3.3 Fast-channel congenital myasthenic syndrome (FCCMS)

The third class of postsynaptic CMS due to mutations in AChR subunit genes is FCCMS. FCCMS is kinetically opposite to SCCMS (Fig. 3). In FCCMS, the closed state of AChR is stabilized compared to the open state which results in abnormally brief channel opening events which, in turn, reduces the amplitude of the endplate potential and impair the safety margin of neuromuscular transmission. The resulting pathophysiology is thus similar to endplate AChR deficiency, but abnormally small endplate potential is a qualitative instead of a quantitative defect in AChR.

FCCMS is an autosomal recessive disorder. One allele carries a missense mutation that confers a fast closure of AChRs, and the other allele usually harbors a low-expressor or null mutation, or the fast channel mutation occurs at homozygosity. As in heterozygous healthy parents of endplate AChR deficiency, we humans may completely lack 50% of each AChR subunit without any clinical symptoms. In FCCMS, a low-expressor or null mutation on one allele unmasks the deleterious effect of the fast-channel mutation on the second allele. Detailed kinetic analyses of FCCMS mutations have revealed special insights into the molecular architectures of the AChR subunits. Three such examples are presented here.

The ε1254ins18 mutation causes a duplication of STRDQE codons at positions 413 to 418 close to the C-terminal end of the long cytoplasmic loop (LCP) linking the third (M3) and fourth (M4) transmembrane domains of the receptor. ε1254ins18-AChR expressed on HEK293 cells opens in three different modes. The opening probabilities of normal AChRs are clustered into a single large peak, whereas the ε1254ins18-AChR shows three different peaks (Milone *et al.*, 1998). In all the three modes, the AChR is activated slowly and inactivated rapidly, which gives rise to an inefficient synaptic response to ACh. Another FCCMS mutation, εA411P in the LCP also destabilizes the channel opening kinetics. The channel opening probabilities of εA411P-AChRs are widely distributed and do not form any discernible peaks (Wang *et al.*, 2000). Our analysis first disclosed that the function of LCP is to stabilize the open conformation of the AChR.

εN436del is a deletion of Asn at the C-terminal end of the LCP. The deletion shortens the LCP and shifts a negatively charged Asp residue at codon 435 against M4. εN436del-AChR decreases the duration of channel opening bursts 2.7-fold compared to the wild type due to a 2.3-fold decrease in gating efficiency and a 2.5-fold decrease in agonist affinity of the diliganded closed state. A series of artificial mutations established that the effects of εN436del are not due to juxtaposition of a negative charge against M4 but to the shortening of the LCP. Deletion of the C-terminal residue of the LCP of the β and δ subunits also results in fast-channel kinetics, but that in the α subunit dictates slow-channel kinetics. Thus, the LCPs of four AChR subunits contribute in an asymmetric manner to optimize the activation of AChRs through allosteric links to the channel and to the agonist binding sites (Shen *et al.*, 2005).

The mutation αV285I introduces a bulky amino acid into the M3 transmembrane domain and causes FCCMS (Fig. 3). Kinetic studies demonstrate that the mutation slows the channel opening rate β and speeds the channel closing rate α, resulting in a 15.1-fold reduction in the channel gating equilibrium constant θ (= β/α). On the other hand, the mutation minimally affects affinity for ACh. The probability of channel openings decreased when we introduced Leu, a bulky amino acid, at position V285, but rather increased when we introduced smaller amino acids such as Thr and Ala. We observed similar effects when we introduced similar

substitutions into the β, δ, and ε subunits. Thus, introduction of bulky amino acids narrows the channel pore, while introduction of smaller amino acids widens the channel pore. Our analysis thus revealed that the M3 domain backs up the channel-lining pore lined by the M2 transmembrane domains and has stereochemical effects on channel gating kinetics (Wang *et al.*, 1999).

FCCMS can be effectively treated with anticholinesterases and 3,4-diaminopyridine. The pharmacologic effects of these drugs were discussed in the section of endplate AChR deficiency (Section 3.1.2).

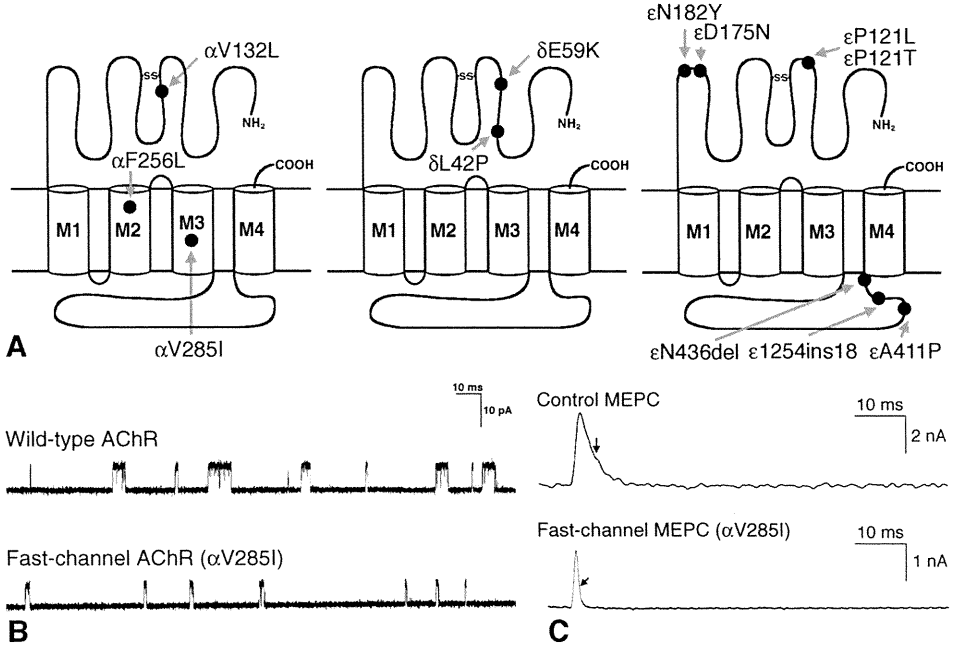

Fig. 3. Fast channel CMS. (A) Schematic diagram of AChR subunits with FCCMS mutations. (B) Single channel currents from wild-type and fast channel (αV285I) AChRs expressed on HEK293 cells. (C) Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring αV285I. The patient's MEPC decays faster than that of the normal control.

3.4 CMS due to defects in skeletal muscle sodium channel, $Na_V1.4$

Another class of postsynaptic CMS is due to mutations in skeletal muscle sodium channel, $Na_V1.4$, encoded by *SCN4A* (Tsujino *et al.*, 2003). Dominant gain-of-function mutations in this gene cause hyperkalemic periodic paralysis (Ptacek *et al.*, 1991), paramyotonia congenita (McClatchey *et al.*, 1992; Ptacek *et al.*, 1992), potassium-aggravated myotonia (Lerche *et al.*, 1993), and hypokalemic periodic paralysis type 2 (Bulman *et al.*, 1999). On the other hand, loss-of-function mutations cause a CMS.

Failure of normal-amplitude endplate potential depolarizing the resting potential to -40 mV in intercostal muscle of a CMS patient with episodes of apnea and myasthenic symptoms

since birth prompted us to search for mutations in *SCN4A*. We identified two heteroallelic missense mutations, S246L and V1442E (Tsujino *et al.*, 2003). Activation kinetics of the mutant $Na_V1.4$ was normal for both S246L and V1442E, but the fast inactivation curves were shifted to hyperpolarization by 7.3 mV for S246L and 33.2 mV for V1442E, indicating that both mutations enhance fast inactivation of the $Na_V1.4$ immediately after it is activated. Moreover, a high proportion of the V1442 channel was in the inactivated state even at a normal resting membrane potential. Recovery from the fast-inactivated state was slowed for both mutations. This was in contrast to gain-of-function mutations in other diseases, which shift the fast inactivation curves to depolarization. Neither S246L nor V1442E affected slow inactivation. Analysis of use-dependent inactivation in HEK293 cells by stimulating at 50 Hz for 3 ms revealed that wild-type and S246L channels decreased the peak current only by 5% and V1442E channel decreased it by 30% during the first few pulses and suggested that the S246L mutation is relatively benign.

4. Synaptic CMS

Defects in three components of the synaptic basal lamina, AChE, β2 laminin and neural agrin, are associated with CMS. The CMS caused by mutations in agrin was discussed above under the postsynaptic CMS (Section 3.1.3) because the site of action of agrin is the LRP4/MuSK complex at the endplate.

4.1 Endplate AChE deficiency due to defects in collagen Q

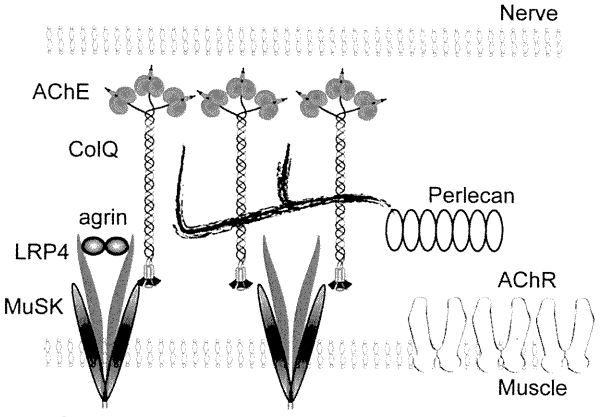

Fig. 4. ColQ anchors to the synaptic basal lamina by binding to perlecan and MuSK.

Three tetramers of catalytic AChE subunits are linked by a triple helical collagen Q (ColQ) to constitute an asymmetric ColQ-tailed AChE (Krejci *et al.*, 1997). ColQ carries three domains (i) an N-terminal proline-rich attachment domain (PRAD) that organizes the catalytic AChE subunits into a tetramer, (ii) a collagenic domain that forms a triple helix, and (iii) a C-terminal domain enriched in charged residues and cysteines. ColQ-tailed AChE is organized in the secretory pathway, excreted, and anchored into the synaptic basal lamina using two domains of ColQ (Fig. 4). First, the collagen domain harbors two heparan sulfate proteoglycan (HSPG) binding domains (Deprez *et al.*, 2003) that bind to HSPG, such as

perlecan (Peng *et al.*, 1999). Second, the C-terminal domain binds to MuSK (Cartaud *et al.*, 2004).

Endplate AChE deficiency is caused by congenital defects of ColQ (Donger *et al.*, 1998; Ohno *et al.*, 1998; Ohno *et al.*, 2000). Congenital defects of ColQ cause endplate AChE deficiency. No mutations have been detected in a gene encoding the catalytic subunit of AChE in CMS or in any other disease. There are three classes of ColQ mutations. First, mutations in the proline-rich attachment domain (PRAD) hinder binding of ColQ to AChE. Sedimentation analysis of AChE species of the patient muscle and transfected cells shows complete lack of ColQ-tailed AChE. Second, mutations in the collagen domain, most of which are truncation mutations, hinder formation of triple helix of ColQ. Sedimentation analysis of muscle and transfected cells demonstrate a truncated single-stranded ColQ associated with a homotetramer of AChE. Third, the mutations in the C-terminal domain have no deleterious effect on formation of the asymmetric ColQ-tailed AChE, but they compromise anchoring of ColQ-tailed AChE to the synaptic basal lamina as elegantly shown in vitro overlay binding of mutant and wild-type human recombinant ColQ-tailed AChE to the frog endplate (Kimbell *et al.*, 2004).

EMG studies show a decremental response as in other CMS. In addition, most patients have a repetitive CMAP response on a single nerve stimulus. The repetitive CMAP decrements faster than the primary CMAP. It can be overlooked unless a well rested muscle is tested by single nerve stimuli. The prolonged dwell time of unhydrolyzed ACh in the synaptic space prolongs the endplate potential; when this exceeds the absolute refractory period of the muscle fiber action potential, it elicits a repetitive CMAP. As mentioned above, a repetitive CMAP also occurs in slow channel syndrome.

Some aspects of the pathophysiology of endplate AChE deficiency resemble those of the SCCMS. As in the SCCMS, neuromuscular transmission is compromised by three distinct mechanisms. First, staircase summation of endplate potentials causes a depolarization block, which inactivates a proportion the voltage-gated skeletal sodium channel, $Na_V1.4$. (Maselli & Soliven, 1991). Second, prolonged exposure of AChR to ACh during physiologic activity desensitizes a fraction of the available AChRs (Milone *et al.*, 1997). Third, repeated openings of AChR cause calcium overloading to the endplate, which culminates in an endplate myopathy (Groshong *et al.*, 2007). Unlike in the SCCMS, the nerve terminals are abnormally small and often encased by Schwann cells. This decreases the quantal content and hence the amplitude of the endplate potential (Engel *et al.*, 1977).

Anticholinesterase medications have no effect on neuromuscular transmission and can cause excessive muscarinic side effects. Quinidine (Fukudome *et al.*, 1997; Harper & Engel, 1997) and fluoxetine (Harper *et al.*, 2003), which shorten the open duration of the AChR channel and benefit the slow-channel syndrome, can increase muscle weakness. A respirator dependent infant with severe endplate AChE deficiency was improved by intermittent blockade of AChR by atracurium, an agent that protects AChR from overexposure to ACh (Breningstall *et al.*, 1996). Ephedrine sulfate at a dose of 150 to 200 mg per day in adults is effective for myasthenic symptoms (Bestue-Cardiel *et al.*, 2005; Mihaylova *et al.*, 2008). Although high concentrations of ephedrine are able to block AChR openings (Milone & Engel, 1996), molecular bases of ephedrine effects in clinical practice remain elusive. As an alternative to ephedrine, albuterol sulfate 8 to 16 mg per day also shows benefit (Liewluck *et al.*, in press).

4.2 CMS due to a defect in β2 laminin

Laminins are cruciform heterotrimeric glycoproteins composed of α, β, and γ chains and are assembled from products of five α, four β, and three γ genes. The laminin molecules are named according to their chain composition. For example, laminin-321 contains α3, β2, and γ1 chains (Aumailley *et al.*, 2005). Three laminins are present at the synaptic basal lamina, laminin-221, laminin-421, and laminin-521. Each contains the β2 subunit. Laminin-421 is restricted to the primary synaptic cleft and promotes the precise alignment of pre- and postsynaptic specializations. Laminin-521 lines the primary and secondary clefts, promotes presynaptic differentiation, and prevents Schwann cells from entering the synaptic cleft. The synaptic laminins provide a stop signal for axons at developing endplates and organize presynaptic differentiation (Sanes, 1997). Mice deficient for *Lamb2* that encodes β2 laminin show reduced terminal branching of presynaptic motor axons, with a decreased number of active zones, no clustering of the synaptic vesicles above the active zones, and extension of Schwann cell processes into the primary synaptic cleft, and decreased spontaneous and evoked quantal release (Noakes *et al.*, 1995; Patton *et al.*, 1998). In addition to its presence at the endplate, β2-laminin is also highly expressed in renal glomeruli and the eye. *LAMB2* mutations in humans cause Pierson syndrome characterized by ocular malformation including small non-reactive pupils, loss of accommodation, and abnormalities of the lens, cornea and retina and by fatal nephrotic syndrome that requires renal transplantation (Zenker *et al.*, 2004).

Maselli and coworkers reported a 20-year-old woman with Pierson syndrome caused by two heteroallelic frameshifting mutations (1478delG and 4804delC) in *LAMB2* who also had a severe CMS (Maselli *et al.*, 2009). The nephrotic syndrome was corrected by a renal transplant at age 15 months. The patient had respiratory distress in infancy, delayed motor milestones, a decremental EMG response, limited ocular ductions, bilateral ptosis, severe proximal limb weakness, scoliosis, and required assisted ventilation at night and sometimes during the day. AChE activity was spared at the NMJ. Electron microscopy of the NMJ showed small axon terminal size and encasement of nerve endings by the Schwann cell, widening of the primary synaptic clefts with invasion of the synaptic space by processes of Schwann cells, moderate simplification of postsynaptic membranes, and decreased number of synaptic vesicles. Both morphological and microelectrode studies were similar to those observed in *Lamb2*-mice (Noakes *et al.*, 1995). Notably, symptoms were worsened by pyridostigmine but were improved by ephedrine.

5. Presynaptic CMS

Choline acetyltransferase (ChAT) is the only presynaptic molecule that is known to be defective in CMS.

5.1 CMS with episodic apnea due to defects in choline acetyltransferase (ChAT)

ACh released from the nerve terminal is hydrolyzed into choline and acetate by AChE at the synaptic basal lamina. Choline is taken up by the nerve terminal by a high-affinity choline transporter on the presynaptic membrane (Apparsundaram *et al.*, 2000; Okuda *et al.*, 2000). ChAT resynthesizes ACh from choline and acetyl-CoA (Oda *et al.*, 1992). After the synaptic vesicles are acidified by the vesicular proton pump (Reimer *et al.*, 1998), the resynthesized cationic ACh is packed into a synaptic vesicle by the vesicular ACh transporter (vAChT) in exchange for protons (Erickson *et al.*, 1994).

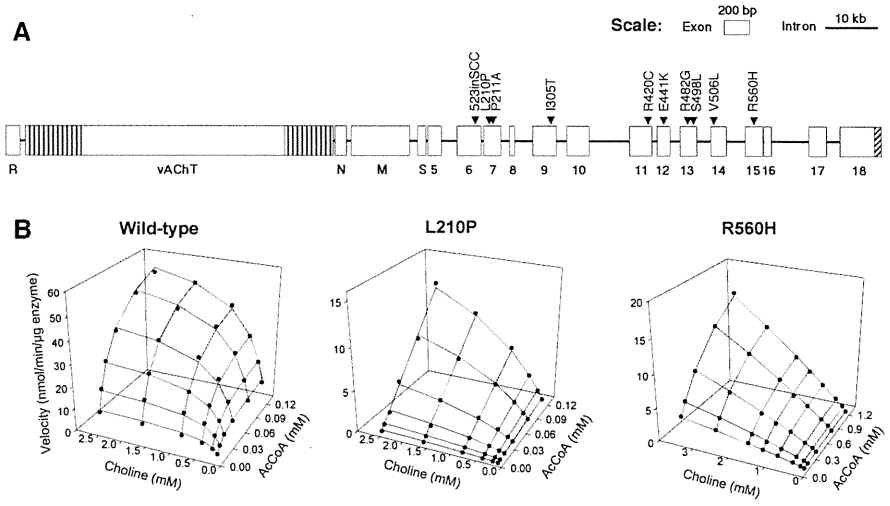

Fig. 5. Choline acetyltransferase (ChAT). (A) Genomic structure of *CHAT* and identified mutations. A gene for vesicular acetylcholine transporter (vAChT) is in the first intron of *CHAT*. (B) Kinetics of wild-type and mutant ChAT enzymes. ChAT synthesizes acetylcholine using choline and acetyl-CoA. L210P abrogates an affinity of ChAT for acetyl-CoA (AcCoA), and R560H abolishes an affinity of ChAT for choline.

We determined the complete genomic structure of *CHAT* encoding ChAT, and identified ten mutations in five CMS patients with the characteristic clinical features of sudden episodes of apnea associated with variable myasthenic symptoms (Ohno *et al.*, 2001). Additional *CHAT* mutations were later reported by other groups (Maselli *et al.*, 2003; Schmidt *et al.*, 2003; Barisic *et al.*, 2005; Mallory *et al.*, 2009; Yeung *et al.*, 2009; Schara *et al.*, 2010). All of our patients showed a marked decrease of the endplate potential after subtetanic stimulation that recovered slowly over 5 to 10 min, which pointed to a defect in the resynthesis or vesicular packaging of ACh at the nerve terminal. Kinetic studies of mutant ChAT enzymes disclosed variable decreases in affinity for choline and/or acetyl-CoA, as well as variable reduction the catalytic rate (Ohno *et al.*, 2001) (Fig. 5). Moreover, some recombinant mutants expressed at a reduced level in COS cells. Two patients carried a functionally null mutation on one allele, but ChAT encoded on the other allele was partially functional. Heterozygous parents that carried the null allele were asymptomatic indicating that humans can tolerate up to but not exceeding 50% reduction of presynaptic ChAT activity. None of our patients has autonomic symptoms or signs of central nervous system involvement other than that attributed to anoxic episodes. This suggests that the ChAT activity and/or substrate availability are rate limiting for ACh synthesis at the motor nerve but not at other cholinergic synapses. Indeed, stimulated quantal release at the endplate is higher than at other cholinergic synapses, which points to selective vulnerability of the NMJ to reduced ACh resynthesis. Crystal structure of ChAT resolved at 2.2 Å revealed that some of the reported *CHAT* mutations in CMS patients are not at the substrate-binding or the catalytic site of ChAT. Hence these mutation exert their effect by an allosteric mechanism or render the enzyme structurally unstable (Cai *et al.*, 2004).

In most patients, anticholinesterase medications are of benefit in ameliorating the myasthenic symptoms and preventing the apneic crises but few patients fail to respond to cholinergic therapy remaining permanently paralyzed and remain respirator dependent. Prophylactic anticholinesterase therapy is advocated even for patients asymptomatic between crises. Parents of affected children must be indoctrinated to anticipate sudden worsening of the weakness and possible apnea with febrile illnesses, excitement, or overexertion. Long-term nocturnal apnea monitoring is indicated in any patient in whom ChAT deficiency is proven or suspected (Byring *et al.*, 2002).

6. Conclusions

We reviewed the clinical and molecular consequences of defects in 11 genes associated with CMS. Molecular studies of CMS began with identification of a missense mutation in the AChR ε subunit in a SCCM patient (Ohno *et al.*, 1995). Since then, mutations in seven postsynaptic, three synaptic, and one presynaptic proteins have been discovered. In some CMS the disease gene has been elusive and await discovery. Resequencing analysis with the next generation sequencers may speed this effort.

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Chapter 8. Molecular defects of acetylcholine receptor subunits in congenital myasthenic syndromes

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ABSTRACT

Congenital myasthenic syndromes (CMS) are caused by mutations in molecules expressed at the neuromuscular junction. Among eight defective molecules identified to date in CMS, mutations in the muscle nicotinic acetylcholine receptor (AChR) subunits are the first to be characterized and are prevalent. Mutations in the AChR subunit genes cause three phenotypes: (i) endplate AChR deficiency in which the number of AChR at the endplate is critically reduced; (ii) slow channel syndrome in which mutations either at the acetylcholine binding sites or at the ion channel pore increases synaptic response to acetylcholine and prolongs AChR channel opening events; and (iii) fast channel syndrome in which mutations either at the acetylcholine binding sites or at the long cytoplasmic loop between the third and fourth transmembrane domains compromise synaptic response to acetylcholine and shortens AChR channel openings. In addition, mutations in AChR subunit genes also cause fetal akinesia deformation sequence, and a single nucleotide polymorphism in the promoter region of the AChR α1 subunit is associated with early onset myasthenia gravis.

INTRODUCTION

Congenital myasthenic syndromes

Congenital myasthenic syndromes (CMS) are heterogeneous disorders caused by congenital defects of molecules expressed at the neuromuscular junction (Fig. 1). Each mutation affects the expression level of the mutant molecule and/or compromises the functional properties of the mutant molecule. The mutant molecules identified to date include (i) acetylcholine receptor (AChR) subunits [1,2], (ii) rapsyn that anchors and clusters AChRs at the neuromuscular junction [3,4], (iii) agrin that is released from the nerve terminal and induces AChR clustering by stimulating the downstream LRP4/MuSK/Dok-7/rapsyn/AChR pathway [5], (iv) MuSK that transmits the AChR-clustering signal from agrin/LRP4 to Dok-7/rapsyn/AChR [6,7], (v) Dok-7 that transmits the AChR-clustering signal from agrin/LRP4/MuSK to rapsyn/AChR [8,9], (vi) skeletal muscle sodium channel type 1.4 ($Na_V1.4$) that spreads depolarization potential from endplate throughout muscle fibers [10], (vii) collagen Q that anchors acetylcholinesterase (AChE) to the synaptic basal lamina [11-13], (viii) choline acetyltransferase (ChAT) that resynthesizes acetylcholine from recycled choline at the nerve terminal [14]. AChR [15], MuSK [16,17], and LRP4 [18] are also targets of myasthenia gravis, in which autoantibody against these molecules compromises the neuromuscular transmission. This review focuses on molecular defects of AChR in CMS.