Figure 1. Schematic of molecules expressed at the neuromuscular junction. Mutated molecules in CMS include acetylcholine receptor (AChR), rapsyn, agrin, MuSK, Dok-7, skeletal muscle sodium channel type 1.4 ($Na_V1.4$), collagen Q, and choline acetyltransferase (ChAT).

Muscle nicotinic acetylcholine receptor

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 [19]. Heteromeric neuronal nicotinic AChRs are comprised of various combinations of α (α2-α7) and β subunits (β2-β4), whereas homomeric AChRs are formed by just one subunit type (e.g., α7-α9) [20]. On the other hand, muscle nicotinic AChRs have only two forms: fetal AChR that carries the α1, β1, δ, and γ subunits encoded by *CHRNA1*, *CHRNB1*, *CHRND*, *CHRNG*, respectively, in the stoichiometry $\alpha1_2\beta1\delta\gamma$; and adult-type AChR that carries the ε subunit instead of the γ subunit in the stoichiometry $\alpha1_2\beta1\delta\varepsilon$ [21]. The ε subunit is encoded by *CHRNE*. The muscle nicotinic AChR harbors two binding sites for ACh at the interfaces between α-δ and α-γ/ε subunits [22,23]. 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 longer time. Cations but no anions 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^+.

Endplate AChR deficiency

Congenital deficiency of endplate AChRs is caused by mutations in genes encoding the AChR subunits. The mutated genes include *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE*, but not *CHRNG*. Endplate AChR deficiency is also caused by mutations in molecules that transmit signals for AChR clustering. These include *AGRN* encoding agrin [5], *MUSK* encoding MuSK [6,7], *DOK7* encoding Dok-7 [8,9], and *RAPSN* encoding rapsyn [3,4]. Mutations in the signaling molecules, however, are not within the scope of this review and are not addressed.

Two different groups of mutations of the AChR subunit genes cause endplate AChR deficiency. The first group includes null mutations in *CHRNE* encoding the AChR ε subunit. The null mutations are caused by frameshifting DNA rearrangements, *de novo* creation of a stop codon, and frameshifting splicing mutations. Large-scale in-frame DNA rearrangements also abolish expression of the AChR ε subunit. Mutations in the promoter region [24] and missense mutations [25] do not completely nullify the expression of ε, but the molecular pathological consequences are indistinguishable from those of null mutations. Lack of the ε subunit can be compensated for by the presence of the γ subunit that is normally expressed in embryos [26]. The patients can survive with γ-AChR even when ε-AChR is lacking. If a null mutation resides on the other AChR subunit genes, the affected individual should have no substituting subunit and cannot survive. Indeed, two such homozygous missense mutations are reported in *CHRNA1* and *CHRND* in lethal fetal akinesia disorders [27]. In general, mutations causing monogenic diseases should be very rare, because a single nucleotide substitution among the 3.0×10^9 nucleotides in a single allele should exhibit a certain phenotype that is recognized as a disease. Most single nucleotide substitutions are likely to be silent or to partially confer a variable phenotype observed in normal individuals. Most of the other nucleotide changes cause early embryonic lethality and we cannot observe such mutations in patients.

The second group of mutations affecting the AChR subunit genes includes missense mutations of *CHRNA1*, *CHRNB*, and *CHRND* encoding the AChR α1, β1, and δ subunits, respectively. These mutations compromise the expression level of the mutant subunit and/or the assembly of AChRs, but do not completely abolish the expression of AChRs. Differences between mutations in *CHRNE* and those in *CHRNA1*, *CHRNB*, and *CHRND* are the tolerance to low expression of the affected subunit. The expression level of the ε subunit may goes to zero, whereas a patient needs a certain amount of AChRs to be expressed at the endplate to survive when a mutation is in a gene for either the α1, β1, or δ subunit. Patients with low-expressor mutations in *CHRNA1*, *CHRNB*, and *CHRND* tend to have a devastating course with high fatality. Some missense mutations in *CHRNA1*, *CHRNB*, *CHRND*, and *CHRNE* also affect the AChR channel kinetics. If a pathological effect due to aberration of the channel kinetics is more than the degree of

aberration of AChR expression, such a mutation is classified as slow channel or fast channel mutation.

In biopsied skeletal muscle, we observed several lines of evidence indicating a decreased number of AChRs at the endplate. Ultrastructural studies demonstrate simplified junctional folds at the endplate and reduced staining for AChRs. Miniature endplate currents (MEPC) are small in amplitude. As the number of ACh in a synaptic vesicle (quanta) is rather increased, the low MEPC amplitude directly indicates a reduced number of AChRs at the endplate. Endplate potentials (EPP) are also small in amplitude. Again, as the number of ACh released by a single nerve stimulus (quantal content) is rather elevated, the low EP amplitude indicates a reduced number of AChRs at the endplate. In patients with null mutations in *CHRNE*, single channel recordings of AChRs at the patient's endplates demonstrate low conductance and prolonged opening bursts, indicating expression of the fetal γ-AChR instead of the adult-type ε-AChR. The conductance of the adult-type ε-AChR is 80 pS, whereas that of the fetal γ-AChR is 60 pS. In patients with low-expressor mutations in either *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 dosages of anticholinesterases. Anticholinesterases inhibit the catalytic activity of AChE, which in turn increases the dwell time of ACh at the synaptic space and enables reassociation of ACh and AChRs. Anticholinesterases are effective in a wide range of diseases where the number of AChRs is reduced independent of its cause. Inadvertent or unexpected overdose of anticholineseterase, however, simulates endplate AChE deficiency [11,13,28]. In endplate AChE deficiency, the neuromuscular signal transduction is compromised by an excessive amount and prologed dwell time of ACh in the synaptic space, which in turn indcues three pathomechanisms: (i) staircase summation of endplate potentials, (ii) excessive desensitization of AChRs, and (iii) endplate myopathy caused by excessive influx of extracellular calcium. The three molecular mechanisms are identical to those observed in the slow channel congenital myasthenic syndrome, as described in the following section.

Slow channel congenital myasthenic syndrome

The second class of CMS due to mutations in the AChR subunit genes is the slow channel congenital myasthenic syndrome (SCCMS) (Table 1). SCCMS is an autosomal dominant disorder, in which a gain-of-function mutation on a single allele compromises the neuromuscular signal transduction [1]. 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 the εL78P mutation minimally prolongs channel opening events and a mutant channel arising from a single allele is not sufficient to cause the disease phenotype [29]. In general, dominantly inherited disorders tend to develop after adolescence, because an individual carrying a mutant allele should get married and transmit the mutant allele to the next generation. In concordance with this notion, SCCMS tend to develop later in life and progresses slowly. Some patients with SCCMS, however, present early in life and become severely disabled even in the first decade.

Table 1. AChR mutations causing slow and fast channel syndromes

Gene	Mutation	Domain	Reference
	Slow channel syndrome		
CHRNA1	α1G153S	ECD	[2,30]
(AChR α1 subunit)	α1V156M	ECD	[30]
	α1N217K	M1	[31-33]
	α1S226Y	M1	[34]
	α1S226F	M1	[34,35]
	α1V249F	M2	[36]
	α1T254I	M2	[30]
	α1S269I	M2-M3 linker	[30]
	α1C418W	M4	[37]
CHRNB1	β1V229F	M1	[33,38]
(AChR β1 subunit)	β1L262M	M2	[39]
	β1V266M	M2	[31,33]

	β1V266A	M2	[40]
CHRND	δS268F	M2	[33,41]
(AChR δ subunit)			
CHRNE	εL78P	ECD	[29,42]
(AChR ε subunit)	εL221F	M1	[29,43]
	εI257F	M2	[44]
	εV259F	M2	[45]
	εV259L	M2	[46]
	εT264P	M2	[1]
	εV265A	M2	[47]
	εL269F	M2	[31,48]
	Fast channel syndrome		
CHRNA1	α1V132L	ECD	[49]
(AChR α1 subunit)	α1F256L	M2	[50]
	α1V285I	M3	[51]
CHRND	δL42P	ECD	[52]
(AChR δ subunit)	δE59K	ECD	[53]
CHRNE	εP121L	ECD	[54]
(AChR ε subunit)	εP121T	ECD	[55]
	εD175N	ECD	[56]
	εN182Y	ECD	[56]
	ε1254ins18	LCP	[57]
	εA411P	LCP	[58]
	εN436del	LCP	[59,60]

ECD, extracellular domain; M1-M4, transmembrane domains 1 to 4; LCP, long cytoplasmic loop.

In SCCMS, neuromuscular transmission defects are caused by three distinct mechanisms. First, staircase summation of endplate potentials causes depolarization of

the membrane potential. Prolonged depolarization makes the voltage-gated skeletal sodium channel less responsive to endplate potential generated by opening of AChR ion channels. Second, mutant AChRs somehow tend to be desensitized [36], 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 triggers the apoptosis pathway and gives rise to endplate myopathy [61]. In normal adult human ε-AChR, 7% of the synaptic current is carried by Ca^{2+}, which is higher than that in fetal human γ-AChR or muscle AChRs from other species [62]. This predisposes to endplate Ca^{2+} overloading when the channel opening events are prolonged. In addition, at least two SCCMS mutations, εT264P [1] and εV259F [45], increase the Ca^{2+} permeability 1.5- and 2-fold, respectively [63].

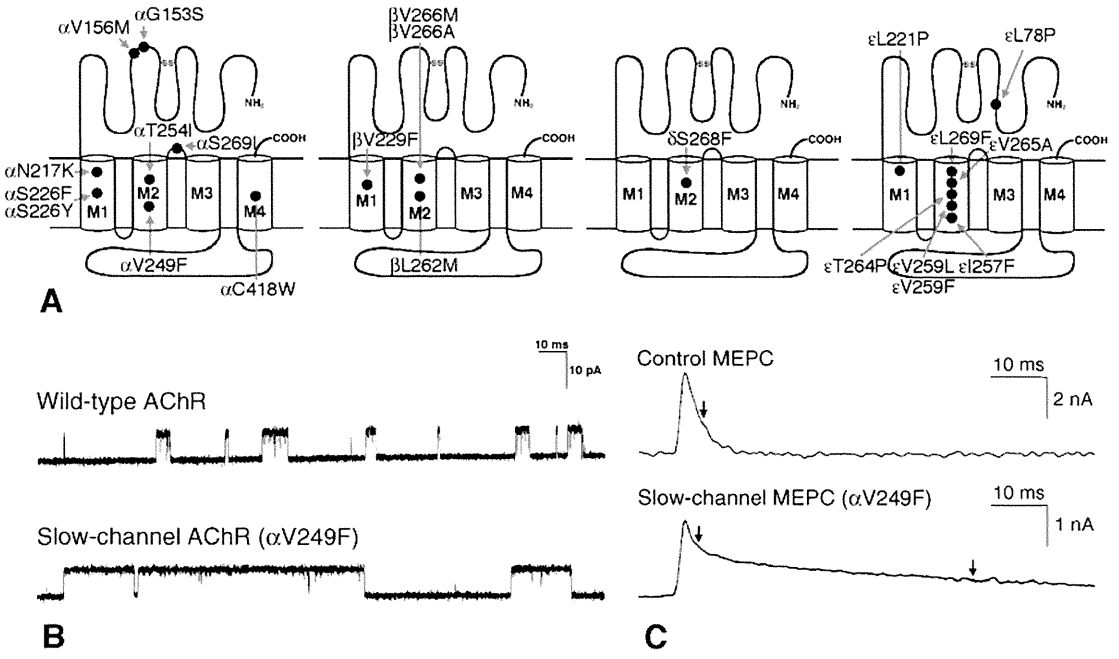

Figure 2. Slow channel syndrome. **(A)** Schematic diagram of AChR subunits with SCCMS mutations. **(B)** Single channel currents from wild-type and slow channel (α1V249F) AChRs expressed on HEK293 cells. **(C)** Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring α1V249F. 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 (Table 1). The first group includes mutations at the extracellular domain like α1G153S [2], as well as at the N-terminal part of the first transmembrane domain like α1N217K [32] and εL221F [43]. 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 biding. 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 [1] introduces a kink into the channel pore, whereas β1V266A [40] and εV265A [47] rather 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 α1V249F [36], εL269F [31], and εT264P [1].

SCCMS can be effectively treated with conventional dosages of long-lived open channel blockers of AChR, such as the antiarrhythmic agent quinidine [64,65] and the antidepressant fluoxetine [66]. Quinidine reduces the prolonged burst duration of SCCMS to the normal level at 5 μM [64]. 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 [65]. 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 [66].

Fast channel congenital myasthenic syndrome

The third class of CMS due to mutations in AChR subunit genes is the fast channel congenital myasthenic syndrome (FCCMS) (Table 1). FCCMS is kinetically opposite to SCCMS (Fig. 3). In FCCMS, the AChR becomes resistant to be transferred to an open state and prematurely comes back to a closed state, which results in insufficient depolarization of the endplate potential. The resulting pathophysiology is thus similar to

endplate AChR deficiency, but the cause of inefficient endplate depolarization is due to qualitative defects of AChRs but not to quantitative defects as in AChR deficiency.

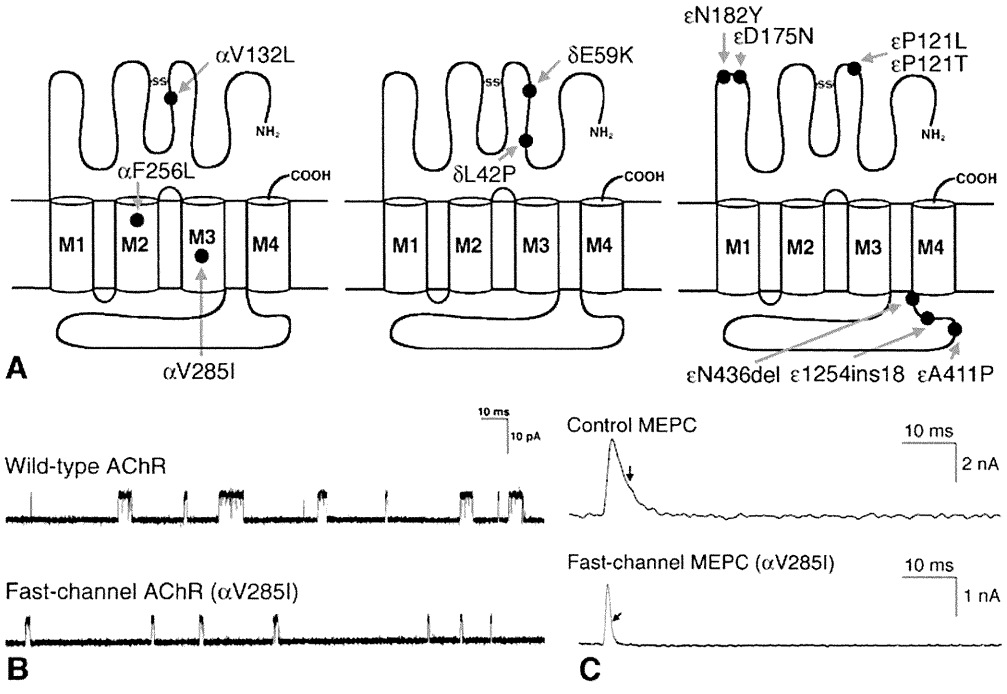

Figure 3. Fast channel syndrome. **(A)** Schematic diagram of AChR subunits with FCCMS mutations. **(B)** Single channel currents from wild-type and fast channel (α1V285I) AChRs expressed on HEK293 cells. **(C)** Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring α1V285I. The patient's MEPC decays faster than that of the normal control.

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. 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 kinetic abnormalities of a FCCMS mutation on the second allele. Detailed kinetic analyses of FCCMS mutations have unmasked yet uncharacterized molecular architectures of the AChR subunits. Three such examples are presented here.

ε1254ins18 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. 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 [57]. 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 [58]. 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 β1 and δ subunits also results in fast-channel kinetics, but that in the α1 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 [59].

The mutation α1V285I 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, which gives rise to 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 β1, 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 first disclosed that the M3 domain backs up the channel-lining pore that is composed by the M2 transmembrane domains and has sterochemical effects on channel gating kinetics [51].

FCCMS can be effectively treated with anticholinesterases and 3,4-diaminopyridine. The mechanism of action of anticholinesterases is described in the section devoted to endplate AChR deficiency. The drug 3,4-diaminopyridine blocks the presynaptic potassium channel, which slows the repolarization of the action potential delivered to the nerve terminal [67]. The enhanced nerve action potential stimulates the presynaptic voltage-gated P/Q-type and N-type Ca^{2+} channels and increases Ca^{2+} influx to the nerve terminal, which then enhances synaptotagmin and the SNARE complex to facilitate the fusion of ACh vesicles to the presynaptic membrane. This increases the amount of ACh released by a single nerve stimulus and enhances AChR channel openings.

Other phenotypes associated with AChR mutations and a single nucleotide polymorphism

Mutations or a single nucleotide polymorphism (SNP) in muscle nicotinic AChR subunits also give rise to phenotypes other than CMS.

The first phenotype is fetal akinesia deformation sequence (FADS). Mutations in the AChR subunit genes cause neuromuscular transmission defects in embryos and restrict intrauterine movements. As human embryos use the fetal γ-AChR by 33 weeks of gestation [68], mutations in *CHRNG* [69,70], as well as in *CHRNA1* and *CHRND* [27], cause FADS.

The second phenotype is early onset myasthenia gravis [71]. Promiscuous expression of a set of self-antigens occurs in medullary thymic epithelial cells to impose T-cell tolerance and to provide protection against autoimmune disorders. The AChR α1 subunit is one of those self-antigens. A SNP in the promoter region of *CHRNA1* compromises expression of the α1 subunit in thymic epithelial cells, which increases the chance of developing myasthenia gravis 2.01- to 2.35-fold in individuals carrying the SNP.

Conclusions

We addressed three types of CMS that are caused by mutations in the AChR subunit genes.

Congenital deficiency of endplate AChRs is caused by mutations in *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE* encoding the AChR α1, β1, δ, and ε subunits, respectively. The mutations are classified into two groups. The first group includes mutations in *CHRNE* that nullify or significantly reduce the expression of the ε subunit. Patients survive with embryonic γ-AChR even when the adult-type ε-AChR is lacking. Null mutations in the other AChR subunit genes are likely to be fatal, which supports a general notion that we have no chance to identify mutations that result in lethal phenotypes. The second group of mutations includes missense mutations of *CHRNA1*, *CHRNB*, and *CHRND*. These mutations compromise the expression level of the mutant subunit and/or the assembly of AChRs, but do not completely abolish the expression of AChRs. Differences between mutations in *CHRNE* and those in *CHRNA1*, *CHRNB*, and *CHRND* are the tolerance to low expression of the affected subunit. As in autoimmune myasthenia gravis, endplate AChR deficiency is well controlled by anticholinesterases.

The slow channel congenital myasthenic syndrome (SCCMS) is an autosomal dominant disorder, in which a gain-of-function mutation causes prolonged AChR channel openings and increases the synaptic response to ACh. In SCCMS, neuromuscular transmission defects are caused by (i) staircase summation of endplate potentials, (ii) excessive desensitization of AChRs, and (iii) endplate myopathy caused by excessive influx of extracellular calcium. SCCMS mutations cause neuromuscular transmission defects either by increasing the affinity of AChR for ACh binding or by retarding the channel closing rate α and variably enhancing the channel opening rate β. SCCMS can be effectively treated with conventional dosages of long-lived open channel blockers of AChR, such as the antiarrhythmic agent quinidine and the antidepressant fluoxetine.

The fast channel congenital myasthenic syndrome (FCCMS) is caused by loss-of-function missense mutations in the AChR subunit genes. The mutations render the AChR resistant to be transferred to an open state and prematurely coming back to a closed state. Detailed kinetic analyses of FCCMS mutations have unmasked yet uncharacterized

molecular architectures of the AChR subunits especially in the third transmembrane domain and in the long cytoplasmic loop. FCCMS can be effectively treated with anticholinesterases and 3,4-diaminopyridine.

Two more clinical phenotypes are associated with variations of the AChR subunit genes. Mutations in *CHRNG* encoding the AChR γ subunit cause another phenotype FADS by restricting intrauterine movement of an embryo. A SNP in the promoter region of *CHRNA1* compromises expression of the α1 subunit in thymic epithelial cells, and increases the chance of developing myasthenia gravis.

Acknowledgements

Works in our laboratories were supported by Grants-in-Aid from the MEXT and the MHLW of Japan (to K.O.), and by the NIH grant (NS6277) and the Muscular Dystrophy Association grant (to A.G.E.).

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MBNL proteins regulate alternative splicing of the skeletal muscle chloride channel *CLCN1*

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Abstract

Increased inclusion of chloride channel 1 (*CLCN-1/CLC-1*) exon 7A is associated with myotonia in myotonic dystrophy type 1 (DM1), a genetic disease caused by the expansion of a CTG repeat. In mouse models, myotonia as well as aberrant splicing of the mouse counterpart of *CLC-1*, *Clcn1*, can be induced by either over-expression of CUG repeat RNAs or knockout of *Mbnl1*, an RNA-binding protein sequestered by CUG repeats in DM1 cells. Here we show that MBNL and CELF proteins regulate the alternative splicing of both human *CLC-1* and mouse *Clcn1*. MBNLs were found to repress the inclusion of exon 7A. This effect was antagonized by the expression of an expanded CUG repeat or CELF4 protein, but not by CUG-BP. MBNL1, which binds directly to regions around the 5' and 3' splice sites of exon 7A, is possibly blocking splicing signals and a putative exonic splicing enhancer located in this region. These results suggest the importance of these proteins in the correct splicing of *Clcn1* and provide molecular evidence for a novel mechanism for splicing regulation.

1. Introduction

Myotonic dystrophy (*dystrophia myotonica* type 1), or DM1, is a genetic disorder with multi-systemic symptoms, such as myotonia, progressive muscle loss, cataracts, cardiac conduction defects, insulin resistance, and cognitive impairments[1)]. DM1 is caused by the expansion of a CTG trinucleotide repeat in the 3' untranslated region (UTR) of the DM protein kinase (*DMPK*) gene[2-4)]. Evidence suggests that the expanded CUG repeats

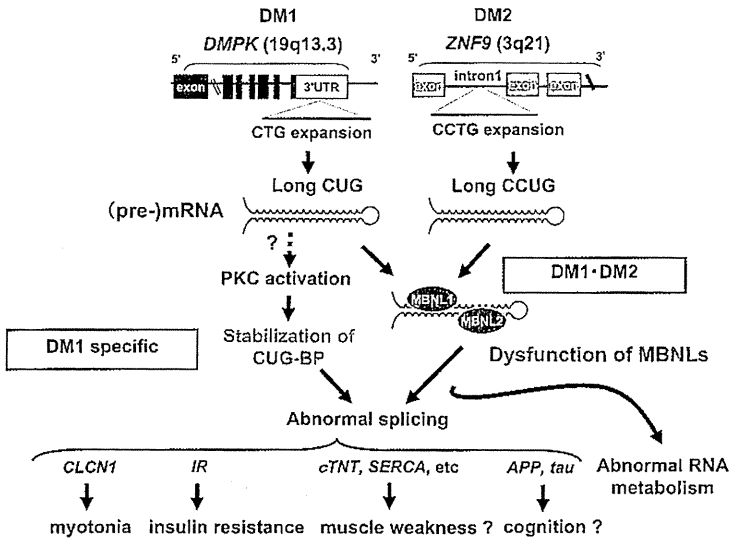

Fig. 1 RNA toxic gain-of-function model for DM

transcribed from a mutated allele cause RNA gain-of-function effects that affect the function of other cellular factors. Recently, a second locus of DM has been identified, and CCTG repeat expansion in intron 1 of the *ZNF9* gene was found to be causative of DM type 2 (DM2)[6)]. Abnormalities in RNA metabolism have been found in the cells of DM patients. Splicing of certain genes is misregulated in DM1. It does not reduce the fidelity of RNA processing or weaken the recognition of constitutive exons. It selectively affects a group of exons that are normally found in fetal or neonatal tissue. These genes include cardiac troponin T (*cTNT/TNNT2*), insulin receptor (*IR*), chloride channel 1 (*CLCN1*), amyloid precursor protein (*APP*), microtubule-associated protein tau (*MAPT*), sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (*SERCA*) 1, and others (**Fig. 1**)[7-11)]. The splicing patterns of some of these genes are also misregulated in DM2 patients. These results suggest that certain RNA-binding proteins that regulate pre-mRNA splicing of these genes are abnormally influenced by the mutant transcripts containing expanded CUG/CCUG repeats[12)].

Two RNA-binding protein families—muscleblind-like (MBNL), and CUG-BP and ETR-3-like factor (CELF) proteins—may play major roles in the pathogenesis of DM. MBNL proteins MBNL1/EXP, MBNL2/MBLL/MLP1, and MBNL3/MBXL/CHCR are orthologs