Mutationsanalysen in Genen des Acetylcholin-Rezeptor-Pathways in Patienten mit Fetal Akinesia Deformation Sequence (FADS)

Publikationsdissertation
zur Erlangung des akademischen Grades
Dr. med.

an der Medizinischen Fakultät
der Universität Leipzig

eingereicht von:
Anne Michalk, geboren am 14.03.1980 in Berlin

angefertigt an der:
Universitätsklinik und Poliklinik für Endokrinologie und Nephrologie der medizinischen Fakultät der Universität Leipzig

Betreuer:
Prof. Dr. med. Tom H. Lindner
Prof. Dr. med. Katrin Hoffmann

Beschluss über die Verleihung des Doktorgrades vom 26.06.2018

Inhaltsverzeichnis

1. Einführung in das Thema

1.1. Prävalenz und Relevanz fetaler Bewegungsstörungen und angeborener Kontrakturen

Fetale Bewegungsstörungen betreffen 15 % aller Schwangerschaften (*McCarthy et al. 2016*). Davon sind die meisten geringgradig ausgeprägt (fetale Hypokinesie). Bei einigen Schwangerschaften liegen jedoch schwerwiegende Bewegungsstörungen (fetale Akinesie) vor. Bewegung ist ein Charakteristikum aller gesunden Lebewesen und beginnt beim Homo sapiens bereits in der achten Schwangerschaftswoche. Fehlende bzw. verminderte Bewegung ist daher ein Alarmzeichen für eine mögliche Schädigung *(Hall et al. 2014)*. Unter dem Konzept, dass Funktion ein essentieller Bestandteil für die normale Entwicklung von Strukturen ist, und jede einzelne wiederum einen Teil eines sorgfältig geplanten und ganzheitlichen Systems darstellt, ist die intrauterine Bewegung notwendig für eine normale Entwicklung des werdenden Lebens *(Hall et al. 1986)*. Die fetale Hypo-/Akinesie ist damit Ausgangpunkt einer Sequenz, die verantwortlich ist für einen Phänotyp, der unter anderem Arthrogryposis, Pterygien, Muskelschwäche, Lungenhypoplasie, respiratorische Insuffizienz, intrauterine Wachstumsretardierung und kraniofaziale Dysmorphien unterschiedlichen Ausmaßes umfasst. In schweren Fällen resultieren prä- bzw. perinatal letale Verläufe.

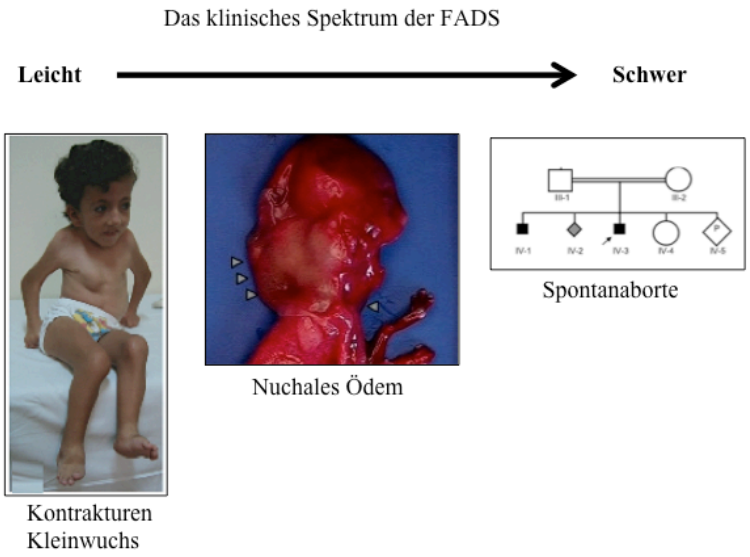

Abb.1 Die Fetal Akinesia Deformation Sequence (FADS) umfasst ein weites klinisches Spektrum. Es reicht von isolierten Kontrakturen, Wachstumretardierung, fetaler Hypo-/Akinesie bis hin zur Lungenhypoplasie, Hydrops fetalis und rezidivierenden Spontanaborten *(Hoffmann et al. 2006 / Michalk et al. 2008)*.

1.2. Das klinische Bild einer FADS

Nahezu immer geht die fetale Akinesie mit Kontrakturen der Gelenke einher. Bei einem Teil der Feten treten in diesem Zusammenhang Kollagenosen und eine Atrophie der gelenknahen Muskeln auf. Die verminderten Kindesbewegungen werden häufig bereits im ersten Trimenon der Schwangerschaft bemerkt und sind fast regelhaft von einer intrauterinen Wachstumsretardierung begleitet. Je früher sich das Krankheitsbild manifestiert, desto gravierender ist das Ausmaß der resultierenden Fehlbildungen (*Hall et al. 2014*). Betroffene zeigen typische, aber nicht spezifische kraniofaziale Auffälligkeiten. Dazu gehören unter anderem eine Mikrognathie, eine Gaumenspalte, ein okulärer Hypertelorismus und tiefsitzende retrovertierte Ohren *(Hammond et al. 1995)*. Weiterhin charakteristisch sind Pterygien, die auch sonographisch intrauterin detektiert werden können. Insbesondere bei den letalen Verlaufsformen der FADS werden extramuskuläre Manifestationen wie ein Polyhydramnion, ein Hydrops fetalis und eine pulmonale Hypoplasie beschrieben. Die Familienanamnese umfasst nicht selten Spontanaborte, Tod- und Fehlgeburten sowie weitere Betroffene.

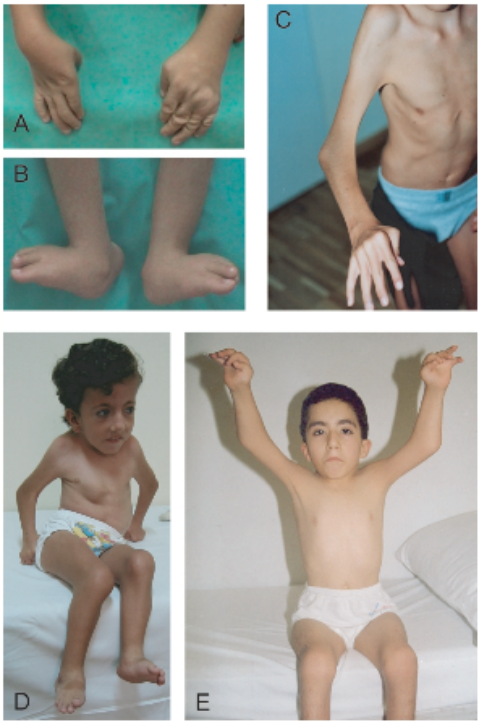

Abb.2 Das klinische Erscheinungsbild der FADS. A Kontrakturen der Hände. **B** Sogenannte „Rocker-bottom-feet“. **C** Pterygien der Ellenbogen und eine muskuläre Atrophie. **D** Ausgeprägte Skoliose. **D+E** Symmetrische Kontrakturen, Pterygien und eine Wachstumsretardierung. Typische, aber nicht spezifische kraniofaziale Dysmorphien sind ein Hypertelorismus, Mikrostomie, Mikrognathie und die tiefsitzenden retrovertierten Ohren. Die mentale Entwicklung kann unbeeinträchtigt sein *(Hoffmann et al. 2006)*.

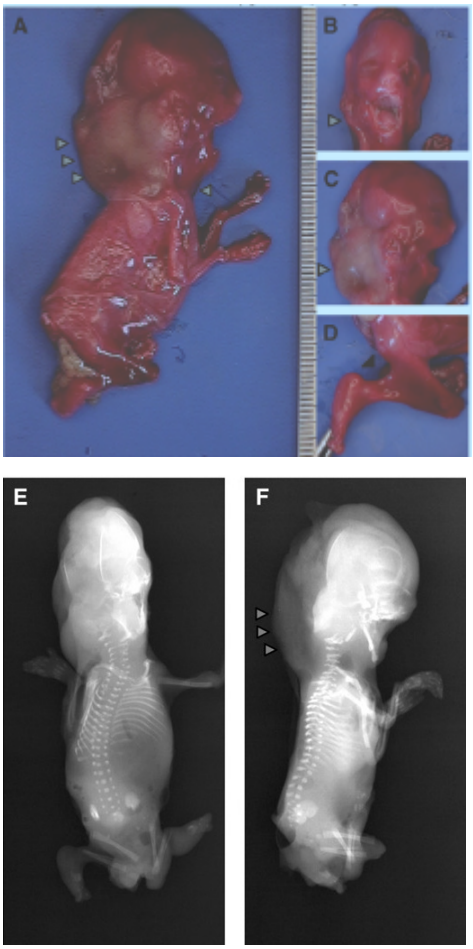

Abb.3 Der Phänotyp von Individuen mit Mutationsnachweis im *CHRNA1* und *CHRND*. A-D Die letalen Verläufe gehen typischerweise mit nuchalem Ödem, Pterygien, Kontrakturen und einem Hydrops fetalis einher. Je ausgeprägter eine intrauterine Bewegungseinschränkung ist, desto wahrscheinlicher sind rezidivierende Aborte in einer Familie. **E+F** Das Fetogramm zeigt eine Skoliose, Schädelfehlbildungen und ein nuchales Hygroma *(Michalk et al.2008)*.

1.3. Ursachen verminderter fetaler Bewegung

Die Ätiologie der FADS ist sehr heterogen. Ursächlich für verminderte fetale Bewegungen kann zum Beispiel ein intrauteriner Platzmangel, wie eine Zwillingsschwangerschaft oder ein Oligohydramnion sein. Platzmangel erhöht die Anfälligkeit für angeborene Kontrakturen. Mütterliche Faktoren, wie Antikörper gegen den fetalen AChR, eine plazentare Minderperfusion oder ein schlecht eingestellter Diabetes mellitus können Auslöser sein. Auf fetaler Seite können Amyoplasien (*Hall et al. 1983*), kongenitale Neuropathien, Synaptopathien, Myopathien, Vaskulopathien, chromosomale Aberrationen oder metabolische Erkrankungen für die klinischen Auffälligkeiten verantwortlich sein *(Hoffmann et al. 2006)*.

Bei der Hälfte der Patienten kann die Ursache aber bis heute nicht geklärt werden. Eine eindeutige klinische und/oder molekulargenetische Diagnose ist jedoch für die Abschätzung der Prognose wichtig. Ebenso können sich aus einer molekulargenetischen Diagnose weit reichende therapeutische Konsequenzen ergeben.

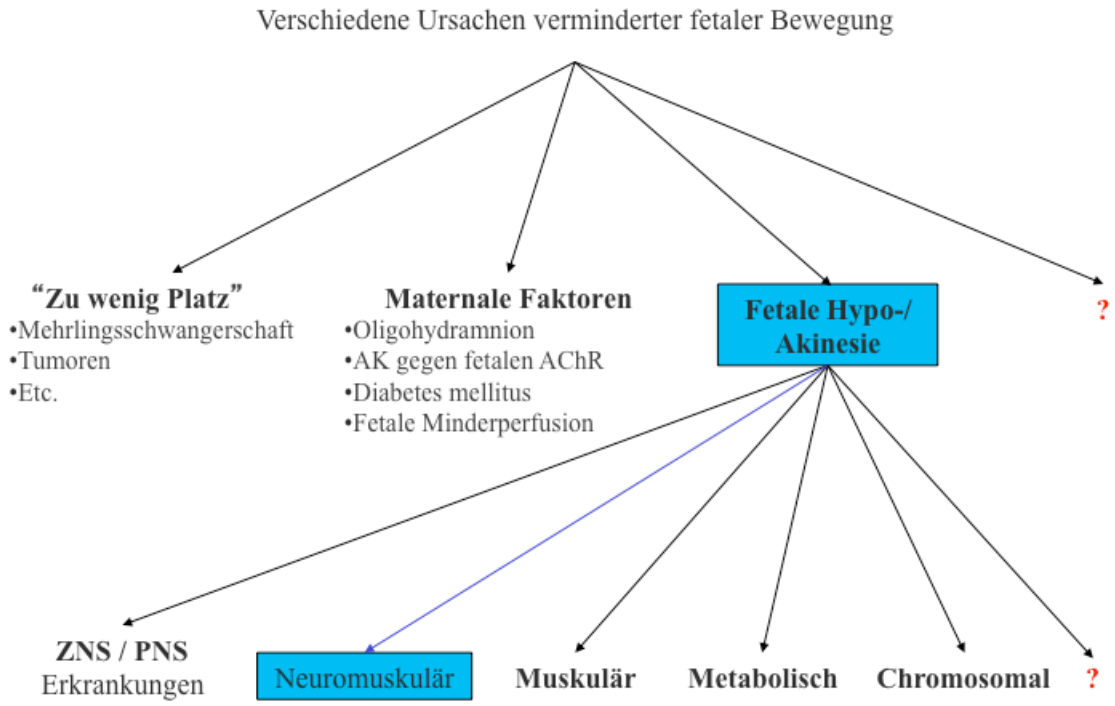

Abb.4 Schematische Darstellung möglicher Ursachen einer fetalen Hypokinesie. Blau markierte Areale verdeutlichen den Fokus der vorgelegten Promotion. Gleichzeitig sind bisher unklare Komponenten mit Fragezeichen versehen.

Aus diesem Grund beschäftigte sich unsere Arbeitsgruppe mit dem Escobar Syndrome (MPS, MIM 265000) und dem letalen Multiple Pterygium Syndrome (LMPS, MIM 253290). Beide Syndrome gehen neben den Kontrakturen vor allem mit Pterygien einher und gelten als Sonderform der Arthrogryposis multiplex congenita. Kopplungsanalysen in den Familien mit Betroffenen führten zur Identifikation von acht Mutationen in der γ-Unterheit (*CHRNG,* MIM 100730) des nicotinergen AChR (*Hoffmann et al. 2006*).

1.4. Der nicotinerge Acetylcholinrezeptor

Der nicotinerge AChR besitzt fünf Untereinheiten. Davon sind zwei α1-, eine β1-, eine δ-Untereinheit sowohl im fetalen als auch im adulten AChR präsent (*CHRNA1*, MIM 100690, *CHRNB1*, MIM 100710, *CHRND*, MIM 100720). Die fetale γ-Untereinheit wird während der 33. Schwangerschaftswoche durch eine adulte ε-Untereinheit ersetzt (*CHRNG*, MIM 100730, *CHRNE*, MIM 100725) (*Moerman et al. 1990*).

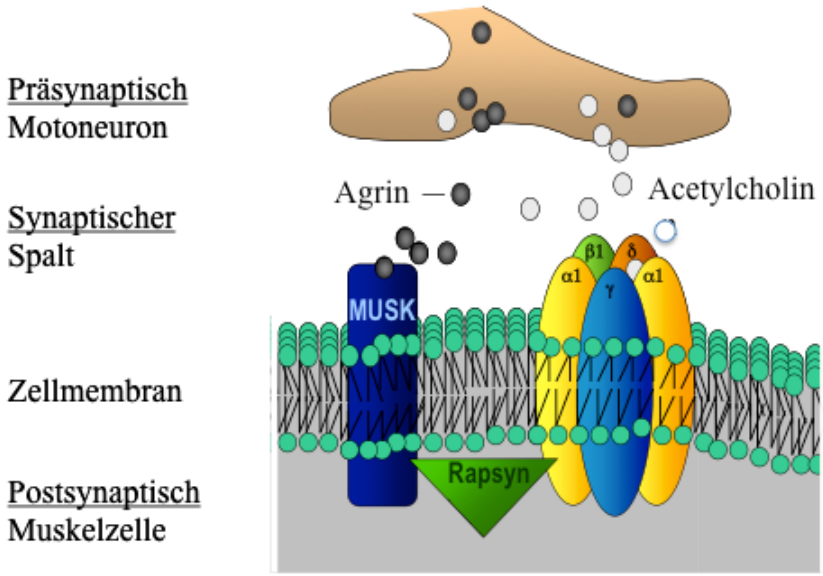

Abb.5 Die schematische Darstellung der neuromuskulären Endplatte mit dem fetalen nicotinergen Acetylcholinrezeptor (nAChR) und ausgewählten Signalweg-Proteinen. Beim nAChR handelt es sich um ein postsynaptisches Membranprotein, bestehend aus fünf transmembranösen Untereinheiten. Rapsyn, ein Protein des Zytoskelettes stabilisiert einerseits die nAChR-Cluster in der postsynaptischen Membran, übermittelt andererseits Signale vom Agrin-aktivierten MUSK *(Michalk et al. 2008)*.

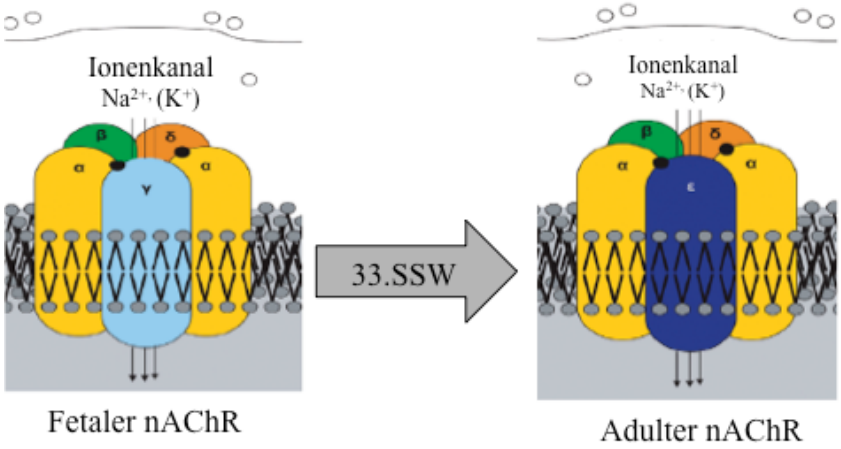

Abb.6 Der Aufbau des fetalen und adulten nicotinergen Acetylcholinrezeptors (nAChR). Man beachtet den Wechsel der fetalen γ-Untereinheit gegen die adulte ε-Untereinheit um die 33. Schwangerschaftswoche *(Hoffmann et al. 2006)*.

Beide, fetaler und adulter Rezeptor, sind essentiell für die neuromuskuläre Signaltransduktion. Der fetale Rezeptor spielt weiterhin eine wichtige Rolle in der Organogenese, beim Aufeinandertreffen von Axon und Muskel (*Hoffmann et al. 2006)*.

Heterozygote Missense- oder Nonsense-Mutationen in den permanenten AChR-Untereinheiten α1, β1, δ und Rapsyn verursachen kongenitale Myasthenie-Syndrome (CMS, MIM 608931) (*Engel et al. 2005*). Diese präsentieren sich klinisch mit variablem Krankheitsbeginn und Schweregrad. Die Betroffenen leiden infolge einer vorzeitigen Ermüdbarkeit der Skelettmuskulatur zum Beispiel an einer Ptosis, Doppelbildern, Schluckstörungen, Atemschwäche und einer verzögerten motorischen Entwicklung.

Klinisch charakteristisch für Mutationen in der fetalen γ-Untereinheit ist das Fehlen myasthener Symptome postnatal. Das ist der große Unterschied zu den Mutationen in der α1-, β1- oder δ- (*CHRNA1, CHRNB1, CHRND*)-Untereinheit des adulten Rezeptors.

Es wurde damals vermutet, dass homozygote Missense- oder Nonsense-Mutationen in den permanenten Untereinheiten des nicotinergen AChR letal verlaufen (*Palace et al. 2007*).

1.5. Mutationssuche in den Genen der α1-, β1- und δ- Untereinheit (*CHRNA1, CHRNB1* und *CHRND*) sowie in dem Rezeptor assoziierten *RAPSN-Gen* bei Patienten mit der FADS

Im Rahmen der vorgelegten Dissertation wurden 63 Patienten mit dem klinischen Bild des Escobar Syndroms, dem letalen Multiple Pterygium Syndrome und anderen Formen der FADS untersucht. Zuerst erfolgte eine Standard-DNA-Sequenzierung der kodierenden Regionen des *CHRNG*-Gens. In drei der Familien fand ich bei Betroffenen krankheitsursächliche Mutationen im *CHRNG*-Gen (nicht Inhalt der vorgelegten Publikation).

In den restlichen 60 Familien vermuteten wir aufgrund des ähnlichen klinischen Bildes und der Familienanamnese Mutationen in anderen Komponenten des Acetylcholinrezeptor-Pathways. Ich sequenzierte die kodierenden Regionen der Gene *CHRNA1, CHRNB1, CHRND* und *RAPSN*.

2. Publikation

Die vorliegende Arbeit bezieht sich auf einen wissenschaftlichen Artikel, der im Folgenden abgedruckt ist.

Autoren: Anne Michalk, Sigmar Stricker et al.

Titel: Acetylcholine Receptor Pathway Mutations Explain Various Fetal Akinesia Deformation Sequence Disorders.

Bibliographie: Am J Hum Genet 82: 464-476 (2008)

ARTICLE

Acetylcholine Receptor Pathway Mutations Explain Various Fetal Akinesia Deformation Sequence Disorders

Anne Michalk,[1,16] Sigmar Stricker,[2,16] Jutta Becker,[3] Rosemarie Rupps,[5] Tapio Pantzar,[5] Jan Miertus,[6] Giovanni Botta,[7] Valeria G. Naretto,[8] Catrin Janetzki,[1] Nausheen Yaqoob,[9] Claus-Eric Ott,[1] Dominik Seelow,[1] Dagmar Wieczorek,[10] Britta Fiebig,[11] Brunhilde Wirth,[3,12] Markus Hoopmann,[4] Marisa Walther,[13] Friederike Körber,[14] Markus Blankenburg,[15] Stefan Mundlos,[1,2] Raoul Heller,[3] and Katrin Hoffmann[1,2,*]

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

[1]Institute for Medical Genetics, Charité University Medicine, Augustenburger Platz 1, D-13353 Berlin, Germany; [2]FG Development & Disease, Max Planck Institute for Molecular Genetics, Ihnestraße 63-73, D-14195 Berlin, Germany; [3]Institute of Human Genetics, [4]Prenatal Medicine, Department of Gynaecology and Obstetrics, University of Cologne, Kerpener Str. 34, D-50931 Cologne, Germany; [5]Departments of Medical Genetics and Pathology, BCCH Children's Hospital, Vancouver, BC V6H3V4, Canada; [6]Medical Genetics Department, St. Elizabeth Cancer Institute, Heydukova 10, Bratislava 812 50, Slovakia; [7]Department of Pathology, "OIRM-S.Anna" Hospital, Corso Spezia, 60, I-10126 Torino, Italy; [8]Azienda Ospedaliera San Giovanni Battista di Torino, Via Santena 19, I-10126 Torino, Italy; [9]Consultant Histopathologist, King Abdul Aziz Specialist Hospital, Taif, Saudi Arabia; [10]Institute of Human Genetics, University of Duisburg-Essen, Campus Essen, Hufelandstr. 55, D-45122 Essen, Germany; [11]Centre for Human Genetics, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany; [12]Institute of Genetics and Centre for Molecular Medicine, University of Cologne, Zülpicher Str. 47, D-50674 Cologne, Germany; [13]Medical Centre of Rheumatology Berlin-Buch, Karower Str. 11, D-13125 Berlin, Germany; [14]Department of Paediatric Radiology, University of Cologne, Joseph-Stelzmann-Strasse 9, D-50931 Cologne, Germany; [15]Department of Neuropaediatrics, Vestische Kinder- und Jugendklinik, University of Witten/Herdecke, Dr.-Friedrich-Steiner Str. 5, D-45711 Datteln, Germany
[16]These authors contributed equally to this work.
*Correspondence: katrin.hoffmann.genetik@charite.de
DOI 10.1016/j.ajhg.2007.11.006.

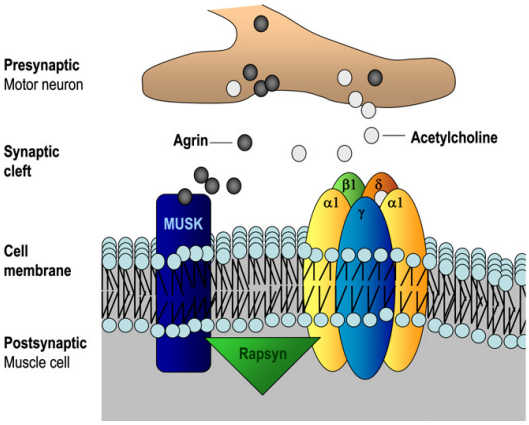

Figure 1. Schematic View of AChR Complex
AChR at the postsynaptic membrane in muscle cells consist of 5 subunits.[11] Two α1, one β1, one δ subunit are always present. The fifth subunit is the fetally expressed γ subunit. By around 33 weeks of gestation in humans, γ subunit expression is stopped switching 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.

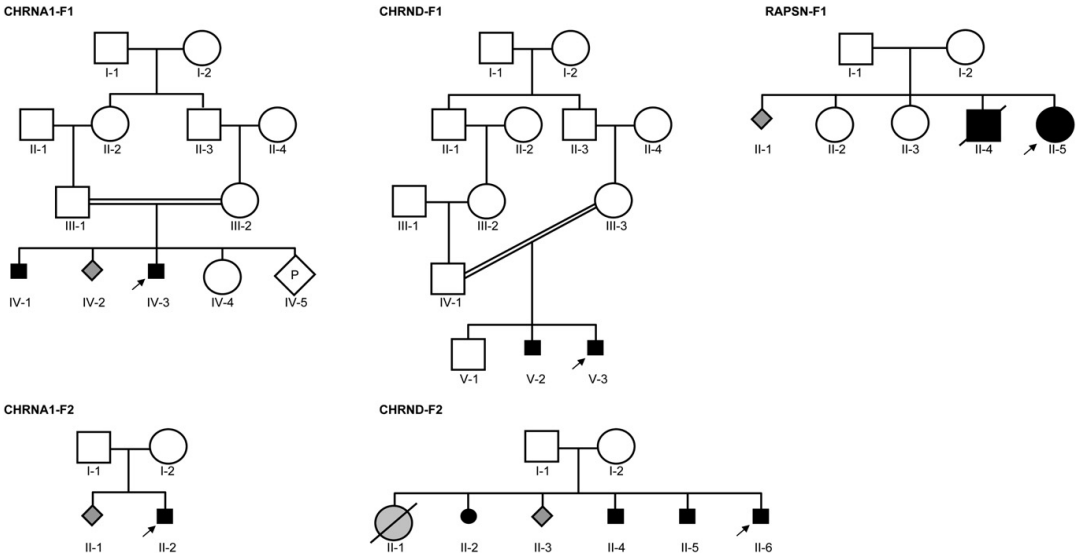

Figure 2. Pedigrees of Described Families with *CHRNA1*, *CHRND*, or *RAPSN* Mutations Indicate Recessive Inheritance and Increased Intrauterine Lethality
Two families with *CHRNA1* mutations, two families with *CHRND* mutations causing lethal multiple pterygium syndrome, and one family with recessive *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, clavicular, 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 gastroesophageal 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

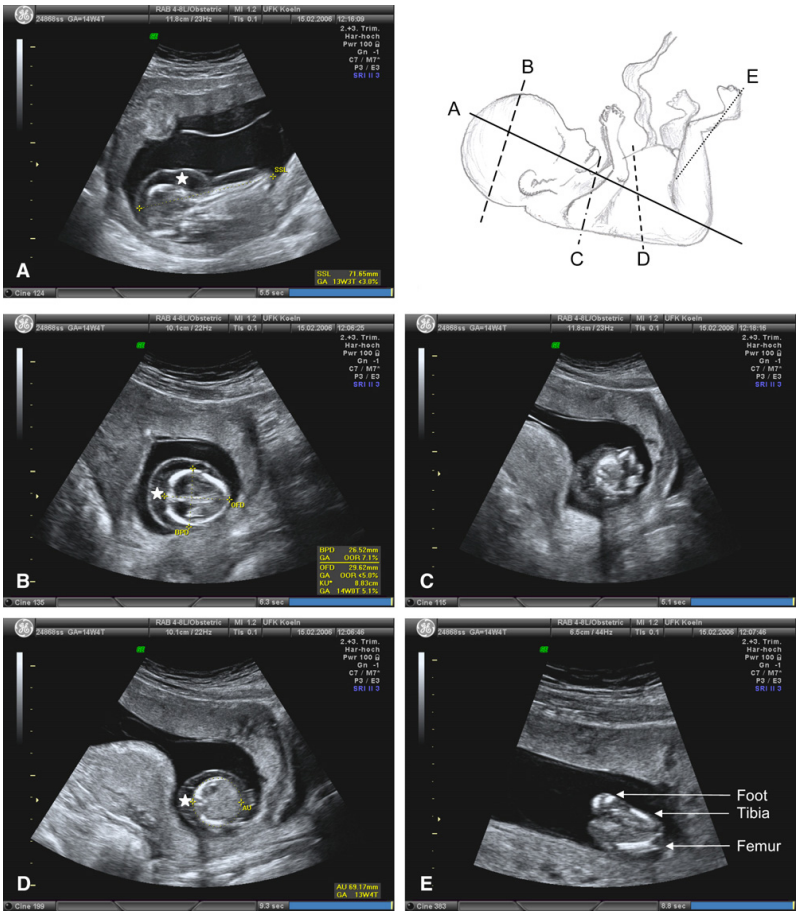

Figure 3. Prenatal Ultrasound Revealed Fetal Hydrops and Contractures in Family CHRND-F1
Extensive generalized edema and flexion contractures of upper and lower limbs in individual V-3 from family CHRND-F1 were identified on prenatal ultrasound at gestational week 13+4 days. Edema separating skin from 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

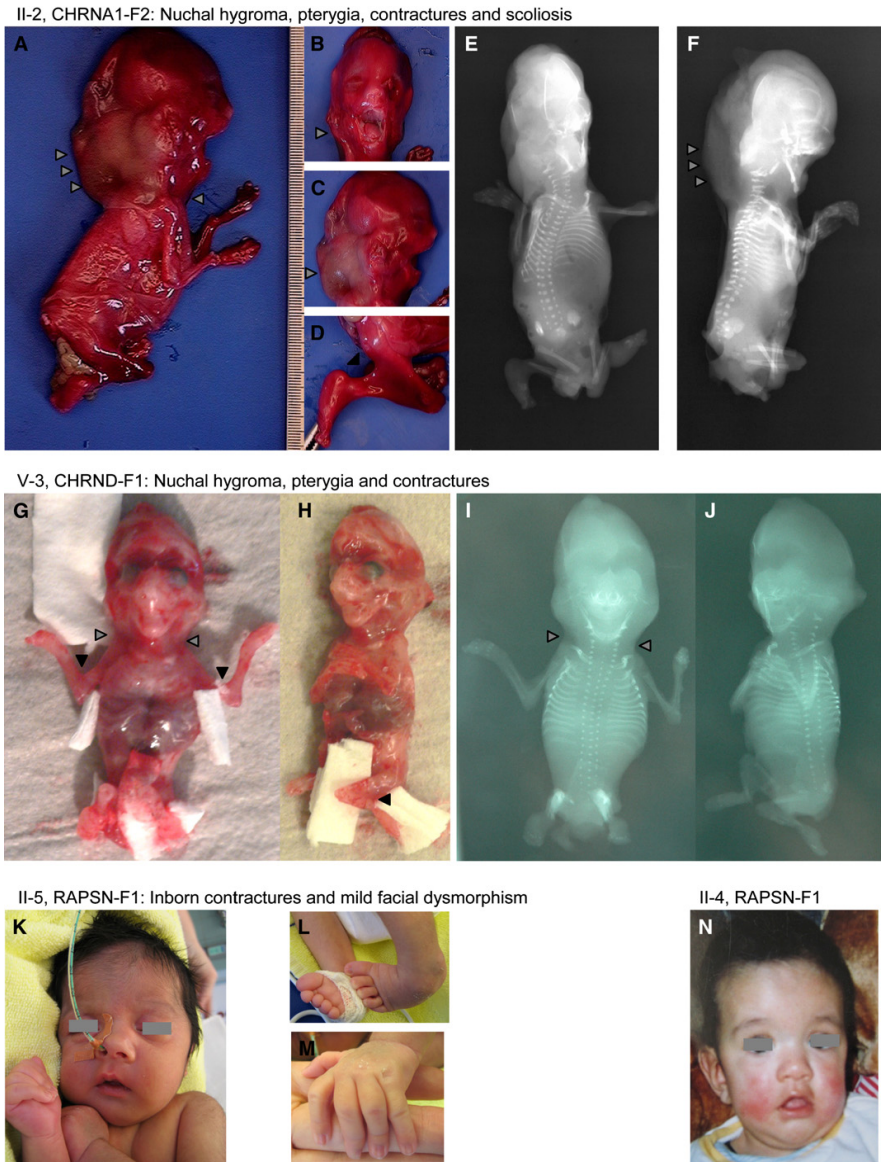

Figure 4. ***CHRNA1*** **and** ***CHRND*** **Mutation-Positive Fetuses Presented with Massive Hydrops, Pterygia, and Contractures on Postmortem Examination whereas Novel Recessive Missense** ***RAPSN*** **Mutations Caused Congenital Arthrogryposis and Life-Threatening Respiratory Distress**

(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).

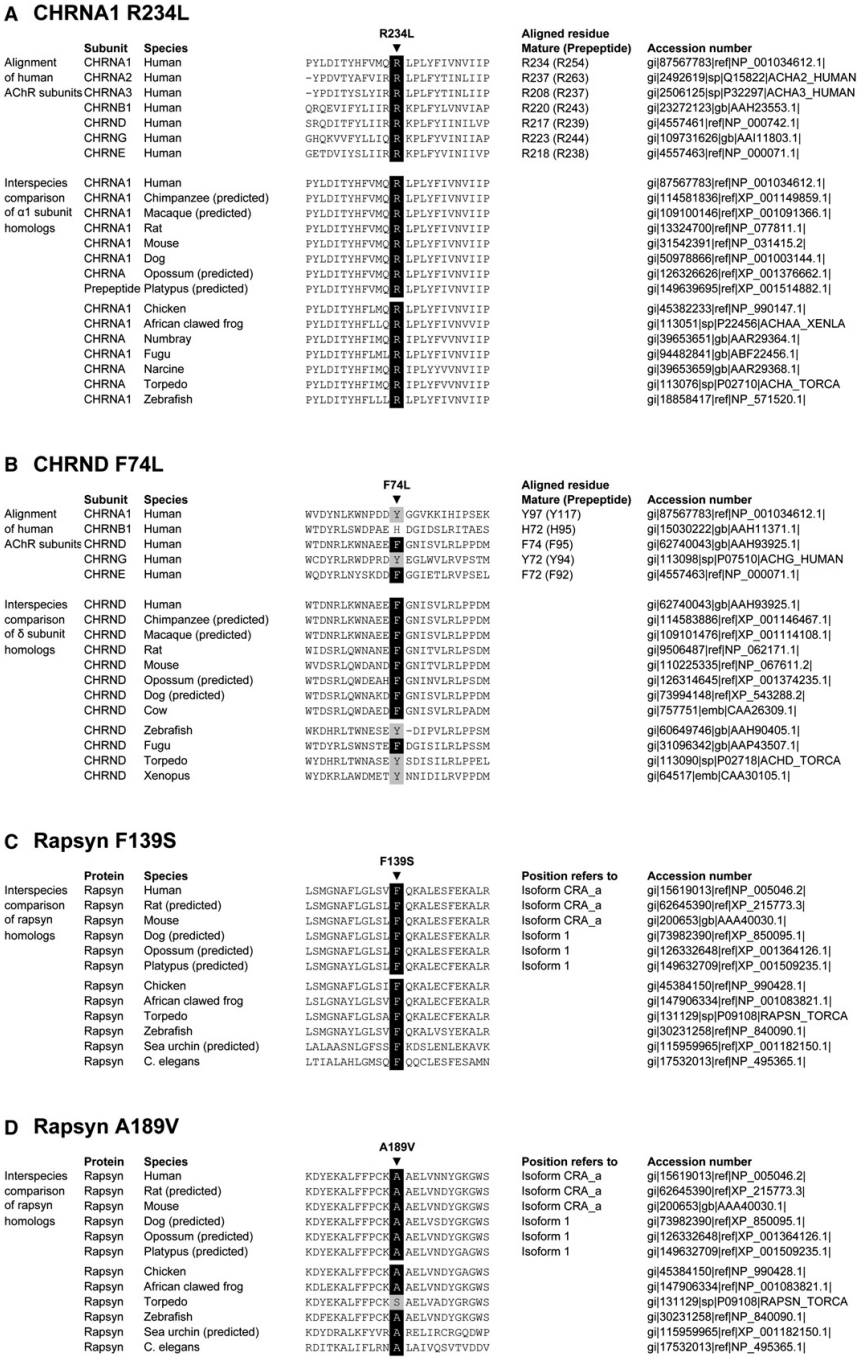

Figure 5. Evolutionary Conservation of CHRNA1, CHRND, and RAPSN Missense Mutations
For all missense mutations, interspecies comparison reveals conservation in homolog positions in all mammals tested. CHRNA1 mutation R234L (A) and both novel *RAPSN* missense mutations (C, D) affect residues that show 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.

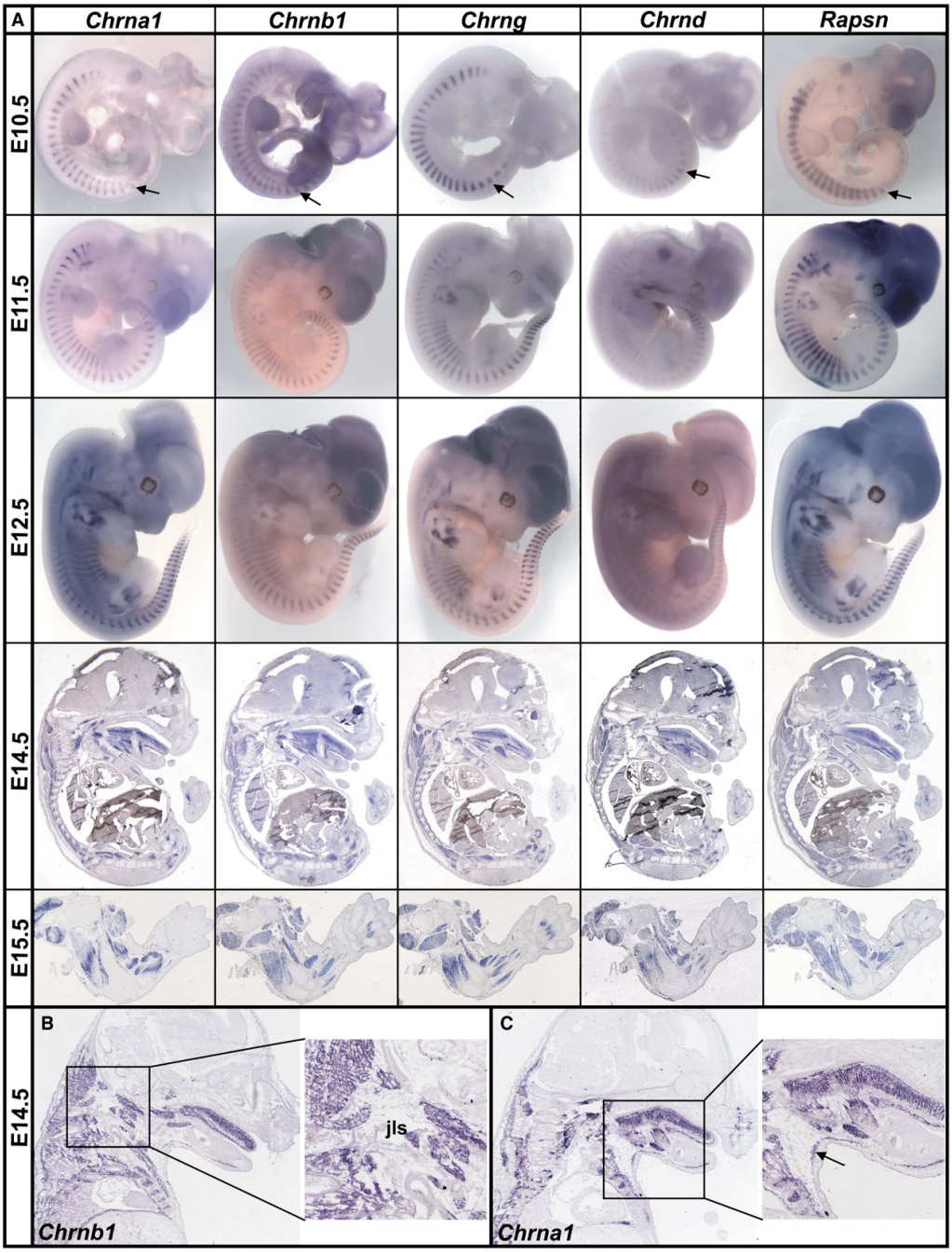

Figure 6. In Situ Hybridization in Mouse Embryos Reveals Early Expression of AChR Subunit Genes and *Rapsn* in Somites and Later in Skeletal Muscle as well as in Hygroma-Relevant Regions

(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/.

Acknowledgments

We thank the patients and their families. We thank Bernhard Hermann for providing clones of in situ probes *Chrna1* and *Chrnb1*. We acknowledge the expert technical assistance by Norbert Brieske, Carola Dietrich, Tanya N. Nelson, and Kathrin Seidel. Friedrich C. Luft, Judith Hall, Eddy Rubin, Martin Digweed, Bernd Wollnik, and Tom H. Lindner critically read the manuscript. The Forschungsförderung of the Charité Medical Faculty supported A.M.; J.M. received a postdoctoral fellowship from the International Centre for Genetic Engineering and Biotechnology, Trieste, Italy. The Deutsche Forschungsgemeinschaft (DFG, SFB 577, project A4) supported K.H., who also received a Rahel Hirsch fellowship from the Charité Medical Faculty.

Received: September 26, 2007
Revised: November 21, 2007
Accepted: November 21, 2007
Published online: February 7, 2008

Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

References

1. Hall, J.G. (1986). Analysis of Pena Shokeir phenotype. Am. J. Med. Genet. *25*, 99–117.
2. Drachman, D.B., and Coulombre, A.J. (1962). Experimental clubfoot and arthrogryposis multiplex congenita. Lancet *2*, 523–526.
3. Polizzi, A., Huson, S.M., and Vincent, A. (2000). Teratogen update: maternal myasthenia gravis as a cause of congenital arthrogryposis. Teratology *62*, 332–341.
4. Dalton, P., Clover, L., Wallerstein, R., Stewart, H., Genzel-Boroviczeny, O., Dean, A., and Vincent, A. (2006). Fetal arthrogryposis and maternal serum antibodies. Neuromuscul. Disord. *16*, 481–491.
5. Hammond, E., and Donnenfeld, A.E. (1995). Fetal akinesia. Obstet. Gynecol. Surv. *50*, 240–249.
6. Romero, N.B., Monnier, N., Viollet, L., Cortey, A., Chevallay, M., Leroy, J.P., Lunardi, J., and Fardeau, M. (2003). Dominant and recessive central core disease associated with RYR1 mutations and fetal akinesia. Brain *126*, 2341–2349.
7. Hall, J.G. (1997). Arthrogryposis multiplex congenita: etiology, genetics, classification, diagnostic approach, and general aspects. J. Pediatr. Orthop. B *6*, 159–166.
8. Morgan, N.V., Brueton, L.A., Cox, P., Greally, M.T., Tolmie, J., Pasha, S., Aligianis, I.A., van Bokhoven, H., Marton, T., Al-Gazali, L., et al. (2006). Mutations in the embryonal subunit of the acetylcholine receptor (CHRNG) cause lethal and Escobar variants of multiple pterygium syndrome. Am. J. Hum. Genet. *79*, 390–395.
9. Hoffmann, K., Muller, J.S., Stricker, S., Megarbane, A., Rajab, A., Lindner, T.H., Cohen, M., Chouery, E., Adaimy, L., Ghanem, I., et al. (2006). Escobar syndrome is a prenatal myasthenia caused by disruption of the acetylcholine receptor fetal gamma subunit. Am. J. Hum. Genet. *79*, 303–312.
10. Hesselmans, L.F., Jennekens, F.G., Van den Oord, C.J., Veldman, H., and Vincent, A. (1993). Development of innervation of skeletal muscle fibers in man: relation to acetylcholine receptors. Anat. Rec. *236*, 553–562.
11. Sanes, J.R., and Lichtman, J.W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat. Rev. Neurosci. *2*, 791–805.
12. Apel, E.D., Roberds, S.L., Campbell, K.P., and Merlie, J.P. (1995). Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron *15*, 115–126.
13. Apel, E.D., Glass, D.J., Moscoso, L.M., Yancopoulos, G.D., and Sanes, J.R. (1997). Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron *18*, 623–635.
14. Witzemann, V. (2006). Development of the neuromuscular junction. Cell Tissue Res. *326*, 263–271.
15. Chen, F., Qian, L., Yang, Z.H., Huang, Y., Ngo, S.T., Ruan, N.J., Wang, J., Schneider, C., Noakes, P.G., Ding, Y.Q., et al. (2007). Rapsyn interaction with calpain stabilizes AChR clusters at the neuromuscular junction. Neuron *55*, 247–260.
16. Engel, A.G., and Sine, S.M. (2005). Current understanding of congenital myasthenic syndromes. Curr. Opin. Pharmacol. *5*, 308–321.
17. Hoffmann, K., Dreger, C.K., Olins, A.L., Olins, D.E., Shultz, L.D., Lucke, B., Karl, H., Kaps, R., Muller, D., Vaya, A., et al. (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.
18. Schwabe, G.C., Trepczik, B., Suring, K., Brieske, N., Tucker, A.S., Sharpe, P.T., Minami, Y., and Mundlos, S. (2004). Ror2 knockout mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome. Dev. Dyn. *229*, 400–410.
19. Brent, A.E., Schweitzer, R., and Tabin, C.J. (2003). A somitic compartment of tendon progenitors. Cell *113*, 235–248.
20. Kues, W.A., Sakmann, B., and Witzemann, V. (1995). Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. Eur. J. Neurosci. *7*, 1376–1385.
21. Ohno, K., and Engel, A.G. (2004). Congenital myasthenic syndromes: gene mutations. Neuromuscul. Disord. *14*, 117–122.
22. Shen, X.M., Ohno, K., Tsujino, A., Brengman, J.M., Gingold, M., Sine, S.M., and Engel, A.G. (2003). Mutation causing severe myasthenia reveals functional asymmetry of AChR signature cystine loops in agonist binding and gating. J. Clin. Invest. *111*, 497–505.
23. Brownlow, S., Webster, R., Croxen, R., Brydson, M., Neville, B., Lin, J.P., Vincent, A., Newsom-Davis, J., and Beeson, D. (2001). Acetylcholine receptor delta subunit mutations underlie a fast-channel myasthenic syndrome and arthrogryposis multiplex congenita. J. Clin. Invest. *108*, 125–130.
24. Shen, X.M., Ohno, K., Fukudome, T., Tsujino, A., Brengman, J.M., De Vivo, D.C., Packer, R.J., and Engel, A.G. (2002). Congenital myasthenic syndrome caused by low-expressor fast-channel AChR delta subunit mutation. Neurology *59*, 1881–1888.
25. Gomez, C.M., Maselli, R.A., Vohra, B.P., Navedo, M., Stiles, J.R., Charnet, P., Schott, K., Rojas, L., Keesey, J., Verity, A., et al. (2002). Novel delta subunit mutation in slow-channel syndrome causes severe weakness by novel mechanisms. Ann. Neurol. *51*, 102–112.
26. Müller, J.S., Baumeister, S.K., Rasic, V.M., Krause, S., Todorovic, S., Kugler, K., Müller-Felber, W., Abicht, A., and Lochmüller, H. (2006). Impaired receptor clustering in congenital myasthenic syndrome with novel RAPSN mutations. Neurology *10*, 1159–1164.
27. Burke, G., Cossins, J., Maxwell, S., Owens, G., Vincent, A., Robb, S., Nicolle, M., Hilton-Jones, D., Newsom-Davis, J., Palace, J., et al. (2003). Rapsyn mutations in hereditary myasthenia: distinct early- and late-onset phenotypes. Neurology *61*, 826–828.
28. Ohno, K., Engel, A.G., Shen, X.M., Selcen, D., Brengman, J., Harper, C.M., Tsujino, A., and Milone, M. (2002). Rapsyn mutations in humans cause endplate acetylcholine-receptor deficiency and myasthenic syndrome. Am. J. Hum. Genet. *70*, 875–885.
29. Ohno, K., and Engel, A.G. (2002). Congenital myasthenic syndromes: genetic defects of the neuromuscular junction. Curr. Neurol. Neurosci. Rep. *2*, 78–88.
30. Hall, J.G. (1984). The lethal multiple pterygium syndromes. Am. J. Med. Genet. *17*, 803–807.

31. Moerman, P., Fryns, J.P., Cornelis, A., Bergmans, G., Vandenberghe, K., and Lauweryns, J.M. (1990). Pathogenesis of the lethal multiple pterygium syndrome. Am. J. Med. Genet. *35*, 415–421.
32. Wilting, J., and Becker, J. (2006). Two endothelial cell lines derived from the somite. Anat. Embryol. (Berl.) *211* (*Suppl 1*), 57–63.
33. Wilting, J., Papoutsi, M., Schneider, M., and Christ, B. (2000). The lymphatic endothelium of the avian wing is of somitic origin. Dev. Dyn. *217*, 271–278.
34. He, L., Papoutsi, M., Huang, R., Tomarev, S.I., Christ, B., Kurz, H., and Wilting, J. (2003). Three different fates of cells migrating from somites into the limb bud. Anat. Embryol. (Berl.) *207*, 29–34.
35. Wilting, J., Aref, Y., Huang, R., Tomarev, S.I., Schweigerer, L., Christ, B., Valasek, P., and Papoutsi, M. (2006). Dual origin of avian lymphatics. Dev. Biol. *292*, 165–173.
36. Sanes, J.R., Apel, E.D., Burgess, R.W., Emerson, R.B., Feng, G., Gautam, M., Glass, D., Grady, R.M., Krejci, E., Lichtman, J.W., et al. (1998). Development of the neuromuscular junction: genetic analysis in mice. J. Physiol. (Paris) *92*, 167–172.
37. Fu, A.K., Ip, F.C., Fu, W.Y., Cheung, J., Wang, J.H., Yung, W.H., and Ip, N.Y. (2005). Aberrant motor axon projection, acetylcholine receptor clustering, and neurotransmission in cyclin-dependent kinase 5 null mice. Proc. Natl. Acad. Sci. USA *102*, 15224–15229.
38. Beeson, D., Higuchi, O., Palace, J., Cossins, J., Spearman, H., Maxwell, S., Newsom-Davis, J., Burke, G., Fawcett, P., Motomura, M., et al. (2006). Dok-7 mutations underlie a neuromuscular junction synaptopathy. Science *313*, 1975–1978.
39. Muller, J.S., Herczegfalvi, A., Vilchez, J.J., Colomer, J., Bachinski, L.L., Mihaylova, V., Santos, M., Schara, U., Deschauer, M., Shevell, M., et al. (2007). Phenotypical spectrum of DOK7 mutations in congenital myasthenic syndromes. Brain *130*, 1497–1506.
40. Palace, J., Lashley, D., Newsom-Davis, J., Cossins, J., Maxwell, S., Kennett, R., Jayawant, S., Yamanashi, Y., and Beeson, D. (2007). Clinical features of the DOK7 neuromuscular junction synaptopathy. Brain *130*, 1507–1515.
41. Antolik, C., Catino, D.H., O'Neill, A.M., Resneck, W.G., Ursitti, J.A., and Bloch, R.J. (2007). The actin binding domain of ACF7 binds directly to the tetratricopeptide repeat domains of rapsyn. Neuroscience *145*, 56–65.

3. Zusammenfassung der Arbeit

Dissertation zur Erlangung des akademischen Grades Dr.med.

Mutationsanalysen in Genen des Acetylcholin-Rezeptor-Pathways in Patienten mit Fetal Akinesia Deformation Sequence (FADS)

eingereicht von:
Anne Michalk

angefertigt an der:
Universitätsklinik und Poliklinik für Endokrinologie und Nephrologie der medizinischen Fakultät der Universität Leipzig

betreut von:
Prof. Dr. med. Tom H. Lindner
Prof. Dr. med. Katrin Hoffmann

eingereicht am: 29.Juni 2017

Mit der vorgelegten Publikationspromotion konnte die Hypothese, dass homozygote Missense- oder Nonsense-Mutationen in den permanenten Untereinheiten des nicotinergen AChR letal verlaufen bestätigt, und das Spektrum an Mutationen im *RAPSN*-Gen erweitert werden.
In der α1-Untereinheit (*CHRNA1*) fanden wir bei Betroffenen die homozygote Missense-Mutation c.761G->T (p.R234L) und die homozygote Frameshift-Mutation c.117-133dup17 (p.H25RfsX19). Weiterhin konnten wir in der δ-Untereinheit (*CHRND*) eine homozygote Nonsense-Mutation *c.234G->A (p.W57X)* und die compound-heterozygote Missense-Nonsense-Mutation c.283T->C (p.F74L) / c.1390C->T (p.R433X) in der δ-Untereinheit (*CHRND*) detektieren.
Die Ergebnisse zeigen, dass eine fetale Akinesie, ein Hydrops fetalis, Kontrakturen und Pterygien durch homozygote und compound-heterozygote funktionelle Nullmutationen im *CHRNA1*- und *CHRND*-Gen verursacht werden. Im Rahmen dieser Arbeit fanden wir

keine Mutation im *CHRNB1*-Gen. Wir vermuten aber, dass homozygote Nullmutationen in der permanenten β1-Untereinheit ebenfalls letal verlaufen.

Bei einem weiteren Patienten mit isolierter fetaler Hypokinesie und Kontrakturen ohne Pterygien wurde in dem Rezeptor-assoziierten *RAPSN*-Gen die bisher unbekannte compound-heterozygote Missense-Mutation c.416T>C (p.F139S) / c.566C->T (p.A189V) nachgewiesen. Diese unterstreicht eindrücklich die Tragweite der Identifizierung einer krankheitsverursachenden Mutation. Der ältere Bruder (II-4) mit gleichem Phänotyp verstarb im Alter von sechs Monaten an den Folgen einer respiratorischen Insuffizienz und konnte nicht von dem Wissen um die Mutation profitieren. Beim zweiten betroffenen Neugeborenen (II-5) konnte nun eine medikamentöse Therapie mit dem Acetylcholinesteraseinhibitor Mestinon® eingeleitet werden. Diese trug nicht nur zu einer neuromuskulären Verbesserung bei, sondern war im Rahmen einer akuten Infektion mit respiratorischer Globalinsuffizienz für das Kind lebensrettend.

Aufgrund dieser klinischen Erfahrungen und der therapeutischen Konsequenzen ist die genetische und funktionelle Diagnostik des Acetylcholinrezeptor–Pathways bei betroffenen Familien mit FADS indiziert.

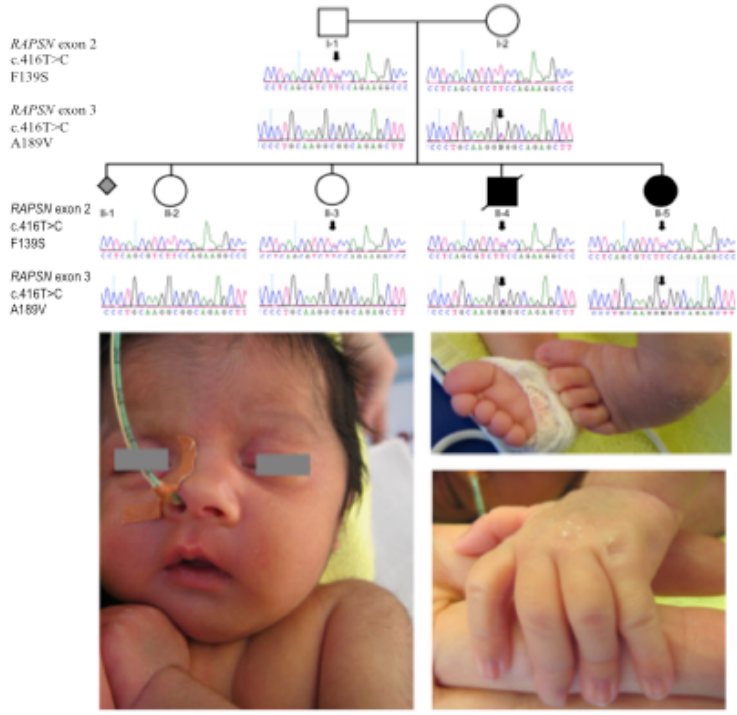

Abb. 7 Identifizierung der Mutation im *RAPSN*-Gen in einer Familie mit einem zuvor verstorbenen männlichen Neugeborenen und einer Totgeburt (II-1). Die Patientin (II-5) bot eine isolierte fetale Hypokinesie und Kontrakturen *ohne* Pterygien. Unter Mestinon®-Therapie kam es zu einer dauerhaften Besserung ihrer muskulären Hypotonie. Während einer Infektions-assoziierten respiratorischen Dekompensation zeigte sie ein sofortiges Ansprechen auf eine Dosiserhöhung des AChE-Inhibitor
(Aus Michalk et al 2008, mit freundlicher Genehmigung von Prof. Heller und Prof. Wieczorek).

Aus humangenetischer Sicht ist es ein wesentliches Ziel, die Diagnose zum frühestmöglichen Zeitpunkt zu stellen und die Prognose möglichst korrekt einzuschätzen. (*Heller et al. 2013*). Das erfordert ein strukturiertes Vorgehen. Unserer Meinung nach ist bei nicht-kritisch kranken Kindern eine Sequenzierung im *CHRNA1-, CHRNB1-, CHRND-, RAPSN-, CHRNG*-Gen und weiterer Pathway-Gene sinnvoll, wenn eine Anamnese mit rezidivierenden Aborten, Hydrops fetalis, Wachstumsretardierung und Arthrogryposis vorliegt. Auch die Suche nach maternalen Antikörpern gegen den fetalen Typ des AChR ist in so einem Fall indiziert. Treten postnatal myasthene Symptome auf, sollte gezielt nach Missense-Mutationen im *CHRNA1-, CHRNB1-, CHRND-, CHRNE*-Gen und in dem Pathway-Gen *RAPSN* gesucht werden.

Die DNA-Sequenzierung mittels diagnostischer Genpanel oder die molekulare Chromosomenanalyse mittels Mikroarray-basierter komparativer genomischer Hybridisierung (CGH) zur genomweiten Detektion von Mikrodeletionen oder Mikroduplikationen sind mittlerweile im klinischen Alltag etabliert (*Kingsmore et al. 2011 / Heller et al. 2013*).

Bis zum heutigen Tag sind mehr als 400 verschiedene Krankheitsbilder mit Arthrogryposis beschrieben. Um zu veranschaulichen, in welche biologischen Prozesse die bisher bekannten 320 krankheitsursächlichen Gene involviert sind, wurde mit Unterstützung der Bioinformatik eine ontologische Analyse durchgeführt (*Hall et al. 2016)*. Die Strukturierung der Daten soll dazu beitragen, die in die Entstehung fetaler Bewegungsstörungen involvierten Mechanismen besser zu verstehen. Genauere Erkenntnisse darüber können uns neue Therapieoptionen eröffnen und präzisere prognostische Aussagen ermöglichen.

Durch die Möglichkeit der Exomsequenzierung entwickelt sich ein heute neues Verständnis für das genetisch sehr heterogene Krankheitsbild der Arthrogryposis. In einem Patientenkollektiv mit Arthrogryposis konnte in 58,3% der Familien eine molekulargenetische Ursache mit Hilfe der Exomsequenzierung ermittelt werden (*Bayram et al. 2016*). Bei unseren Patienten gelang dies mit der Standard-DNA-Sequenzierung nur in circa 8-10% der Patienten. Neben bereits bekannten arthrogryposis-assoziierten Genen konnten so zusätzlich weitere Genloci identifiziert werden. Diese können eine Erklärung für die fehlende Genotyp-Phänotyp-Korrelation und die große inter- und intrafamiläre Variabilität der Erkrankung sein. Desweitern detektiert man neue krankheitsverursachende Gene. Die Exom- bzw. Genomsequenzierung als diagnostische Methode bietet uns daher die Möglichkeit, die Genetik und die molekulargenetischen

Mechanismen besser zu verstehen und somit ein tieferes Verständnis für die vielseitige Ätiologie der Arthrogryposis zu bekommen.

In den kommenden Jahren könnte sich bei kranken Neugeborenen neben dem etablierten Neugeborenenscreening eine Exom- bzw. Genomsequenzierung durchsetzen (*Kingsmore et al. 2015 /Smith et al. 2017/ Berg et al. 2017*). Bisher durchlaufen Neugeborene mit angeborenen Fehlbildungen oder syndromalen Erscheinungsbildern einen aufwendigen und oft langwierigen diagnostischen Prozess, der nicht selten ohne endgültige Diagnose endet. Gerade in Akutsituationen auf einer neonatologischen Intensivstation kann eine definitive Diagnose ein gezieltes Vorgehen ermöglichen. Bestenfalls wie im Falle unserer Familie, bei der nach Detektion der krankheitsverursachenden Mutation im *RAPSN*-Gen eine effektive medikamentöse Therapiemöglichkeit resultierte. Jede neue Erkenntnis erweitert unser Verständnis um dieses heterogene Krankheitsbild und eröffnet eine gezielte, interdisziplinäre medizinische Begleitung der betroffenen Familien. Im Falle einer schnellen genetischen Diagnose kann man bei letaler Diagnose gezielt palliative Maßnahmen einleiten und Familien in ihrem Trauerprozess unterstützen.

Die zeitnahe Diagnose einer seltenen monogenen Erkrankung bei schwerstkranken Neugeborenen ermöglicht mitunter eine gezielte perinatale Behandlung, eine bessere Prognosebeurteilung oder eben auch eine palliative Betreuung (*Kingsmore et al. 2011).*

Literaturverzeichnis

Web Ressource:

OMIM: Online Mendelian Inheritance www.ncbi.nlm.nih.gov/omim/.

pubMed: www.ncbi.nlm.nih.gov/m/pubmed/.

UpToDate: www.uptodate.com.

Referenzen:

1. Antolik C, Catino DH, O'Neill AM, Resneck WG, Ursitti JA, Bloch RJ. The actin binding domain of ACF7 binds directly to the tetratricopeptide repeat domains of rapsyn. Neuroscience 145: 56-65 (2007).
2. Apel ED, Roberds SL, Campbell KP, Merlie JP. Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron 15: 115-126 (1995).
3. Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD, Sanes JR. Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron 18: 623-635 (1997).
4. Bayram et al. Molecular etiology of arthrogryposis in multiple families of mostly Turkish origin. J Clin Invest. 126(2):762-778 (2016).
5. Beeson D, Higuchi O, Palace J, Cossins J, Spearman H, Maxwell S, Newsom-Davis J, Burke G, Fawcett P, Motomura M, et al. Dok-7 mutations underlie a neuromuscular junction synaptopathy. Science 313: 1975-1978 (2006).
6. Berg et al. Newborn Sequencing in Genomic Medicine and Public Health. Pediatrics. Feb;139(2). pii: e20162252 (2017).
7. Bokhoven H, Marton T, Al-Gazali L, et al. Mutations in the embryonal subunit of the acetylcholine receptor (*CHRNG*) cause lethal and Escobar variants of multiple pterygium syndrome. Am J Hum Genet 79: 390-395 (2006).
8. Brent AE, Schweitzer R, Tabin CJ. A somitic compartment of tendon progenitors. Cell 113: 235-248 (2003).
9. Brownlow S, Webster R, Croxen R, Brydson M, Neville B, Lin JP, Vincent A, Newsom Davis J, Beeson D. Acetylcholine receptor delta subunit mutations underlie a fast-channel myasthenic syndrome and arthrogryposis multiplex congenita. J Clin Invest 108: 125-130 (2001).
10. Brueton LA, Huson SM, Cox PM, Shirley I, Thompson EM, Barnes PR, Price J, Newsom-Davis J, Vincent A.Asymptomatic maternal myasthenia as a cause of the Pena-Shokeir phenotype. Am J Med Genet. 92: 1-6 (2000).
11. Brugnoni R, Maggi L, Canioni E, Moroni I, Pantaleoni C, D'Arrigo S, Riva D, Cornelio F, Bernasconi P, Mantegazza R. Identification of previously unreported mutations in *CHRNA1*, *CHRNE* and *RAPSN* genes in three unrelated Italian patients with congenital myasthenic syndromes. J Neurol 257: 1119-1123 (2010).

12. Burke G, Cossins J, Maxwell S, Owens G, Vincent A, Robb S, Nicolle M, Hilton-Jones D, Newsom-Davis J, Palace J, et al. Rapsyn mutations in hereditary myasthenia: distinct early-and late-onset phenotypes. Neurology 61: 826-828 (2003).
13. Chen F, Qian L, Yang ZH, Huang Y, Ngo ST, Ruan NJ, Wang J, Schneider C, Noakes PG, Ding YQ, et al. Rapsyn interaction with calpain stabilizes AChR clusters at the neuromuscular junction. Neuron 55: 247-260 (2007).
14. Croxen R, Newland C, Beeson D, Oosterhuis H, Chauplannaz G, Vincent A, Newsom-Davis J. Mutations in different functional domains of the human muscle acetylcholine receptor alpha subunit in patients with the slow-channel congenital myasthenic syndrome. Hum. Molec. Genet. 6: 767-774 (1997).
15. Dalton P, Clover L, Wallerstein R, Stewart H, Genzel-Boroviczeny O, Dean A, Vincent A. Fetal arthrogryposis and maternal serum antibodies. Neuromuscul Disord 16: 481-491 (2006).
16. Donker M, Eijckelhof B, TanG, de Vries. Serial postural and motor assessment of FADS, Early Human Development 85: 785-790 (2009).
17. Drachman DB, Coulombre AJ. Experimental clubfoot and arthrogryposis multiplex congenita. Lancet 2: 523-526 (1962).
18. ENCODE Project Consortium. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447: 799-816 (2007).
19. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 489: 57-74 (2012).
20. Engel AG, Lambert EH, Gomez MR. A new myasthenic syndrome with end-plate acetylcholinesterase deficiency, small nerve terminals, and reduced acetylcholine release. Ann Neurol. 1: 315-330 (1977).
21. Engel AG, Ohno, K., Milone, M., Wang, H.-L., Nakano, S., Bouzat, C., Pruitt, J. N., II, Hutchinson, D. O., Brengman, J. M., Bren, N., Sieb, J. P., Sine, S. M. New mutations in acetylcholine receptor subunit genes reveal heterogeneity in the slow-channel congenital myasthenic syndrome. Hum Molec Genet 5: 1217-1227 (1996).
22. Engel AG, Sine SM. Current understanding of congenital myasthenic syndromes. Curr Opin Pharmacol 5: 308-321 (2005).
23. Engel AG, et al. Current status of the congenital myasthenic syndromes. Neuromuscul Disord 2: 99-111 (2012).
24. Froehner SC, Gulbrandsen V, Hyman C, Jeng AY, Neubig RR, Cohen JB. Immunofluorescence localization at the mammalian neuromuscular junction of the Mr 43,000 protein of Torpedo postsynaptic membranes. Proc Natl Acad Sci USA 78: 5230-5234 (1981).
25. Fu AK, Ip FC, Fu WY, Cheung J, Wang JH, Yung WH, Ip NY Aberrant motor axon projection, acetylcholine receptor clustering and neurotransmission in cyclin dependent kinase 5 null mice. Proc Natl Acad Sci USA 102: 15224-15229 (2005).
26. Gomez CM, Maselli RA, Vohra BP, Navedo M, Stiles JR, Charnet P, Schott K, Rojas L, Keesey J, Verity A, et al. Novel delta subunit mutation in slow – channel

syndrome causes severe weakness by novel mechanisms. Ann Neurol 51: 102-112 (2002).
27. Hall JG et al. Amyoplasia: a common, sporadic condition with congenital contractures. Am J Med Genet 15:571-590 (1983).
28. Hall JG et al. The lethal multiple pterygium syndromes. Am J Med Genet 17: 803-807 (1984).
29. Hall JG et al. Analysis of Pena Shokeir phenotype. Am J Med Genet 25: 99-117 (1986).
30. Hall JG et al. Arthrogryposis multiplex congenita: etiology, genetics, classification, diagnostic approach and general aspects. J Pediatr Orthop B 6: 159-166 (1997).
31. Hall JG et al. Arthrogryposis (multiple congenital contractures): diagnostic approach to etiology, classification, genetics and general principles. Eur J Med Genet 57: 464-472 (2014).
32. Hall et al Arthrogryposis as a Syndrome: Gene Ontology Analysis. Mol Syndromol. 7(3):101-109 (2016).
33. Hammond E, Donnenfeld AE. Fetal akinesia. Obstet Gynecol Surv 50: 240-249 (1995).
34. He L, Papoutsi M, Huang R, Christ B, Kurz H, Wilting J. Three different fates of cells migrating from somites into the limb bud. Anat Embryol (Berl) 207: 29-34 (2003).
35. Hesselmans LF, Jennekens FG, Van den Oord CJ, Veldman H, Vincent A. Development of innervation of skeletal muscle fibers in man: relation to acetylcholine receptors. Anat Rec 236: 553-562 (1993).
36. Hoellen et al. APC and Pena – Shokeir Phenotyp: Challenge of Prenatal Diagnosis-Report of 21 Cases, Anten Findings and Review, Fetal Diagn Ther 30: 289-298 (2011).
37. Hoffmann K, Dreger CK, Olins AL, Shultz LD, Karl H, Kaps R, Muller D, Vaya A, et al. Mutations in the gene encoding the lamin B receptor produce an altered nuclear morphology in granulocytes (Pelger-Huet anomaly). Nat Genet 31: 410-414 (2002).
38. Hoffmann K, Muller JS, Stricker S, Megarbane A, Rajab A, Lindner TH, Cohen M, Chouery E, Adaimy L, Ghanem I, et al. Escobar syndrome is a prenatal myasthenia caused by disruption of the acetylcholine receptor fetal gamma subunit. Am J Hum Genet 79: 303-312 (2006).
39. Kinali M, Beeson D, Pitt MC, Simonds AK, Aloysius A, Cockerill H, Davis T, Palace J, Manzur AY, Sewry C, Muntoni F, Robb SA. CMS in childhood: Diagnostic and management challenges, J Neuroimmunology 201: 6-12 (2008).
40. Kingsmore SF, Dinwiddie DL, Miller NA, Soden SE, Saunders CJ. Comprehensive genetic testing of mendelian diseases of childhood by next-generation sequencing. Expert Rev Mol Diagn 11: 855-868 (2011).
41. Kingsmore SF, Petrikin J, Willig LK, Guest E. Emergency medical genome: a breakthrough application of precision medicine. Genome Med. 7:82 (2015).

42. Koenen M, Peter C, Villarroel A, Witzemann V, Sakmann B. Acetylcholine receptor channel subtype directs the innervation pattern of skeletal muscle. EMBO Rep 6: 570-576 (2005).
43. Kues WA, Sakmann B, Witzemann V. Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. Eur J Neurosci 7: 1376-1385 (1995).
44. Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF. RNA regulation of epigenetic processes. Bioessays 31: 51-59 (2009).
45. McCarthy et al. Perinatal outcomes of reduced fetal movements: a cohort study. BMC Pregnancy and Childbirth 160:169 (2016).
46. Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS. Specific expression of long noncoding RNAs in the mouse brain. Proc Natl Acad Sci USA 105: 716-721 (2008).
47. Michalk A, Stricker S, Becker J, Rupps R, Pantzar T, Miertus J, Botta G, Naretto VG, Janetzki C, Yaqoob N, Ott CE, Seelow D, Wieczorek D, Fiebig B, Wirth B, Hoopmann M, Walther M, Körber F, Blankenburg M, Mundlos S, Heller R, Hoffmann K. Acetylcholine receptor pathway mutations explain various fetal akinesia deformation sequence disorders. Am J Hum Genet 82: 464-476 (2008).
48. Milone, X.M. Shen, D. SelcenK. Ohno, J. Brengman, S.T. Iannaccone C.M. Harper, A.G. Engel. Myasthenic syndrome due to defects in rapsyn, Neurology 73: 228-235 (2009).
49. Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature. 321: 406-411(1986).
50. Moerman P, Fryns JP, Cornelis A, Vandenberghe K, Lauweryns JM. Pathogenesis of the lethal multiple pterygium syndrome. Am J Med Genet 35: 415-421 (1990).
51. Monnier N, Lunardi J, Marty I, Mezin P, Labarre-Vila A, Dieterich K, Jouk PS. Absence of ß-Tropomyosin is a new cause of Escobar syndrome associated with nemaline myopathy Neuromuslar Disorders 19: 118-123 (2008).
52. Morgan NV, Brueton LA, Cox P, Greally MT, Tolmie J, Pasha S, Aligianis IA, van Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2: 791-805 (2001).
53. Müller JS, Baumeister SK, Rasic VM, Krause S, Todorovic S, Kugler K, Müller-Felber W, Abicht A, Lochmüller H. Impaired receptor clustering in congenital myasthenic syndrome with novel RAPSN mutations. Neurology 67: 1159-1164 (2006).
54. Müller JS, Baumeister SK, Schara U, et al. Impaired receptor clustering in congenital myasthenic syndrome with novel RAPSN mutation. Brain 129: 2784-2793 (2006).
55. Müller JS, Herczegfalvi A, Vilchez JJ, Colomer J, Bachinski LL, Mihaylova V, Santos M, Schara U, Deschauer M, Shevell M, et al. Phenotypical spectrum of DOK7 mutations in congenital myasthenic syndromes. Brain 130: 1497-1506 (2007).
56. Ohno K, Engel AG. Congenital myasthenic syndromes: genetic defects of the neuromuscular junction. Curr Neurol Neurosci Rep 2: 78-88 (2002).

57. Ohno K, Engel AG, Shen XM, Selcen D, Brengman J, Harper CM, Tsujino A, Milone M. Rapsyn mutations in humans cause endplate acetylcholine-receptor deficiency and myasthenic syndrome. Am J Hum Genet 70: 875-885 (2003).
58. Ohno K, Engel AG. Congenital myasthenic syndromes: gene mutations. Neuromuscul Disord 14: 117-22 (2004).
59. Palace J, Lashley D, Newsom – Davis J, Cossins J, Maxwell S, Kennett R, Jayawant S, Yamanashi Y, Beeson D. Clinical features of the DOK7 neuromuscular junction synaptopathy. Brain 130: 1507-1515 (2007).
60. Petronis A, et al. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature 465: 721-727 (2010).
61. Polizzi A, Huson SM, Vincent A. Teratogen update: maternal myasthenia gravis as a cause of congenital arthrogryposis. Teratology 62: 332-341 (2000).
62. Sanes JR, Apel ED, Burgess RW, Emerson RB, Feng G, Gautam M, Glass D, Grady RM, Krejci E, Lichtman JW, et al. Development of the neuromuscular junction: genetic analysis in mice. J Physiol Paris 92: 167-172 (1998).
63. Schwabe GC, Trepczik B, Suring K, Brieske N, Tucker AS, Sharpe PT, Minami Y, Mundlos S. Ror2 knockout mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome. Dev Dyn 229: 400-410 (2004).
64. Shen XM, Ohno K, Fukudome T, Tsujino A, Brengman JM, De Vivo DC, Packer RJ, Engel AG. Congenital myasthenic syndrome caused by low-expressor fast-channel AChR delta subunit mutation. Neurology 59: 1881-1888 (2002).
65. Shen XM, Ohno K, Tsujino A, Brengman JM, Gingold M, Sine SM, Engel AG. Mutation causing severe myasthenia reveals functional asymmetry of AChR signature cystine loops in agonist binding and gating. J Clin Invest 111: 497-505 (2003).
66. Shen XM, Brengman, JM, Edvardson S, Sine SM, Engel AG. Highly fatal fast-channelsyndrome caused by AChR-epsilon subunit mutation at the agonist binding site. Neurology 79: 449-454 (2012).
67. Sine SM, Ohno K, Auerbach A, Milone M, Pruitt JN, Engel AG. Mutation of the acetylcholine receptor alpha subunit causes a slow-channel myasthenic syndrome by enhancing agonist binding affinity. Neuron 15: 229-239 (1995).
68. Slatkin M. Epigenetic inheritance and the missing heritability problem. Genetics 182: 845-850 (2009).
69. Smith et al. Whole-Exome Sequencing and Whole-Genome Sequencing in Critically Ill Neonates Suspected to Have Single-Gene Disorders. Cold Spring Harb Perspect Med. 6(2):a023168 (2016).
70. Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS. Non-coding RNAs: regulators of disease. J Pathol 220: 126-139 (2010).
71. Takahashi M, Kubo T, Mizoguchi A, Carlson CG, Endo K, Ohnishi K. Spontaneous muscle action potentials fail to develop without fetal-type acetylcholine receptors. EMBO Rep. 3: 674-681 (2002).
72. Uchitel O, Engel AG, Walls TJ, Nagel A, Atassi MZ, Bril V. Congenital myasthenic syndromes: II. Syndrome attributed to abnormal interaction of acetylcholine with its receptor. Muscle Nerve. 16: 1293-301 (1993).
73. Vernet – der Garabedian, et al. Development 124: 5075-5086 (1994).

74. Vogt J, Harrison B, Spearman H, Cossins J, Vermeer S, Naudin ten Cate, Morgan N, Beeson D, Maher E. Mutation Analysis of *CHRNA1*, *CHRNB1*, *CHRND* und *RAPSN* Genes in MPS/FADS Patients, AJHG 82: 222-227 (2008).
75. Vogt J, Morgan NV, Marton T, Maxwell S, Harrison BJ, Beeson D, Maher ER. Germline mutation in DOK7 associated with fetal akinesia deformation sequence. J. Med Genet 46: 338-340 (2009).
76. Vogt J, et al. *CHRNG* genotype-phenotype correlations in the multiple pterygium syndromes, J Med Genet 49: 21-26 (2012).
77. Wang K, Li M, Hakonarson H. Analysing biological pathways in genome-wide association studies. Nat Rev Genet 11: 843-854 (2010).
78. Wilting J, Papoutsi M, Schneider M, Christ B. The lymphatic endothelium of the avian wing is of somitic origin. Dev Dyn 217: 271 – 280 (2000).
79. Wilting J, Aref Y, Huang R, Tomarev SI, Schweigerer L, Christ B, Valasek P, Papoutsi M. Dual origin of avian lymphatics. Dev Biol 292: 165-173 (2006).
80. Wilting J, Becker J. Two endothelial cell lines derived from the somite. Anat Embryol (Berl) 211 Suppl 1: 57-63 (2006).
81. Witzemann V, et al. Development of the neuromuscular junction. Cell Tissue Res 326: 263-271 (2006).

Anlagen

Mutationsanalysen in Genen des Acetylcholin-Rezeptor-Pathways in Patienten mit Fetal Akinesia Deformation Sequence (FADS)

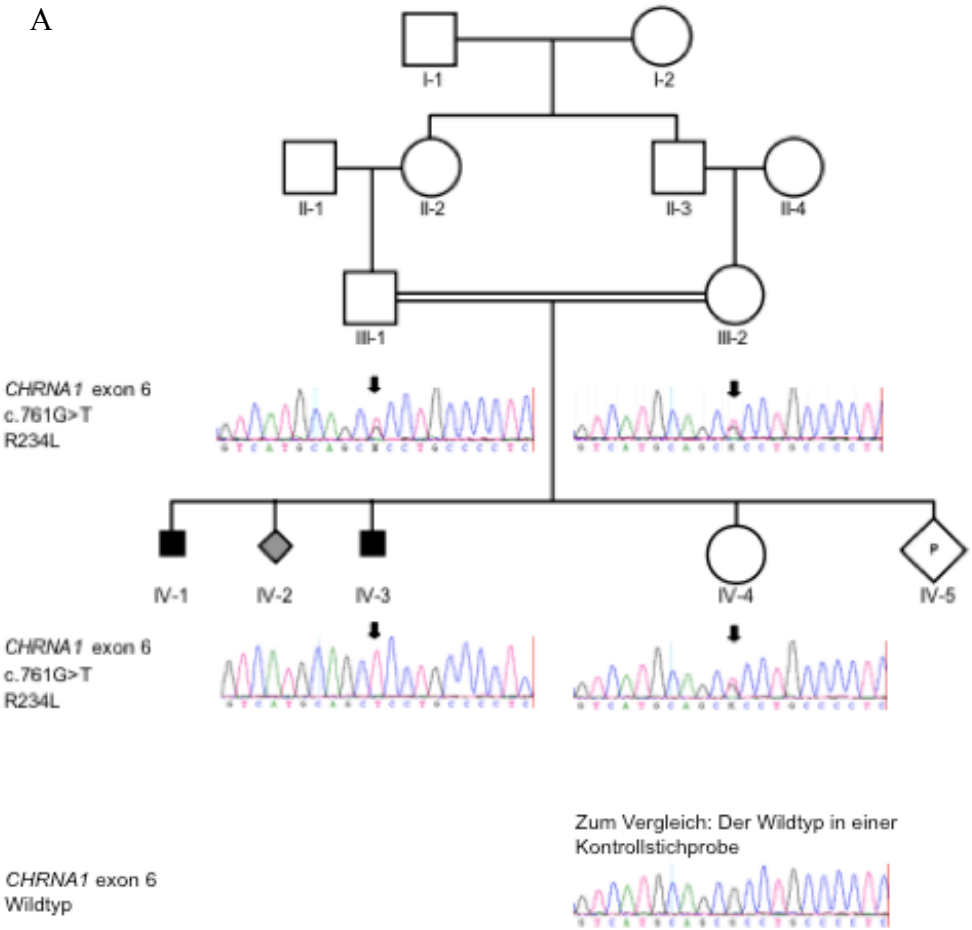

Abb. A Sequenzanalysen und Stammbaum der Familie *CHRNA1*-F1. Der Stammbaum dieser konsanguinen Familie zeigt Betroffene mit fetaler Akinesie und letalen Multiple Pterygium Syndrome. Die Sequenzierung offenbart die Missense-Mutation *CHRNA1* c.761G>T (R234L) im Exon 6 des Gens. Der betroffene Fetus IV-3 war homozygot für diese Mutation, wohingegen beide Eltern und ein gesundes Geschwisterkind heterozygote Träger der genetischen Alteration sind. Die Kontrollprobe zeigt den Wildtyp der Sequenz.

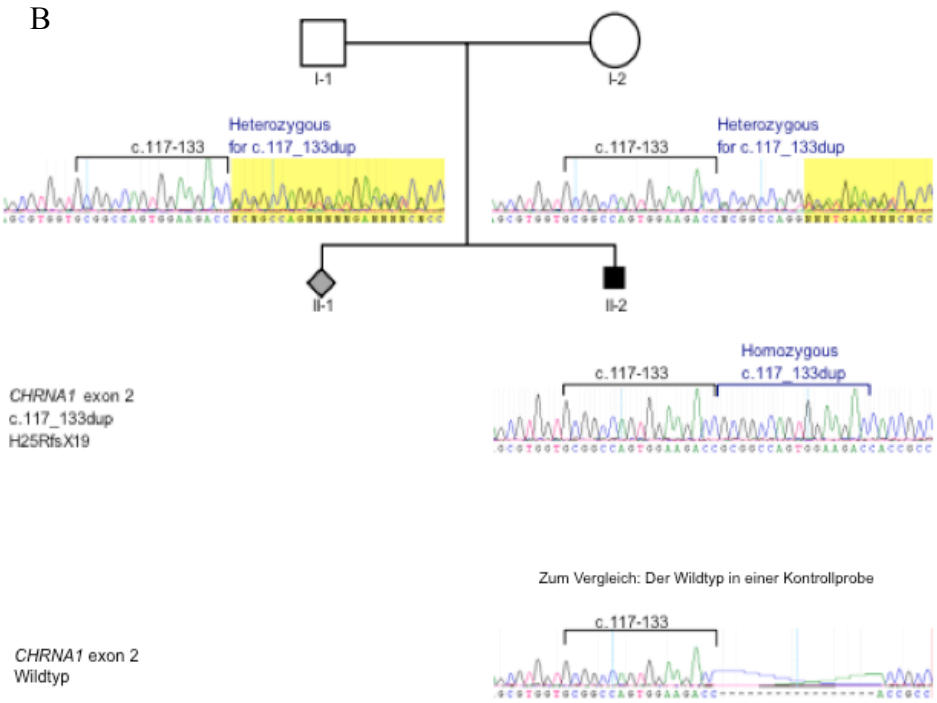

Abb. B Sequenzanalysen und Stammbaum der Familie *CHRNA1*-F2. Die Sequenzierung offenbart bei dem betroffenen Kind II-2 die homozygote Nonsense-Mutation *CHRNA1* c.117_133dup (H25RfsX19) im Exon 2 des Gens. Es resultiert eine Framshift-Mutation mit vorzeitigem Stopcodon. Beide Eltern I-1 und I-2 sind heterozygote Träger der Mutation. Die Kontrollprobe zeigt den Wildtyp der Sequenz.

C

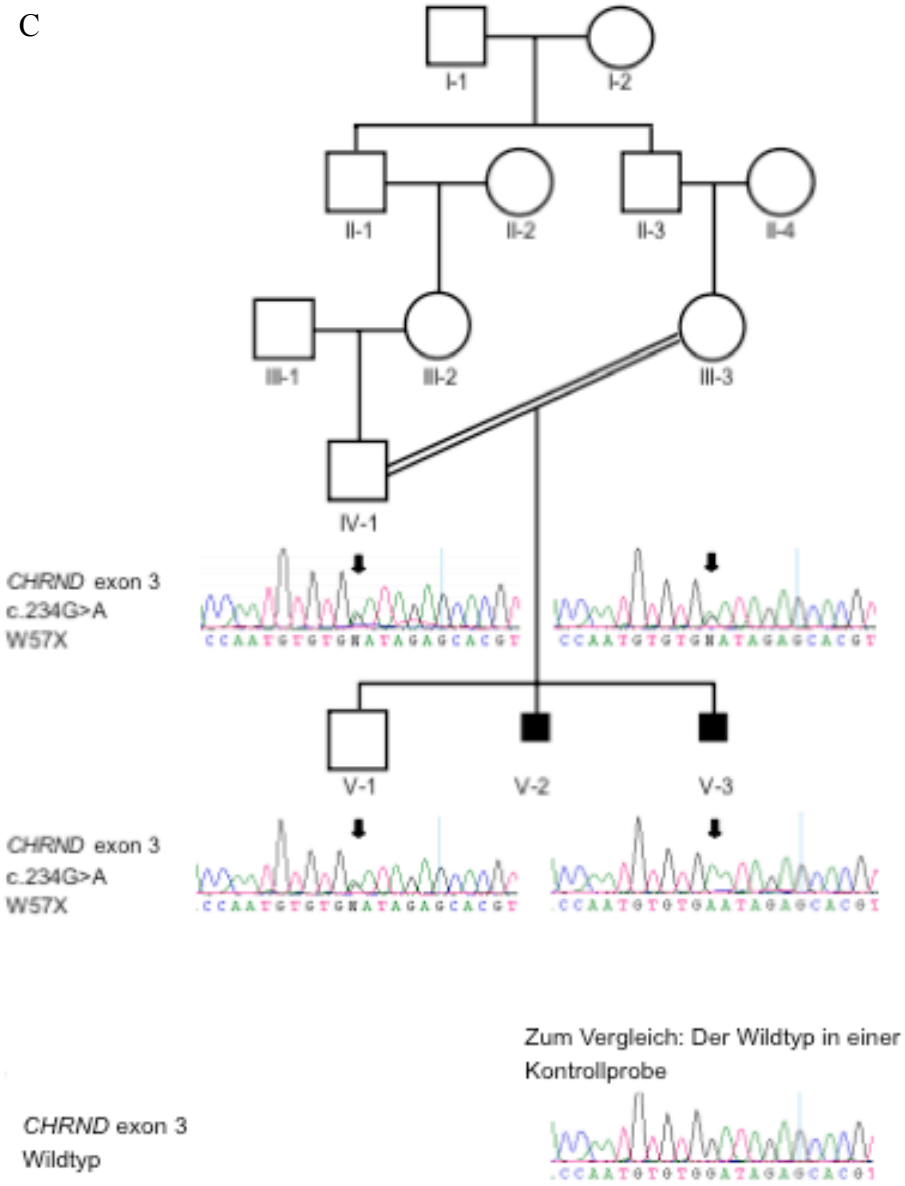

Abb. C Sequenzanalysen und Stammbaum der Familie *CHRND*-F1. Der Stammbaum dieser konsanguinen Familie zeigt zwei Betroffene mit dem letalen Multiple Pterygium Syndrome. Die Sequenzierung offenbart bei dem betroffenen Fetus V-3 die homozygote Nonsense-Mutation *CHRND* c.234G>A (W57X) im Exon 3 des Gens. Beide Eltern IV-1 und III-3 und ein gesundes Geschwisterkind V-1 sind hingegen heterozygote Träger der genetischen Alteration. Die Kontrollprobe zeigt den Wildtyp der Sequenz.

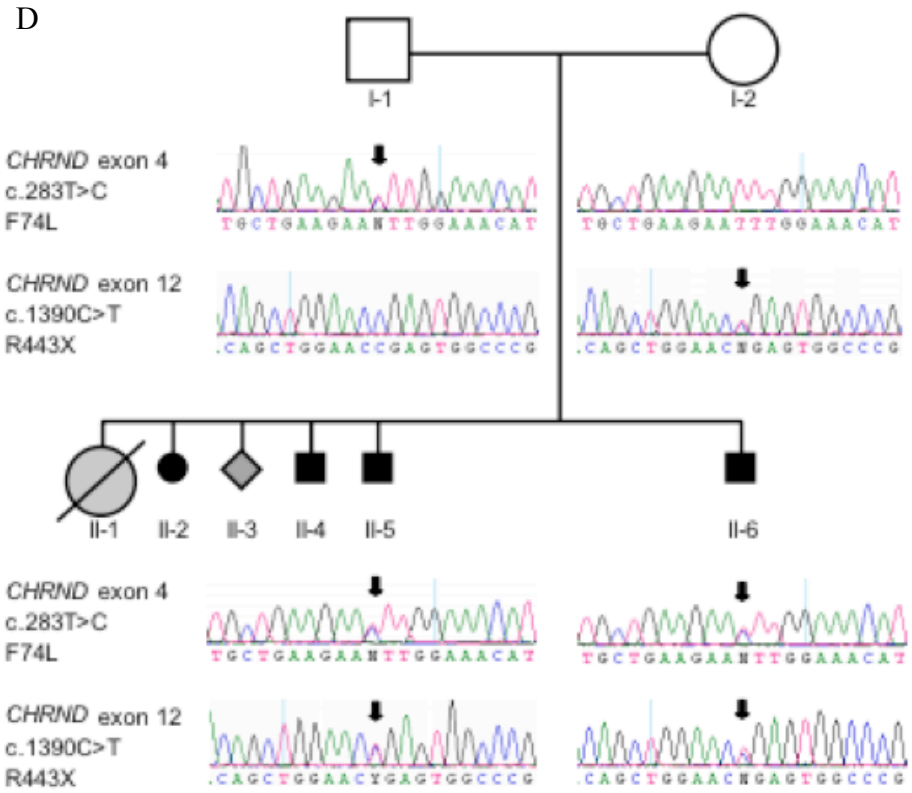

Abb. D Sequenzanalysen und Stammbaum der Familie *CHRND*-F2. Der Stammbaum dieser Familie zeigt zahlreiche Betroffene mit fetaler Akinesie und letalen Multiple Pterygium Syndrome. Die betroffenen Feten II-5 und II-6 sind compound heterozygot für die Missense-Mutation *CHRND* c.283T>C (F74L) in Exon 4 und die Nonsense-Mutation *CHRND* c.1390C>T (R443X) in Exon 12 des Gens. Beide Eltern I-1 und I-2 sind jeweils heterozygoter Träger einer dieser Mutationen.

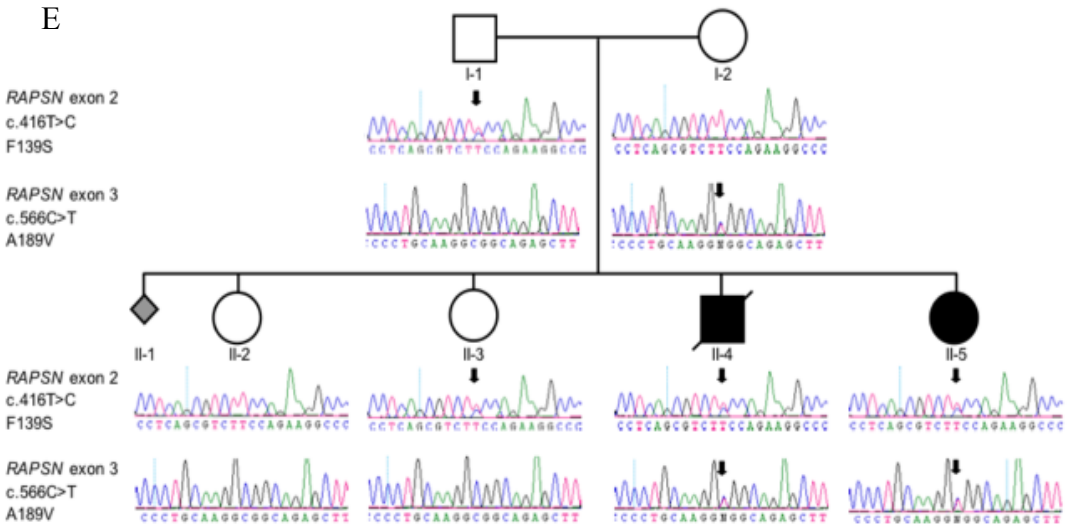

Abb. E Sequenzanalysen und Stammbaum der Familie *RAPSN*-F1. Der Stammbaum zeigt neben zwei gesunden Mädchen II-2 und II-3, einen Frühabort II-1 und zwei Betroffene II-4 und II-5 mit fetaler Akinesie. Diese weisen die compound heterozygote Missense-Mutation *RAPSN* c.416T>C (F139S) im Exon 2 und *RAPSN* c.566C->T (A189V) im Exon 3 des Gens auf. Die genetische Diagnosestellung ermöglichte eine Therapie des zweiten betroffenen Kindes II-5 der Familie, während das erste betroffene Kind II-4 an respiratorischer Insuffizienz verstarb. Beide Eltern I-1, I-2 und eines der gesunden Geschwister II-3 sind jeweils heterozygoter Träger dieser Mutationen. Ein gesundes Mädchen II-2 trägt keine der heterozygoten Veränderungen.

Ergebnisse am Mausmodell (Arbeiten von S. Stricker)

Im Mausmodell wiesen wir die Expression dieser AChR-Pathway-Komponenten im Skelettmuskel aber auch in sehr frühen Entwicklungsstadien in Somiten nach. Das könnte wegweisend für die Frage nach der Genese der Frühaborte und der häufig assoziierten frühen Fehlbildungen wie Ödeme, Hydrops, Herzfehler und Nierenfehlbildungen bei Patienten mit fetalem Akinesie-Syndrom sein. Die Studien könnten dafür sprechen, dass die einzelnen Untereinheiten noch vor der Zusammenlagerung zum klassischen Acetylcholinrezeptor in einer anderen Funktion agieren.

Übersichtstabelle der detektierten Mutationen im *CHRNA1, CHRND, RAPSN* und *CHRNG-Gen*.

	Gen	Exon	Veränderung auf Nukleotidebene	Veränderung auf Proteinebene (ohne Signalpeptid)
1.	*CHRNA1*	2	c.117_133dup	H25RfsX19
2.		6	c.761G>T	R234L
3.	*CHRND*	3	c.234G>A	W57X
4.		4+12	c.283T>C	F74L
5.			c.1390C>T	R433X
6.	*RAPSN*	2+3	c.416T>C	F139S
7.			c.566C>T	A189V
8.	*CHRNG*	1	c.13C>T	p.Q-18X
9.		4+5	c.256C>T	p.R64C
10.			c.481G>A	p.W139X
11.		4+5	c.300dup(9)	78dup(3)
12.			c.1408C>T	R448X
13.		4+5	c.446G>A	A127T
14.		6	c.259G>A	E169E
15.		7	c.715C>T	R217C
16.		8	c.807insT	Δ248-274,275X
17.		10	c.1249G>C	Δ395-418,419X
18.		10	1242G>C	E414Q
19.		12	c.1408C>T	A448X
20.		12	c.1509C>T	P481S

Tabelle 1 Übersichtstabelle der detektierten Mutationen. Im Rahmen der Arbeit am Institut für medizinische Genetik wurden eine Vielzahl an krankheitsverursachenden Mutationen bei Patienten mit der fetalen Akinesia Deformation Sequence (FADS) detektiert. Diese sind hier tabellarisch zusammengetragen. Bisher nicht publizierte Mutationen sind blau hinterlegt.

Darstellung des eigenen wissenschaftlichen Beitrags

American Journal of Human Genetic

Acetylcholine Receptor Pathway Mutations Explain Various Fetal Akinesia Deformation Sequence Disorders

Anne Michalk, Sigmar Stricker**, Jutta Becker, Rosemarie Rupps, Tapio Pantzar, Jan Miertus, Giovanni Botta, Valeria G. Naretto, Catrin Janetzki, Nausheen Yaqoob, Claus-Eric Ott, Dominik Seelow, Dagmar Wieczorek, Britta Fiebig, Brunhilde Wirth, Markus Hoopmann, Marisa Walther, Friederike Korber, Markus Blankenburg, Stefan Mundlos, Raoul Heller and Katrin Hoffmann.

**contributed equally*

Anne Michalk forschte während ihrer Promotionsarbeit auf dem Gebiet der fetalen Bewegungsstörungen. Ein Teil ihrer Ergebnisse erschien im American Journal of Human Genetic. Sie hat dazu folgende wissenschaftliche Beiträge geleistet:

1. Die genetische Feinkartierung und Haplotypisierung in den betroffenen Familien.
2. Die Sequenzierung positioneller und funktioneller Kandidatengene, namentlich *CHRNA1 CHRNB, CHRND, CHRNG, RAPSN, MUSK, AGRIN* und *DOK7*.
    - Primer-Design
    - Etablierung und Sequenzierung
    - Sequenz-Auswertung
3. Die Interpretation detektierter Veränderungen.
    - Datenbank-Recherchen
    - Evolutionäre Konservierung
    - Segregationsanalysen innerhalb der Familien
    - Untersuchung von 300 Kontroll-Chromosomen z.A. eines Polymorphismus
4. Mitarbeit beim Genotyp-Phänotyp-Vergleich.
5. Mitarbeit bei der Manuskripterstellung inklusive der Erstellung von:
    - Abbildung 1. Schematische Darstellung des AChR-Komplexes
    - Abbildung 2. Stammbäume der Familien mit Mutationen
    - Abbildung 5. Evolutionäre Konservierung der Missense-Mutationen
    - Tabelle 1. Klinische Merkmale der Patienten mit schwerer FADS
    - Tabelle 2. AChR-Pathway Mutationen in Familien mit schwerer FADS

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

.....	.....
Datum	Unterschrift

Lebenslauf

Danksagung

Ich bedanke mich herzlich bei allen teilnehmenden Patienten. Ich hoffe, dass die Ergebnisse meiner Arbeit einigen Familien Antworten auf ihre Fragen geben können und wichtige, wenn auch kleine Stufen auf dem Weg zu einer verbesserten genetischen Beratung und einer spezifischen Therapiemöglichkeit darstellen.

Danken möchte ich auch den Kollegen der Arbeitsgruppe am Institut für Medizinische Genetik und allen Koautoren für ihre konstruktiven Kommentare und ihr stets offenes Ohr.

Ich bedanke mich besonders bei Frau Prof. Dr. med. Hoffmann, dass ich in ihrer Arbeitsgruppe am Institut für Medizinische Genetik der Charité mitarbeiten und in diesem Rahmen promovieren durfte. Ich blicke zurück auf eine sehr spannende, intensive Zeit, in der sie immer für Fragen, Hilfestellungen und Ratschläge offen war. Durch ihre große Expertise in der genetischen Forschung und ihr umfassendes Verständnis für anspruchsvolle wissenschaftliche Publikationen hat sie mich weiter vorangebracht, als ich je erwartet hätte. Ein ganz besonderer Dank gilt ihrer fast endlosen Geduld.

Weiterhin möchte ich mich sehr bei meinem Chefarzt Prof. Dr. med. Haas für sein Engagement und seine Beharrlichkeit bedanken.

Letztendlich gilt mein Dank Herrn Professor Dr. med. Lindner, der mir die Möglichkeit gab diese schöne Arbeit zu einem guten Ende zu bringen.