

Escobar Syndrome Is a Prenatal Myasthenia Caused by Disruption of the Acetylcholine Receptor Fetal γ Subunit

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Escobar syndrome is a form of arthrogryposis multiplex congenita and features joint contractures, pterygia, and respiratory distress. Similar findings occur in newborns exposed to nicotinic acetylcholine receptor (AChR) antibodies from myasthenic mothers. We performed linkage studies in families with Escobar syndrome and identified eight mutations within the γ -subunit gene (*CHRNG*) of the AChR. Our functional studies show that γ -subunit mutations prevent the correct localization of the fetal AChR in human embryonic kidney–cell membranes and that the expression pattern in prenatal mice corresponds to the human clinical phenotype. AChRs have five subunits. Two α , one β , and one δ subunit are always present. By switching γ to ϵ subunits in late fetal development, fetal AChRs are gradually replaced by adult AChRs. Fetal and adult AChRs are essential for neuromuscular signal transduction. In addition, the fetal AChRs seem to be the guide for the primary encounter of axon and muscle. Because of this important function in organogenesis, human mutations in the γ subunit were thought to be lethal, as they are in γ -knockout mice. In contrast, many mutations in other subunits have been found to be viable but cause postnatally persisting or beginning myasthenic syndromes. We conclude that Escobar syndrome is an inherited fetal myasthenic disease that also affects neuromuscular organogenesis. Because γ expression is restricted to early development, patients have no myasthenic symptoms later in life. This is the major difference from mutations in the other AChR subunits and the striking parallel to the symptoms found in neonates with arthrogryposis when maternal AChR auto-antibodies crossed the placenta and caused the transient inactivation of the AChR pathway.

Escobar or multiple pterygia syndrome (MIM 609339 and 265000), a form of arthrogryposis multiplex congenita, is an autosomal recessive condition characterized by excessive webbing (pterygia), congenital contractures (arthrogryposis), and scoliosis.^{1,2} Variable other features include intrauterine death, congenital respiratory distress, short stature, faciocranial dysmorphism, ptosis, low-set ears, arachnodactyly, and cryptorchism in males. Congenital contractures are common and may be caused by reduced fetal movements at sensitive times of development.^{3–5} Possible causes of decreased fetal mobility include space constraints such as oligohydramnion, drugs, metabolic conditions, or neuromuscular disorders including myasthenia gravis (MG [MIM 254200]).⁶ Myasthenia is characterized by intermittent muscle weakness, most commonly caused by autoantibodies binding to nicotinic acetylcholine receptor (AChR).⁷ During pregnancy, autoantibodies may cross the placenta and cause transient muscle weakness or, more seriously, an arthrogryposislike syndrome.⁸ Hereditary causes of congenital myasthenic syndrome (e.g.,

MIM 608931, 601462, 254210, and 608930]) have also been identified, mostly mutations in *CHRNA1* (MIM 100690), *CHRNA1* (MIM 100710), *CHRND* (MIM 100720), and *CHRNE* (MIM 100725), the genes encoding the α , β , δ , and ϵ subunits of AChRs, respectively.⁹

The AChR is composed of five subunits; two α , one β , and one δ subunit are invariably present. The γ subunit is present before the 33rd wk of gestation in humans but is replaced by an ϵ subunit in the late fetal and perinatal period, thereby forming the adult AChR (fig. 1).^{10–13} The fetal AChR helps to establish the primary encounter of muscle and axon.¹⁴ Thus, the γ subunit not only contributes to neuromuscular signal transduction but is also important for neuromuscular organogenesis. The importance of the fetal AChR subtype for neuromuscular development is underscored by the lethal phenotype of γ inactivation in mice.¹⁵ We identified *CHRNG* (MIM 100730) mutations in families with Escobar syndrome and showed that the trait is a congenital dysmorphism caused by the transient inactivation of the neuromuscular end plate.

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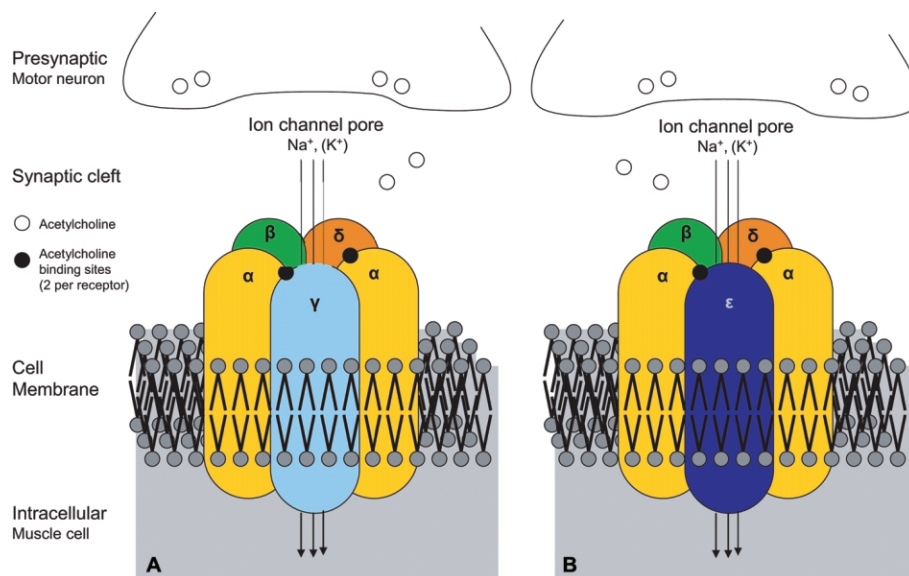


Figure 1. Structure and subunit composition of the fetal and adult AChR at muscle cells. Acetylcholine release from nerve terminals results in activation of the AChR at the postsynaptic membrane. This triggers an end-plate potential that activates voltage-dependent sodium channels and finally generates an action potential in the muscle. An AChR consists of a pentamer of paralogous subunits. Two types of skeletal-muscle AChR are identified by their different functions and subunit compositions. *A*, Fetal AChR. A fetal type of AChR has 2 α , β , γ , and δ subunits and is synthesized before week 33 of gestation in humans and before postnatal day P9 in mice.^{10–12} *B*, Adult AChR. Adult-type AChRs are formed through a gradual replacement of the fetal γ by the adult ϵ subunit.^{12,13}

Methods

Patients

We studied seven families with Escobar syndrome and control individuals. The families came from Germany, Lebanon (three families), Oman, Switzerland, and Turkey (table 1 and fig. 2). Five families were consanguineous. Our ethics committee approved the study, and written, informed consent was obtained from all participants or their legal guardians. Patients did not give consent to a muscle biopsy for scientific purposes.

Genomewide Scan, Fine Mapping, and Sequencing

We used the 10K Affymetrix SNP chip for our genome scan and analyzed the data using ALLEGRO v1.2c,¹⁶ GENEHUNTER v2.1r5,¹⁷ and easyLINKAGE v5.03.¹⁸ We assumed a recessive model

with complete penetrance, 0.001 disease-allele frequency, and equally distributed marker-allele frequencies. Fine mapping with microsatellites was done as described elsewhere.¹⁹ We included all available family members and additional families, and we reconstructed haplotypes by GENEHUNTER and manually. We next sequenced functional candidate genes within the linkage interval with standard sequencing procedures.¹⁹ Primer sequences are available on request. All described *CHRNA3* mutations were tested in control chromosomes and for correct segregation within the patients' families.

Human Embryonic Kidney (HEK) Cell-Expression Studies

We cloned amplified cDNA sequences of wild-type and mutant mouse AChR subunits into the expression vector system pRC/

Table 1. *CHRNA3* Mutations in Escobar Syndrome

Characteristic	Findings for γ Subunit of AChR by Family and Mutation						
	EG-1	EG-2	EG-3	EG-4	EG-5	EG-6	EG-7
Location (exon)	1	8	4 and 5	4 and 12	10	7	7
Position cDNA	13C→T ^a	807insT ^a	256C→T/481G→A	300dup(9)/1408C→T	1249G→C ^a	715C→T ^a	715C→T ^a
Residue in mature protein ^b	γ Q-18X	γ Δ 248–274, 275X	γ R64C/ γ W139X	γ 78dup(3)/ γ R448X	γ Δ 395–418,419X ^c	γ R217C	γ R217C
Family data:							
Origin	Oman	Lebanon	Switzerland	Germany	Lebanon	Lebanon	Turkey
Consanguinity	+	+	–	–	+	+	+
Family history of abortions	NA	–	–	–	+	+	+

NOTE.—EG = Escobar syndrome by *CHRNA3* mutation; NA = not available.

^a Homozygous.

^b After cleavage of signal peptide.

^c Or γ E395Q.

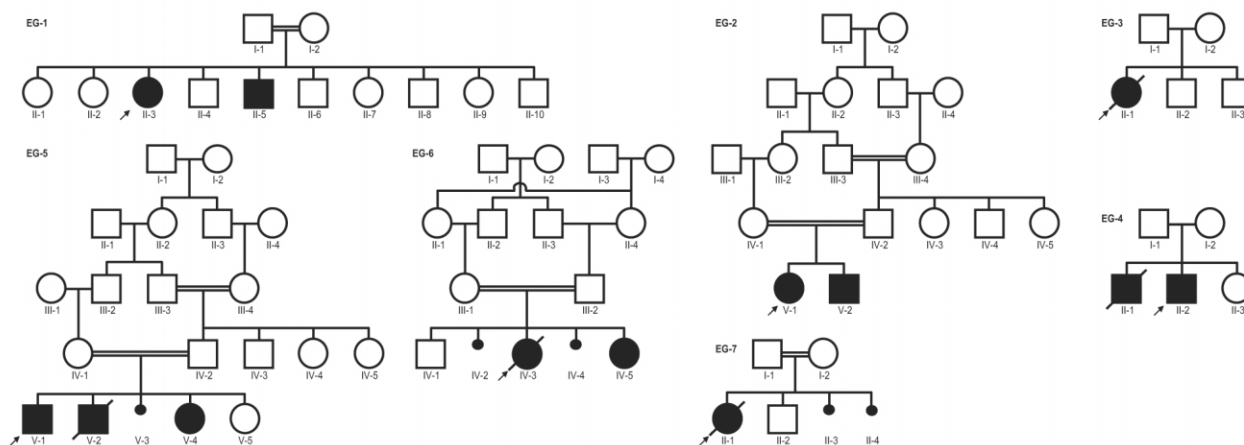


Figure 2. Pedigrees of families with Escobar syndrome caused by *CHRN3* mutations

CMV2 (Invitrogen). The human mutations 78dup(3), R217C, and R448X were introduced into the wild-type γ AChR vector by PCR-based mutagenesis. Since the mouse cytoplasmic loop of the γ subunit is 2 aa longer than the human form, human amino acid R448 corresponds to R450 in mice.

HEK293 cells were grown at 37°C on uncoated glass cover slips in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum; 24 h after plating, we transfected cells with α , β , δ , and either wild-type or mutant γ subunit vector constructs, at a ratio of 2:1:1:1, with 5 μ g as the amount of α subunit plasmid. Forty-eight hours after transfection, cells were rinsed with PBS and were fixed with 4% paraformaldehyde. To visualize expression of AChR, we then incubated with 2 μ g/ml α -bungarotoxin and Alexa Fluor 594 conjugate (Invitrogen), for 1 h at room temperature. After rinsing with PBS, cells were mounted on specimen supports (DAKO Fluorescent Mounting Medium) and were analyzed by fluorescence microscopy with use of a Leica DM RBE microscope.

Section In Situ Hybridization

We prepared mouse embryos of stages of embryonic day 14.5 (E14.5), cut frozen 15- μ m sections, and used the semi-automated TECAN GenePaint system.²⁰ Probes for AChR subunits were generated by RT-PCR from mouse E14.5 whole cDNA (γ) and P9 whole hindlimb cDNA (ϵ). Antisense riboprobes were transcribed with T7 polymerase with the Roche Dig-RNA labeling kit according to the manufacturer's instructions. For each subunit, we generated two independent probes, hybridizing to different portions of the RNA to verify specificity of the signal. Primer sequences are available on request.

Results

We studied seven families that had children with Escobar syndrome (tables 1 and 2 and fig. 2). All patients had clinical features consistent with arthrogryposis multiplex congenita and multiple pterygia (fig. 3A–3C) and presented faciocranial dysmorphism, short stature, and cryptorchidism in male. Scoliosis, arachnodactyly, facial weak-

ness, and respiratory distress were variably expressed (fig. 3D–3E). Most patients had high-arched palate and low-set ears. Family history of reduced prenatal movement and/or spontaneous abortions was frequently reported. All surviving patients had growth retardation but normal mental development. Interestingly, features of myasthenia were absent. Electromyography (EMG) in patient II-2 of family EG-4 did not reveal spontaneous activity while at rest. Early recruitment of low-amplitude and polyphasic muscle action potentials were noted. Motor nerve–conduction velocity and distal latency of the right median nerve (56 m/s, 1.8 ms) and sensory nerve–conduction velocity and amplitude of the right median nerve (66 m/s, 31 mV) were normal. Repetitive stimulation of the right median nerve with 3 Hz did not reveal a pathological decrement. Thus, there was no sign of a neuromuscular transmission defect in this patient, but there was some indication of myogenic lesion by electrophysiological standards. Similar results were obtained for families EG-1, EG-2, and EG-6.

Testing of pulmonary function in patient II-2 of family EG-4 at age 18 years revealed a severely reduced vital capacity (0.7 liter, corresponding to 27% of age-, sex-, and height-matched normal value); cardiac function was normal on echocardiography. Cardiac and pulmonary function tests were not available for the other families. However, two patients (patients II-1 in family EG-3 and II-1 in family EG-4) showed congenital eventration of the diaphragm on chest x-ray, indicating diaphragmatic muscle weakness. One (from family EG-3) also had congenital pulmonary hypoplasia.

We found a region with significant linkage to chromosome 2q37 (multipoint parametric LOD score 3.26) in family 1, described elsewhere by Rajab et al.² Subsequent microsatellite analyses revealed a LOD score of 3.51. We confirmed that region in another consanguineous family, EG-1, with two affected and eight unaffected offspring (LOD = 3.25). Fine mapping and haplotyping narrowed

Table 2. Clinical Features in Patients with Escobar Syndrome

Characteristic	Findings for Escobar Syndrome by Individual ^a												
	EG-1 II-3	EG-1 II-5	EG-2 V-1	EG-2 V-2	EG-3 II-1	EG-4 II-1	EG-4 II-2	EG-5 V-1	EG-5 V-4	EG-6 IV-3	EG-6 IV-5	EG-7 II-1	
Sex	F	M	F	M	F	M	M	M	F	F	F	F	
Current age ^b	12	5	21	16	Died at age 3 mo	Died at age 3 mo	18	14	7	Died at age 13 d	7	Stillborn	
Congenital problems:													
Decreased movements	NA	NA	NA	+	+	+	+	+	–	–	+	Stillborn	
Facial weakness	+	+	–	–	+	NA	+	–	–	NA	+	Stillborn	
Respiratory distress	+	+	–	–	+	+	–	–	–	–	–	Stillborn	
Dysmorphic signs:													
Arthrogryposis multiplex	+	+	+	+	+	+	+	+	+	+	+	+	
Pterygia	+	+	+	+	+	+	+	+	+	NA	+	+	
Short stature	+	+	+	+	+	NA	+	+	+	NA	+	NA	
Kyphosis/scoliosis	+	+	+	+	–	+	+	–	–	NA	+	NA	
Faciocranial dysmorphism	+	+	+	+	+	NA	+	+	+	+	+	NA	
High-arched palate	+	+	+	+	+	NA	+	+	+	NA	+	NA	
Cleft palate	–	–	–	–	–	NA	–	–	–	–	+	NA	
Arachnodactyly	+	+	+	+	–	+	+	+	+	+	+	NA	
Cryptorchism	...	+	...	+	...	+	+	+	
Postnatal myasthenic features:													
Clinical myasthenic symptoms	–	–	–	–	–	NA	–	–	–	NA	–	Stillborn	
Myasthenic signs on EMG	–	–	–	NA	NA	NA	–	NA	NA	NA	–	Stillborn	

NOTE.—The first sign diagnosed by ultrasound or reported by mothers is reduced fetal movement. At birth, the children come to medical attention because of variable joint contractures, multiple pterygia, and facial dysmorphism with long face, high-arched palate, small mouth, and retrognathism. Respiratory distress is a frequent life-threatening complication. Later in life, patients frequently are affected by reduced muscular mass but do not show myasthenic symptoms and have normal EMG, except for unspecific indication of chronic myopathy. The phenotype can vary between mutations and also within one class of mutations. NA = not available.

^a EG = Escobar syndrome by *CHRNA3* mutation.

^b In years, unless otherwise specified.



Figure 3. Clinical phenotype of patients with Escobar syndrome. The consistent major signs of Escobar syndrome are multiple contractures (arthrogryposis) and multiple pterygia. We show hand contractures (A), rocker-bottom feet with prominent heels (B), and an elbow web with muscular atrophy (C). The phenotype is variable, as shown for two patients from different families. Patient IV-5, from family EG-6, with homozygous mutation γ R217C is more severely affected (D) than patient V-1, from family EG-5, with homozygous mutation γ 1249G→C (E). The general appearance—with elongated face, small mouth with downturned corners, mild ptosis, down-slanting palpebral fissures, multiple pterygia, and muscular hypotrophy—is present in both patients. Scoliosis is severe in one patient (D) and absent in the other (E).

the region to a 10-cM interval between markers *D2S172* and *D2S336* (235.07–245.44 cM, according to Marshfield genetic maps).

Within that region resides the gene encoding the fetally expressed γ subunit of the AChR. Results of mutation screening are given in figure 4 and table 1. We identified three nonsense (γ Q18X, γ W139X, and γ R448X), one putative splice-site (γ 1249G→C), and one frameshift (γ 807insT) mutation in *CHRNA3*. These mutations are pre-

dicted to truncate major parts of the γ subunit or to result in premature mRNA nonsense-mediated decay. Mutations γ R64C, γ R217C, and γ 78dup(3) affect evolutionary highly conserved residues, indicating functional relevance (fig. 5). The mutations cosegregated with the phenotype in the families and were not found in 292 control chromosomes, thus making a polymorphism unlikely. In two other families, including the family of the initial genomewide scan, we did not identify mutations, either within the coding

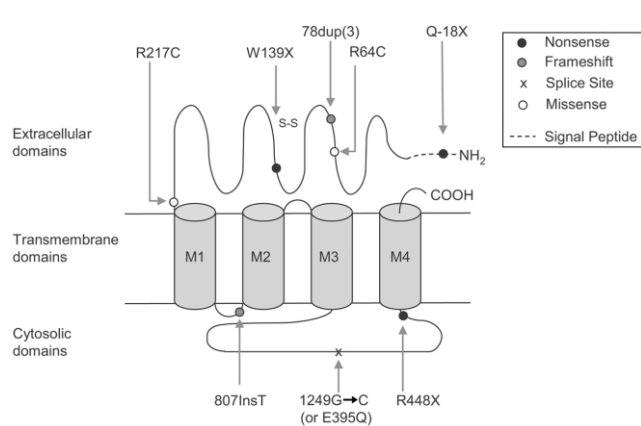


Figure 4. Schematic structure of the AChR γ subunit and localization of the identified mutations. The γ subunit and the other subunits contain an extracellular large N-terminus where acetylcholine binds and drugs and toxins dock (nicotine, muscle relaxants, *D*-tubocurarine, and α -bungarotoxin). Then, four transmembrane domains (M1–M4) follow with a large cytoplasmic domain (CD2) between M3 and M4 and an extracellular short C-terminus.^{21,22} Subunit structure was adapted from the work of Engel and Sine.⁹ The γ subunit consists of 517 aa, starting with the start methionine. Residues were counted, in accordance with traditional nomenclature, from the first amino acid following the signal peptide. S-S marks an important cysteine loop.

part and introns of *CHRNA1* or in the 5' and 3' UTRs. We assume that, for these families, the disease-causing mutation likely affects a regulatory element farther apart from the 5' and 3' end of *CHRNA1*, is due to a cytogenetic rearrangement affecting *CHRNA1* expression, or is due to a mutation in another gene within the linked region. We excluded the coding part of *CHRNA1* that is located within the linked region.

We transfected HEK cells with expression vectors for α , β , γ , and δ subunits that resembled the wild-type situation in the developing fetus. This resulted in regular AChR assembly and positioning at the cellular surface (fig. 6A). In contrast, without the γ subunit-expression vector, the complex made by all other subunits did not move to the cell surface, since there was no cell surface bungarotoxin-staining (fig. 6J). This state of affairs corresponds to the γ Q-18X mutation. Introduction of the mutations γ 78dup(3), γ R217C, or γ R448X into the γ -expression vector also prevented AChR positioning at the cellular surface (fig. 6C, 6E, and 6G, respectively). The muscle-type nicotinic AChR subunits assemble into functional $\alpha_2\beta\gamma\delta$ pentamers in the endoplasmic reticulum (ER) through a series of folding and oligomerization events.²¹ Assembly intermediates can be detected in the ER of transfected cells as soon as one of the bungarotoxin-binding sites forms—for example, α - δ dimers. This is the case also in cells transfected with wild-type α , β , δ , and mutant γ subunits, where a staining of the ER by bungarotoxin was achieved when cells were permeabilized (fig. 6D, 6F, 6H, and 6J). In sum-

mary, we see in the tested γ mutations that there is a partial subunit assembly in the ER but no AChR expression at the cellular surface.

To illustrate the AChR expression at a time putatively sensitive to the development of arthrogryposis in embryogenesis, we studied the expression of the γ and ε subunits in mouse embryos at E14, in limb sections at E15, and in limb muscle sections at postnatal days P1, P4, and P7. As expected, the ε subunit was not expressed before birth (data not shown). At E14, robust γ expression was observed in limb, paravertebral, facial, intercostal, and neck musculature and in the diaphragm (fig. 7A). Individual muscle-fiber staining is shown for E15 (fig. 7B and 7C). No expression was detected in the heart or the intestine. Postnatally, the expression of the γ subunit was strong and focal on P1 (fig. 7D) and P4 (not shown) but decreased by P7 (fig. 7E). The ε subunit was not visible on P1 (fig. 7F), was visible on P4, and was robustly expressed on P7 (fig. 7G), supporting earlier data.¹¹

Discussion

Mutations in the genes encoding the α (*CHRNA1*), β (*CHRNA1*), δ (*CHRND*), and ε (*CHRNE*) AChR subunits cause congenital myasthenic syndrome.⁹ In contrast, patients with Escobar syndrome have normal muscle tone and no myasthenic symptoms later in life. We present compelling evidence that *CHRNA1* mutations are responsible for the arthrogryposis multiplex congenita observed in Escobar syndrome. The mutations result in an impaired, truncated, or absent γ subunit. We show that fetal AChR cannot be expressed on the surface of HEK cells when the γ subunit is mutated or missing. We also demonstrate that the γ subunit is expressed at sites and at a time in fetal life corresponding to the development of the clinical phenotype. Impaired γ -subunit structure results in improper AChR function in fetal life, with reduced prenatal muscle strength and movement. This explains dysmorphic consequences such as contractures, pterygia, high-arched palate, and cryptorchism in male. Since the γ subunit generally disappears in late fetal development and is replaced by the ε subunit, the patients exhibit almost no progression and no signs of myasthenia after birth.

The five AChR subunits have to be fully assembled in the ER before the receptor is inserted into the membrane.^{21,25} If one subunit is missing, the receptor cannot reach the cell surface, which results in AChR deficiency, as shown for nonsense and some missense mutations in the ε subunit.⁹ The γ nonsense mutations described here are expected to have similar effects. This hypothesis is supported by our in vitro experiments showing lack of fetal AChR at the cell surface in the absence of the γ subunit. The expression studies in transfected HEK cells indicate that the mutations γ 78dup(3), γ R217C, and γ R448X lead to AChR deficiency at the cell surface as well. The pathogenic effect of these mutations results from either

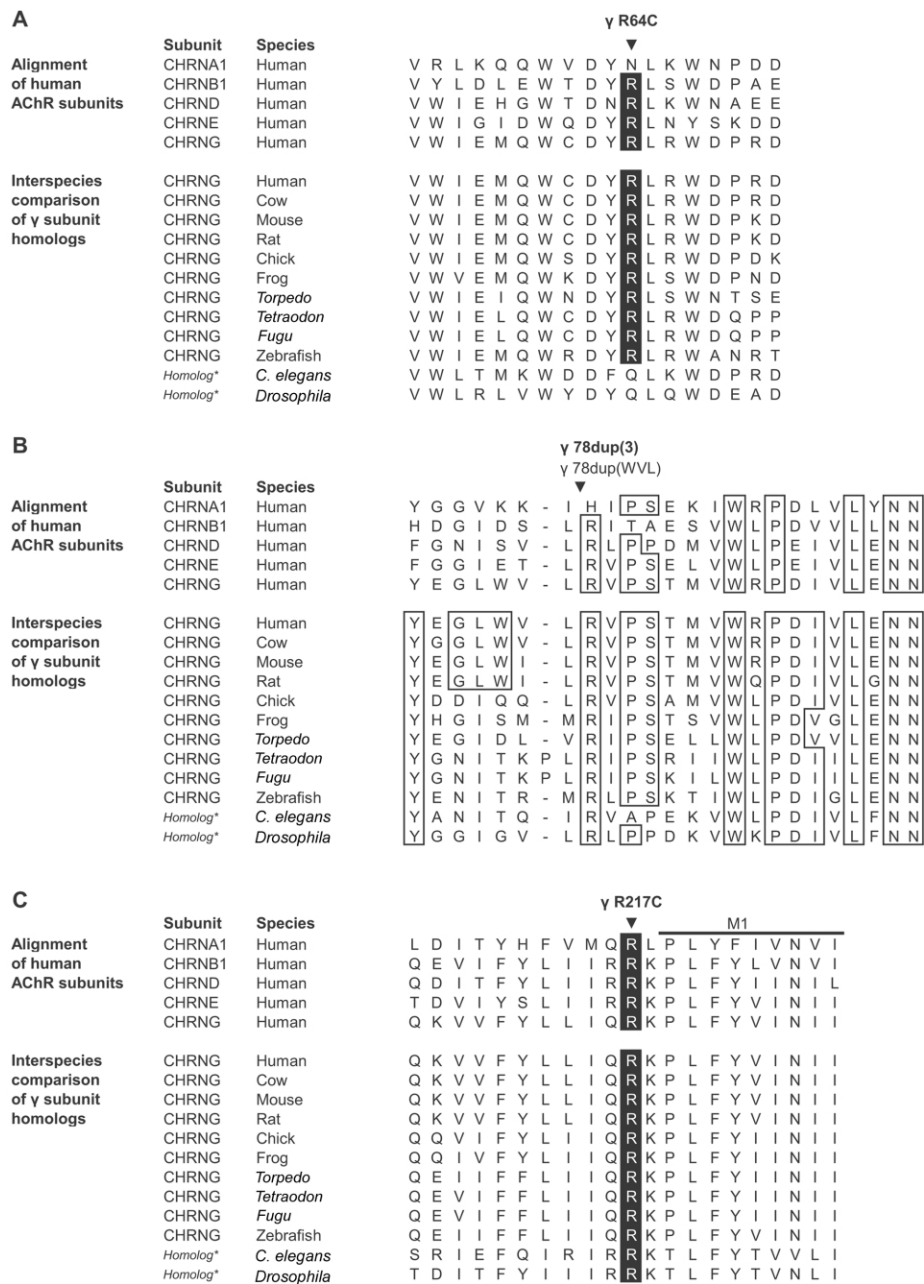


Figure 5. Evolutionary conservation of residues relevant for identified missense and duplication mutations. *A*, Evolutionary conservation of residue γ R64 relevant for mutation γ R64C. The strongly basic residue arginine R is conserved in human β , δ , γ , and ϵ subunits, as well as among vertebrate γ subunits. Furthermore, insertion of an additional cysteine residue instead of arginine might interfere with the cysteine loops relevant in and between AChR subunits. *B*, Evolutionary conservation of residues relevant for mutation γ 78dup(3). The duplication of residues WVL is located before an R that is evolutionarily completely conserved among γ subunits of all species analyzed and close to a conserved W_PDI_L-motif. The analogous position in the ϵ subunit ϵ L78 was shown to locate at the interface of α and ϵ subunits in snail AChR and seems to provide a hydrogen bond between the β sheets involved in acetylcholine binding.^{23,24} *C*, Evolutionary conservation of residue γ R217 relevant for mutation γ R217C. Residue γ R217 is completely conserved in all human subunits as well as within all species analyzed. Furthermore, insertion of an additional cysteine residue instead of arginine might interfere with the cysteine loops relevant in and between AChR subunits. The line marked "M1" depicts the beginning of the transmembrane domain M1. GenBank accession numbers for alignment of human AChR subunits are as follows: NP_000070.1 (CHRNA1), NP_000738.2 (CHRNB1), NP_000742.1 (CHRND), NP_000071.1 (CHRNE), and NP_005190.4 (CHRNA1). GenBank accession numbers for interspecies comparison of γ subunit homologs are as follows: NP_005190.4 (*Homo sapiens*), P13536 (*Bos taurus*), P04760 (*Mus musculus*), P18916 (*Rattus norvegicus*), P02713 (*Gallus gallus*), P05376 (*Xenopus laevis*), P02714 (*Torpedo californica*), Q4RV81 (*Tetraodon nigroviridis*), Q7T2Y7 (*Fugu rubripes*), F09E8.7* (*Caenorhabditis elegans*), and P04755* (*Drosophila melanogaster*). The Ensembl number is ENSDART00000028118** (*Danio rerio*). (Those in the figure marked with an asterisk [*] are the closest homologs to the human CHRNA1 found in *D. melanogaster* and *C. elegans*, respectively.)

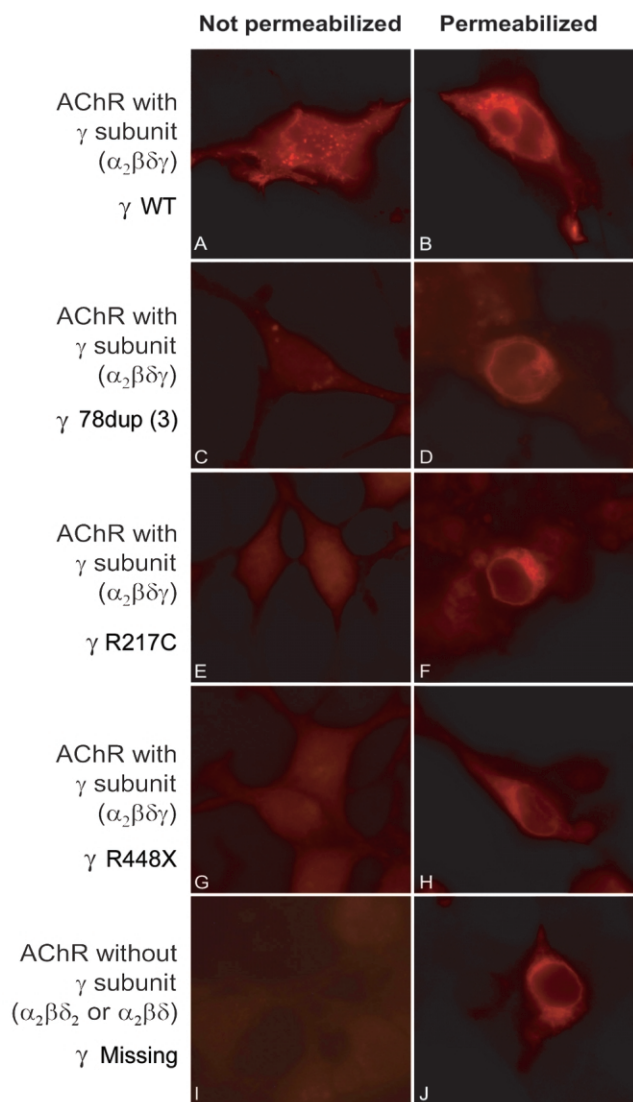


Figure 6. AChR expression. Altered or missing γ subunit prevents surface expression of fetal AChRs in HEK-cell studies. Transfection with the α , β , γ , and δ subunits results in regular assembly and positioning of the fetal AChR at the cellular surface (A). No AChR surface expression is seen when the γ transfection vector carries mutations γ 78dup(3) (C), γ R217C (E), or γ R448X (G) or is completely missing (I). There might be a partial AChR subunit assembly within the cell, since after-permeabilization bungarotoxin stains the ER (D, F, H, and J). (Receptors were visualized with bungarotoxin staining.)

severely reduced expression levels of the mutated γ subunits or from impaired AChR assembly or transport.

Although all patients consistently showed arthrogryposis multiplex and multiple pterygia, severity of the clinical phenotype varied significantly among and within families. For example, homozygous mutation γ R217C was associated with an affected stillbirth in family EG-7 as well as with neonatal death and a viable though severe phenotype in two patients from family EG-6. We also suspect

that this homozygous mutation caused the history of abortions in these families. In family EG-4, one affected boy died at age 3 mo, and his 18-year-old brother is severely affected. Therefore, not only the status of the mutations but also genetic background and interindividual differences at the time of the γ - ϵ switch might be of relevance.

In situ hybridization at mouse E14 showed that the γ subunit is expressed in skeletal muscles of the limbs, head, paravertebral trunk, and in the diaphragm. This pattern corresponds to the major sites of human disease. Postnatally, γ expression decreased along with a reciprocal increase in the ϵ subunit, indicating that the disease is mainly relevant for intrauterine neuromuscular development. We conclude that the observed postnatal symptoms—namely arthrogryposis, pterygia, and scoliosis—are a result of improper AChR function in utero. There is little or no progression of the disease after birth, although the

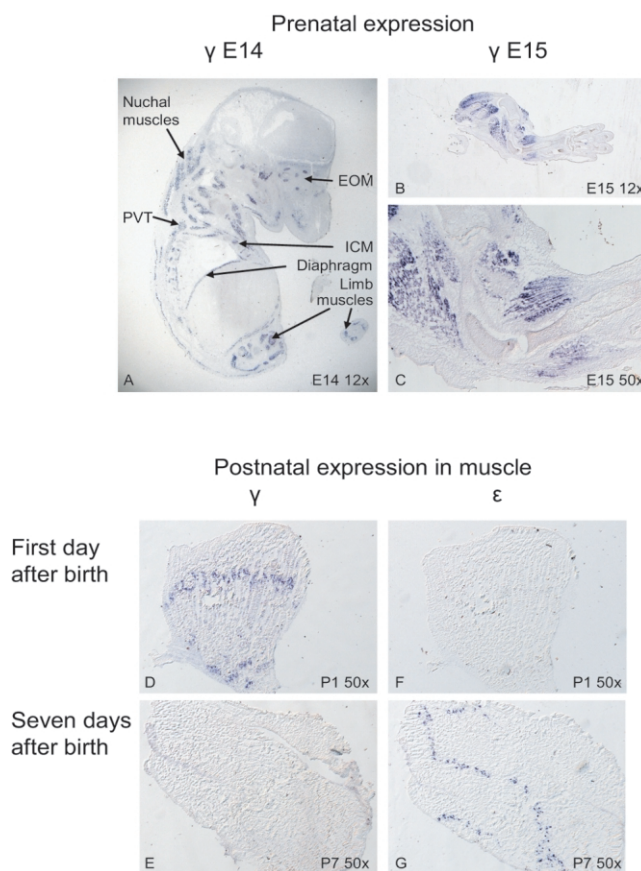


Figure 7. In situ hybridization. In situ hybridization at mouse E14.5 shows a significant expression of the γ subunit in skeletal muscles of the limbs, neck, and head, paravertebrally as well as in the diaphragm (A–C). This corresponds to the major sites of the phenotype observed in human Escobar syndrome. Postnatally, the γ subunit expression decreases (D and E), with reciprocal increase of the ϵ subunit (F and G). EOM = Extraocular muscles; ICM = intercostal muscles; PVT = paravertebral trunk.

deformities may nevertheless lead to lethal complications. This phenomenon explains the lack of myasthenia in patients with γ mutations after birth. Accordingly, lack of the γ subunit in mice caused muscular weakness, hindlimb paralysis, feeding problems, and stillbirth and death within 48 h because of respiratory failure.¹⁵ Homozygous ϵ -knockout mice showed normal development in prenatal and early neonatal life but later developed progressive impairment of neuromuscular transmission with muscular weakness and death around age 2–3 mo.^{26,27} The late onset and rather mild phenotype are due to inadequate but partly compensatory γ expression postnatally.²⁷ In contrast, no compensatory increase of ϵ expression was observed in mice lacking the γ subunit, resulting in a near absence of AChR in muscle fibers.¹⁵

We found that individuals with *CHRNA1* nonsense mutations can survive, although an increased frequency of stillbirth and abortions is noted in their families. In contrast to the postnatal switch in mice, expression of the ϵ subunit starts already prenatally at ~7 mo of pregnancy in the bovine diaphragm and ~33 wk of gestation in humans.^{13,10} The subunit switch in early postpartal time in mice and in late gestation in humans indicates that mice might be born with fetal AChR, whereas humans and cows already have adult AChR. A prenatal start of ϵ expression, especially in respiratory muscles, might be the life-saving difference.

Antibodies in myasthenia gravis could be directed against any subunit. However, there is some evidence that women whose babies are affected by maternal AChR antibody-induced myasthenia have higher titers of antibodies and a higher proportion that bind to fetal AChR epitopes.⁸ These women can be asymptomatic, but their children are affected with severe arthrogryposis and hypotonia.^{28–32} Almost all features—such as decreased fetal and congenital movements, arthrogryposis, facial weakness, respiratory distress, pulmonary hypoplasia, kyphosis/scoliosis, intrauterine growth retardation, faciocranial dysmorphism, low-set ears, high-arched palate, and cryptorchidism—we have seen in our patients were also reported in stillbirths or newborns after intrauterine exposure to fetal AChR antibodies.^{29–32} Pathogenicity of these antibodies was also demonstrated by exposing rodents to human maternal AChR antibodies. Most rodent pups showed multiple contractures, craniofacial dysmorphism, and multiple pterygia, similar to the human phenotype.⁸ Fetal exposure to D-tubocurarine blocks fetal AChR and results in arthrogryposis multiplex congenita and pterygia.^{4,33–35} Taken together, features seen in our patients with *CHRNA1* mutations are mimicked by drug or antibody inhibition of the fetal AChR.

In our patients, *CHRNA1* mutations are frequently associated with congenital diaphragmatic muscle weakness, pulmonary hypoplasia, or respiratory distress. This might suggest that γ -subunit alterations can lead to respiratory dysfunction, probably by both decreased muscle movement and disturbed lung development. This is supported

by a case report in which intrauterine exposure to fetal AChR antibodies led to pulmonary hypoplasia and malsegmentation of pulmonary lobes.³²

We identified functional null mutations in the γ subunit of AChR in Escobar syndrome that presumably result in the inability to assemble the receptor and thus the downregulation of the entire fetal AChR complex. We conclude that Escobar syndrome is an inherited fetal myasthenic disease that also affects neuromuscular organogenesis. Because γ expression is restricted to early development, patients have no myasthenic symptoms later in life, in contrast to those with mutations in other AChR subunits. Our findings are of relevance not only for the rare inherited Escobar syndrome but also for other fetal akinesia/hypokinesia sequences, congenital myasthenic syndrome, and myasthenia gravis. They also support the pathogenetic views of arthrogryposis and pterygia in general and the complex regulation and function of AChR subunits during and after development. We think that Escobar syndrome elucidates the missing link among inherited AChR-subunit disorders and appears to be a phenocopy of the most severe form of neonatal myasthenia gravis.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Ensembl, <http://www.ensembl.org/index.html> (for *Danio rerio* prediction [accession number ENSDART0000028118])
GenBank, <http://www.ncbi.nih.gov/Genbank/> (for *Homo sapiens* AChR subunits CHRNA1 [accession number NP_000070.1], CHRNB1 [accession number NP_000738.2], CHRND [accession number NP_000742.1], and CHRNE [accession number NP_000071.1], CHRNG [accession number NP_005190.4] and interspecies comparison of γ subunit homologs *H. sapiens* [accession number NP_005190.4], *Bos taurus* [accession number P13536], *Mus musculus* [accession number P04760], *Rattus norvegicus* [accession number P18916], *Gallus gallus* [accession number P02713], *Xenopus laevis* [accession number P05376], *Torpedo californica* [accession number P02714], *Tetraodon nigroviridis* [accession number Q4RV81], *Fugu rubripes* [accession number Q7T2Y7], *C. elegans* [accession number F09E8.7], and *D. melanogaster* [accession number P04755])
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi>

.nlm.nih.gov/Omim/ (for Escobar or multiple pterygia syndrome, MG, congenital myasthenic syndrome, *CHRNA1*, *CHRN1*, *CHRN2*, *CHRN3*, and *CHRN4*)

References

1. Escobar V, Bixler D, Gleiser S, Weaver DD, Gibbs T (1978) Multiple pterygium syndrome. *Am J Dis Child* 132:609–611
2. Rajab A, Hoffmann K, Ganesh A, Sethu AU, Mundlos S (2005) Escobar variant with pursed mouth, creased tongue, ophthalmologic features, and scoliosis in 6 children from Oman. *Am J Med Genet A* 134:151–157
3. Hammond E, Donnenfeld AE (1995) Fetal akinesia. *Obstet Gynecol Surv* 50:240–249
4. Drachman DB, Coulombre AJ (1962) Experimental clubfoot and arthrogryposis multiplex congenita. *Lancet* 2:523–526
5. Murray PD, Drachman DB (1969) The role of movement in the development of joints and related structures: the head and neck in the chick embryo. *J Embryol Exp Morphol* 22:349–371
6. Hall JG (1997) Arthrogryposis multiplex congenita: etiology, genetics, classification, diagnostic approach, and general aspects. *J Pediatr Orthop B* 6:159–166
7. Patrick J, Lindstrom J (1973) Autoimmune response to acetylcholine receptor. *Science* 180:871–872
8. Polizzi A, Huson SM, Vincent A (2000) Teratogen update: maternal myasthenia gravis as a cause of congenital arthrogryposis. *Teratology* 62:332–341
9. Engel AG, Sine SM (2005) Current understanding of congenital myasthenic syndromes. *Curr Opin Pharmacol* 5:308–321
10. Hesselmans LF, Jennekens FG, Van den Oord CJ, Veldman H, Vincent A (1993) Development of innervation of skeletal muscle fibers in man: relation to acetylcholine receptors. *Anat Rec* 236:553–562
11. Missias AC, Chu GC, Klocke BJ, Sanes JR, Merlie JP (1996) Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR γ -to- ϵ switch. *Dev Biol* 179:223–238
12. Yumoto N, Wakatsuki S, Sehara-Fujisawa A (2005) The acetylcholine receptor γ -to- ϵ switch occurs in individual endplates. *Biochem Biophys Res Commun* 331:1522–1527
13. Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature* 321:406–411
14. Koenen M, Peter C, Villarroel A, Witzemann V, Sakmann B (2005) Acetylcholine receptor channel subtype directs the innervation pattern of skeletal muscle. *EMBO Rep* 6:570–576
15. Takahashi M, Kubo T, Mizoguchi A, Carlson CG, Endo K, Ohnishi K (2002) Spontaneous muscle action potentials fail to develop without fetal-type acetylcholine receptors. *EMBO Rep* 3:674–681
16. Gudbjartsson DF, Jonasson K, Frigge ML, Kong A (2000) Allegro, a new computer program for multipoint linkage analysis. *Nat Genet* 25:12–13
17. Kruglyak L, Daly MJ, Lander ES (1995) Rapid multipoint linkage analysis of recessive traits in nuclear families, including homozygosity mapping. *Am J Hum Genet* 56:519–527
18. Hoffmann K, Lindner TH (2005) easyLINKAGE-Plus—automated linkage analyses using large-scale SNP data. *Bioinformatics* 21:3565–3567
19. Hoffmann K, Dreger CK, Olins AL, Olins DE, Shultz LD, Lucke B, Karl H, Kaps R, Muller D, Vaya A, Aznar J, Ware RE, Sotelo Cruz N, Lindner TH, Herrmann H, Reis A, Sperling K (2002) Mutations in the gene encoding the lamin B receptor produce an altered nuclear morphology in granulocytes (Pelger-Huet anomaly). *Nat Genet* 31:410–414
20. Visel A, Thaller C, Eichele G (2004) GenePaint.org: an atlas of gene expression patterns in the mouse embryo. *Nucleic Acids Res* 32:D552–D556
21. Wanamaker CP, Christianson JC, Green WN (2003) Regulation of nicotinic acetylcholine receptor assembly. *Ann N Y Acad Sci* 998:66–80
22. Shen XM, Ohno K, Sine SM, Engel AG (2005) Subunit-specific contribution to agonist binding and channel gating revealed by inherited mutation in muscle acetylcholine receptor M3-M4 linker. *Brain* 128:345–355
23. Beeson D, Webster R, Ealing J, Croxson R, Brownlow S, Brydson M, Newsom-Davis J, Slater C, Hatton C, Shelley C, Colquhoun D, Vincent A (2003) Structural abnormalities of the AChR caused by mutations underlying congenital myasthenic syndromes. *Ann N Y Acad Sci* 998:114–124
24. Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* 411:269–276
25. Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci* 2:791–805
26. Witzemann V, Schwarz H, Koenen M, Berberich C, Villarroel A, Wernig A, Brenner HR, Sakmann B (1996) Acetylcholine receptor ϵ -subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. *Proc Natl Acad Sci USA* 93:13286–13291
27. Missias AC, Mudd J, Cunningham JM, Steinbach JH, Merlie JP, Sanes JR (1997) Deficient development and maintenance of postsynaptic specializations in mutant mice lacking an “adult” acetylcholine receptor subunit. *Development* 124:5075–5086
28. Vernet-der Garabedian B, Lacokova M, Eymard B, Morel E, Faltin M, Zajac J, Sadovsky O, Dommergues M, Tripon P, Bach JF (1994) Association of neonatal myasthenia gravis with antibodies against the fetal acetylcholine receptor. *J Clin Invest* 94:555–559
29. Cantagrel S, Maury L, Yamamoto AM, Maheut J, Toutain A, Castelnau P (2002) Akinesia, arthrogryposis, craniosynostosis: a presentation of neonatal myasthenia with fetal onset. *Am J Perinatol* 19:297–301
30. Vincent A, Newland C, Brueton L, Beeson D, Riemersma S, Huson SM, Newsom-Davis J (1995) Arthrogryposis multiplex congenita with maternal autoantibodies specific for a fetal antigen. *Lancet* 346:24–25
31. Brueton LA, Huson SM, Cox PM, Shirley I, Thompson EM, Barnes PR, Price J, Newsom-Davis J, Vincent A (2000) Asymptomatic maternal myasthenia as a cause of the Pena-Shokeir phenotype. *Am J Med Genet* 92:1–6
32. Barnes PR, Kanabar DJ, Brueton L, Newsom-Davis J, Huson SM, Mann NP, Hilton-Jones D (1995) Recurrent congenital arthrogryposis leading to a diagnosis of myasthenia gravis in an initially asymptomatic mother. *Neuromuscul Disord* 5:59–65
33. Kopta C, Steinbach JH (1994) Comparison of mammalian adult and fetal nicotinic acetylcholine receptors stably expressed in fibroblasts. *J Neurosci* 14:3922–3933
34. Jago RH (1970) Arthrogryposis following treatment of maternal tetanus with muscle relaxants. *Arch Dis Child* 45:277–279
35. Moessinger AC (1983) Fetal akinesia deformation sequence: an animal model. *Pediatrics* 72:857–863