CHRND mutation causes a congenital myasthenic syndrome by impairing co-clustering of the acetylcholine receptor with rapsyn

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The objective of this study was to analyse the mutations of the acetylcholine receptor (AChR) delta subunit gene (CHRND) in a patient with sporadic congenital myasthenic syndrome (CMS). Mutations in various genes encoding proteins expressed at the neuromuscular junction may cause CMS. Mutations of AChR subunit genes lead to end-plate AChR deficiency or to altered kinetic properties of the receptor. Mutations in the alpha, beta and delta subunits of the AChR are less frequent than mutations of the epsilon subunit; mutations in these subunits leading to AChR deficiency are often associated with a severe phenotype. A sporadic patient from Germany was studied, who presented with an early onset CMS associated with feeding difficulties, ptosis, a moderate general weakness responsive to anticholinesterase treatment and recurrent episodes of respiratory insufficiency provoked by infections. The CHRND gene was screened for mutations by RFLP, long-range PCR and sequence analysis. Subsequently, we conducted functional studies of AChR mutants co-transfected with rapsyn in HEK 293 cells. Heterozygously to a 2.2 kb microdeletion disrupting the CHRND gene, we identified a novel point mutation in the long cytoplasmic loop, CHRND E381K. The cytoplasmic loop of the AChR subunits is known to be essential for AChR–rapsyn co-clustering. We therefore studied the interaction of AChR containing the CHRND E381K mutation with rapsyn by evaluating expression and co-localization of rapsyn and mutated AChR subunits in co-transfected HEK 293 cells. Interestingly, the mutated receptor showed severely reduced cluster formation compared with the wild-type receptor. In contrast, the corresponding amino acid substitution in the cytoplasmic loop of the AChR epsilon (CHRNE E376K) as well as a recently reported CMS mutation affecting this domain (CHRNE N436del) had no impact on cluster formation. CHRND mutations are a rare cause for CMS but should be considered in patients with a severe, early onset disease form, clinically resembling a rapsyn phenotype with recurrent episodic apnoeas. Our results suggest that impairment of AChR–rapsyn co-clustering—a well-known molecular mechanism for rapsyn mutations—could also result from mutations in the delta subunit. Introduction of the same mutation in the epsilon subunit had no effect on AChR clustering indicating a special role of the delta subunit in AChR–rapsyn interactions.

Keywords: congenital myasthenic syndrome; AChR delta-subunit mutation; chromosomal deletion; neuromuscular junction; rapsyn

Abbreviations: AChR = acetylcholine receptor; CMS = congenital myasthenic syndrome

CHRND mutation in congenital myasthenia

Introduction

The synaptic transmission at the neuromuscular junction is mediated by muscle nicotinic acetylcholine receptors (AChRs). Critical for efficient transmission is the highly specialized organization of AChRs into high-density clusters at the post-synaptic membrane. One of the key proteins in AChR clustering is rapsyn, a 43 kDa cytoplasmic protein that co-localizes precisely with AChRs at the crests of the junctional folds (Froehner et al., 1981; Gautam et al., 1995). The exact model of how rapsyn associates with the AChR and recruits AChR into clusters is still under investigation. Little is known about the sites within the AChR that are responsible for its interaction with rapsyn. On the basis of studies of recombinant proteins expressed in heterologous cells, rapsyn has been proposed to interact directly with the large intracellular domain between transmembrane domains M3 and M4 of the homologous AChR subunits (Maine and Merlie, 1993; Yu and Hall, 1994; Huebsch and Mainone, 2003). Which of the four AChR subunits (alpha, beta, delta and epsilon) interacts with rapsyn in vivo and which sequence elements are important for cluster formation remain to be studied.

In a subgroup of congenital myasthenic syndromes (CMS), a human genetic disease with impaired neuromuscular transmission (Hantai et al., 2004; Beeson et al., 2005; Engel and Sine, 2005), mutations within the rapsyn gene (RAPSN) result in reduced co-clustering of AChRs and rapsyn (Ohno et al., 2002). Interestingly, patients with RAPSN mutations exhibit a distinct clinical phenotype with the most prominent feature of sudden apnoeas in early childhood (Ohno et al., 2002; Burke et al., 2003; Müller et al., 2003; Richard et al., 2003). Mutations of different AChR subunit genes in CMS patients have been shown to alter AChR channel kinetics and/or to cause end-plate (EP-) AChR deficiency (Engel and Sine, 2005). However, an impairment of the rapsyn-induced clustering of AChR has never been demonstrated for any of the numerous reported AChR mutations.

We report on a CMS patient with a clinical phenotype highly suggestive for an underlying RAPSN mutation who nevertheless carries two mutations (Δ2.2 kb and E381K) in the delta AChR subunit. For one of the mutations, E381K in the large cytoplasmic loop of the delta subunit, we were able to demonstrate a reduced co-clustering with rapsyn in a heterologous expression system.

Material and methods

DNA samples

Venous blood samples were obtained from the CMS patient as well as from his unaffected parents, from his brother and his sister. All studies were carried out with informed consent of the patient’s parents and were approved by the institutional ethics review board. Genomic DNA was isolated using a blood and tissue culture DNA extraction kit according to the manufacturer’s recommendations (Wizard Genomic DNA Purification Kit, Promega, Mannheim, Germany).

Sequence analysis, RFLPs

PCR primers were designed according to the published genomic structure (GenBank accession number AF307337/gi:10732831) of the CHRND gene. Nucleotide positions correspond to the genome assembly of May 2004 from the University of California Santa Cruz Genome Bioinformatics Site (http://genome.ucsc.edu). The Repeat Masker program provided by this website was used to analyse the nature of the sequences flanking the deletion breakpoints. In the patient, all 12 exons, flanking intronic regions, and the promoter of the CHRND gene were amplified by PCR and sequenced. PCR-amplified fragments were purified with the NucleoSpin Extract kit (Macherey-Nagel, Düren, Germany) and sequenced using an Applied Biosystems model 3100 Avant DNA sequencer and fluorescence-labelled dideoxy terminators (Perkin-Elmer, Foster City, CA, USA). In addition, all 12 exons, adjacent intronic regions and the promoter region of the AChR ε-subunit gene (CHRNE, GenBank accession number AF105999/gi:4580858) and all 8 exons, flanking intronic and promoter regions of the gene encoding for rapsyn (RAPSN, GenBank accession number AC090559/gi:22002211, mRNA AF449218/gi:19310212) were sequenced and scrutinized for mutations. Possible mutations in the CHAT gene were excluded by haplotype analysis according to Schmidt et al. (2003).

Screening for the mutation E381K (1141G > A) was performed by restriction analysis using TaqI. By this method, the patient’s family and 100 healthy controls were tested for the mutation.

Determination of the deletion breakpoints

The long-range polymerase chain reaction was applied to the genomic DNA of the patient and his family using the Expand Long Template PCR System from Roche (Basel, Switzerland). Specific primers in sense and antisense direction were used to determine the deletion breakpoints: D4 forward 5’-cctgctggatgtgcctgc, D6 forward 5’-tccgacctagttatgtgatg, D8 reverse 5’-tcgggtgccccctgctggatt.

Cloning procedures

In order to generate the mutations CHRND E381K, D377K, M379A, F380A, E381L, K382E and E385K, the plasmid pBlueScript SK, which contains the gene encoding the wild-type delta subunit of the mouse AChR, was used as a template for PCR-based mutagenesis. Primer sequences and cloning strategies can be obtained on request. To confirm the presence of the introduced mutations, and to rule out any errors, the inserts were sequenced. For expression in HEK 293 cells, the mutated delta subunits were subcloned into the pRC/CMV2 vector (Invitrogen, Karlsruhe, Germany) using HindIII and NotI.

The mutation CHRNE E376K was directly introduced into the wild-type mouse epsilon subunit cDNA in the vector pRC/CMV2 by PCR-based mutagenesis. In order to generate the mutation CHRNE N436del, the mouse epsilon subunit cDNA in the vector pBlueScript SK was used as a template for site-directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit, Stratagene, Amsterdam, The Netherlands; primers 5’-ggagcccttggtctgattttgg and 5’-cccaaaaaaacatcacgggcttcc). For expression in HEK 293 cells, the mutated epsilon subunit was subcloned into the pRC/CMV2 vector using NotI and ApaI.

Expression in HEK 293 cells

Wild-type and mutant mouse AChR subunits in the vector pRC/CMV2 (Invitrogen, Karlsruhe, Germany) were used for transfection.
studies. The plasmid RAPSN-eGFP-N1, harbours the genes encoding for human wild-type rapsyn and enhanced green fluorescent protein.

HEK cells were grown at 37°C on uncoated glass cover slips in six-well plates in DMEM supplemented with 10% foetal bovine serum. Transfection was carried out 24 h after plating using the calcium phosphate method. Cells were transfected with either wild-type or mutant AChR-delta-subunit cDNA (2.5 μg), wild-type alpha, beta, epsilon AChR-subunit cDNA (at a ratio of 2 : 1 : 1 with 5 μg alpha subunit) and wild-type rapsyn cDNA (2.5 μg). Forty-eight hours after transfection, cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS. To visualize expression of AChR, cells were then incubated with 2 μg/ml α-bungarotoxin, Alexa Fluor® 594 conjugate (Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. After rinsing with PBS, cells were mounted on specimen supports using DAKO® Fluorescent Mounting Medium.

To analyse the cells and to obtain images, fluorescence microscopy (microscope: Leica DM RBE) was used. After transfections, each cover slip was systematically traversed and searched for co-transfected cells, displaying AChR and rapsyn distributed either diffusely or in clusters. A total of 300 co-transfected cells per mutation collected from 6 independent transfection experiments were imaged and evaluated for the wild-type, as well as the mutations δE381K, eE376K and eN436del. For the mutations δD377K, δM379A, δF380A, δE381L, δK382E and δE385K we evaluated a total of 150 cells per mutation from three independent transfection experiments. For each mutation the proportion of cells displaying AChR–rapsyn clusters was determined. Statistical evaluation (calculation of mean values and standard deviations) was done with the program WinSTAT3.1.

For confocal imaging cells were examined using a Leica DM IRB/E microscope (Leica Microsystems, Mannheim, Germany) equipped with a Krypton-Argon Laser (Leica TCS NT).

Characterization of surface expression

Wild-type and mutant AChR δ-subunit cDNAs, in combination with wild-type α-, β- and ε-subunit cDNAs, were transfected into HEK 293 cells grown on six-well tissue culture plates using polyethylenimine. Surface AChR expression was determined 2 days post-transfection by incubating cells in 10 nM 125I-α-bungarotoxin (125I-α-BuTx) with 1 mg/ml BSA for 30 min. Cells were washed four times with PBS and extracted in 1.25% Triton X-100, in 60 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride and 125I-α-BuTx binding determined using a gamma counter.

Results

Clinical data

The German patient, currently 7 years of age, is the third child of non-consanguineous healthy parents. His two older siblings are healthy. The index patient was born after an uneventful 38 week pregnancy by caesarean section. Side by side he had a high arched palate, neither facial dysmorphism nor congenital contractures were noted. However, difficulties in feeding and sucking with frequent chokes developed post-partum. In addition, an intermittent left-side eyelid ptosis, facial weakness and a daytime-dependent abnormal fatigue and muscular hypotonia were noted in the neonatal period. Motor development was moderately delayed. Repeatedly, mild viral infections of the upper airways led to exacerbation of symptoms with increased general weakness, swallowing difficulties for solid food and respiratory distress. At 7 months of age, a viral infection led to respiratory insufficiency that required assisted ventilation for 3 days. At the age of 18 months, a neuromuscular transmission defect was diagnosed on the basis of a positive intravenous edrophonium (Camsilon®) test and a decremental response to 3 Hz repetitive stimulation (N. ulnaris, 16%, N. medianus, 14%, N. accessorius, 11%). A subsequent therapy with increasing doses of pyridostigmine bromide (Mestinon®) was started and resulted in significant clinical improvement of ptosis and hypotonia at a final dose of 7 mg/kg/day. In particular, the child was now able to eat solid food, and motor development improved. By intermittent increase of the pyridostigmine dosage or by decrease of the dosing intervals, subsequent infections were successfully managed without any further respiratory crises. Over the next years, the general course of disease was favourable. Pyridostigmine bromide dosage had to be readjusted several times up to presently 8 mg/kg/day. A current neurological examination revealed age-appropriate motor and mental development, and a mild exercise-induced, generalized weakness of limb muscles, predominantly involving proximal muscles. Slight bilateral ptosis without restriction of extraocular movements and bulbar weakness apparent by a minor weakness of facial muscles were observed. The anti-AChR antibody test was negative. Repetitive stimulation (3/s) of two distal (right N. medianus and right N. ulnaris) as well as two motor nerves (right and left Nn. accessorii) revealed a decremental response of the compound muscle action potential (CMAP) at rest (11–16%). However, the decremental response was abolished by anticholinesterase treatment.

Mutational analysis

Mutations of the RAPSN and the CHRNE gene were excluded by direct sequencing of the genes. Haplotype analysis excluded linkage to the CHAT locus, but indicated linkage to the CHRND gene. Subsequent sequence analysis of the coding and the promoter region of the CHRND gene revealed indeed a heterozygous A-to-G transition in exon 10 at nucleotide position 1141 (1141G>A) in the index patient II/1 of our German CMS pedigree. The mutation leads to an amino acid exchange (E381K) that, to our knowledge, has not been described before. Restriction analysis revealed that unaffected family members either carry the mutation E381K heterozygously (I/2) or do not carry the mutation (I/1, II/2 and II/3; Fig. 1A). This is in accordance with an autosomal recessive trait. In 200 control alleles, the mutation E381K was not detected.

Since no additional mutation was found by PCR-based sequence analysis of the coding and promoter regions of the patient’s CHRND gene, we concluded that a chromosomal deletion or a chromosomal rearrangement of the CHRND gene might have been inherited from the patient’s father.
Fig. 1 (A) Results of the mutation analysis. Restriction enzyme analysis of the CMS family (upper panel). To detect the \textit{CHRND} mutation E381K (1141G→A), a PCR fragment of 576 bp containing exon 10 was amplified. Owing to the mutation, a restriction site for \textit{Taq}I is abolished. As a consequence, the mutated allele remains undigested, whereas the wild-type allele yields two fragments (341 and 235 bp). The patient (II/1) and his unaffected mother (I/2) are heterozygous for the mutation E381K. The patient’s father does not carry the mutation E381K. Lower panel: Long-range PCR performed in the family with primers D4 forward (located in intron 5) and D8 reverse (located at the end of exon 12). For the mother (I/2) and the unaffected sister (II/3) of the patient, the expected product of 6746 bp was obtained. For the father (I/1) and the patient (II/1), an additional shorter product of 4528 bp was amplified, placing the chromosomal microdeletion between intron 5 and exon 12. Only the patient (II/1) carries both the mutation E381K and the microdeletion in the \textit{CHRND} gene. (B) Schematic representation of the genomic organization of the \textit{CHRND} gene and the position of the deletion characterized in this study. The 12 exons of the gene are represented by filled boxes and are numbered. Striped boxes symbolize the promoter region and 3’-UTR of the gene; the \textit{AluSc} element is shown as a white box. A 2.2 kb portion (indicated by orange shading) between exon 8 and the \textit{AluSc} element in intron 9 is deleted in the patient. \textit{cen} = centromere, \textit{tel} = telomere, \textit{Sc} = \textit{AluSc} element. (C) Alignment of amino acid sequences of human, bovine, mouse, rat, frog, chicken, \textit{danio rerio}, \textit{electric ray} and \textit{takifugu rubripes} for the AChR delta subunit. Glutamate at position 381 and neighbouring amino acids are highly conserved among delta subunits of different species. In the human epsilon subunit, glutamate is conserved. The human gamma subunit shows glutamine at the corresponding position. Identical amino acid residues are shown in black, similar residues are coloured in blue, and residues not conserved are marked in green.
Identification and characterization of the deletion

In order to map putative chromosomal deletions/rearrangements, a long-range PCR amplification was carried out on genomic DNA of the patient encompassing exon 10 of the CHRN D gene that contains the mutation E381K. In the patient (I/2), as well as in his unaffected father (I/1) and brother (II/2), a long-range PCR with primers D4 forward (located in intron 5) and D8 reverse (located at the end of exon 12 of the CHRN D gene) revealed the presence of a second band of 4.5 kb in addition to the expected band of 6746 bp (Fig. 1A). This suggested a chromosomal deletion of \( \sim 2.2 \) kb in length. In a sequencing reaction with primer D6 forward (located in intron 7), this fragment proved to be the result of a deletion starting in exon 8 (breakpoint is located 79 bp behind the start of exon 8) and extending into intron 9 (end of deletion is located 282 bp before the start of exon 10). The total deletion of 2218 bp results in the loss of half of exon 8 and the entire exon 9 of CHRN D. The end of the deletion is located 49 bp within an AluSc element (Fig. 1B).

Functional characterization of CHRN D E381K

Each AChR subunit consists of four transmembrane regions (M1–M4), a large N-terminal extracellular domain and a long cytoplasmic loop between transmembrane regions 3 and 4. E381K is located within the major cytoplasmic loop of the \( \delta \) subunit and is the first mutation reported in this domain of CHRN D. The glutamic acid in position 381 is highly conserved among the \( \delta \) subunits of various species as well as in the \( \epsilon \) subunit (Fig. 1C). In the \( \epsilon \) subunit the corresponding E is located at position 376.

To assess the functional impact of this missense mutation we cloned E381K into \( \delta \) subunit cDNA and expressed the mutated receptor in HEK 293 cells. Receptor molecules containing the mutated delta subunit were detected at a similar level, and with a similar distribution in dot-like structures at the cell surface as the wild-type receptor (Fig. 2A). Because transfected cells were not permeabilized before staining with \( \alpha \)-bungarotoxin, only AChR at the cell surface were stained. In control cells only transfected with wild-type \( \alpha \), \( \beta \) and \( \epsilon \) subunits, no AChRs were detected at the cell surface (data not shown).

To confirm the results for channels harbouring the \( \delta E381K \) mutation we expressed mutant and wild-type AChRs in HEK 293 cells and quantified surface expression by labelling with \( ^{125} \)I-\( \alpha \)-bungarotoxin. Expression of the mutant channels was reduced to \( \sim 70\% \) of wild-type (Fig. 2B).

When co-transfected with rapsyn cDNA, wild-type AChR form receptor–rapsyn clusters at the cell membrane (Froehner et al., 1990). As the cytoplasmic loop of the AChR subunits is known to be involved in this clustering process (Huebsch and Maimone, 2003) we co-transfected AChR harbouring the \( \delta \) subunit mutation with rapsyn-EGFP and subsequently examined the formed clusters.

When co-transfected with rapsyn, AChR reorganizes into higher density clusters at the cell membrane; these clusters contain both AChR and rapsyn. For the wild-type AChR, 72.9 ± 9.75% of all transfected cells displayed high-density AChR–rapsyn clusters at the cell membrane. In contrast, only in 32.5 ± 7.2% of the cells transfected with AChR containing E381K AChR–rapsyn clusters were detected (Figs 2C and 3). Furthermore, in most of the cells co-localization was weak (defined by only 1–2 common clusters per cell). Therefore, we concluded that presence of the mutation E381K interferes with AChR–rapsyn clustering. As this amino acid is conserved also in the AChR epsilon subunit, we introduced the corresponding mutation E–K into the large cytoplasmic loop of the epsilon subunit (CHRNE E376K) and analysed subsequent cluster formation. In addition, we examined a second mutation of the epsilon subunit cytoplasmic loop (N436del; 1304del3) that has been previously reported in CMS patients (Shen et al., 2005). N436del leads to the loss of the last amino acid of the loop and is known to be a fast-channel mutation combined with a minor reduction in AChR expression. AChR containing one of these epsilon subunit mutations are expressed in transfected HEK 293 cells in a similar pattern compared with the wild-type receptor (Fig. 2A). When co-transfected with rapsyn-EGFP, cluster formation of these AChR was indistinguishable from wild-type AChR by quantity and/or appearance (Figs 2C and 3).

To investigate further the relevance of the amino acid \( \delta E381 \) for AChR–rapsyn interaction, we mutated neighbouring residues of the delta subunit cytoplasmic loop. In charged residues, charge was reversed (D377K, K382E and E385K); uncharged amino acids were mutated to alanine (M379A, F380A). In addition, E381 itself was modified to an uncharged amino acid (E381L). Subsequently, we evaluated co-clustering with rapsyn in co-transfected HEK 293 cells for these six mutations. The mutations K382E and E385K had no influence on cluster formation; we found AChR–rapsyn clusters in 73 and 70.7% of cells expressing both AChR and rapsyn (Fig. 4). In contrast, all modifications of residues N-terminal of E381 and modification of E381 itself reduced the proportion of cells with AChR–rapsyn clusters to well below 50% (\( \delta D377K \): 38.9%, \( \delta M379A \): 43.5%, \( \delta F380A \): 46.9%, \( \delta E381L \): 36.5%; Fig. 4).

Discussion

We investigated a CMS patient with clinical features characteristic of AChR deficiency due to mutations in the AChR-clustering protein rapsyn. Surprisingly, this patient did not have mutations in RAPSN but was a compound heterozygote for a microdeletion \( \delta \Delta 2.2 \) kb and a missense mutation \( \delta E381K \) in CHRN D, the AChR \( \delta \)-subunit gene. Functional studies of \( \delta E381K \) show that this mutation impairs AChR–rapsyn co-clustering, implying that defective AChR clustering contributes to the pathogenic mechanism of the mutation. This is the first reported example of
Fig. 2 (A) Confocal images of the expression of wild-type, δE381K, εE376K, and εN436del-AChR in HEK cells. AChR molecules were stained with Alexa Fluor® 594 conjugated α-bungarotoxin; the cells were not permeabilized before staining. All AChR variants are abundantly expressed at the cell surface. Bar: 10 μm. (B) Expression of AChR containing wild-type or mutant δ subunit in HEK 293 cells. Total ¹²⁵I-α-BuTx binding to the surface of HEK 293 cells transfected with cDNAs encoding wild-type αβδε, wild-type αβε and αβε plus the mutant δ subunit. Results are normalized for ¹²⁵I-α-BuTx binding to αβδε and represent the mean ± standard deviation of six transfections. Control cells were transfected with pcDNA3.1. BuTx = bungarotoxin. (C) Confocal images of the distribution of surface AChR (red signal, left column), rapsyn-EGFP (green signal, middle column) and superimposed images (right column) in cells co-transfected with wild-type rapsyn-EGFP and wild-type AChR (upper row), AChR with δE381K (second row), AChR with εE376K (third row) and AChR with εN436del (bottom row), respectively. Yellow signals indicate AChR–rapsyn co-localization; typical clusters are indicated by arrows. Whereas in the case of wild-type AChR and the two epsilon subunit mutations AChR–rapsyn clusters were detected in the majority of the co-transfected cells, only one-third of cells transfected with δE381K AChR displayed co-localization with rapsyn. Row two shows a typical example where no co-localization was present. Bar: 10 μm.
an AChR CMS mutation affecting clustering, and should provide a basis for the identification of sites of direct interaction between the AChR and rapsyn, which thus far has proved elusive.

To our knowledge, only seven different mutations have been reported for the AChR delta subunit gene (CHRND). These affect different domains of the protein but none of them is located in the large cytoplasmic loop of the delta subunit. Slow-channel kinetics have been demonstrated for the missense mutations S268F (Gomez et al., 2002) and V93L (Shen et al., 2003) while P250Q (Shen et al., 2002), E59K (Brownlow et al., 2001) and L42P (Shen et al., 2003) have been characterized as fast-channel mutations. Two other mutations of CHRND reduce [I58K; (Shen et al., 2003)] or abolish [756del2; (Brownlow et al., 2001)] the expression of the delta subunit.

Clinically, the most remarkable features in our patient were sudden exacerbations of weakness in infancy provoked by respiratory infections that repeatedly culminated in respiratory distress. Sudden apnoeas are rarely observed in patients with CHRNE mutations but are a hallmark of patients with CHAT and RAPSN mutations. Beside early onset apnoeas, absence of ophthalmoparesis and favourable response to anticholinesterase medication resulting in regular motor development are other observations in our patient that are characteristic for underlying RAPSN mutations. Although our patient strongly resembled the early onset phenotype of patients with underlying mutations in the RAPSN gene (Burke et al., 2003; Müller et al., 2003), mutations in this gene were excluded.

For AChR delta subunit mutations, a full description of clinical data is available for only five patients. These patients reveal different phenotypes that can be largely attributed to the respective functional characteristics of their underlying mutation. A severe and progressive phenotype is reported for both the slow-channel mutation CHRND S268F and the homozygous fast-channel mutation P250Q in three Arab patients (Gomez et al., 2002; Shen et al., 2002). Similar to our case, the patient harbouring the fast-channel mutation E59K appears to be affected less severely (Brownlow et al., 2001). However, different from our patient she had congenital joint contractures, and a marked limitation of eye movements. Episodic apneas or respiratory distress in early infancy were only reported for the E59K fast-channel patient (Brownlow et al., 2001), but not for the other patients with CHRND mutations. Interestingly, in some of the families with CHRND fast-channel mutations, there were similarly affected relatives reported who died from respiratory complications in early infancy (Brownlow et al., 2001; Shen et al., 2003).

In our patient, the phenotypic features are likely to be caused by the missense mutation E381K. As the deletion on the second allele causes the loss of one part of exon 8 and the entire exon 9 it most likely represents a null mutation. However, no muscle tissue of the patient was available for confirmation in vivo. This is, to our knowledge, the first...
microdeletion described for the CHRNDE gene. So far, only two other microdeletions were detected in CMS genes [Δ1290 bp in CHRN(E) (Abicht et al., 2002) and Δ4.5 kb in RAPSN (Müller et al., 2004)]. In all three cases, Alu elements were involved in either homologous or non-homologous illegitimate recombinations. Such deletions are not detectable when genomic DNA is amplified exon by exon and tested by sequence or heteroduplex analysis; therefore, the frequency of deletions may be underestimated. However, the proportion of deletion mutations in the relatively small CMS genes certainly does not warrant a standard analysis by quantitative PCR techniques as it is the case for the large DMD gene where deletions account for up to 60% of all Duchenne muscular dystrophy cases (Prior and Bridgeman, 2005). Nevertheless, a deletion or duplication should be considered in CMS patients where only one heterozygous pathogenic mutation is found in the coding region and adjacent intronic and regulatory regions of a gene.

The mutation CHRNDE E381K is the first mutation that has been identified in the cytoplasmic loop of the AChR delta subunit. In contrast, mutations in the cytoplasmic loop of the epsilon and the beta subunit have been previously described, and the underlying pathogenic mechanisms were elucidated in cell culture experiments: B426delEQE impairs AChR assembly by disrupting a specific interaction between beta and delta subunits and is a low-expressor mutation (Quiram et al., 1999). CHRNNE R311W (Ohno et al., 1997) and P331L (Croxen et al., 2001) are low-expressor mutations, too, whereas CHRNNE A411P, N436del, 1254ins18 show fast-channel kinetics combined with a reduction in expression (Milone et al., 1998; Wang et al., 2000; Engel and Sine, 2005; Shen et al., 2005). Quantitative analysis of AChR expression with δE381K in vitro revealed a slight reduction of surface expression (to 70% of wild-type), which may not be sufficient to fully explain the phenotype. However, we did not obtain a motor point biopsy of the patient. Since expression may be different at the end-plate as compared with the heterologous in vitro assay, we cannot exclude lower than 70% of normal expression at the end-plate.

A refined model of the Torpedo AChR at 4 Å resolution (Unwin, 2005) shows that the cytoplasmic loop of each AChR subunit contains at its C-terminal end an a-helix structure (MA) that contributes to gating and cation selectivity of the channel. The mutations CHRNNE A411P, N436del, 1254ins18 are part of the MA helix of the epsilon subunit, supporting their role for channel opening. The N-terminal part of the cytoplasmic loop appears to be disordered and is not directly seen in the X-ray structure (Unwin, 2005). For the delta subunit, this N-terminal loop domain encompasses residues 313–415 including the region with the analysed delta mutations. This domain seems exposed and freely accessible by interaction partners such as rapsyn.

The cytoplasmic loop of the AChR subunits is known to be necessary for the interaction with rapsyn and the subsequent rapsyn-dependent clustering at the cell membrane. Missense mutations of rapsyn have been shown to reduce AChR–rapsyn clustering (Ohno et al., 2002). Interestingly, the
clinical history of our patient was suggestive for rapsyn deficiency. Vice versa, we wanted to investigate whether missense mutations in the cytoplasmic loop of AChR subunits might have the same effect. Therefore, we studied the functional consequences of the CHRN D mutation E381K on the interaction of AChRs with rapsyn. Interestingly, we observed a markedly reduced ability of the mutated receptor to cluster with rapsyn. This effect was unexpected, as previous work (Huebsch and Maimone, 2003) indicated that each AChR subunit on its own can co-cluster with rapsyn when co-expressed in cell culture. If the cytoplasmic loop of the respective subunit was deleted, the ability to co-cluster with rapsyn was completely lost. Therefore, Huebsch and Maimone (2003) hypothesized that the intact cytoplasmic loop of one single AChR subunit is sufficient to assure clustering with rapsyn. However, they were not able to prove this hypothesis by experiments with AChR-pentamers containing four deleted and only one wild-type subunit with cytoplasmic loop, as those modified subunits did not assemble into pentamers if co-transfected. Moreover, in cells expressing all AChR subunits rapsyn might interact preferably with one specific subunit only.

The two mutations introduced into the cytoplasmic loop of the AChR e subunit did not impair clustering, hinting at an asymmetric function of the AChR subunits in the interaction process with rapsyn. Subsequently, we mutated several residues adjacent to E381 in the cytoplasmic loop of the delta subunit in order to map a putative interaction domain of the delta subunit with rapsyn. Studies of co-clustering of AChRs harbouring these delta subunit mutations revealed that a region critical for clustering might start upstream and stretch up to amino acid E381. AChRs harbouring the mutations D377K, D379A, D380A, D381L and D381K show impaired AChR–rapsyn co-clustering compared with the wild-type receptor, whereas the changes introduced downstream of E381 did not seem to impair co-clustering.

Further studies are needed to define in detail the differential roles of the AChR subunits in the interaction process with rapsyn and—given a special role for the delta subunit cytoplasmic loop—to further narrow down the protein domains essential for co-clustering.

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CHRN D mutation in congenital myasthenia


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