Fig. 4 *CHRNA1* carries a 75-nt exon P3A. Its inclusion generates a nonfunctional alpha subunit of the acetylcholine receptor. hnRNP H and PTB silence recognition of exon P3A and induce its skipping. The IVS3-8G>A mutation identified in a patient with congenital myasthenic syndrome weakens the binding of hnRNP H and causes inclusion of exon P3A. Tannic acid facilitates the expression of PTB and partially ameliorates aberrant splicing due to IVS3-8G>A

that encodes the survival of motor neuron 1. Humans carry almost identical *SMN1* and *SMN2* genes both on chromosome 5q13. *SMN2* carries a C-to-T transition at position 6 of exon 7 compared to *SMN1*, which results in loss of an SF2/ASF-dependent ESE activity (Cartegni et al., 2006). In addition, *SMN2* carries an A-to-G transition at position +100 of intron 7, which creates a high-affinity hnRNP A1-binding site and promotes skipping of exon 7 (Kashima et al., 2007). Skipping of exon 7 in *SMN2* can be ameliorated by therapeutic doses of valproic acid (Brichta et al., 2003, 2006) and of salbutamol (Angelozzi et al., 2008).

4 Skipping of Multiple Exons Caused by a Single Splicing Mutation

4.1 Skipping of Multiple Contiguous Exons

A mutation disrupting a splicing *cis*-element generally affects splicing of a single exon or intron, but sometimes generates aberrant transcripts affecting multiple neighboring exons. Skipping of multiple contiguous exons is accounted for by ordered removal of introns and consequent clustering of neighboring exons (Schwarze et al., 1999; Takahara et al., 2002).

4.2 Nonsense-Associated Skipping of a Remote Exon (NASRE)

A single mutation infrequently causes skipping of a remote exon. In a patient with congenital myasthenic syndrome, we found that a 7-nt deletion in exon 7 of *CHRNE* causes complete skipping of the preceding exon 6. *CHRNE* exon 6 is composed of 101 nucleotides. It carries weak splicing signals and is partially skipped even in normal subjects. The exon 6-skipped transcript, however, is removed by the nonsense-mediated mRNA decay (NMD) mechanism. The 7-nt deletion in exon 7 restores the open reading frame of the exon 6-skipped transcript and renders it immune to NMD. On the other hand, the normally spliced transcript carries a
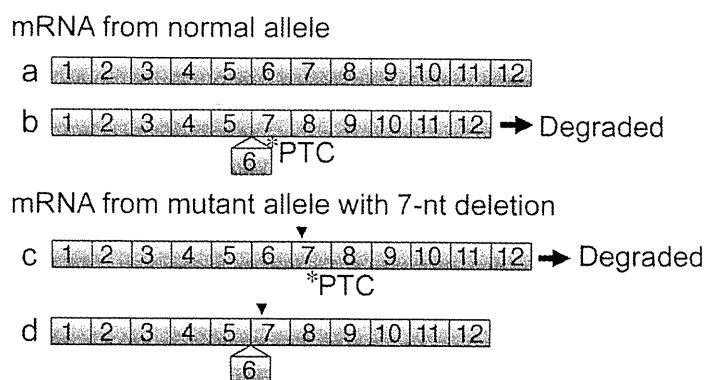
Fig. 5 NASRE. Wild-type *CHRNE* generates the normally spliced transcript (a) and the exon 6-skipped transcript (b), because exon 6 carries weak splicing signals. The exon-skipped transcript carries a premature termination codon (PTC) and is degraded by NMD. A 7-nt deletion (*arrowhead*) in exon 7 generates a PTC in the normally spliced transcript (c) and is degraded by NMD. The deletion resumes the open reading frame from the exon 6-skipped transcript, and the transcript escapes NMD (d)

premature stop codon (PTC) after the 7-nt deletion, and is degraded by NMD[1] (Fig. 5). We dubbed this mechanism NASRE, and found that it is in effect in *SLC25A20* (Hsu et al., 2001), *DBT* (Fisher et al., 1993), *BTK* (Haire et al., 1997), and *MLH1* (Clarke et al., 2000).

# 5 Disorders Associated with Dysregulation of Splicing *Trans*-Factors

5.1 Myotonic Dystrophy

Myotonic dystrophy is an autosomal dominant multisystem disorder affecting skeletal muscles, eye, heart, endocrine system, and central nervous system. The clinical symptoms include variable degrees of muscle weakness and wasting, myotonia, cataract, insulin resistance, hypogonadism, cardiac conduction defects, frontal balding, and intellectual disabilities (Harper and Monckton, 2004). Myotonic dystrophy is caused by abnormally expanded CTG repeats in the 3' untranslated region of the *DMPK* gene encoding the dystrophia myotonica protein kinase on chromosome 19q13 (myotonic dystrophy type 1, DM1) (Brook et al., 1992) or by abnormally expanded CCTG repeats in intron 1 of the *ZNF9* gene encoding the zinc finger protein 9 on chromosome 3q21 (myotonic dystrophy type 2, DM2) (Liquori et al., 2001). In DM1, normal individuals have 5–30 repeats, mildly affected patients

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[1] Nonsense-mediated mRNA decay (NMD). NMD is a quality-assurance mechanism that degrades mRNAs harboring a premature termination codon (PTC) (Chang et al., 2007). Proteins translated from mRNAs harboring PTCs potentially have dominant-negative or deleterious activities. In pre-mRNA splicing, an exon–junction complex (EJC) is deposited 20–24 nucleotides upstream of each exon–exon junction. Ribosomes remove EJCs, but, in the presence of a PTC, EJCs stay on the transcript and trigger the NMD pathway in the cytoplasm.

have 50–80 repeats, and severely affected individuals have 2000 or more copies of CTG (Gharehbaghi-Schnell et al., 1998). In DM2, the size of expanded repeats is extremely variable, ranging from 75 to 11,000 repeats, with a mean of 5000 CCTG repeats (Liquori et al., 2001).

In both DM1 and DM2, expanded CTG or CCTG repeats in the noncoding regions sequestrate a splicing *trans*-factor muscleblind encoded by *MBNL1* to intranuclear RNA foci harboring the mutant RNA, and somehow upregulate another splicing *trans*-factor CUG-binding protein encoded by *CUGBP1* (Ranum and Cooper, 2006) (Fig. 6). Dysregulation of the two splicing *trans*-factors then causes aberrant splicing of their target genes. The aberrantly spliced genes identified to date in skeletal and cardiac muscles include *ATP2A1* (*SERCA1*) exon 22, *ATP2A2* (*SERCA2*) intron 19, *CAPN3* exon 16, *CLCN1* intron 2 and exons 6b/7a, *DMD* exons 71 and 78, *DTNA* exons 11A and 12, *FHOD1* (*FHOS*) exon 11a, *GFPT1* (*GFAT1*) exon 10, *INSR* exon 11, *KCNAB1* exons 2b/2c, *LDB3* (*ZASP*) exon 11 (189-nt exon 7 according to RefSeq Build 36.3), *MBNL1* exon 7 (54-nt exon 6 according to RefSeq), *MBNL2* exon 7 (54 nt, no exonic annotation in RefSeq), *MTMR1* exons 2.1 and 2.2, *NRAP* exon 12, *PDLIM3* (*ALP*) exons 5a/5b, *RYR1* exon 70, *TNNT2* exon 5, *TNNT3* fetal exon, *TTN* exons Zr4 and Zr5 (138-nt exon 11 and 138-nt exon 12 according to RefSeq), and *TTN* exon Mex5 (303-nt exon 315 according to RefSeq) (Philips et al., 1998; Savkur et al., 2001; Kimura et al., 2005; Lin et al., 2006). Lin and colleagues report that alternative transcripts observed in myotonic dystrophy are all fetal isoforms (Lin et al., 2006). Muscleblind normally translocates
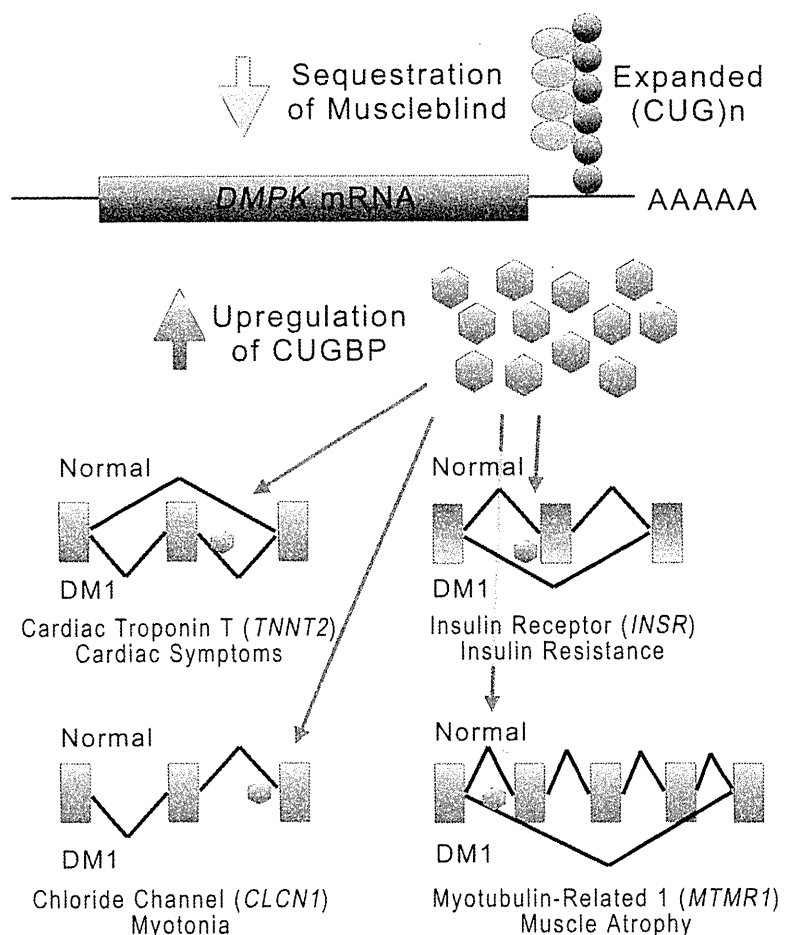
Fig. 6 In DM1, expanded CUG repeats in the 3' UTR of DMPK sequestrate muscleblind and upregulates CUG-binding protein. Dysregulation of these splicing *trans*-factors causes aberrant splicing of their inherent target genes. Four representative target genes are indicated

from cytoplasm to nucleus in the postnatal period to induce adult-type splicings, and lack of muscleblind in nucleus due to sequestration to RNA foci recapitulates fetal splicing patterns.

5.2 Alzheimer's Disease (AD) and Frontotemporal Dementia with Parkinsonism Linked to Chromosome 17 (FTDP-17)

AD is the most common neurodegenerative disease representing dementia. It is characterized by intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques. NFTs are composed of aggregates of the hyperphosphorylated tau protein encoded by MAPT. The amyloid plaques are composed of amyloid β peptide (Aβ) that originates from enzymatic cleavage of the amyloid precursor protein (APP) by β-secretase followed by γ-secretase (LaFerla et al., 2007). The γ-secretase is an enzyme complex composed of presenilin-1 (PS1) or presenilin-2 (PS2), as well as nicastrin, anterior pharynx defective (APH-1), and presenilin enhancer 2 (PEN-2) (Takasugi et al., 2003). Autosomal dominant forms of AD constitute ~5% of AD and are caused by mutations in APP, PS1, or PS2 (Bertram and Tanzi, 2008).

Although the pathomechanisms underlying sporadic AD remain mostly unknown, PS2 exon 5 is exclusively skipped in brains of sporadic AD, which is mediated by overexpression of a splicing trans-factor, HMGA1a (Sato et al., 1999; Manabe et al., 2003). As hypoxia induces the overexpression of HMGA1a, the upregulation of HMGA1a in sporadic AD may or may not represent an agonal state of AD, in which respiratory insufficiency possibly associated with pneumonia frequently becomes the cause of death.

Mutations in MAPT are not observed in AD, but are present in FTDP-17. MAPT exon 10 is alternatively spliced in normal brain. N279K, K280del, and L284L mutations on exon 10 provoke aberrant splicing of exon 10 by disrupting or enhancing exonic splicing cis-elements, and cause FTDP-17 (D'Souza et al., 1999) (Fig. 7). The splicing trans-factors for these cis-elements are also identified (Jiang et al., 2004; Kondo et al., 2004).

Fig. 7 Mutations on MAPT exon 10 cause excessive skipping (N279K and L284L) or inclusion (K280del) of exon 10
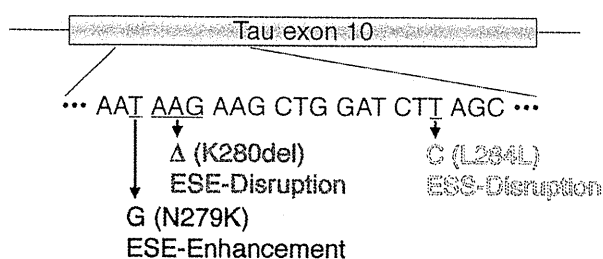
5.3 Facioscapulohumeral Muscular Dystrophy (FSHD)

FSHD is the third most common hereditary muscular dystrophy after Duchenne muscular dystrophy and myotonic dystrophy. As its name represents, the disease predominantly affects the face, the scapulae, and the proximal arm muscles. In

FSHD, the number of a 3.3 kb repeat in the subtelomeric region of 4q (4q35), designated D4Z4, are abnormally reduced (Wijmenga et al., 1992). Loss of D4Z4 causes upregulation of FRG1 located upstream of D4Z4 (Gabellini et al., 2002). FRG1 is a splicing trans-factor, and its overexpression causes aberrant splicing of TNNT3 encoding the troponin T type 3 of fast skeletal muscle and MTMR1 encoding the myotubularin-related protein 1 (Gabellini et al., 2006). The reported splicing aberrations in FSHD, however, have not been confirmed by us (unpublished data) or by the other groups (personal communications).

5.4 Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS)

Fragile X mental retardation syndrome is caused by abnormal expansion of a CGG repeat in the 5' untranslated region of FMR1, which culminates in hypermethylation of FMR1 and silences its expression (Kremer et al., 1991). On the other hand, moderate expansion of the CGG repeat in FMR1 causes FXTAS, which is characterized by intention tremor, Parkinsonism, cognitive decline, and neuropathy (Hagerman and Hagerman, 2004). In FXTAS, CGG-binding proteins including hnRNP A2 and muscleblind are excessively bound to the expanded CGG repeats of FMR1 and are depleted from the cellular pool (Iwahashi et al., 2006), which results in the loss their functions in other regulatory processes (Jacquemont et al., 2007).

5.5 Prader–Willi Syndrome (PWS)

PWS is an autosomal dominant disorder characterized by obesity, muscular hypotonia and weakness, mental retardation, short stature, hypogonadotropic hypogonadism, and small distal extremities. The proximal long arm of chromosome 15 (15q11-q13) is normally imprinted in order to achieve parent-specific monoallelic gene expressions. Some genes in this region are expressed only from the maternal allele, and some others are only from the paternal allele. Lack of a functional paternal copy of 15q11-13 causes PWS, whereas lack of a functional maternal copy of UBE3A in the same region results in Angelman syndrome (Horsthemke and Wagstaff, 2008). PWS is caused by a deletion of the paternal 15q11-q13 or by maternal uniparental disomy 15.

A snoRNA HBII-52 is located in the defective region of PWS. HBII-52 binds to an ESS in exon Vb of HTR2C encoding the serotonin receptor 2C, and its disruption in PWS causes aberrant splicing of HTR2C and potentially accounts for dysfunctional serotonergic system in PWS (Kishore and Stamm, 2006).

5.6 Rett Syndrome

Rett syndrome is a neurodevelopmental disorder in females, which is characterized by loss of speech, stereotypical movements of hands, microcephaly, seizures, and

mental retardation. Rett syndrome is caused by a mutation in *MECP2* encoding the metyl-CpG-binding protein 2 (Amir et al., 1999). MeCP2 binds to a splicing *trans*-factor *YB-1* and the abnormal regulation of YB-1 causes aberrant splicing of its target genes (Young et al., 2005).

5.7 Spinocerebellar Ataxia Type 8 (SCA8)

SCA8 is caused by an abnormal expansion of CTA/CTG repeats in the protein-noncoding *ATXN8OS*, which represents the *ATXN8* opposite strand (Ikeda et al., 2008). Expanded CUG repeats on the *ATXN8OS* transcript potentially bind to and sequestrate CUG-binding proteins, as we observe in myotonic dystrophy (Mutsuddi and Rebay, 2005). In addition, *ATXN8* on the opposite strand of *ATXN8OS* encodes the Kelch-like 1, and the expanded CAG repeats on *ATXN8* give rise to a polyglu-tamine tract that forms a cytotoxic aggregate in neuronal cells (Moseley et al., 2006). Furthermore, expression of *ATXN8OS* is colocalized with that of *ATXN8* (Chen et al., 2008). *ATXN8OS* thus potentially serves as an antisense RNA for *ATXN8*, and the abnormal CTA/CTG expansion in *ATXN8OS* may dysregulate the expression of *ATXN8* (Fig. 8).

Fig. 8 Expanded CTG on *ATXN8OS* exerts three toxic effects on the bidirectional transcripts
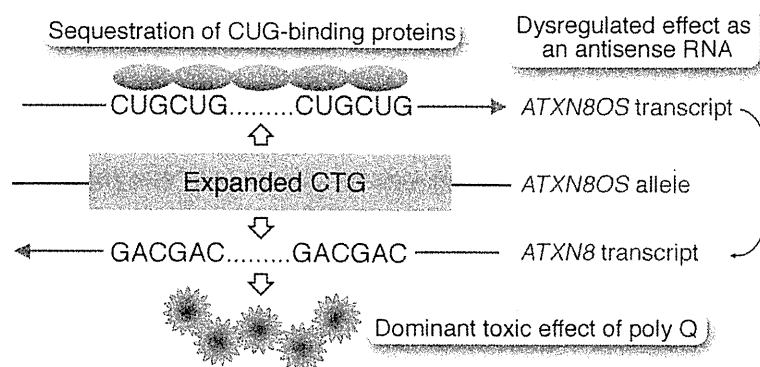
5.8 Paraneoplastic Neurological Disorders (PND)

In PND, tumors outside of the nervous system excrete humoral factors such as hormones and cytokines, or provoke an immune response against specific molecules expressed in tumors, and cause a wide range of neurological symptoms. In paraneo-plastic opsoclonus myoclonus ataxia (POMA), autoantibodies are raised against the Nova family of neuron-specific splicing *trans*-factor (Jensen et al., 2000; Ule et al., 2003, 2006; Licatalosi et al., 2008). In paraneoplastic encephalomyelitis and sensory neuropathy (PEN/SN or Hu syndrome), autoantibodies recognize the Hu family of RNA-binding protein (Szabo et al., 1991), a human homologue of the *Drosophila* splicing *trans*-factor *Elav* (Koushika et al., 2000; Soller and White, 2003). In both disorders, autoantibodies downregulate the splicing *trans*-factors and cause aberrant splicing in neuronal cells.

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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 $\alpha 1$ 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

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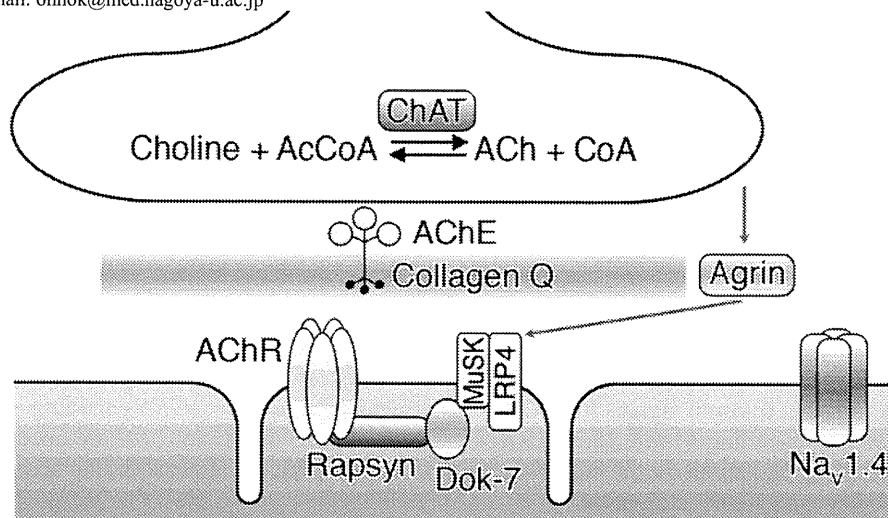
Figure 1. Schematic of molecules expressed at the neuromuscular junction. Mutated molecules in CMS include acetylcholine receptor (AChR), rapsyn, agrin, MuSK, Dok-7, skeletal muscle sodium channel type 1.4 ($Na_V1.4$), collagen Q, and choline acetyltransferase (ChAT).

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.

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 ($\alpha2$-$\alpha7$) and β subunits ($\beta2$-$\beta4$), whereas homomeric AChRs are formed by just one subunit type (e.g., $\alpha7$-$\alpha9$) [20]. On the other hand, muscle nicotinic AChRs have only two forms: fetal AChR that carries the $\alpha1$, $\beta1$, 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

$\alpha 1_2\beta 1\delta\varepsilon$ [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 $\alpha 7$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*, *CHRNB1*, and *CHRND* encoding the AChR $\alpha 1$, $\beta 1$, 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*, *CHRNB1*, 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 $\alpha 1$, $\beta 1$, or δ subunit. Patients with low-expressor mutations in *CHRNA1*, *CHRNB1*, and *CHRND* tend to have a devastating course with high fatality. Some missense mutations in *CHRNA1*, *CHRNB1*, *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 induces 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]
	εI254ins18	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,
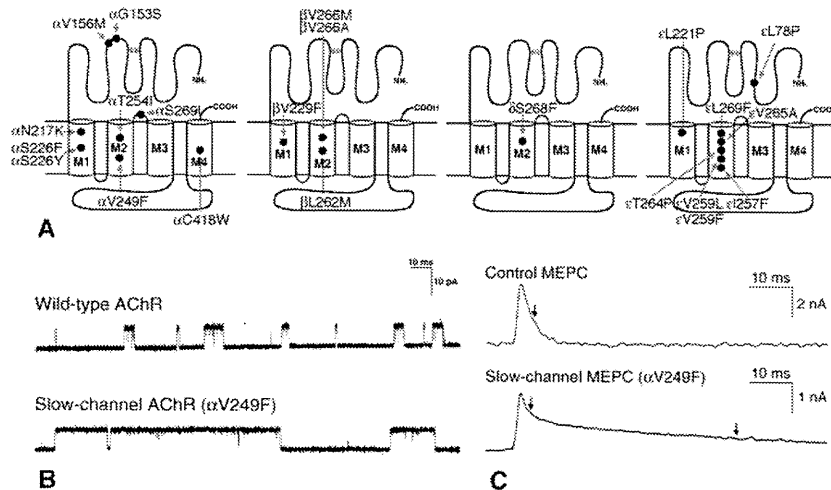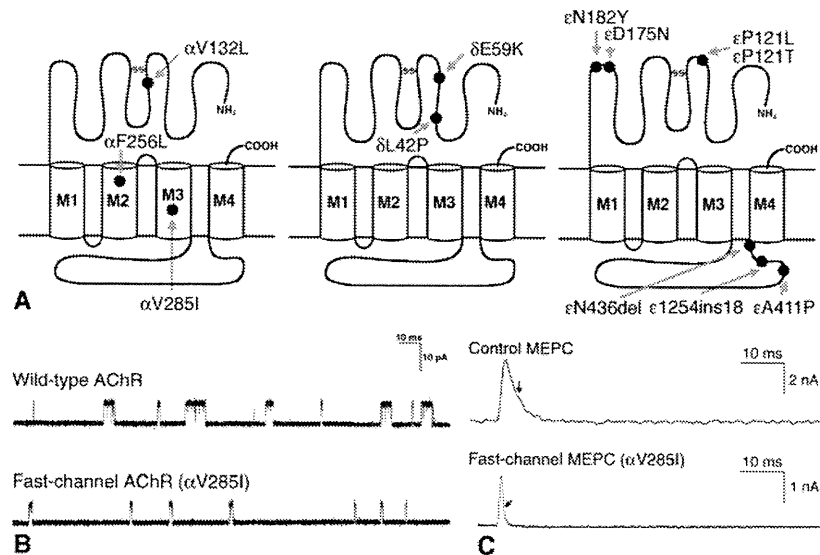
Figure 2. Slow channel syndrome. (A) Schematic diagram of AChR subunits with SCCMS mutations. (B) Single channel currents from wild-type and slow channel (α1V249F) AChRs expressed on HEK293 cells. (C) Miniature endplate current (MEPC) recorded from endplates of a control and a patient harboring α1V249F. The patient's MEPC decays biexponentially (arrows) due to expression of both wild-type and mutant AChRs.

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].

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

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.

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.

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 α1subunit 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, and δ and εsubunits, 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*, *CHRNB1*, 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*, *CHRNB1*, 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.

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