2.2 Physiology of the nicotinic muscle AChR

Nicotinic AChRs are pentameric ligand-gated ion channels. The family of pentameric ligand-gated ion channels includes cationic AChRs, cationic serotonergic receptors ($5HT_3$), anionic glycine receptors, and anionic $GABA_A$ and $GABA_C$ receptors (Keramidas *et al.*, 2004). Heteromeric neuronal nicotinic AChRs are comprised of various combinations of α (α2-α7) and β subunits (β2-β4), whereas homomeric AChRs are formed only by a single α subunit (e.g., α7-α9) (Mihailescu & Drucker-Colin, 2000). On the other hand, nicotinic muscle AChRs have only two forms: fetal AChR that carries the α, β, δ, and γ subunits encoded by *CHRNA1*, *CHRNB1*, *CHRND*, *CHRNG*, respectively, in the stoichiometry $\alpha_2\beta\delta\gamma$; and adult-type AChR that carries the ε subunit instead of the γ subunit in the stoichiometry $\alpha_2\beta\delta\varepsilon$ (Mishina *et al.*, 1986). The ε subunit is encoded by *CHRNE*. Nicotinic muscle AChR harbors two binding sites for ACh at the interfaces between the α–δ and α–γ/α–ε subunits (Lee *et al.*, 2009; Mukhtasimova *et al.*, 2009). Binding of a single ACh molecule opens the channel pore but for a short time. Binding of two ACh molecules stabilizes the open state of AChR, and AChR stays open for a longer time. Only cations pass through the channel pore of nicotinic AChRs. Unlike sodium, potassium, or calcium channels, AChRs, in general, have no selectivity for cations, but α7 AChRs have 10-20 times higher permeability for Ca^{2+} than for Na^+.

3. Postsynaptic CMS

Postsynaptic CMS is classified into four phenotypes: (i) endplate AChR deficiency due to defects in AChR, rapsyn, agrin, MuSK, Dok-7, plectin, glutamine-fructose-6-phosphate aminotransferase 1, (ii) slow-channel congenital myasthenic syndrome, (iii) fast-channel congenital myasthenic syndrome, and (iv) sodium channel myasthenia.

3.1 Endplate AChR deficiency

Endplate AChR deficiency is caused by defects in AChR, rapsyn, agrin, MuSK, Dok-7, plectin, and GFPT1.

3.1.1 Endplate AChR deficiency due to defects in AChR subunits

Endplate AChRs deficiency can arise from mutations in *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE*, but not *CHRNG*.

Two different groups of mutations of the AChR subunit genes cause endplate AChR deficiency. The first group includes null mutations in *CHRNE* encoding the ε subunit. The null mutations are caused by frameshifting DNA rearrangements, *de novo* creation of a stop codon, and frameshifting splice-site mutations, or mutations involving residues essential for subunit assembly. Large-scale in-frame DNA rearrangements also abolish expression of the AChR ε subunit (Abicht *et al.*, 2002). Mutations in the promoter region (Ohno *et al.*, 1999) and most missense mutations (Ohno *et al.*, 1997) do not completely abolish expression of the ε subunit but the molecular consequences are indistinguishable from those of null mutations. Lack of the ε subunit can be compensated for by the presence of the fetal γ subunit that is normally expressed in embryos (Engel *et al.*, 1996). The patients can survive with γ-AChR even in the absence of ε-AChR. If a null mutation resides in the other AChR

subunit genes, the affected individual will have no substituting subunit and cannot survive. Indeed, two homozygous missense low expressor or null mutations in *CHRNA1* and *CHRND* caused lethal fetal akinesia (Michalk *et al.*, 2008).

The second group of mutations affecting the AChR subunit genes includes missense mutations of *CHRNA1*, *CHRNB1*, and *CHRND*. These mutations compromise expression of the mutant subunit and/or the assembly of AChRs, but do not completely abolish AChRs expression. The main difference between mutations in *CHRNE* and those in *CHRNA1*, *CHRNB1*, and *CHRND* is tolerance to low or no expression of the ε subunit whereas similar mutations in other subunits generally have devastating consequences and cause high fatality. Some missense mutations in *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE* also affect the AChR channel kinetics and vice versa. The kinetic effects will predominate if the second mutation is a low expressor, or if the kinetic mutation has slow-channel features with dominant gain-of function effects.

In endplate AChR deficiency, the postsynaptic membrane displays a reduced binding for peroxidase- or ^{125}I-labeled α-bungarotoxin and the synaptic response to ACh, reflected by the amplitude of the miniature endplate potential, endplate potential, and endplate current, is reduced. In some but not all cases the postsynaptic region is simplified. In most cases, the muscle fibers display an increased number of small synaptic contacts over an extended length of the muscle fiber. In some patients quantal release is higher than normal. In patients with null mutations in *CHRNE*, single channel recordings of AChRs at patient endplates reveal prolonged opening bursts that open to an amplitude of 60 pS, indicating expression of the fetal γ-AChR in contrast to the adult ε-AChR that has shorter opening bursts and opens to an amplitude of 80 pS. In contrast, in most patients with low-expressor mutations in the *CHRNA1*, *CHRNB1*, or *CHRND*, single channel recordings demonstrate no or minor kinetic abnormalities.

As in autoimmune myasthenia gravis, endplate AChR deficiency is generally well controlled by regular doses of anticholinesterases. Anticholinesterase medications inhibit the catalytic activity of AChE; this prolongs the dwell time of ACh in the synaptic space and allows each ACh molecule to bind repeatedly to AChR.

3.1.2 Endplate AChR deficiency due to defects in rapsyn

Congenital defects of rapsyn also cause endplate AChR deficiency. Rapsyn makes a homomeric cluster and binds to AChR as well as to β-dystroglycan, and forms AChR clusters at the endplate (Froehner *et al.*, 1990; Cartaud *et al.*, 1998; Ramarao & Cohen, 1998; Ramarao *et al.*, 2001). The structural domains of rapsyn include an N-terminal myristoylation signal required for membrane association (Ramarao & Cohen, 1998), seven tetratrico peptide repeats at codons 6 to 279 that subserve rapsyn self-association (Ramarao & Cohen, 1998; Ramarao *et al.*, 2001), a coiled-coil domain at codons 298 to 331 that binds to the long cytoplasmic loop of each AChR subunit (Bartoli *et al.*, 2001), a Cys-rich RING-H2 domain at codons 363-402 that binds to the cytoplasmic domain of β-dystroglycan (Bartoli *et al.*, 2001) and mediates the MuSK induced phosphorylation of AChR (Lee *et al.*, 2008), and a serine phosphorylation site at codon 406. Transcription of rapsyn in muscle is under the control of helix-loop-helix myogenic determination factors that bind to the *cis*-acting E-box sequence in the *RAPSN* promoter (Ohno *et al.*, 2003).

Loss-of-function mutations in *RAPSN* have been reported in the coding region (Ohno *et al.*, 2002; Burke *et al.*, 2003; Dunne & Maselli, 2003; Maselli *et al.*, 2003; Muller *et al.*, 2003; Banwell *et al.*, 2004; Yasaki *et al.*, 2004; Cossins *et al.*, 2006; Muller *et al.*, 2006) as we as in the promoter region (Ohno *et al.*, 2003). N88K in *RAPSN* is one of the most frequently observed mutations in CMS (Muller *et al.*, 2003; Richard et al., 2003). We reported lack of a founder haplotype for N88K (Ohno & Engel, 2004), but analysis of markers closer to *RAPSN* later revealed possible presence of a shared haplotype (Muller et al., 2004) suggesting that N88K is an ancient founder mutation but subsequent multiple recombination events and divergence of microsatellite markers have narrowed the shared haplotype region. Functional analysis L14P, N88K, and 553ins5 disclosed that these mutations have no effect on self-association of rapsyn but impair colocalization of rapsyn with AChR (Ohno *et al.*, 2002). Analysis of A25V, N88K, R91L, L361R, and K373del later revealed diverse molecular defects affecting colocalization of rapsyn with AChR, formation of agrin-induced AChR clusters, self-association of rapsyn, and expression of rapsyn (Cossins *et al.*, 2006). Although there are no genotype-phenotype correlations in mutations at the coding region, arthrogryposis at birth and other congenital malformations occurs in nearly a third of the patients. In addition, the -38A>G mutation affecting an E-box in the promoter region observed in Near-Eastern Jewish patients exhibits unique facial malformations associated with prognathism and malocclusion (Ohno *et al.*, 2003).

Most patients respond well to anticholinesterase medications. Some patients further improve with addition of 3,4-diaminopyridine, ephedrine, and albuterol (Banwell *et al.*, 2004). The drug 3,4-diaminopyridine blocks the presynaptic potassium channel, which slows the repolarization of the presynaptic membrane (Wirtz *et al.*, 2010) enhancing the influx of Ca^{2+} through the presynaptic voltage-gated P/Q-type and N-type channels. This, in turn, facilitates the exocytosis of synaptic vesicles and the quantal content of the endplate potential.

3.1.3 Endplate AChR deficiency due to a defect in agrin

Neural agrin released from the nerve terminal is a key mediator of synaptogenesis at the NMJ. A reported homozygous G1709R agrin mutation, however, did not cause AChR deficiency but mutations in agrin are potential causes of AChR deficiency by interfering with the activation of MuSK and by impeding synaptic maturation.

The patient harboring the G1709R mutation was a 42-year-old woman with right lid ptosis since birth, no oculoparesis, and mild weakness of facial, hip-girdle and anterior tibial muscles, and refractoriness to pyridostigmine or 3,4-diaminopyridine (Huze *et al.*, 2009). The mutation is in the laminin G-like 2 domain, upstream of the neuron-specific y and z exons that are required for MuSK activation and AChR clustering. AChR and agrin expression at the endplate were normal. Structural studies showed endplates with misshaped synaptic gutters partially filled by nerve endings and formation of new endplate regions. The postsynaptic regions were preserved. Expression studies in myotubes using a mini-agrin construct revealed the mutation did not affect MuSK activation or agrin binding to α-dystroglycan. Forced expression of the mutant mini-agrin gene in mouse soleus muscle induced changes similar to those at patient endplates. Thus, the observed mutation perturbs the maintenance of the endplate without altering the canonical function of agrin to induce development of the postsynaptic compartment.

3.1.4 Endplate AChR deficiency due to defects in MuSK

MuSK and LRP4 form a heteromeric receptor for agrin. Five *MUSK* mutations have been reported in three papers. The first report describes heteroallelic frameshift (220insC) and missense (V790M) mutations in a patient with respiratory distress in early life, mild ptosis, decreased upward gaze, and fatigable weakness of the cervical and proximal more than distal muscles. The symptoms were worsened by pregnancy. Treatment with pyridostigmine and 3,4-diaminopyridine was ineffective (Chevessier *et al.*, 2004). The frameshift mutation prevents MuSK expression and the missense mutation decreases MuSK expression and impairs its interaction with Dok-7. Forced expression of the mutant protein in mouse muscle decreased AChR expression at the endplate and caused aberrant axonal outgrowth (Chevessier *et al.*, 2004). Interestingly, mice homozygous for MuSK V789M (which corresponds to the human MuSK V790M) are normal but mice hemizygous for V789M are severely affected suggesting that MuSK V790M in humans is a haploinsufficient only when accompanied by a null mutation (Chevessier *et al.*, 2008).

A second report describes heteroallelic M605I and A727V mutations in MuSK in a patient with severe myasthenic symptoms since early life that improved after puberty but worsened after menstrual periods. The MEPP and MEPC amplitudes in anconeus muscle were reduced to about 30% of normal and the EPP quantal content was half-normal. Synaptic contacts were small and electron microscopy showed simplified postsynaptic regions with too few secondary synaptic clefts. The patient failed to respond to pyridostigmine, ephedrine or 3,4-diaminopyuridine but responded partially to albuterol (Maselli *et al.*, 2010).

A third report describes a homozygous P31L mutation in the extracellular domain of MuSK in 5 patients in a consanguineous Sudanese kinship. The findings included ptosis from an early age, partial ophthalmoparesis, and weakness of torso and limb girdle muscles. Pyridostigmine therapy gave only slight benefit (Mihaylova *et al.*, 2009).

3.1.5 Endplate AChR deficiency due to defects in Dok-7

Phosphorylated MuSK recruits a noncatalytic adaptor protein, Dok-7. Recruited Dok-7 further facilitates phosphorylation of MuSK (Okada *et al.*, 2006). Dok-7 is highly expressed at the postsynaptic region of skeletal muscle and in heart. It harbors an N terminal pleckstrin homology domain (PH) important for membrane association, a phosphotyrosine-binding (PTB) domain, and C-terminal sites for phosphorylation. The PH and PTB domains are required for association with and phosphorylation of MuSK. Phosphorylation of two C terminal residues is a requisite for Dok-7 activation by Crk and Crk-L (Hallock *et al.*, 2010).

Numerous mutations have been identified in *DOK7* (Beeson *et al.*, 2006; Muller *et al.*, 2007; Anderson *et al.*, 2008; Selcen *et al.*, 2008; Vogt *et al.*, 2009; Ben Ammar *et al.*, 2010). Nearly all patients carry a common 1124_1127dupTGCC mutation in exon 7. This and other mutations upstream of the C-terminal phosphorylation sites abrogate the ability of Dok-7 to associate with Crk1/Crk1L and hence its activation (Hallock *et al.*, 2010; Wu *et al.*, 2010). Mutations disrupting or eliminating the PH and PTB domains of Dok-7 prevent dimerization and association of Dok-7 with MuSK (Bergamin *et al.*, 2010).

3.1.6 Endplate AChR deficiency due to defects in plectin

Plectin, encoded by *PLEC*, is a highly conserved and ubiquitously expressed intermediate filament-linking protein concentrated at sites of mechanical stress, such as the postsynaptic membrane of the endplate, the sarcolemma, Z-disks in skeletal muscle, hemidesmosomes in skin, and intercalated disks in cardiac muscle. Pathogenic mutations in *PLEC* result in epidermolysis bullosa simplex, a progressive myopathy (Smith *et al.*, 1996), and, in some patients, myasthenic syndrome (Banwell *et al.*, 1999; Selcen *et al.*, 2011). We reported two cases of CMS associated with plectin deficiency (Banwell *et al.*, 1999; Selcen *et al.*, 2011). The dystrophic changes in muscle are attributed to dislocation of the fiber organelles no longer anchored by the cytoskeletal intermediate filaments and to sarcolemmal defects allowing Ca^{2+} ingress into the muscle fibers. The myasthenic syndrome is attributed to destruction of the junctional folds lacking adequate cytoskeletal support.

3.1.7 Endplate AChR deficiency due to defects in glutamine-fructose-6-phosphate aminotransferase 1 (GFPT1)

Glutamine-fructose-6-phosphate transaminase 1, encoded by *GFPT1*, catalyzes transfer of an amino group from glutamine onto fructose-6-phosphate, yielding glucosamine-6-phosphate and glutamate. GFPT1 is a rate-limiting enzyme that controls the flux of glucose into the hexosamine biosynthesis pathway. GFPT1 thus initiates formation of UDP-N-acetylglucosamine (UDP-GlcNAc), which is a source of multiple glycosylation processes including addition of N-acetylglucosamine to serine or threonine residues (O-linked GlcNAc) (Wells *et al.*, 2001). The disease gene was discovered by linkage analysis and homozygosity mapping of 13 kinships with a limb-girdle CMS often associated with tubular aggregates in skeletal muscle (Senderek *et al.*, 2011). Immunoblots of muscle of affected patients revealed decreased expression of O-linked GlcNAc, but the responsible molecule(s) causing CMS remain elusive.

3.2 Slow-channel congenital myasthenic syndrome (SCCMS)

The second class of postsynaptic CMS due to mutations in the AChR subunit genes is SCCMS. SCCMS is an autosomal dominant disorder, in which a gain-of-function mutation on a single allele compromises the neuromuscular signal transduction (Ohno *et al.*, 1995). The mutation causes prolonged AChR channel openings and increases the synaptic response to ACh (Fig. 2). There is a single reported case of autosomal recessive SCCMS, in which an εL78P mutation minimally prolongs channel opening events but the mutant channel arising from a single allele is not sufficient to cause disease (Croxen *et al.*, 2002). In general, dominantly inherited disorders, including SCCMS, tend to present after adolescence and have a relatively mild course. Some patients with SCCMS, however, present early in life and become severely disabled even in the first decade.

In SCCMS, neuromuscular transmission is compromised by three distinct mechanisms. First, staircase summation of endplate potentials causes depolarization block of the postsynaptic membrane by rendering the voltage-gated skeletal muscle sodium channel go into an inactivated state and thereby inhibit action potential generation (Maselli & Soliven, 1991). Second, some mutant AChRs are prone to become desensitized (Milone *et al.*, 1997), which reduces the number of AChRs that respond to the released ACh quanta. Third,

prolonged opening of AChR causes excessive influx of extracellular calcium, which results in focal degeneration of the junctional folds as well as apoptosis of some of the junctional nuclei (Groshong *et al.*, 2007). In normal adult human ε-AChR, 7% of the synaptic current is carried by Ca^{2+}, which is higher than that carried by the human fetal γ-AChR or by muscle AChRs of other species (Fucile *et al.*, 2006). This predisposes endplate to Ca^{2+} overloading when the channel opening events are prolonged. In addition, at least two SCCMS mutations, εT264P (Ohno *et al.*, 1995) and αV259F (Fidzianska *et al.*, 2005), increase the Ca^{2+} permeability 1.5- and 2-fold, respectively (Di Castro *et al.*, 2007).

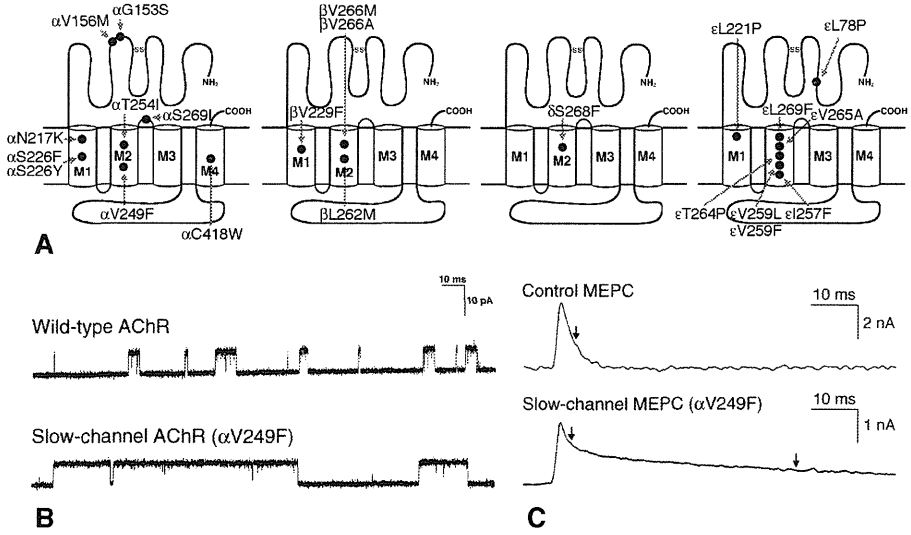

Fig. 2. Slow channel CMS. (A) Schematic diagram of AChR subunits with SCCMS mutations. (B) Single channel currents from wild-type and slow channel (αV249F) AChRs expressed on HEK293 cells. (C) Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring αV249F. The patient's MEPC decays biexponentially (arrows) due to expression of both wild-type and mutant AChRs.

Slow channel mutations can be divided into two groups. The first group includes mutations at the extracellular domain like αG153S (Sine *et al.*, 1995), as well as at the N-terminal part of the first transmembrane domain like αN217K (Wang *et al.*, 1997) and εL221F (Hatton *et al.*, 2003). These mutations increase the affinity for ACh binding, probably by retarding the dissociation of ACh from the binding site, which gives rise to repeated channel openings after a single event of ACh binding. The second group includes mutations at the second transmembrane domain (M2) that lines the ion channel pore. These mutations mostly introduce a bulky amino acid into the channel lining face, but εT264P (Ohno *et al.*, 1995) introduces a kink into the channel pore, whereas βV266A (Shen *et al.*, 2003) and εV265A (Ohno *et al.*, 1998) introduce a smaller amino acid into the pore. Mutations in M2 retard the channel closing rate α and variably enhance the channel opening rate β. Some mutations in M2 also increase affinity for ACh, which include αV249F (Milone *et al.*, 1997), εL269F (Engel *et al.*, 1996), and εT264P (Ohno *et al.*, 1995).

SCCMS can be treated with conventional doses of long-lived open channel blockers of AChR, such as the antiarrhythmic agent quinidine (Fukudome *et al.*, 1998; Harper & Engel, 1998) and the antidepressant fluoxetine (Harper *et al.*, 2003). Quinidine reduces the prolonged burst duration of SCCMS to the normal level at 5 μM (Fukudome *et al.*, 1998). As the concentration of quinidine in the treatment of cardiac arrhythmia is 6-15 μM, 5 μM is readily attainable in clinical practice and indeed demonstrates significant effects (Harper & Engel, 1998). Similarly, fluoxetine reduces the prolonged burst duration to the normal level at 10 μM, which is clinically attainable without adverse effects at 80 to 120 mg/day of fluoxetine (Harper *et al.*, 2003).

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).

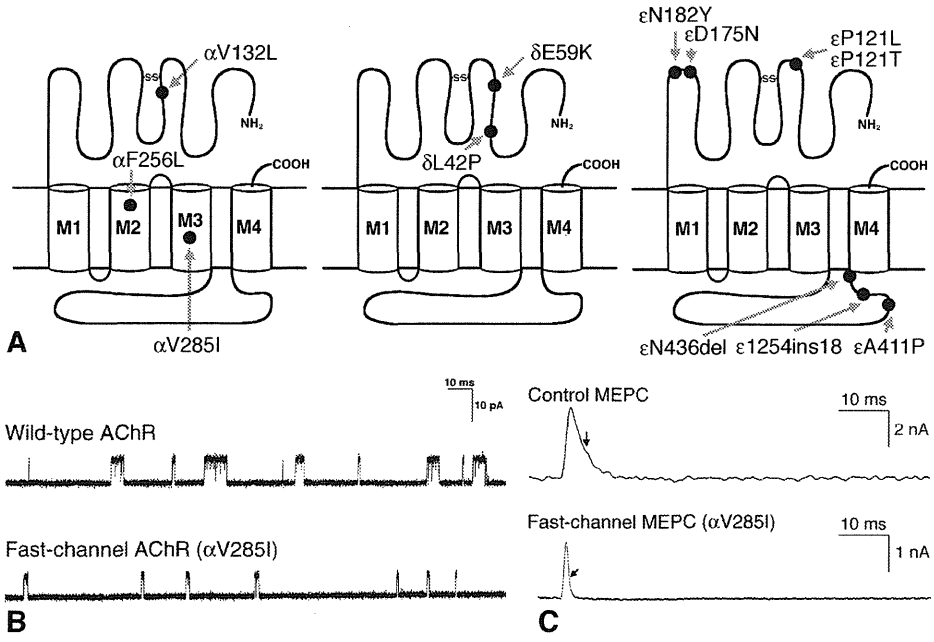

Fig. 3. Fast channel CMS. (A) Schematic diagram of AChR subunits with FCCMS mutations. (B) Single channel currents from wild-type and fast channel (αV285I) AChRs expressed on HEK293 cells. (C) Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring αV285I. The patient's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

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).

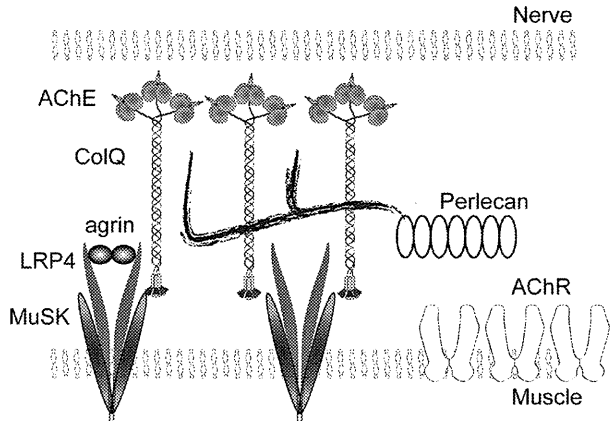

Fig. 4. ColQ anchors to the synaptic basal lamina by binding to perlecan and MuSK.

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).

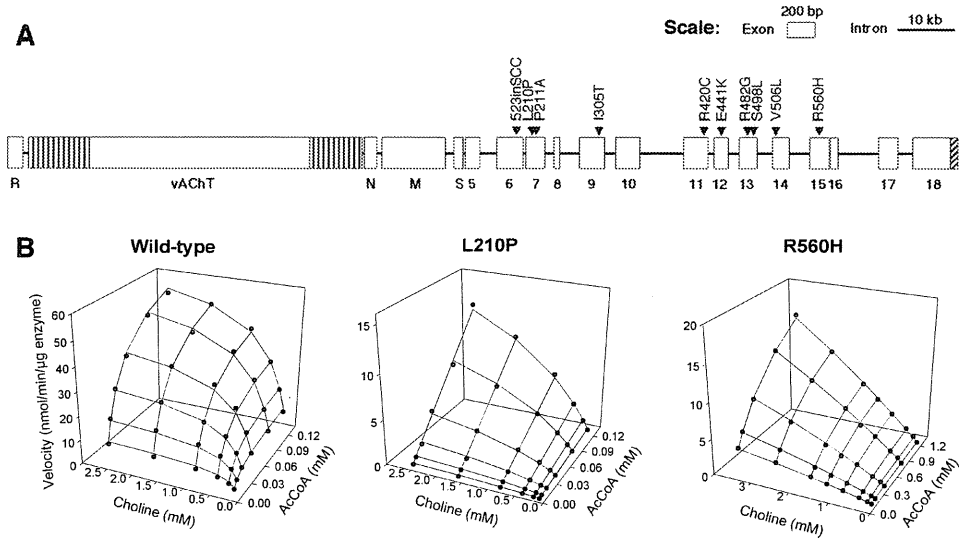

Fig. 5. Choline acetyltransferase (ChAT). (A) Genomic structure of *CHAT* and identified mutations. A gene for vesicular acetylcholine transporter (vAChT) is in the first intron of *CHAT*. (B) Kinetics of wild-type and mutant ChAT enzymes. ChAT synthesizes acetylcholine using choline and acetyl-CoA.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