Acetylcholine Receptor Pathway Mutations Explain Various Fetal Akinesia Deformation Sequence Disorders

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Impaired fetal movement causes malformations, summarized as fetal akinesia deformation sequence (FADS), and is triggered by environmental and genetic factors. Acetylcholine receptor (AChR) components are suspects because mutations in the fetally expressed γ subunit (*CHRNG*) of AChR were found in two FADS disorders, lethal multiple pterygium syndrome (LMPS) and Escobar syndrome. Other AChR subunits α1, β1, and δ (*CHRNA1, CHRNB1, CHRND*) as well as receptor-associated protein of the synapse (*RAPSN*) previously revealed missense or compound nonsense-missense mutations in viable congenital myasthenic syndrome; lethality of homozygous null mutations was predicted but never shown. We provide the first report to our knowledge of homozygous nonsense mutations in *CHRNA1* and *CHRND* and show that they were lethal, whereas novel recessive missense mutations in *RAPSN* caused a severe but not necessarily lethal phenotype. To elucidate disease-associated malformations such as frequent abortions, fetal edema, cystic hygroma, or cardiac defects, we studied *Chrna1*, *Chrnb1*, *Chrnd*, *Chrng*, and *Rapsn* in mouse embryos and found expression in skeletal muscles but also in early somite development. This indicates that early developmental defects might be due to somite expression in addition to solely muscle-specific effects. We conclude that complete or severe functional disruption of fetal AChR causes lethal multiple pterygium syndrome whereas milder alterations result in fetal hypokinesia with inborn contractures or a myasthenic syndrome later in life.

Introduction

Fetal movement is a precondition for normal fetal development and growth. Depending on severity, intrauterine movement restriction causes growth retardation, fetal hydrops, polyhydramnios, pulmonary hypoplasia, multiple joint contractures with or without webbing (pterygia), and other features, giving rise to the term fetal akinesia deformation sequence (FADS [MIM 208150]).[1] Fetal akinesia occurs in many genetic and acquired disorders. Examples of environmental causes are curare exposure and circulating maternal antibodies against the fetal acetylcholine receptor.[2–4] Restricted intrauterine space in twin pregnancies or oligohydramnios also increases susceptibility. Genetic entities include trisomy 18, myotonic dystrophies, central core myopathy, and synaptopathies.[5–9]

Synaptopathies are diseases of the neuromuscular junction. Key molecules necessary for neuromuscular signal transduction include presynaptic acetylcholine transferase (CAT), acetylcholine esterase (AChE) in the synapse, and postsynaptic muscular nicotinic acetylcholine receptor (AChR). During fetal development, AChR consists of two α1, one β1, one δ, and one γ subunit, whereas after 33 weeks gestation, the γ subunit is replaced by an ε subunit (CHRNA1 [MIM 100690], CHRNB1 [MIM 100710], CHRND [MIM 100720], CHRNG [MIM 100730], CHRNE [MIM 100725]).[10] For neuromuscular signal transduction, all subunits must be functional. In addition, the AChR must be correctly assembled, clustered, anchored, activated, and linked. This complex process requires many contributing proteins such as agrin (AGRN [MIM 103320]), muscle skeletal tyrosine kinase (MUSK [MIM 601296]), the muscle-intrinsic activator of MUSK named DOK7 (downstream of tyrosine kinase 7 DOK7 [MIM 610285]), and the receptor-associated protein, rapsyn (RAPSN [MIM 601592]), that is involved in AChR assembly and localization to the cell membrane (Figure 1).[11]

Genetic disorders of the postsynaptic complex present as recessively or dominantly inherited myasthenic syndromes with variable age of onset and clinical severity.[8,9,16] Mutations in the acetylcholine receptor γ subunit cause two prenatal myasthenic syndromes belonging to the

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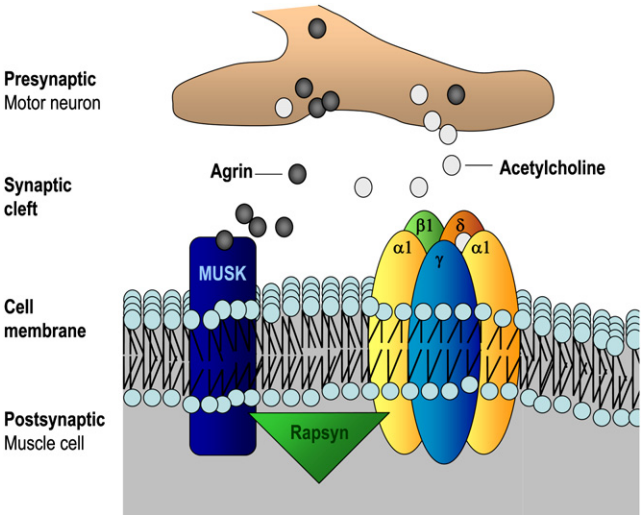

Figure 1. Schematic View of AChR Complex

AChR at the postsynaptic membrane in muscle cells consist of 5 subunits.[11] Two α1, one β1, one δ subunit are always present. The fifth subunit is the fetally expressed γ subunit. By around 33 weeks of gestation in humans, γ subunit expression is stopped switching to ε, thereby replacing fetal-type AChR by adult-type AChR.[10] Rapsyn is a cytoskeletal membrane protein colocalizing with AChR. Functionally, rapsyn is considered to transduce signals from the agrin-activated MUSK. Not shown here, rapsyn connects the receptor with the cytoskeletal dystrophin-glycoprotein-complex (DGC) and stabilizes AChR clusters by interaction with calpain.[12–15]

FADS spectrum, lethal multiple pterygium syndrome (LMPS [MIM 253290]) and Escobar syndrome, a severe condition with inborn contractures, pterygia, and respiratory distress (MIM 265000).[8,9] The AChR γ subunit is expressed in fetal development and is replaced by an ε subunit in late gestation.[10] The fetal *CHRNG* expression explains the severe intrauterine phenotype with contractures, pterygia, and increased intrauterine lethality. The physiological substitution of the γ by the ε subunit explains the absence of myasthenic symptoms after birth. This γ-ε switch is exceptional because the other AChR subunits α1, β1, and δ do not have a substitute and remain throughout fetal and adult life. Mutations in *CHRNA1*, *CHRNB1*, *CHRND*, and *RAPSN* cause congenital myasthenic syndromes with muscular hypotonia or increased fatigue later in life.[16] The previously published patients either had dominant myasthenic syndrome, recessive missense alterations, or compound genotypes of nonsense-missense mutations in *CHRNA1*, *CHRNB1*, or *CHRND*.[16] Lethality of homozygous null mutations in nonsubstituted AChR subunits was predicted but never proven.[16]

We analyzed another 63 patients with lethal multiple pterygium syndrome, Escobar syndrome, or other severe forms of FADS but found *CHRNG* mutations in only three of them. In the remaining 60 families, we suspected mutations in other AChR pathway genes. We therefore sequenced the genes for rapsyn and for the other fetally expressed AChR subunits α1, β1, and δ.

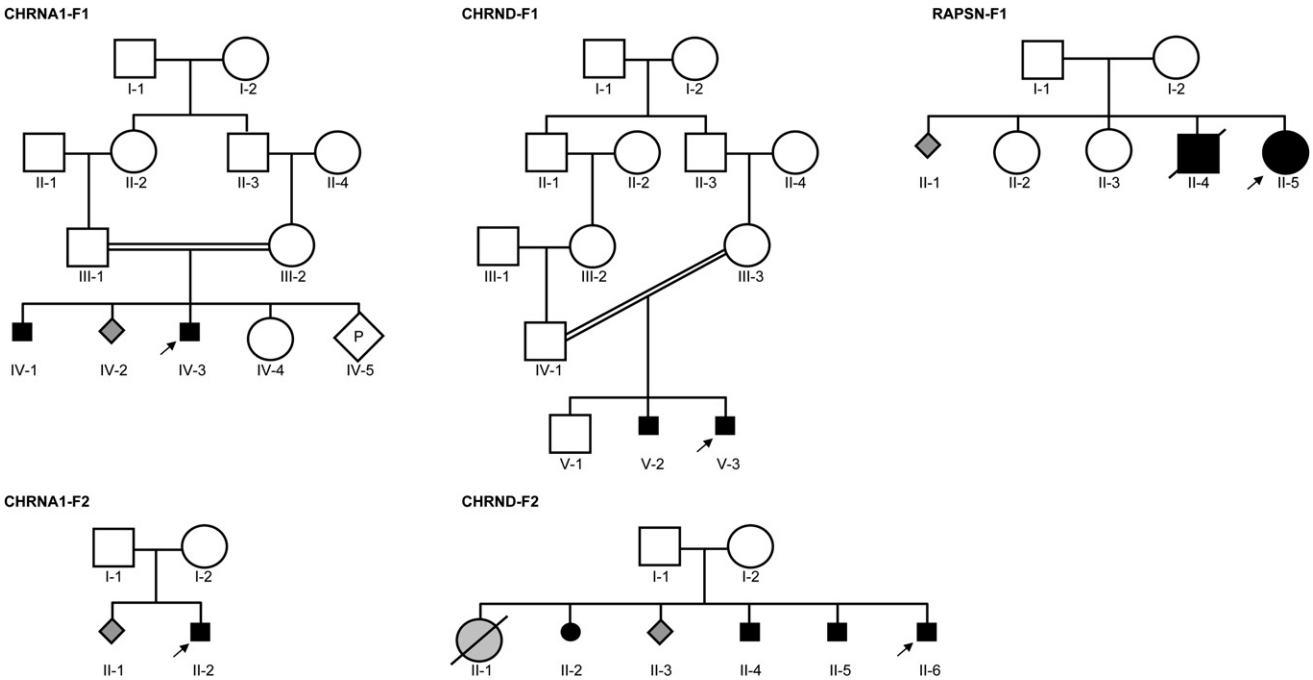

Figure 2. Pedigrees of Described Families with *CHRNA1*, *CHRND*, or *RAPSN* Mutations Indicate Recessive Inheritance and Increased Intrauterine Lethality

Two families with *CHRNA1* mutations, two families with *CHRND* mutations causing lethal multiple pterygium syndrome, and one family with recessive *RAPSN* mutations resulting in fetal akinesia syndrome with inborn contractures are shown. Arrow: index patient. Small symbols represent abortions, intrauterine death, or termination of pregnancy because of severe affection.

Table 1. Clinical Features in Patients with Severe Fetal Akinesia Sequence

Family ID	CHRNA1-F1		CHRNA1-F2		CHRND-F1		CHRND-F2					RAPSN-F1	
Family history of abortions	yes (IV-2)		−		−		yes (II-3)					yes (II-1)	
Patients, General Data													
Patient ID	CHRNA1-F1; IV-1	CHRNA1-F1; IV-3	CHRNA1-F2; II-1	CHRNA1-F2; II-2	CHRND-F1; V-2	CHRND-F1; V-3	CHRND-F2; II-1	CHRND-F2; II-2	CHRND-F2; II-4	CHRND-F2; II-5	CHRND-F2; II-6	RAPSN-F1; II-4	RAPSN-F1; II-5
Gender	M	M	?	M	M	M	F	F	M	M	M	M	F
Sample for mutation analysis	−	+	−	+	−	+	−	−	−	+	+	+	+
Duration of pregnancy	24 gw	20 gw	28 gw	17 gw	15 gw	15 gw	23 gw	19 gw	12 gw	13 gw	?	36 gw+6	38 gw+3
Current age	stillbirth	TP	stillbirth	TP	TP	TP	died 2 d	TP	TP	TP	TP	died 10 m	10 m
Intrauterine Problems													
Growth retardation	+	+	NA	+	+	+	NA	NA	NA	+	+	−	−
Edema/cystic hygroma	+/+	+/+	NA	+/+ (7 gw)	+/NA	+/+	+/NA	NA/+	NA/+	NA/+	+/+	−/−	−/−
Decreased movements	+	+	NA	+	+	+	NA	NA	+	+	NA	+	+
Joint contractures	+	+	NA	+	+	+	NA	+	+	+	+	+	+
Poly/oligohydramnion	−	−	NA	−	−	−	NA	NA	NA	NA	NA	−	−
Postnatal or Autopsy Findings													
Faciocranial dysmorphism	+	+	NA	+	NA	NA	NA	+	NA	NA	+	+	+
High arched palate	NA	NA	NA	−	NA	NA	NA	NA	NA	NA	NA	+	+
Cleft palate	+	+	NA	−	NA	NA	NA	NA	NA	NA	NA	−	+
Downslanting palpebral fissures	NA	NA	NA	+	NA	NA	NA	NA	NA	NA	+	+	+
Hypertelorism	NA	NA	NA	NA	NA	NA	NA	+	NA	NA	+	+	+
Depressed nasal bridge	NA	NA	NA	+	NA	NA	+	+	NA	NA	+	−	−
Micrognathia	+	NA	NA	+	NA	NA	NA	+	NA	+	+	+	+
Low-set ears	NA	NA	NA	+	NA	NA	+	+	NA	+	+	+	+
Reduced muscle bulk/hypoplasia	+	+	NA	NA	NA	+	NA	NA	NA	NA	NA	Hypotonia	Hypotonia
Scoliosis	+	+	NA	−	NA	+	NA	NA	NA	NA	NA	−	−
Contractures	+	+	NA	+	NA	+	NA	+	+	+	+	+	+
Pterygia	+	+	NA	+	NA	+	(generalized edema)	+	+	+	+	−	−
Other features		rocker bottom feet	infarction of placenta			pectus excavatum; broad ribs, claviculae, and Os metatarsale I	big atrial septal defect; lung hypoplasia; hydrothorax; ascites	rocker bottom feet; lung hypoplasia; hydrothorax; pericardial effusion; shortened ribs				respiratory problems; feeding problems; cryptorchism	respiratory problems; feeding problems; weak cry

Included are lethal multiple pterygium, Escobar-like syndrome, and fetal akinesia with inborn arthrogryposis. Data on prenatal ultrasound are available from some patients and consistently report growth retardation and contractures, detected as early as 12 weeks of gestation. Severe fetal akinesia, pterygia, generalized hydrops, and nuchal hygroma were found in patients from families CHRNA1-F1/F2 and CHRND-F1/F2, but not in family RAPSN-F1. Increased nuchal translucency or fetal hydrops were early diagnostic but unspecific sonographic signs toward the end of the first trimester. In second trimester, frank fetal hypo-/akinesia and multiple contractures were detectable. Early abortions and stillbirths were noted in families CHRNA1-F1/F2 and CHRND-F1/F2, indicating intrauterine lethality (Figure 2). Subsequent pregnancies with severely affected fetuses in these families were terminated. Micrognathia and low-set ears occurred in all patients. Cleft palate was reported in families CHRNA1-F1/F2 as well as in family RAPSN-F1 (submucous type); no information was available for families CHRND-F1/F2. Hypertelorism, downslanting palpebral fissures, and scoliosis were reported in various combinations, but it has not been consistently possible (especially in the prenatally deceased patients) to ascertain the presence or absence of these features. Autopsy and X-ray examination when performed did not reveal any vertebral anomalies. None of the affected fetuses from families CHRNA1-F1/F2 and CHRND-F1/F2 reached birth at term. Newborn II-5 from family RAPSN-F1 presented with respiratory distress, arthrogryposis multiplex congenita (AMC), proximal muscular hypotonia, and distal muscular hypertonia. The neonate did not grimace nor cry and required breathing assistance by constant positive airway pressure (CPAP). Because of persisting feeding difficulties and pronounced gastresophageal reflux, percutaneous endoscopic gastrostomy (PEG) feeding was started at the age of 7 months. Her affected brother II-4 (RAPSN-F1) had similar symptoms and died by 10 months resulting from respiratory insufficiency. None of the parents presented symptoms indicative for neuromuscular disease. Expert baseline neurological examinations were normal for parents from families CHRNA1-F1/F2, CHRND-F1, and RAPSN-F1 (not available for family CHRND-F2). However, an up to 60% decrement of the muscle action potential after repetitive stimulation with 5 Hz revealed subclinical myasthenia in the father of family RAPSN-F1 who is heterozygous for the novel *RAPSN* mutation c.416C→T. Abbreviations: F, female; M, male; NA, not available; gw, gestational week; m, month; d, day; TP, termination of pregnancy; indicates severely affected fetus.

We report the first homozygous nonsense mutations in the two nonsubstituted subunits α1 and δ that were indeed lethal. This shows that first trimester fetal akinesia with hydrops, contractures, pterygia, and a fatal prognosis can be due to recessive null mutations in *CHRNA1* and *CHRND* whereas novel recessive missense mutations in *RAPSN* caused isolated fetal hypokinesia and contractures without pterygia. We suggest that a spectrum of FADS phenotypes ranging from recurrent spontaneous abortions and lethal multiple pterygium syndrome (severe) to arthrogryposis multiplex congenita (mild) are prenatal myasthenic syndromes that warrant genetic and functional diagnostic investigations of the AChR pathway.

Material and Methods

We studied 63 families referred for molecular genetic analysis of FADS. Our ethics committee approved the study. Written, informed consent was obtained from all participants or their legal guardians.

We performed standard DNA sequencing.[17] We sequenced coding regions of *CHRNG* initially and found mutations in three of 63 families (data not shown). In the 60 remaining families, we analyzed coding regions of functional candidate genes *CHRNA1, CHRNB1, CHRND*, and *RAPSN*. Primer sequences are available on request. All newly identified mutations were tested for correct segregation in the patient's families and in 300 control chromosomes to exclude a previously undescribed polymorphism. Standard sequencing procedures do not exclude disease-contributing mutations in promoter regions, other regulatory elements, and exon spanning deletions or inversions of the sequenced genes *CHRNA1, CHRNB1, CHRND, CHRNG*, and *RAPSN*. However, the phenotypic severity at least of the lethal phenotypes suggests more pronounced effects than noncoding mutations would exercise by modifying gene expression.

In Situ Hybridization

We generated probes for the α1, β1, and δ subunits and for rapsyn by RT-PCR from mouse E14.5 whole cDNA and received two other probes for α1 and δ from Bernhard Hermann and Heiner Schrewe (MPI for Molecular Genetics, Berlin). Primer sequences are available on request. Antisense riboprobes were transcribed with SP6 or T7 polymerase with the Roche Dig-RNA labeling kit according to the manufacturer's instructions. Probes for AChR subunits γ and ε as well as protocols for whole-mount in situ hybridizations and in situ hybridizations on paraffin sections were previously described.[9,18,19]

Results

Pedigrees of five families with positive mutation analysis indicated recessive inheritance (Figure 2). Clinical characteristics of these patients with new mutations are summarized in Table 1. Prenatal ultrasound showed hypokinesia and growth retardation. Frequent features were joint contractures, cleft palate, and facial dysmorphism with low-set ears, hypertelorism, and micrognathia. All affected individuals had reduced muscle mass compared to normally

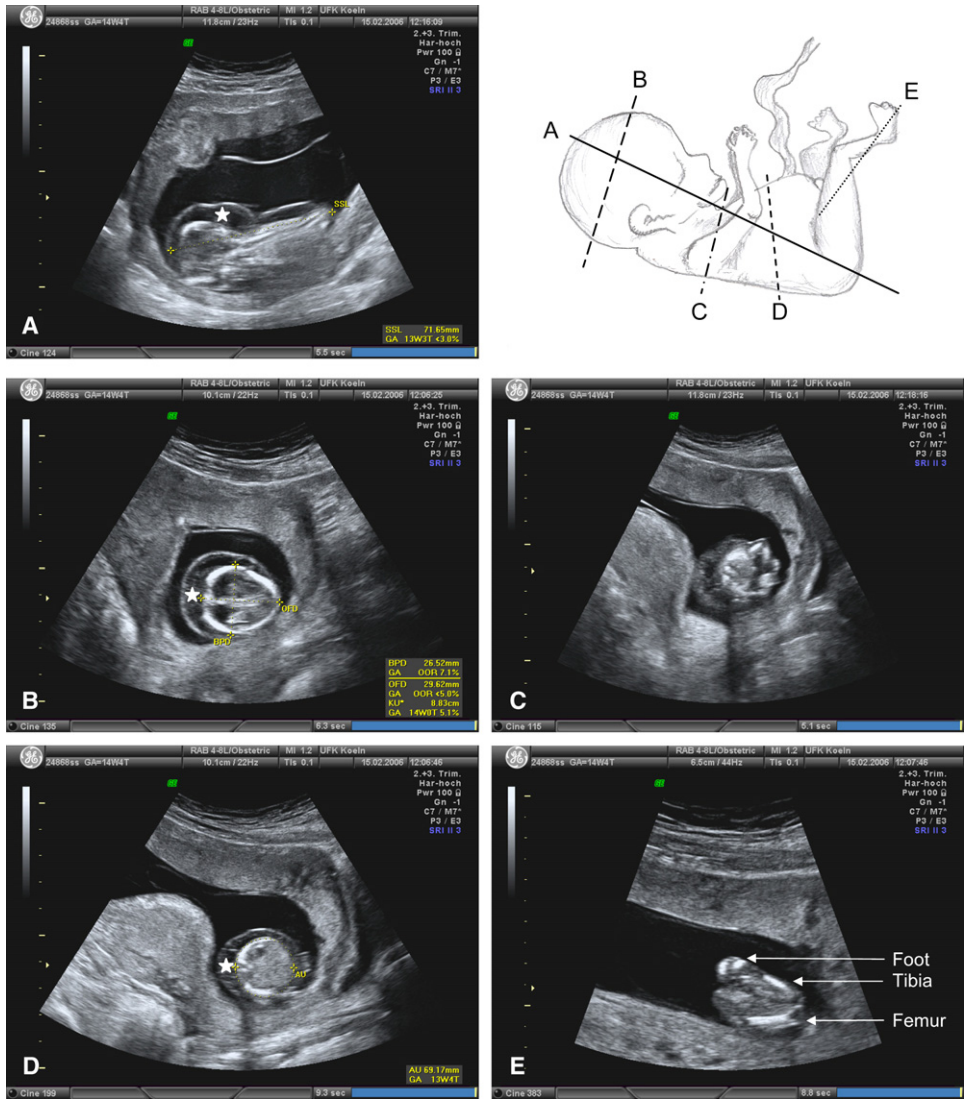

Figure 3. Prenatal Ultrasound Revealed Fetal Hydrops and Contractures in Family CHRND-F1

Extensive generalized edema and flexion contractures of upper and lower limbs in individual V-3 from family CHRND-F1 were identified on prenatal ultrasound at gestational week 13+4 days. Edema separating skin from underlying structures extended from head and neck to trunk and is marked by an asterisk.

(A) Pathologic nuchal edema extending down the back.

(B) Planum frontooccipitale with noticeable hygroma.

(C) Cross section through shoulder region reveals flexion contractures with adducted forearms and hands.

(D) Upper abdominal cross section shows extensive edema.

(E) Flexion contracture of lower limb with pes equines deformity.

developed fetuses. Affected subjects in families CHRNA1-F1/F2 and CHRND-F1/F2 had edema and pterygia (Figures 3 and 4). Joint contractures and pterygia affected upper and lower extremities, variably, but predominantly shoulders, elbows, wrists, hips, knees, and ankles were involved (Figure 4). Occasionally, webbing between fingers was detected. Most affected fetuses did not reach term because of spontaneous abortion, early intrauterine death, or pregnancy termination. If born alive, neonatal respiratory distress was observed (preterm patient II-1 in family CHRND-F2 and both patients from family RAPSN-F1). Though restricted by tissue maceration, pathological examination revealed additional features such as hygroma (CHRNA1-F1/F2, CHRND-F1/F2; Table 1 and Figure 4), generalized hydrops (CHRNA1-F1/F2, CHRND-F1/F2; Figures 3 and 4), pulmonary hypoplasia (CHRND-F2, RAPSN-F1), and atrial septal defect (CHRND-F2).

We sequenced functional candidate genes *CHRNA1, CHRNB1, CHRND,* and *RAPSN* in 60 families without *CHRNG* mutations. We identified seven recessive mutations in five families (Table 2). All mutations segregated with disease and were not found in 300 control chromosomes. Family CHRNA1-F1 revealed homozygosity for the missense mutation *CHRNA1* c.761G→T predicted to cause

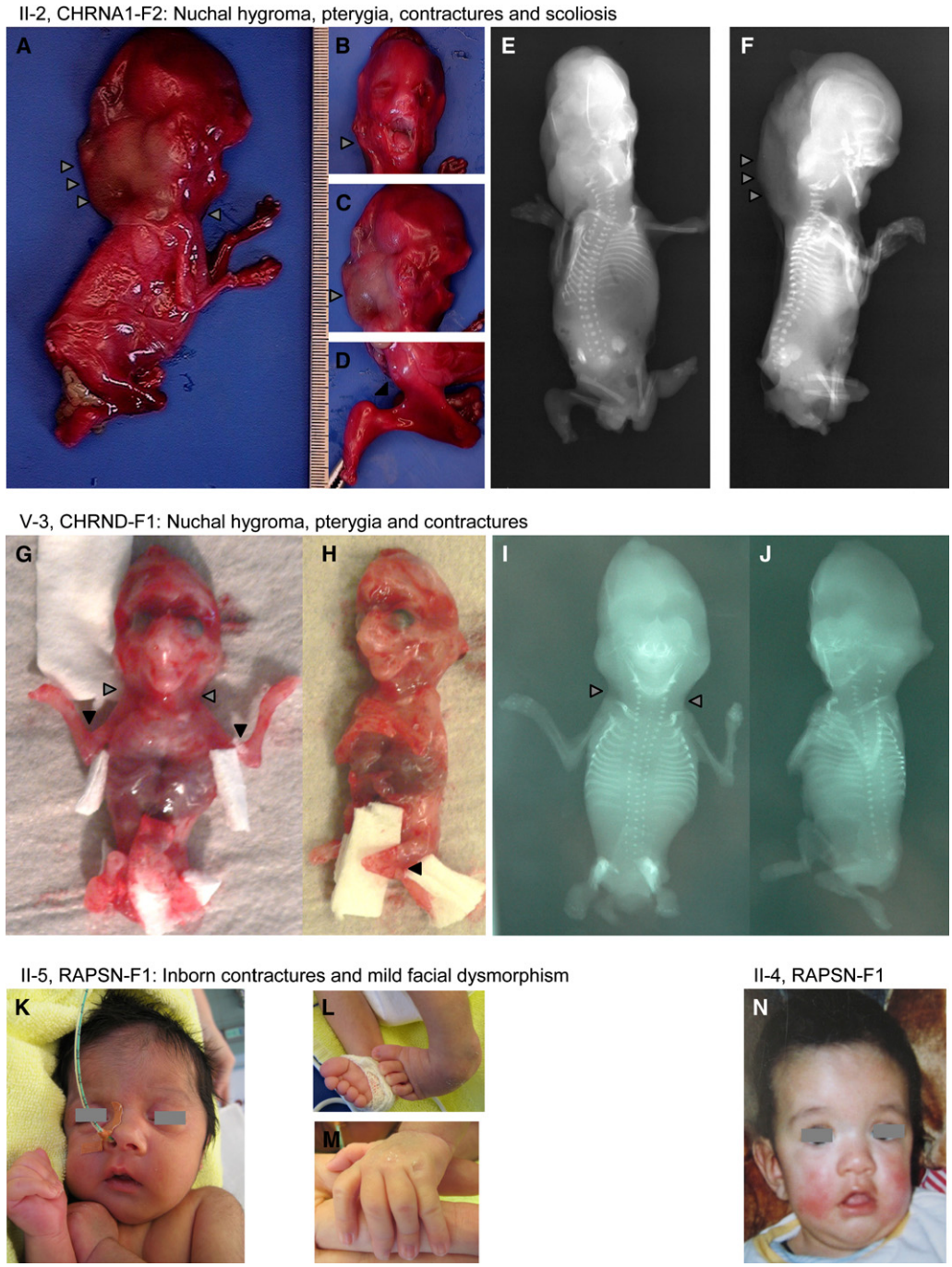

Figure 4. ***CHRNA1*** **and** ***CHRND*** **Mutation-Positive Fetuses Presented with Massive Hydrops, Pterygia, and Contractures on Postmortem Examination whereas Novel Recessive Missense** ***RAPSN*** **Mutations Caused Congenital Arthrogryposis and Life-Threatening Respiratory Distress**

(A–F) Individual II-2 from family CHRNA1-F2 shows generalized edema most extreme at neck and head (gray arrowheads in [A] and [B]), pterygia at elbows and knees (black arrowheads in [A] and [D]), and severe joint contractures (A, D–F) after induced abortion at 17 gestational weeks. X-ray reveals scoliosis, malformed head, and soft tissue swelling by nuchal hygroma (E, F).

(G–J) Individual V-3 from family CHRND-F1 is the same patient as in ultrasound Figure 3 and shown after induced abortion at gestational age 14+6. Generalized edema (gray arrowheads in [G] and [I]), pterygia at elbows and knees (black arrowheads in [G] and [H]), and severe joint contractures (G–J) were detected on postmortem inspection. The fetogram (I, J) showed relatively broad clavicles, ribs, and left metatarsal bone I, but did not reveal any severe skeletal anomalies.

(K–N) In contrast, patients with *RAPSN* mutations were born at term (family RAPSN-F1). Patient II-5 (K–M) was born with severe respiratory problems and inborn contractures (L, M). Her affected brother II-4 (N) had similar clinical manifestations and deceased because of respiratory insufficiency at the age of 10 months. Both patients had down-slanting palpebral fissures, mild hypertelorism, a wide nasal bridge, low-set ears, micrognathia, and small mouth with tented lips (K, N).

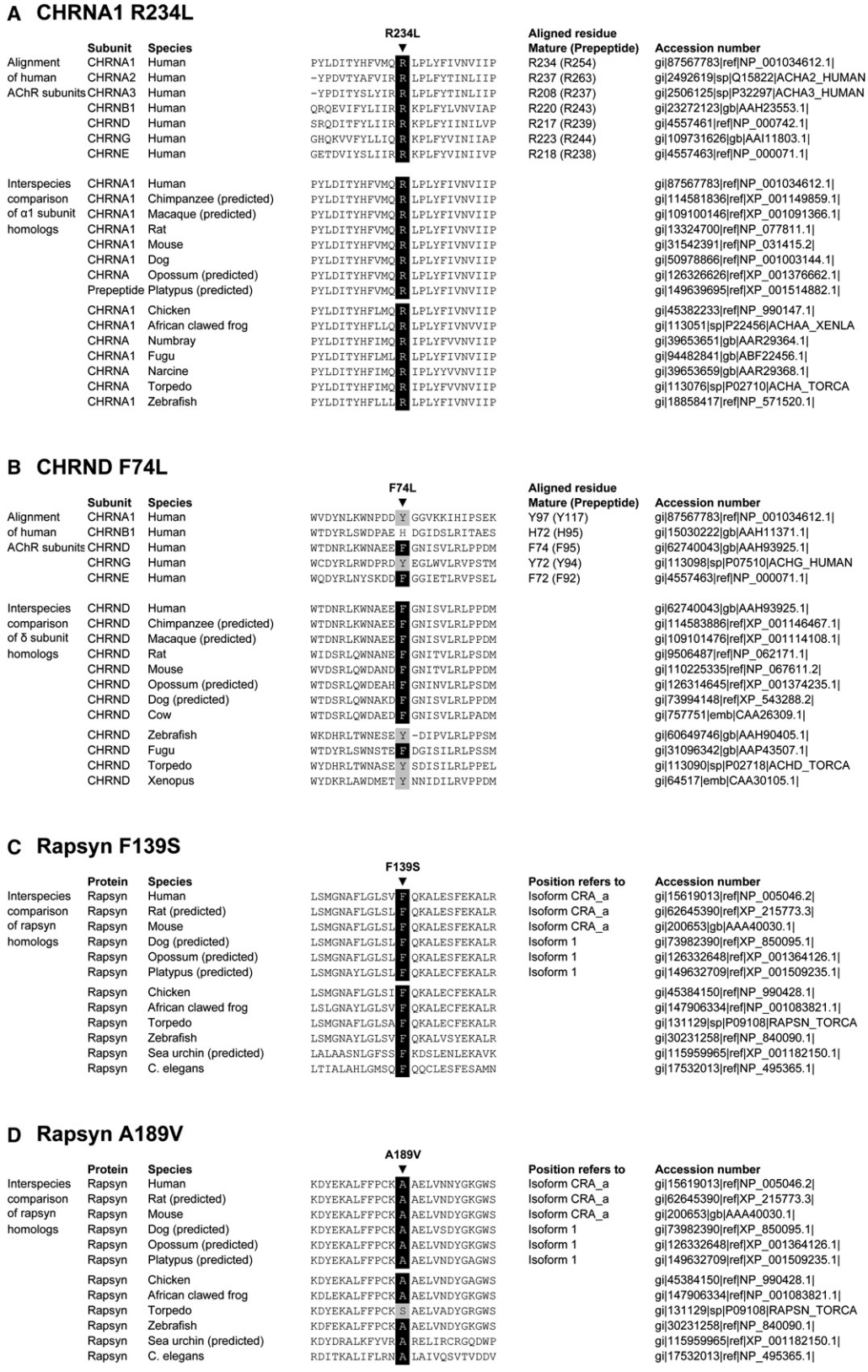

Figure 5. Evolutionary Conservation of CHRNA1, CHRND, and RAPSN Missense Mutations

For all missense mutations, interspecies comparison reveals conservation in homolog positions in all mammals tested. CHRNA1 mutation R234L (A) and both novel *RAPSN* missense mutations (C, D) affect residues that show evolutionary conservation even beyond mammals. In addition, residue of CHRNA1 mutation R234L (A) is also conserved in other human AChR subunits β1, δ, γ, and ε as well as other α-type subunits α2 and α3 in nonmuscular AChR.

Table 2. AChR Pathway Mutations in Families with Severe Fetal Akinesia Sequence

Family ID	CHRNA1-F1	CHRNA1-F2	CHRND-F1	CHRND-F2	RAPSN-F1
Mutation in gene	*CHRNA1*	*CHRNA1*	*CHRND*	*CHRND*	*RAPSN*
Location	exon 6	exon 2	exon 3	exon 4/exon 12	exon 2/exon 3
Position cDNA	c.761G→T homozygous	c.117-133 dup17 homozygous	c.234G→A homozygous	c.283T→C/c.1390C→T	c.416T→C/c.566C→T
Residue in mature protein (residue before cleavage of signal peptide)	R234L (R254L in precursor)	H25RfsX19 (H45RfsX19 in precursor)	W57X (W78X in precursor)	F74L/R443X (F95L/R464X in precursor)	F139S/A189V
Origin	Pakistani	African	Turkish	German	Pakistani
Consanguinity	+	−	+	−	−

Included are lethal multiple pterygium, Escobar-like syndrome, and fetal akinesia with inborn arthrogryposis.

amino acid substitution α1.R234L. Supporting the functional relevance of position α1.R234L, this residue is completely conserved across species as well as in the homologous positions of the human β1, δ, γ, ε, α2, and α3 subunits (Figure 5A). In family CHRNA1-F2, the affected fetus carried a homozygous duplication of 17 base pairs c.117-133 dup17 in *CHRNA1*, resulting in a frameshift mutation with a subsequent premature stop codon (α1.H25RfsX19). A homozygous nonsense mutation *CHRND* c.234G→A in family CHRND-F1 introduced a premature stop (δ.W57X). Family CHRND-F2 is compound heterozygous for *CHRND* mutations c.1390C→T and c.283T→C. The former encode the nonsense mutation δ.R443X and the missense mutation δ.F74L. Residue δ.F74L is conserved among mammals (Figure 5B). Both patients from family RAPSN-F1 were compound heterozygous for the *RAPSN* missense mutations c.416T→C and c.566C→T, which encode F139S and A189V, respectively. Both residues are conserved across species, indicating functional relevance (Figures 5C and 5D).

Patients with lethal multiple pterygium syndrome because of *CHRNG* mutations not only had severe prenatal myasthenic symptoms, but also showed associated extramuscular findings such as generalized edema, nuchal hygroma, situs inversus, or cardiac defects.[8,9] Extramuscular manifestations could indicate disruptive effects in late embryonic and early fetal development and also occurred in individuals with the new *CHRNA1*, *CHRND*, and *RAPSN* mutations described above. Therefore, we wanted to know whether or not AChR subunit genes could contribute to earlier developmental processes. To study this question, we analyzed the expression of *Chrna1*, *Chrnb1*, *Chrnd*, *Chrng*, and *Rapsn* in mouse embryos before (E10.5, E11.5) and during (E12.5, E14.5) muscle development as well as in limb sections with advanced muscle development (E15.5) (Figure 6). Interestingly, all studied subunits and rapsyn are expressed in early somites as early as E10.5. At E11.5, expression of *Chrna1*, *Chrnb1*, *Chrnd*, *Chrng*, and *Rapsn* begins in the upper developing limb and seems to proceed proximal further into the developing muscle bulks at E12.5. At E14.5, expression corresponds to the muscle anlagen in the trunk, neck, limbs, and diaphragm, confirming earlier studies.[9,20] We also noticed strong expression of all analyzed subunit genes in the nuchal musculature, including those in close proximity to the jugular lymphatic sac as well as in subcutaneous muscle layers (Figures 6B and 6C).

Discussion

We identified the first (to our knowledge) recessive null mutations in the nonsubstituted AChR subunits α1 and δ. The reported *CHRNA1* and *CHRND* mutations are null mutations or affect evolutionary conserved amino acids and caused lethal multiple pterygium syndrome. The novel *RAPSN* missense mutations were clinically associated with inborn contractures and respiratory distress that was lethal in one of the affected sibs. We found that *Chrna1*, *Chrnb1*, *Chrnd*, *Chrng*, and *Rapsn* are expressed in somites in early murine embryonic development and later also in skeletal muscle. These findings support their pathogenic relevance to FADS.

All patients with *CHRNA1*, *CHRND*, and *RAPSN* mutations presented here had fetal akinesia, intrauterine growth retardation, micrognathia, and contractures consistent with fetal akinesia deformation sequence.[1,5] We wondered what distinguishes AChR pathway mutations causing a relatively mild phenotype from other mutations with prenatal death and what factors contribute to the presence or absence of pterygia and the occurrence of additional findings such as cardiac defects and pulmonary hypoplasia. *CHRNG* mutations cause prenatal myasthenia that improves in late pregnancy and does not progress or persist in surviving patients after birth.[8,9] This is due to the physiological γ to ε switch around 33 weeks gestation in humans, when ε begins to function.[10] Mutations in genes encoding other components of the postsynaptic AChR pathway should lead to

(A) CHRNA1 R234L.
(B) CHRND F74L.
(C) RAPSN F139S.
(D) RAPSN A189V.

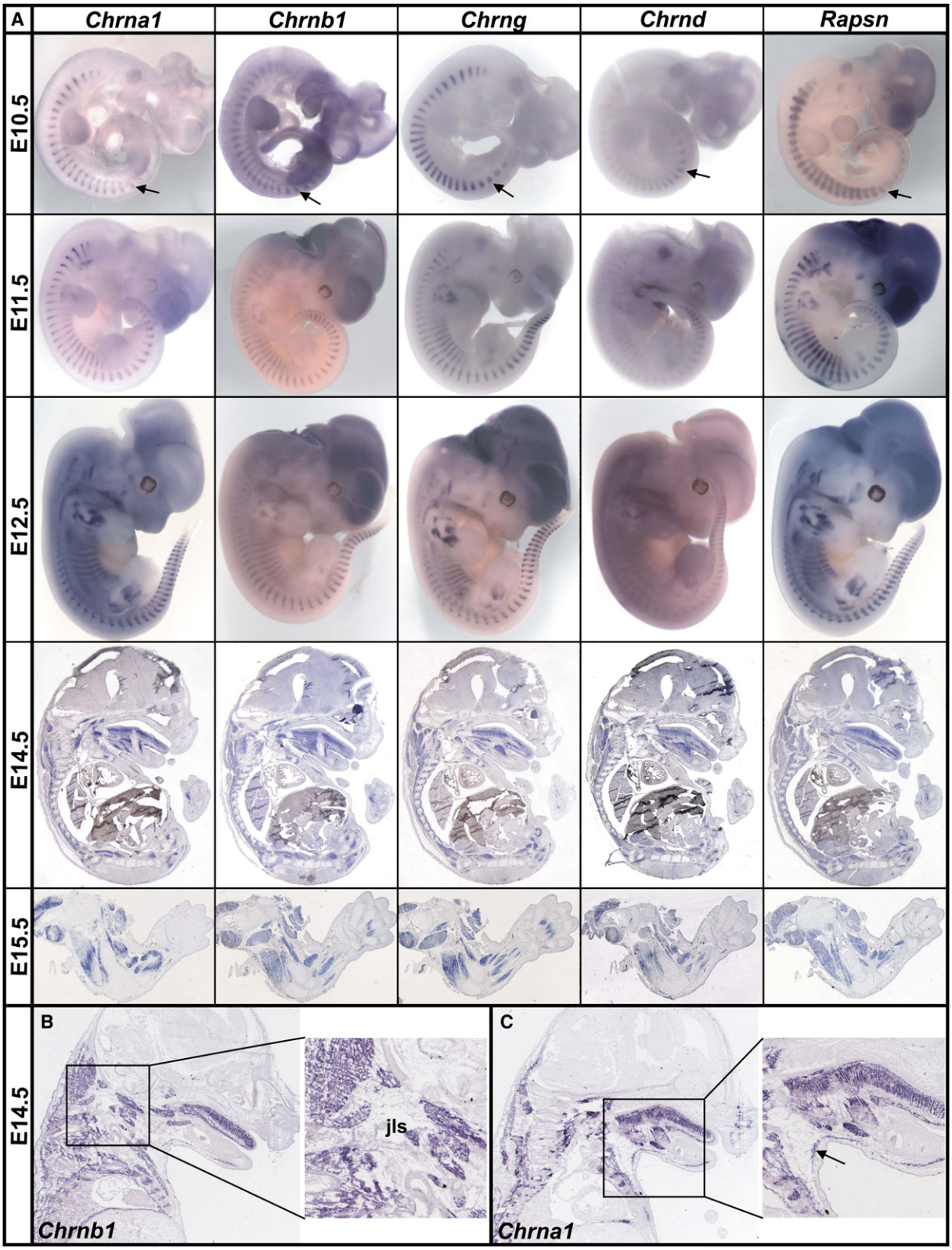

Figure 6. In Situ Hybridization in Mouse Embryos Reveals Early Expression of AChR Subunit Genes and *Rapsn* in Somites and Later in Skeletal Muscle as well as in Hygroma-Relevant Regions

(A) Expression of *Chrna1, Chrnb1, Chrnd, Chrng*, and *Rapsn* in mouse development. Probes and embryonic stages are as indicated. E10.5, E11.5, and E12.5 are shown as whole-mount in situ hybridization (ISH), E14.5 and E15.5 are shown as section-ISH. *Chrna1, Chrnb1, Chrnd, Chrng*, and *Rapsn* are distinctly expressed in early somites as early as E10.5 (arrows), corresponding to human developmental age of 32 days (46 gestational day, Carnegie stage 14). At E11.5, expression of *Chrna1, Chrnb1, Chrnd, Chrng*, and *Rapsn* starts in upper developing

a phenotype that is more pronounced or even lethal because of lack of a substituting subunit for all other AChR subunits. Our data confirm this view.

Accordingly, the homozygous frameshift mutation α1.H25RfsX19 in *CHRNA1* in family CHRNA1-F2 predicts a complete shutdown of fetal AChR function and was associated with intrauterine death. This finding is, to our knowledge, the first homozygous nonsense mutation reported in this subunit. Severe edema was detected as early as the 7th week of pregnancy and persisted to the 14th gestational week when the pregnancy was terminated. Severe fetal hydrops, extensive nuchal hygroma, and extreme webbing were found. A homozygous missense mutation α1.R234L caused a similar phenotype in family CHRNA1-F1, indicating that a change of the evolutionary conserved residue α1.R234 has the same lethal functional effect as the nonsense mutation α1.H25RfsX19. In comparison, previously reported missense mutations in *CHRNA1* cause viable slow or fast channel congenital myasthenic syndrome (CMS [e.g., MIM 60893, 601462, 254210]).[21] A severely affected female with fast channel myasthenic syndrome and newborn respiratory distress harbored compound heterozygosity for a truncating and a missense mutation (*CHRNA1*.381 delC/α1.V132L).[22] This patient might represent an intermediate type between milder myasthenic syndrome of postnatal onset and the severe prenatal effects we observed in lethal multiple pterygium syndrome.

In the δ subunit, our mutation δ.W57X is, to our knowledge, the first reported homozygous truncating mutation and causes a severe intrauterine phenotype with massive edema, contractures, and pterygia in family CHRND-F1. Family CHRND-F2 presented with an equally severe phenotype with spontaneous abortion, massive hydrops, pterygia, and pulmonary hypoplasia. The affected individuals were compound heterozygous for nonsense mutation δ.R443X and missense mutation δ.F74L. The mutations in the δ subunit reported here represent the severe end of the spectrum. Other investigators have reported compound heterozygosity for a missense and nonsense mutation (δ.E59K/δ.756ins2) in a viable patient with inborn contractures at an intermediate level, and a myasthenic syndrome of postnatal onset with two other missense mutations.[23–25] Their patients likely represent the milder end of the disease spectrum.

Our findings indicate that homozygosity for functional null mutations of AChR subunits α1 and δ, which are not substituted in fetal development, causes a severe phenotype with intrauterine hydrops, pterygia, and a high likelihood for intrauterine death. Our data confirm the hypothesis that complete functional loss of nonsubstituted subunits would be lethal.[16] Although we did not find *CHRNB1* mutations in our patient sample, we postulate that this situation also applies for recessive functional null mutations in the β1 subunit that has a similar functional impact.

In comparison, the *RAPSN* missense mutations F139S and A189V were associated with a milder phenotype with inborn contractures and life-threatening respiratory distress. Previously, only one patient with a homozygous missense mutation other than N88K, namely R164C in exon 2 of the *RAPSN* gene, presented with contractures, muscular hypotonia, feeding, and respiratory problems.[26] Thus, our mutations extend the spectrum of *RAPSN* missense mutations associated with congenital contractures. Commonly, recessive *RAPSN* missense mutations cause congenital myasthenic syndrome with associated AChR deficiency whereas compound heterozygosity of a missense

limb and seems to further proceed from proximal into the developing muscle bulks at E12.5 days. At E14.5, expression corresponds to muscle anlagen at trunk, neck, limbs, and diaphragm. Whereas *Chrna1*, *Chrnb1*, *Chrng*, and *Rapsn* are stably expressed throughout the embryonic stages analyzed, *Chrnd* exhibits a more dynamic expression pattern. *Chrnd* shows strong expression in the posterior (newly formed) somites at E10.5 and E11.5. Expression apparently decreases around E12.5, to barely detectable levels in whole-mount and section in situ hybridization in somites (not shown). At E14.5, the muscles still show relatively weak expression. However, robust expression is reappearing in differentiated muscles at E15.5.

Comparative developmental stages adapted from Wessels and Markwald.

Mouse E10.5 ≈ Carnegie stage 14 ≈ human developmental age of 32 days (post conception) ≈ 46 days gestation (post menstruation)
Mouse E11.5 ≈ Carnegie stage 16 ≈ human developmental age of 37 days (post conception) ≈ 51 days gestation (post menstruation)
Mouse E12.5 ≈ Carnegie stage 18 ≈ human developmental age of 44 days (post conception) ≈ 58 days gestation (post menstruation)
Mouse E14.5 ≈ Carnegie stage 23 ≈ human developmental age of 54–56 days (post conception) ≈ 68–70 days gestation (post menstruation)

(B and C) Prenatal expression of AChR subunit genes and *Rapsn* in hygroma-relevant regions. Expression of α1 at E14.5 is representative also for β1, δ, and γ (data not shown). Note strong expression in nuchal area, close proximity to jugular lymphatic sac (marked as "jls" in higher magnification in [B]), and subcutaneous muscle layers (marked by an arrow in [C]). Mutations might contribute to edema by, for example, decreased or absent muscle contractures and subsequently impaired transport of lymphatic fluid. The lymphatic system develops from two structures. Deep parts of the jugular lymphatic sacs derive from tissue around jugular veins. Superficial parts of the jugular lymphatic sacs and peripheral lymphatic vessels develop from local lymphangioblasts originating from mesodermal anlagen.[31–34] As long as no connection is made between the jugular lymphatic sacs and the jugular veins, transient nuchal edema is physiological. If the lymphojugular junction connection is delayed and/or volume increases, abnormal nuchal edema or cystic hygroma occurs. A possible AChR-related pathomechanism for fetal edema is that defective neuromuscular signal transduction causes lack of muscle contractions and thus impairs lymphatic fluid movement. Other potential mechanisms include altered muscle development affecting subsequent development and differentiation of lymphatic vessels. Finally, both muscle and lymphatic anlagen may depend on similar AChR-related signals or components. AChR and rapsyn expression in premuscular and muscular tissues and close anatomical proximity to lymphatic structures both in embryonic development and fetal differentiation is consistent with this view.

and a truncating defect were associated with contractures.[16,26–29]

We were curious what differentiates mutations presenting with pterygia from those that only reduce intrauterine movements and cause contractures, because impaired fetal movement seems to trigger both contractures and pterygia. In our families, hypokinesia and contractures were first detected by ultrasound at 11–14 weeks of gestation. At that time, pterygia in patients with *CHRNA1* or *CHRND* mutations were already visible at postmortem examination. The onset of pterygia is therefore likely to occur earlier. In family CHRNA1-F2, fetal hydrops and cystic nuchal hygroma appeared on ultrasound in the 7th gestational week; the fetus later presented with severe webbing (Figure 4). In this case, fetal edema could have predisposed to subsequent webbing. A pathogenic association of pterygia with previous fetal edema has long been suspected.[30] This notion is supported by observations in our families.

Mutations in AChR subunit genes are also associated with developmental anomalies in other tissues that are not primarily associated with neuromuscular junctions.[8,9] Cardiac defects, renal malformation, and intestinal malrotation are features of the lethal multiple pterygium syndrome.[1,5] We observed edema, cardiac defects, and pulmonary hypoplasia in our patients. This association indicates that the mutations might interfere with normal embryogenesis. Our in situ data show expression not only in later developmental stages when muscle differentiation is evident, but also in early developmental stages as demonstrated in mouse E10.5. Mouse developmental stage E10.5 corresponds to human developmental day 32, at which time *Chrna1, Chrnb1, Chrnd*, *Chrng*, and *Rapsn* are expressed in early somites. Somites are segmental regions where mesodermal structures develop in the trunk. This indicates that mutations in the AChR subunit genes may have an effect on early mesodermal structures such as muscle anlagen. Also, peripheral lymphangioblasts are the organizers for lymphatic vessels in extremities and develop from these regions in the somites.[31–35] Thus, very early prenatal findings, such as frequent abortions or generalized edema, could be attributed to a defect of early AChR subunit expression in somites in addition to muscle-specific effects alone. However, at this point the evidence is speculative, because so far no role for AChR expression has been demonstrated in early embryonic development or mesodermal differentiation. In later developmental stages, the classical function of AChR in establishing, maintenance, and signal transduction of neuromuscular junctions might contribute to hypokinesia and growth retardation.

Accordingly, other genes encoding AChR pathway components may harbor mutations in FADS patients without mutations in AChR subunits or RAPSN. AChR-related candidates are Agrin, MUSK, ARIA, the postsynaptic cytoskeletal components dystroglycan and actin, as well as a growing number of more or less direct contributors such as recently reported for CDK5, calpain, DOK7, and ACF7.[11,15,36–41]

Awareness of the variable clinical presentation of AChR pathway mutations might be life-saving, as in our family RAPSN-F1. At birth, both affected children had arthrogryposis but were not diagnosed for neuromuscular transmission defects. After identification of *RAPSN* mutations, the surviving child was started on AChE inhibitors (4 × 1 mg/kg/d Mestinone). Her motor function and hypotonia improved significantly. However, feeding problems have been persisting; a percutaneous endoscopic gastrostomy (PEG) tube is still required. The positive therapeutic effect was even more obvious when the patient had to be admitted to the ICU because of an infection-triggered myasthenic crisis with a decrease of capillary O_2 saturation to 60% and respiratory acidosis. Remarkably, only 1 day after increasing the dose of Mestinone to 6 mg/kg/d, her symptoms and respiratory parameters improved to an extent that she could leave the ICU. After her infection and the subsequent stop of the antibiotic treatment, the dose of Mestinone was reduced back to the initial 4 mg/kg/d without any side effects. This underlines the therapeutic relevance of early clinical and genetic consideration of AChR pathway mutations.

We conclude that AChR pathway mutations are relevant for a wide FADS disease spectrum. Our results confirm the idea that fetal akinesia predisposes to the development of contractures. Additional first trimester fetal edema seems to predispose to pterygia development. Consequently, the AChR pathway contributes to a broad spectrum of intrauterine phenotypes and should be examined functionally and genetically in patients with recurrent spontaneous abortions, fetal akinesia, hydrops, pterygia, or inborn contractures.

Supplemental Data

One supplemental figure showing sequence traces and segregation within patient's families can be found with this article online at http://www.ajhg.org/.

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Web Resources

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