD2

The most conspicuous effect of TMD2 mutations is a dramatic increase in duration of ACh-induced openings. Mean open durations increase from 0.5 ms for wild-type AChR to approx 50 ms for mutations in TMD2 *(42,44,46).* Also conspicuous are frequent channel openings in the absence of ACh *(42,46)* which occur rarely in wild-type type AChR. In addition to increasing open duration, the CMS mutation αV249F increases affinity of ACh for the resting state of the AChR *(46).* A complete set of activation rate constants has not been determined for receptors with mutations in TMD2 due to difficulty assigning the multiple closed time components to steps in a kinetic scheme *(46).* Finally, mutations in TMD2 enhance desensitization of the AChR, as shown by increased ACh affinity following equilibrium exposure to agonist *(44,46).*

Mechanistic Consequences of Mutations in TMD2

These are best described by considering the following allosteric description of acetylcholine receptor activation *(53,54):*



Scheme 1

Here, resting (R) and active (R*) states of the receptor spontaneously interconvert in the absence of agonist, and activation is driven by progressive occupancy of the sites together with tighter binding of agonist to the active compared to the resting state (i.e., the equilibrium dissociation constants K* are much smaller than K). Given tighter binding to the active state, the equilibrium constant θ between resting and active states increases with increasing agonist occupancy such that $\theta_2 >> \theta_1 >> \theta_0$. Thus, for muscle AChR, θ_0 is approximately 10^{-6}, θ_1 is approxy 10^{-2} and θ_2 ranges from 25–100. Thus in wild-type AChR, the essential task of the receptor is to bind the agonist more tightly to the active, rather than to the resting, state in order to overcome the unfavorable equilibrium constant θ_0 *(54).*

Increased spontaneous openings due to mutations in TMD2 indicate and increase of θ_0 in Scheme 1. A small value of θ_0 is vital for minimizing cation influx at rest and for maximizing the range of the response to ACh. The first ACh binding step thus begins with a mutant receptor already prone to opening, and the enhanced affinity of ACh for the active

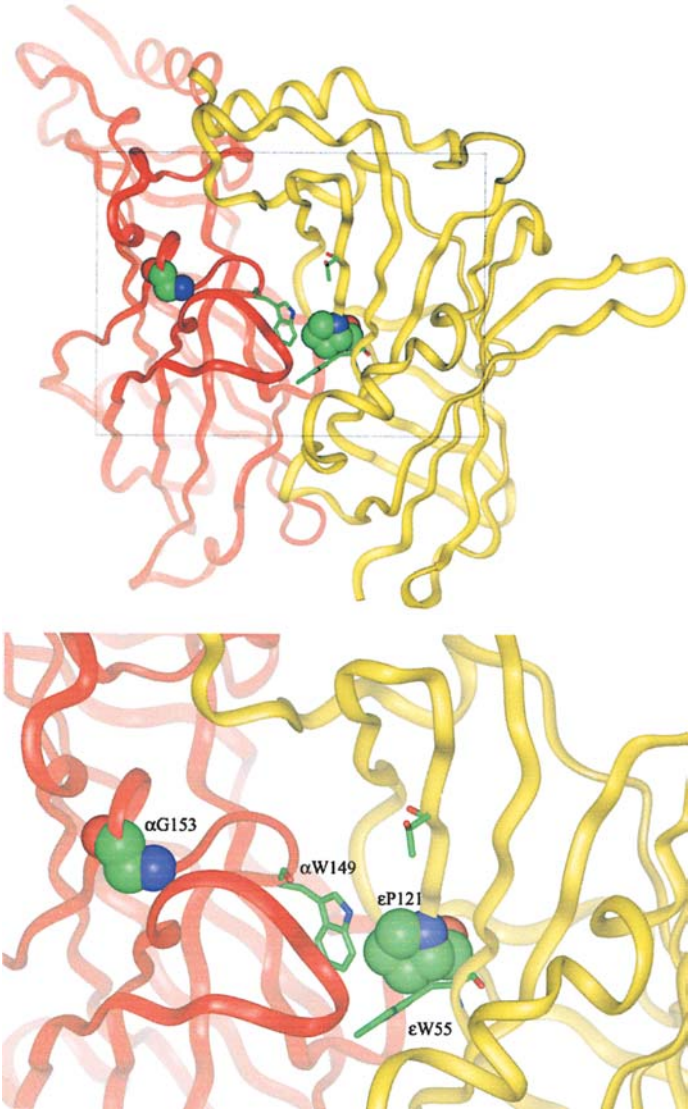


Fig. 5. Structural model of ligand-binding regions of human α and ε subunits generated by scanning mutagenesis and homology modeling. The α subunit portion of the ligand-binding site is shown in red ribbons, and the ε subunit portion is shown in yellow ribbons. Rendered in space-filling format are mutant residues in slow-channel (αG153) and fast-channel (εP121) congenital myasthenic syndromes. For orientation, key binding site residues (αW149 and εW55) are rendered in stick format. Lower panel is enlargement of boxed part of upper panel. Adapted from Sine, S. M., Wang, H-L. and Bren, N. (2002) Lysine scanning mutagenesis delineates structural model of the nicotinic receptor ligand binding domain, *J. Biol. Chem.* **277,** 29,210–29,233.

over the resting state further promotes opening of the mono-liganded receptor according to. $\theta_1 = \theta_0 K_1/K_1^*$. Analogously, the increase of θ_1 propagates to the doubly-liganded AChR, increasing the corresponding opening equilibrium constant θ_2 according to $\theta_2 = \theta_1 K_2/K_2^*$. Rates of channel opening are also increased by mutations in TMD2, the most obvious being

the ACh-independent opening rate β_0. The doubly-liganded opening rate β_2 also increases with mutations in TMD2 *(46)*, although the extent of the increase is uncertain because the opening rate for wild-type AChR approaches resolution limits of the patch clamp. The doubly-liganded closing rate α_2 slows with mutations in TMD2 owing to stabilization of the open state relative to the transition state. Finally, desensitization is enhanced to varying degrees for the different mutations in TMD2. A likely mechanistic explanation is increase of the equilibrium constant governing desensitization in the absence of ACh, analogous to that just described for the channel opening process; enhanced affinity of ACh for the desensitized over the resting state promotes desensitization of singly- and doubly-liganded receptors. Thus formutations in TMD2, channel activation is enhanced upon short-term exposure to ACh, whereas desensitization is enhanced upon long-term exposure.

Mechanistic Implications of Mutations in TMD2

Because TMD2 forms the ion channel, functional consequences of mutating this domain likely result from direct perturbation of the gating apparatus. Although the gating apparatus comprises TMDs from all five subunits, and gating comprises synchronous movement of all five, TMD2 mutations in individual subunits appear independent of the other subunits *(55)*. The overall results of mutating TMD2 indicate that its structure is essential in wild type AChR for stabilizing the channel in the closed state relative to the active and desensitized states; closed state stability prevents unwanted cation influx at rest, and also optimizes the fraction of activatable receptors. The structure of TMD2 is also vital for tuning the stability of the open relative to the transition state to provide millisecond EP current decay times inherent in wild type AChR. Because TMD2 couples tightly to the ACh binding site, mutations in TMD2 that affect ACh binding affinity likely exert their actions through allosteric propagation of the initial perturbation in the channel via a coupling structure.

Functional Consequences of the SCCMS Mutation αG153S

αG153S was the first CMS mutation discovered at the ACh binding site, and localized to one of three key regions of the α subunit that contribute to the site *(43)*. Flanking the key aromatic residues αW149 and αY151 *(56)* at the ligand binding site (*see* Fig. 5), αG153S greatly slows the rate of ACh dissociation from the doubly-liganded closed state of the AChR, increasing ACh affinity for this inactive state. In the αG153S AChR, the doubly-liganded closed state opens repeatedly during a single ACh occupancy because the rate of channel opening is some 46-fold greater than the competing rate of ACh dissociation, as opposed to 3.5-fold greater in wild-type AChR. αG153S also stabilizes the open channel and desensitized states, suggesting increased ACh affinity for these functional states. Increased stability of the open state is reflected by slowing of the channel closing rate, while increased stability of the desensitized state is demonstrated by tighter binding of ACh to receptors desensitized by the local anesthetic proadifen. Thus αG153S enhances ACh affinity for resting, active and desensitized states of the AChR.

Mechanistic Consequences of αG153S

By slowing the rate of ACh dissociation k_{-2} (Scheme 1), αG153S prolongs individual AChR activation episodes, known as bursts, according to $\tau_B = \tau_O (1 + \beta/k_{-2})$, where τ_B is mean burst duration and τ_O is mean open interval duration. Burst duration increases further due to the slower rate of channel closing α, which increases mean open duration according to $\tau_O=1/\alpha$. The probability that a doubly-liganded receptor will open increases according to $P= \beta/(\beta + k_{-2})$, predicting an increased peak response following instantaneous delivery of ACh. Desensitization is enhanced by αG153S, owing to tighter binding of ACh to this refractory state. Thus by increasing residence time of ACh at the bind-

ing site in all states of the AChR, αG153S produces a greater peak response, prolongs burst duration, and enhances desensitization.

Fast-Channel Syndromes

The fast-channel CMS are caused by recessive, loss-of-function mutations. Eight fast-channel mutations have been indentified *(41,57–63)* (shaded symbols in Fig. 4A). In each case, the mutated allele causing the kinetic abnormality is accompanied by a null mutation in the second allele so that the kinetic mutation dominates the clinical phenotype.

Low-Affinity Fast-Channel Syndromes

Three identified kinetic mutations fall in this group. Each mutation results in abnormally brief channel events, reduces the amplitude of the quantal response by decreasing the probability of channel openings, and decreases the number of channel reopenings in bursts of openings. Two mutations in the extracellular domain of the ε subunit, εP121L and εP121T, involve substitution of proline residue 121 by a leucine or a threonine *(57,58).* Detailed analysis of the effects of εP121L indicate that it diminishes diliganded open state affinity and reduces gating efficiency (*see* below) *(57).*

The third mutation in this group, αV132L, occurs in the highly conserved cys-loop of the α subunit, a disulfide-bridged β-hairpin formed between cystines 128 and 142. This mutation markedly reduces closed-state affinity for ACh and impairs gating efficiency by speeding the rate of channel closing *(59).*

Functional Consequences of the Fast-Channel CMS Mutation εP121L

The fast channel CMS mutation, εP121L, localizes within loop F of the ε subunit that contributes to the ACh binding site (*see* Fig. 5). Located at the carboxyl-terminal boundary of a series of residues that affect competitive antagonist binding *(30)* including εY111 and εT117, εP121L does not affect ACh affinity for the resting state of the AChR *(57).* Instead, εP121L markedly reduces probability of channel opening at saturating ACh concentrations. Accompanying this reduced opening probability, the rate of channel opening of the doubly-liganded AChR slows by nearly 500-fold, while the rate of channel closing increases about two-fold. Desensitization, on the other hand, develops more slowly and to a lesser extent due to reduced ACh affinity for the α–ε site in the desensitized state. Reduced ACh affinity for the desensitized state results in a nearly steady stream of single channel events even at high concentrations of ACh. Thus εP121L impairs entry of the AChR into its two functional states, active and desensitized.

Mechanistic Consequences of εP121L

Kinetic analysis of currents through AChRs containing εP121L provides a complete set of rate constants describing the activation process *(57).* Thus the conclusion of normal affinity for ACh in the resting state follows directly from the measured rate constants. Open-state affinity can be determined by applying the principle of microscopic reversibility to the second cycle in Scheme 1, using the measured closed-state affinity and gating steps for singly- and doubly-liganded receptors. Double-liganded open-state affinity, given by $K_2^* = K_2\,\theta_1/\theta_2$, decreases from 35 n*M* for wild-type to 1.5μ*M* for εP121L *(57).* Thus εP121 selectively stabilizes ACh bound to the open state of wild-type AChR. Additionally, the dramatic slowing of the rate of opening of the doubly-liganded AChR, β_2, suggests that εP121 is critical in forming the transition state in the path toward the open state. Impaired desensitization by εP121L is explained analogously to that just described for activation: loss of tight binding of ACh to the desensitized state allows the resting state to predominate even at high ACh concentrations. Thus the results from the εP121L mutation suggest that the proline 121 is critical in contributing to a binding site structure that better complements ACh bound to open and desensitized states relative to the resting state. The overall findings show that in wild-type AChR, tighter binding of ACh to func-

tional states is the fundamental driving force underlying agonist-induced activation and desensitization.

Fast-Channel Syndrome Due to a Selective Gating Abnormality

Replacement of a valine by an isoleucine at residue 285 in TMD3 of the α subunit (αV285I) selectively reduces gating efficiency by depressing the channel-opening rate β and enhancing the channel-closing rate α. The αV285I mutation also decreases AChR expression, which further impairs the safety margin of neuromuscular transmission *(60).*

Fast-Channel Syndrome Associated With Unstable (Mode-Switching) Kinetics

Two mutations causing unstable channel kinetics have been identified. The ε1254ins18 mutation, which is an inframe duplication of codons 413 to 418 (STRDQE) in the long cytoplasmic loop of ε, also reduces AChR expression at the EP *(41).* At the EP, ε1254ins18-AChR shows abnormally brief activation episodes during steady-state ACh application. When expressed in HEK cells and exposed to desensitizing concentrations of ACh, gating by ε1254ins18-AChR is not uniform but changes abruptly between at least three inefficient modes in which the receptor opens more slowly and closes more rapidly than normal. In this disorder, the reduced gating efficiency and decreased AChR expression are partially offset by expression of fetal AChR harboring the γ instead of the ε subunit (γ-AChR); this improves electrical activity at EP and likely rescues the phenotype *(41).*

The second mutation that destabilizes channel kinetics is a nearby missense mutation in the ε subunit, εA411P. When this mutation is expressed in HEK cells, different clusters of channel openings differ widely in their gating efficiency, so that the spread in the distribution of the channel openings and closing rates is greatly expanded *(61).* That both ε1254ins18 and εA411P occur in the amphipathic helix region of the TMD3/TMD4 long cytoplasmic loops of the ε subunit, implicates this region of the ε subunit in the modulation of channel-gating kinetics.

Other Fast-Channel Mutations

Both εN436del at the C-terminal end of the long cytoplasmic loop of ε *(62)* and δE59K in the extracellular domain of the δ subunit *(63)* result in abnormally brief channel currents. A detailed analysis of their effects on the kinetics of channel activation has not been published to date. δE59K, however, is of special interest because the affected infant, who also harbored a null mutation in the second δ subunit allele, was born with joint contractures (arthrogryposis), indicating hypomotility in utero.

CMS Caused by AChR Deficiency With or Without Minor Kinetic Abnormality

CMS with severe EP AChR deficiency result from different types of homozygous or, more frequently, heterozygous recessive mutations in AChR subunit genes. The mutations are concentrated in the ε subunit. A likely reason for this is that expression of the fetal type γ subunit, although at a low level, may compensate for absence of the ε subunit *(41,64,65),* whereas patients harboring null mutations in subunits other than ε might not survive for lack of a substituting subunit. In addition, the gene encoding the ε subunit, and especially exons coding for the long cytoplasmic loop, have a high GC content that could predispose to DNA rearrangements.

Different types of recessive mutations causing severe EP AChR deficiency have been identified:

(1) Mutations causing premature termination of the translational chain — these mutations are frameshifting *(64–72),* occur at a splice site *(66,67,69),* or produce a stop codon directly *(65).*
(2) Point mutations in the promoter region of a subunit gene (ε-155G→A *[73]* and ε-156C→T *[74]*).
(3) Missense mutations in a signal peptide region (εG-8R *[57]* and εV-13D *[69]*).
(4) Mutations involving residues essential for assembly of the pentameric receptor. Mutations of this type were observed in the ε subunit at an *N*-glycosylation site (εS143L) *(57),* in cysteine

128 (εC128S), a residue that is an essential part of the C128-C142 disulfide loop in the extracellular domain *(41),* in arginine 147 (εR147L) in the extracellular domain, which lies between isoleucine 145 and threonine 150, residues that contribute to subunit assembly *(65),* and in threonine 51 (εT51P) *(69);* and with a 3 codon deletion in the long cytoplasmic loop of the β subunit *(75).* (5) Missense mutations affecting both AChR expression and kinetics. For example, εR311W in the long cytoplasmic loop between M3 and M4 decreases *(65),* whereas εP245L in the M1 domain increases *(65),* the open duration of channel events. In the case of εR311W and εP245L, the kinetic consequences are modest and are likely overshadowed by the reduced expression of the mutant gene. For a list of AChR subunit gene mutations and the appropriate references, the reader is referred to a recently published gene table *(76).*

CMS Caused by Mutations in Rapsyn

In a subset of CMS patients with EP AChR deficiency but no mutations in AChR, the genetic defect has been elusive. Rapsyn, a 43 kDa postsynaptic protein, plays an essential role in clustering AChR at the EP. Seven tetratricopeptide repeats (TPRs) of rapsyn subserve self-association, a coiled-coil domain binds to AChR, and a RING-H2 domain associates with β-dystroglycan and links rapsyn to the subsynaptic cytoskeleton. Rapsyn self-association precedes recruitment of AChR to rapsyn clusters. In four patients with EP AChR deficiency but no mutations in AChR, the authors recently identified three recessive rapsyn mutations: one patient carries L14P in TPR1 and N88K in TPR3; two are homozygous for N88K; and one carries N88K and 553ins5 that frameshifts in TPR 5. EP studies in each case show decreased staining for rapsyn as well as AChR and impaired postsynaptic morphologic development. Expression studies in HEK cells reveal that none of the mutations hinders rapsyn self-association but all three diminish co-clustering of AChR with rapsyn. That missense mutations in TPR domains decrease co-clustering of rapsyn with AChR implies that effects of these mutations propagate downstream to the coiled-coil domain, or that the mutations have an allosteric effect on the conformation of the coiled-coil or RING-H2 domains.

Other Possible Causes of EP AChR Deficiency

Although EP AChR deficiencies have now been traced to mutations in AChR subunits and rapsyn, it is important to note that mutations in other EP specific proteins involved in regulating AChR expression or aggregation at the EP remain a potential cause of CMS. These proteins include neural agrin *(77,78),* MuSK *(79),* the predicted protein MASC that enables agrin to bind to MuSK *(79),* the predicted protein RATL that functionally couples MuSK to rapsyn *(80),* Src, Fyn, and Yes kinases signaling downstream from MuSK *(81),* β-dystroglycan *(82),* S-NRAP *(83),* neuregulin and its signaling molecules *(84–86),* neurotrophin-4 *(87)* and its receptor TrkB *(88),* as well as α-dystrobrevin *(89),* utrophin *(90),* and α-syntrophin *(91).*

CMS Associated with Plectin Deficiency

Plectin is a highly conserved and ubiquitously expressed intermediate filament-linking protein concentrated at sites of mechanical stress, such as the postsynaptic membrane of the EP, the sarcolemma, Z-disks in skeletal muscle, hemidesmosomes in skin, and intercalated disks in cardiac muscle. Pathogenic mutations in plectin are associated with a simplex variety of epidermolysis bullosa, a progressive myopathy with dystrophic features, and a myasthenic syndrome without EP AChR deficiency *(92).* The exact mechanism by which the plectin deficiency impairs neuromuscular transmission is still not known.

Final Comments

Characterization of the CMS has proceeded from clinical observation to electrophysio-

logic and ultrastructural studies, mutation analysis, and then to investigation of the effects of the observed mutations on the properties of the mutant proteins. For each identified CMS, this approach has explained the mechanism that impairs neuromuscular transmission and has pointed to rational therapy. Moreover, mutations detected in proteins critical to neuromuscular transmission have highlighted functionally significant domains of the affected proteins and yielded precise structure–function correlations. That the advances in the CMS have been so rapid in the past decade can be attributed to more than one factor: the ease with which transmission at single neuromuscular synapses can be studied in vitro; identification of genes encoding subunits of muscle AChR, subunits of EP AChE, as well as rapsyn, and choline acetyltransferase; the advent of mammalian expression studies for defining the properties of the mutant proteins; and increasing sophistication of patch-clamp analysis allowing dissection of the kinetics of AChR activation.

Studies of CMS also have general implications for both future investigations of neurological disease and the basic science of neurotransmission. In the near term, understanding neurological diseases of unknown etiology will likely require careful clinical diagnosis combined with morphologic, physiologic, genetic, and biophysical analyses. There are likely many such disorders of unknown origin, because by comparison, the neuromuscular junction is under far greater selective pressure and has fewer compensatory mechanisms than synapses in the central nervous system. In the long term, rapid evaluation of the genome may quickly pinpoint candidate genes, but biophysical and functional analysis will still be required. That is actually beneficial from the standpoint of advancing basic science, as clinical investigation of CMS has proven to be a rich source of basic science insights. It seems evolution has provided a novel search beam for revealing cornerstones of function, whether it is a key part of the protein sequence or the key macromolecule itself. For example, physiologists long theorized that endplate current decay kinetics depend not only on the kinetics of opening and closing of the channel, but also on the rate of dissociation of agonist. The importance of agonist dissociation was clearly illustrated by a CMS mutation due to slowed dissociation of agonist, which prolonged the decay rate. Even unforeseen insight emerges that may generalize to many other channels as well as enzymes, such as the existence of structures that maintain fidelity of channel gating revealed by a CMS. Basic scientists must be ready to take advantage of the profound opportunities offered by clinical medicine. The future brims with many exciting opportunities where clinical and basic science partner in a virtuous cycle in which clinical medicine benefits basic science, which in turn benefits clinical medicine.

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