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Dihydropyridine receptor (DHPR) congenital myopathy

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Abstract

Muscle contraction upon nerve stimulation relies on excitation-contraction coupling (ECC) to promote the rapid and generalized release of calcium within myofibers. In skeletal muscle, ECC is performed by the direct coupling of a voltage-gated L-type Ca^{2+} channel (dihydropyridine receptor; DHPR) located on the T-tubule with a Ca^{2+} release channel (ryanodine receptor; RYR1) on the sarcoplasmic reticulum (SR) component of the triad. Here we characterize a novel class of congenital myopathy at the morphological, molecular and functional level. We describe a cohort of 11 patients from 7 families presenting with perinatal hypotonia, severe axial and generalized weakness. Ophthalmoplegia is present in 4 patients. The analysis of muscle biopsies demonstrated a characteristic intermyofibrillar network due to SR dilatation in some samples, internal nuclei and areas of myofibrillar disorganization. Exome sequencing revealed 10 recessive or dominant mutations in *CACNA1S* ($Ca_v1.1$), the pore-forming subunit of DHPR in skeletal muscle. Both recessive and dominant mutations correlated with a consistent phenotype, a decrease in protein level, and with a major impairment of Ca^{2+} release induced by depolarization in cultured myotubes. While dominant *CACNA1S* mutations were previously linked to malignant hyperthermia susceptibility or hypokalemic periodic paralysis, our findings strengthen the importance of DHPR for perinatal muscle function in human. These data also highlight CACNA1S and ECC as therapeutic targets for the development of treatments that may be facilitated by previous knowledge accumulated on DHPR.

Keywords: DHPR; Congenital myopathy; excitation–contraction coupling; triad; centronuclear myopathy; core myopathy; myotubular myopathy

Introduction

Excitation–contraction coupling (ECC) is the process by which an action potential triggered by nerve-induced muscle membrane depolarization causes the muscle fiber to contract. In skeletal muscle, ECC relies on a direct coupling between a Ca^{2+} release channel (the ryanodine receptor; RYR1) located on the sarcoplasmic reticulum (SR), and a voltage-gated L-type Ca^{2+} channel (the dihydropyridine receptor ; DHPR) on the T-tubule [27]. ECC occurs at the triad, a specialized membrane structure formed by the apposition of a sarcolemma invagination (the T-tubule) and two SR saccules containing RyRs [9]. Upon depolarization, activation of the DHPR induces the opening of RyR1 and the release of Ca^{2+} from the SR stores, subsequently triggering muscle contraction.

The *CACNA1S* ($Ca_v1.1$) gene encodes the pore-forming subunit of DHPR in skeletal muscle. It is composed of 44 exons and has an embryonic splice variant lacking the in-frame exon 29. CACNA1S is a 1873 amino acids protein with four transmembrane domains (I-IV), each with six transmembrane segments (S1-S6) [33]. The loop between domains II and III is instrumental in mediating the activation of RYR1 [18,27,7]. The importance of CACNA1S in ECC has been demonstrated *in cellula* and also *in vivo*. Cells lacking CACNA1S have impaired ECC, and mice and drosophila with defective CACNA1S (*Dmca1D* ortholog in the fly) are embryonically lethal [8,15,4]. In adult mice, CACNA1S appears to control muscle mass as acute downregulation of *Cacna1s* leads to massive atrophy, although ECC was functional [24].

Heterozygous dominantly acting *CACNA1S* mutations have been previously associated either with malignant hyperthermia susceptibility (MHS5; MIM#601887) triggered by volatile anaesthetics [20], hypokalemic periodic paralysis (HOKPP; MIM#170400) characterized by periodic attacks of muscle weakness associated with a decrease in the serum potassium level [14,26], and thyrotoxic periodic paralysis (TTPP1; MIM#188580) [17]. Moreover, alternative splicing of exon 29 is repressed in patients with myotonic dystrophy and correlates with increased channel conductance and voltage sensitivity [31]. However, the link between mutations of DHPR and a myopathy is not yet fully established. More generally, triad defects due to mutations in other genes encoding for the ECC machinery have been reported in a sub-class of myopathies known as triadopathies, while secondary triad abnormalities may be observed in several other muscle diseases [1,5].

In this study we analyzed a cohort of patients with congenital myopathy, a clinically and genetically heterogeneous group of muscle diseases of variable severity, characterized by antenatal, neonatal or early onset, ranging from severe fetal akinesia to milder forms of hypotonia and muscle weakness, in conjunction with the presence of characterisitic morphological hallmarks on skeletal muscle biopsy [21,29]. Histopathologically and ultrastructurally, they can be sub-classified into nemaline myopathies with rods inclusions and/or protein aggregates, core myopathies with well-defined muscle fiber areas devoid of oxidative enzyme activity, centronuclear myopathy with several fibers with internal and centralized nuclei. Fiber type disproportion with a bias toward smaller and more abundant type 1 fibers is a feature common in several forms [22]. Advances in recent genetic technologies, combined with extensive studies of large families and well-defined cohorts led to the identification of approximately 30 causative congenital myopathy genes (www.musclegenetable.fr), of which a majority encode for proteins associated with the sarcomere structure or its stability [21]. Despite these advances, approximately half of patients with congenital myopathies remain without a confirmed genetic diagnosis, which is essential for appropriate disease management, prognosis, genetic counseling and development of therapies [2].

Here we identified both recessive and dominant *CACNA1S* mutations by exome sequencing as a cause of a congenital myopathy characterized by specific morphological hallmarks in a cohort of 11 patients from 7 families.

Material and methods

Patients

Eleven patients from 7 unrelated families from Caucasian, Argentinean or Vietnamese origin were included in the present study. The cohort comprises 3 sporadic cases (patients P2, P5 and P9), 2 families with dominant inheritance (P6-8; P10 and P11), and 2 families with several affected siblings and recessive inheritance (P1; P3 and P4). All patients presented with a clinical history of a congenital myopathy and underwent detailed clinical examinations. Clinical, histopathological and genetic features are summarized in Table 1. Sample collection was performed with written informed consent from the patients or parents according to the declaration of Helsinki.

Muscle Imaging

CT scan for P1 and MRI for P2, P4, P5, P7, P8, P9, P10, P11 were performed. T1-STIR was performed for P4, P5, P7, P8, P9, P10 and P11.

Exome sequencing and analysis

Genomic DNA of patients and relatives was isolated from peripheral blood by phenol-chloroform extraction. DNA storage and usage was IRB approved (DC-2012-1693 and 12-N-0095 for NIH). Exome sequencing (ES) was performed for all living patients, the parents of families 1 and 6, the mother of P3 and P4 in family 3, the father of P7 and P8 in family 5. ES was performed at CNG (Evry, France), at DeCode as part of Neuromics (Reykjavik, Iceland), at the Broad Institute (Cambridge, USA) and at the UCLA (Los Angeles, USA). Fragmented genomic DNA was enriched for exons using the SureSelect Human all Exon 50Mb capture library v5 (Agilent, Santa Clara, USA) and sequenced 90nt paired-end on Illumina HiSeq2000/2500 sequencers.

Sequence data were aligned to the GRCh37/hg19 reference genome using the Burrows-Wheeler aligner software (http://bio-bwa.sourceforge.net), and variant calling was performed with SAMtools or the UnifiedGenotyper (https://www.broadinstitute.org/gatk). The following databases were used for variant annotation and filtering: Exome Variant Server (http://evs.gs.washington.edu/EVS/), ExAC Browser (http://exac.broadinstitute.org/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 genomes (http://www.1000genomes.org/), as well as in-house exome databases. Variants ranking was performed with VaRank [11].

Confirmation of variants was performed by Sanger sequencing. Haplotype analysis between families 1 and 2 was performed using four (CA)n microsatellites flanking the *CACNA1S* gene, which are localized 39kb and 25 kb upstream and 3.5kb and 18 kb downstream respectively (coordinates, primers and conditions are available on request).

Morphological studies

An open deltoid muscle biopsy was performed for patients P1, 2, 4, 6, 7, 9 following informed consent. Age at muscle biopsy varied from 6 months to 60 years. A needle quadriceps biopsy was performed on patient 5 at 9 months and a bicep brachii biopsy was performed for P10 at 44 years of age. For conventional histochemical techniques 10 µm cryostat sections were stained with haematoxylin and eosin (H&E), modified Gomori trichrome (mGT), Periodic acid Schiff technique (PAS), Oil red O, reduced nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH), Cytochrome Oxidase (COX), the Menadione-nitro blue tetrazolium and Adenosine triphosphatase (ATPase) preincubated at pH 9.4, 4.63, or 4.35. Immunocytochemistry analysis using antibodies against different myosins was performed for patient 5.

Digital photographs were obtained with a Zeiss AxioCam HRc linked to a Zeiss Axioplan Bright Field Microscope and processed with the Axio Vision 4.4 software (Zeiss, Germany) for the majority of patients with exception of patient 5 where a digital Leica scanner was used.

Electron microscopy

Electron microscopy analysis was performed in patients P1, 2, 4, 6, 7, 9, 10 Muscle specimens were fixed with glutaraldehyde (2.5%, pH 7.4), post fixed with osmium tetroxide (2%), dehydrated and embedded in resin (EMBed-812, Electron Microscopy Sciences, USA). Ultra-thin sections from three blocks from each patient were stained with uranyl acetate and lead citrate. The grids were observed using a Philips CM120 electron microscope (80 kV; Philips Electronics NV, Eindhoven, The Netherlands) and were photo documented using a Morada camera (Soft Imaging System, France).

Immunohistochemistry and immunofluorescence

Frozen muscle samples for immunohistochemical and immunofluorescence analyses were available for 5 patients (P1, 5, 6, 7, 9). Fast, slow, developmental, neonatal myosin heavy chain (NCL-MHCn, Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom) antibodies were applied to 10 µm thick cryosections, and revealed by immunoperoxidase techniques (ROCHE-Benchmark XT). For patient 5 was used a manual labelling with a biotinylated secondary conjugated to peroxidase. Additionally, immunofluorescence analyses were assessed on 8-µm-thick cryosections overnight at 4°C using different antibodies as Myosin alpha and beta-slow heavy chain, fast 2A heavy chain, and 2X myosin heavy chain (BA-D5, SC-71, and 6H1, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA), CACNA1S antibody (ab2862 mouse monoclonal, Abcam, Cambridge, UK), Ryanodine Receptor 1 (C-terminal rabbit polyclonal), triadin (kind gift from Isabelle Marty, rabbit polyclonal) [28]. Subsequently, sections were incubated with appropriate conjugated secondary antibodies during one hour. A set of control slides was prepared with omission of the primary antibodies, and using aged-matched non-myopathic muscles.

RNA studies

RNA was extracted from muscle samples of P1 and P3 and from myotubes differentiated from fibroblasts infected by a MyoD expressing lentivirus for P5 using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, USA) according to the manufacturer's instructions. Reverse transcription was done using random and oligo-d(T) primers. Amplification of the regions of interest was performed with specific *CACNA1S* primers (Supplementary table 1) and PCR products were Sanger-sequenced.

Western Blot

Cryostat sections of fresh frozen muscle tissue were lysed in a buffer containing 2% SDS, 5% beta-mercaptoethanol, and 62.5mM tris HCL ph6.8 with a mix of protease inhibitors (Sigma). Samples were quickly micro-centrifuged. Laemmli buffer was added and samples were denatured at room temperature. Total protein extract were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes, blocked with nonfat milk, and incubated overnight with anti-CACNA1S antibody. Immunoblots were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Saint Quentin in Yvelines, France) on a G-Box system using GeneSnap software (Ozyme, Montigny le Bretonneux, France).

Myotubes from patient P1 and from healthy controls were lysed in a buffer containing 50mM Tris, 100 mM NaCl, 1 mM EGTA, 0,5% NP-40, 0,5% Triton-X100, 0,1% SDS, 1mM DTT, 1mM PMSF and a mix of protease inhibitor (Complete EDTA-free, Roche) and denatured in Laemmli buffer at 95°C. Total protein extracts were separated on 6% SDS-PAGE gel and transferred to a nitrocellulose membrane. Detection of CACNA1S was done with an antibody targeting the middle part of the protein (HPA048892, Sigma, St-Louis, USA). Loading control was verified with the use of a stain-free technique (Trichloroethylene, Sigma, St-Louis, USA) and imaged were acquired with the ChemiDoc Touch Imaging System (Bio-Rad).

Cell culture and imaging

Muscle cells were established from patient P1 by the Cochin Center (Paris, France). Cells were plated on a thin matrigel layer on 35 mm dishes (Ibidi, Martinsried, Germany) with proliferation medium (DMEM/HAMF10 (1:1), 20% FCS, HEPES 10 mM, Penicillin 100 UI/mL, Streptomycin 100 µg/mL), then differentiated into myotubes at 80% confluence by changing the medium (DMEM, 2% HS, Penicillin 100 UI/mL, Streptomycin 100 µg/mL). The next day, an upper layer of matrigel was added and the differentiation medium was changed every two days for 10 days. Myotubes were fixed with 4% PFA and incubated with antibodies against CACNA1S (HPA048892, Sigma, St-Louis, USA) and RYR1 (R129, Sigma St-Louis, USA).

Intracellular calcium measurements

Patient P1, P10 and control muscle cells were transferred onto glass coverslips and allowed to proliferate in growth medium until visible groups of > 10 cells were formed. The medium was then switched to differentiation medium, and Ca^{2+} measurements were performed 7–10 days later. Free

cytosolic [Ca^{2+}] was determined using the fluorescent Ca^{2+} indicator Fura-2 as described.[3] Coverslips were mounted onto a 37 °C thermostat chamber, which was continuously perfused with Krebs Ringer medium. Individual cells were stimulated with a 12-way 100-mm diameter quartz micromanifold computer-controlled microperfuser (ALA Scientific) as previously described.[32] On-line (340 nm, 380 nm, and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a x20 water-immersion FLUAR objective (0.17 numerical aperture) with filters (BP 340/380, FT 425, and BP 500/530) and attached to a Cascade 125+ charge-coupled device camera. The cells were analyzed using Metamorph (Molecular Devices) imaging system, and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm. Ca^{2+} calibration was performed as previously decribed (Sekulic-Jablanovic et al., 2016).

Results

Characterization of a cohort of patients with congenital myopathy

Our cohort is composed of 11 patients from 7 unrelated families and includes 4 females (P3, P4, P6, P11) and 7 males (P1, P2, P5, P7, P8, P9, P10). Ages range from 8 to 60 years. Clinical summary, histopathological features and genetic characterization of all patients is provided in Table 1. Family history reveals potentially recessive inheritance for 2 families (F1, F3) and 2 families had a clear dominant transmission (F5 and F7), while P2, P5 and P9 were sporadic cases. All patients presented with a congenital myopathy involving congenital or early-onset hypotonia, delayed motor milestones and progressive muscle weakness, with prominent axial involvement in most patients. Decreased fetal movement and breech presentation suggested antenatal involvement in several patients. In addition, one patient (P5) also had periodic paralysis and loss of speech during periods of illness and stress. These symptoms improved significantly after treatment with acetazolamide, a carbonic anhydrase inhibitor known to be beneficial for some HOKPP patients [19]. All patients had mild facial involvement, 4 patients had ophthalmoplegia and one patient had ptosis. The respiratory system is heterogeneously affected ranging from normal to severely impaired. Most of the patients had mild to severe swallowing issues. There was no cardiac involvement nor clear involvement of other non-neuromuscular functions. Serum creatine kinase (CK) levels were within normal range in 9 patients but elevated in the 2 patients from family 7 (1000-2000 U/l range).

Morphological study has been done on muscle biopsies for individuals in 6 families (Fig. 1, Supplementary Fig. S1). Histological analyses of the muscle biopsies of 4 families (F1, F2, F5, F6) showed an alveolar aspect of the intermyofibrillar network on NADH-TR staining (Fig. 1a-b). Fiber size variability was present in 5 families (F1, F4, F5, F6, F7) (Fig. 1a-d). Centralized or internalized nuclei were observed in 4 families (F1, F2, F5, F7) and core-like or focal disorganisation structures in 4 families (F1, F2, F4, F7). Uniformity of type 1 (consistent by immunofluorescence, not shown) was observed in F1, F2, F6 and predominance of type 1 fibers in family 5 was noted. The biopsy of patient P10 in family F7 showed a dystrophic aspect, with mild increase in endomysial connective tissue, scattered split fibers, in addition to an increase in number of centralized nuclei and occasional fibers with degeneration or regeneration, which is consistent with the finding of increased serum CK to levels more than 1000 IU/l.

The ultrastructure of muscles from patients P1, P2, P3, P6, P7, P9 and P10 was investigated with electron microscopy and uncovered common features such as dilated T-tubules and SR (Fig. 2b,d,e,g,h,i) and focal zones of myofibrillar disorganization (Fig. 2c). In P1, increased frequency of triads per sarcomere has been observed (Fig. 2a). Long T-tubules and small areas of altered sarcomere striation could be seen in P2 (Fig. 2d-f). Muscle sections from P6 and P7 displayed less abnormalities and only some SR and T-tubules were dilated. However, the junctional feet between SR and T-tubules appeared normal in all patients.

The overall histological and ultrastructural findings were suggestive of a diagnosis of centronuclear or core myopathy. Exome analysis and targeted sequencing of the known related genes including *MTM1*, *BIN1*, *DNM2* and *RYR1* did not reveal any pathogenic variations.

Muscle MRI or CT scan of the lower limbs was performed on nine patients (Fig. 3). Symmetric involvement of anterior and posterior compartments at the level of the thigh was seen in all, varying from mild atrophy in patients P7 and P8 to a marked atrophy in P5 and severe and extensive fatty infiltration throughout all muscle groups in patients P5 and P10. Anterior compartment muscles were slightly more involved than hamstrings in most. In some patients (P1, P2, P9) the semitendinosus muscle was selectively more affected than the biceps femoris and semimembranosus. In Patients P10 and P11, there was also relative sparing of the vastus intermedius and adductor magnus. Where available, imaging at the level of the leg showed findings ranging from mostly absence of involvement to a pattern with relative sparing of the tibialis anterior and posterior muscles as compared to soleus and gastrocnemii. Overall, there is no specific pattern observed on muscle imaging compared to other congenital myopathies.

In summary, all patients display a consistent phenotype of neonatal or early-onset progressive muscle weakness with facial involvement and peculiar structural alterations on the muscle biopsy, with variable extra-ocular involvement and CK elevation, suggesting a common genetic basis of the disease.

Identification of dominant and recessive mutations in *CACNA1S*

To identify the genetic cause of the disease, we performed exome sequencing on all available affected individuals, as well as one or both parents of families 1, 3, 5 and 6. With the use of different bioinformatics pipelines, we filtered and ranked the best candidate genes and identified several rare variants in *CACNA1S,* the gene coding the alpha 1 subunit of the L-type voltage-dependent Ca^{2+} channel, a major protein for excitation-contraction coupling in skeletal muscle. All variants were validated by Sanger sequencing in all available family members and segregation with the disease was confirmed.

Four families (F1 to F4) displayed recessive disease segregation with compound heterozygous mutations (Fig. 4a). P1 from Family 1 has two frameshift mutations in exons 9 (p.S397Pfs*3; c.1189_1190del) and 40 (p.L1656Rfs*67; c.4967del). P2 from Family 2 carries a nonsense mutation in exon 37 (p.Q1485*; c.4453C>T) and the same exon 40 frameshift as P1. The shared c.4967del mutation in Families 1 and 2 is likely to be from a different origin considering that they do not share a common haplotype for 2 upstream microsatellites loci, the nearest being 25 kb from the gene. The two siblings P3 and P4 from Family 3 carry a missense mutation in exon 6 (p.F275L; c.825C>A) and a frameshift in exon 18 (p.L791Cfs*37; c.2371delC). P5 from Family 4 carries two predicted missense mutations in exons 30 (p.Q1265H; c.3795G>T) and 3 (p.E100K; c.298G>A). The respective parents of the patients are carrier of one mutation each, and the 2 unaffected siblings in family 1 carry a single variant, consistent with recessive inheritance.

In the remaining 3 unrelated families, 3 novel heterozygous variants were identified in 5 patients (Fig. 4b). The affected individuals P6, P7, and P8 from family 5 carry a missense mutation in exon 16 (p.P742Q; c.2225C>A) that appears *de novo* in the mother and segregates in her two affected sons. Patient P9 from family 6 carries a different d*e novo* missense affecting the same amino acid (p.P742S; c.2224C>T). Another *de novo* missense mutation was identified in both affected father (P10) and daughter (P11) from family 7 (p.L1348V; c.4099C>G). None of the missense mutations were found in unaffected family members, consistent with dominant inheritance.

All variants are present on both CACNA1S isoforms, and affect different domains of the protein (Fig. 4c). All variants were novel with the exception of the c.825C>A (p.F275L) and c.3795G>T (p.Q1265H) variants. They were both found in recessive families 3 and 4 respectively, and are listed in a heterozygous state at very low frequency in the ExAC exome database (3.10^{-5} and 8.10^{-6}, respectively). With regards to the predicted missense changes in our recessive families, the p.F275L substitution in family 3 affects a conserved amino acid of the pore-forming loop L5, which is in close proximity to the selectivity filter. The c.3795G>T variant found in family 4 not only involves an amino acid substitution, but is in addition predicted to impact on splicing, resulting in a shift of the reading frame and a premature stop codon (p.Q1265Hfs*57, outlined below). The second missense variant found in family 4 (p.E100K; c.298G>A) affects a glutamic acid that may interact with arginine 174 for the connection of transmembrane segments in the voltage-sensing domain, as modeled in the recently resolved 3D structure of DHPR (Supplementary Fig. S2).

Concerning the missense mutations in the dominant families, proline 742 is affected by two different mutations in two unrelated families (p.P742Q and p.P742S). This residue is conserved in mouse and zebrafish and appears to be specific for the skeletal muscle alpha 1 subunit of DHPR, as it is not conserved in other alpha 1 subunits expressed in other tissues as CACNA1C or CACNA1D (Fig. 4D). This residue is localized in the loop between domains II and III involved in DHPR-RyR1 coupling, and a previous study demonstrated that the artificial P742T mutation strongly reduced ECC [16].

Altogether, those findings indicate that mutations in *CACNA1S* cause early-onset myopathy with dominant or recessive disease inheritance.

Impact of mutations on CACNA1S transcript and protein

In total, we identified 10 different *CACNA1S* mutations including 3 frameshift, 1 nonsense, and 6 missense mutations including 3 confirmed *de novo* mutations. The truncating variants are predicted to remove the C-terminal 217, 388 or 1476 amino acids from 1873 in the adult protein isoform. In order to assess if the premature stop codons lead to nonsense-mediated mRNA-decay (NMD), we extracted, reverse transcribed, and sequenced *CACNA1S* muscle RNA from patients P1 (Family 1) and P3 (Family 3). For patient P1, we detected amplicons of both alleles in comparable amounts, ruling out major mRNA-decay (Fig. 5a). In contrast, the p.L791Cfs*37 mutation in P3 leads to mRNA-decay as only the other allele, carrying the missense mutation, was detected. As mentioned above, one of the missense mutation in patient P5 (predicted protein impact p.Q1265H) is also predicted to disrupt the donor splice site of exon 30. Sequencing of *CACNA1S* RNA extracted from myotubes differentiated from P5 myoblasts indicated retention of 68 nucleotides from intron 30 leading to the frameshift p.Q1265Hfs*57 and the deletion of the last 608 amino acids (Fig. 5a). Both alleles were detected, illustrating that mRNA decay does not occur. RNA from other patients were not available. In summary, all recessive cases described here carry at least one mutation involving a premature stop codon, although not all appear to trigger mRNA decay.

In order to verify the impact of the mutations on the protein level, we performed western blot on muscle biopsies and myotubes extracts. CACNA1S was barely detectable by western-blot in myotubes extracts from patient P1 with compound truncations (Fig. 5b). Similar results were obtained from muscle extracts of P1 (Fig. 5b). Detectable protein level was observed in muscle extracts from patient P3, harbouring one truncation and one missense mutations. Analysis of muscle extracts from P6, P7 and P9 (all three from dominant families) revealed a strongly reduced level of CACNA1S (Fig. 5b). These data support overall decreased protein stability following missense or frameshift mutations, in both dominant and recessive cases.

The subcellular localization of some of the mutated CACNA1S proteins was investigated in cultured myotubes from patients and in skeletal muscle. In contrast to control myotubes, a very low fluorescent signal was noted with an antibody against the N-terminal domain of CACNA1S in differentiated myotubes from patient P1 myoblasts, supporting western blot findings that overall CACNA1S is strongly reduced in this patient carrying recessive mutations (Fig. 5c). RyR1 signals appeared as longitudinally aligned dots in both control and patient myotubes, suggesting that the CACNA1S mutations do not impact on RyR1 localization, consistent with previous studies showing that *Cacna1s* knock out myotubes still show triadic localization of RyR1 [25]. The muscle sections from the recessive patient P1 and the dominant patient P6 revealed decreased CACNA1S immunoreactivity and showed a peculiar alveolar aspect reminiscent of the observations with oxidative staining (Fig. 1; Fig. 5d). Aberrant diffuse accumulations of CACNA1S were also observed in the patient's muscles (Fig. 5d). Triadin (junctional SR marker) labelling appeared mildly decreased with lumps or irregular appearance in patients P1, whereas the signal was slightly decreased in patients P6 and P9 (Fig. 5d).

In conclusion, most recessive and dominant mutations we investigated in this study are associated with a decrease in CACNA1S protein level and an abnormal localization pattern in available mature muscle.

Physiopathological insights into CACNA1S myopathy

CACNA1S is a subunit of the voltage-dependent Ca^{2+} channel (DHPR) regulating Ca^{2+} release from the SR stores. To assess the functional impact of *CACNA1S* mutations, we investigated Ca^{2+}

homeostasis in myotubes from the recessive patient P1 and the dominant patient P10 (Fig. 6). Resting cytosolic Ca^{2+} levels were similar between both patients and control myotubes, showing that the mutations do not affect resting Ca^{2+} homeostasis.

We next studied Ca^{2+} release induced by KCl depolarization. Although the dose response curves have similar shapes in patients and controls (EC_{50} 14.3+/-8.5 for control and 13.2+/-8.3 for patient cells; Fig. 6), the maximum level of Ca^{2+} released is 2.5 times lower in patients compared to controls. The reduction of the depolarization-induced calcium release was not due to a reduction of the size of intracellular Ca^{2+} stores, since there was no significant difference in the total amount of calcium released caused by thapsigargin and ionomycin (Supplementary Fig. 3). These results strongly suggest that the CACNA1S mutations impair excitation-contraction coupling, potentially through a decreased activation of RyR1 due to the strong decreased content of CACNA1S protein. These data demonstrate that recessive truncations and the dominant p.L1348V mutation induce similar alterations in the ECC function of CACNA1S.

Consistent with defects in the ECC, the patients´ muscle fibers showed several defects of the triad, including dilated T-tubules and SR (Fig. 2).

Overall, these data indicate that the function of CACNA1S and the muscle ultrastructure are similarly altered in both recessive and dominant patients. Pathomechanistically, both mutation types are associated with altered calcium homeostasis and reduced ECC with SR dilatation and myofibrillar disorganisation.

Discussion

In the present study we describe a clinical and histopathological cohort of patients with a consistent phenotype of early-onset myopathy and mutations in the skeletal muscle alpha 1 subunit of DHPR, CACNA1S. Mutations segregated either through recessive or dominant modes of inheritance, or were found as *de novo* mutations in singletons. Two of the mutations, one dominant and one recessive were tested functionally and both resulted in a significant decrease of EC coupling.

DHPR myopathy and DHPR-related diseases

The described 11 patients from 7 families all display severe early-onset myopathy with progressive, generalized predominately axial muscle weakness, facial involvement with or without ophthalmoplegia, elevated CK in one family and episodes of periodic paralysis in one patient. The morphological and ultrastructural analyses revealed centralized or internalized nuclei and focal zones of sarcomeric disorganisation in several patients. Taken together, this phenotype is reminiscent of findings observed in the myotubular or centronuclear myopathy and in core myopathy, conditions in which ophthalmoplegia (an uncommon feature in other congenital myopathies) is often observed. Those myopathies are also associated with dominant and recessive mutations in *RYR1* [5,21] and the identification of CACNA1S mutations in patients with overlapping phenotype is not unexpected, as DHPR directly regulates the RyR1 Ca^{2+} release channel. The “alveolar” aspect of the intermyofibrillar network observed in the majority of the patients and highlighted with SR labelling both with specific antibodies or oxidative staining and appearing as a dilated SR by electron microscopy, is a peculiar and possibly a histopathological hallmark for *CACNA1S* mutations.

Muscle imaging of the lower limbs recapitulates some patterns seen in CNM and congenital myopathies. While some patients show relatively mild and nonspecific atrophy of muscle groups, three patients from different families had selective involvement of the semitendinosus muscle in the posterior thigh, a finding reported both in early-onset CNM due to *DNM2* mutations and in *TTN*-related myopathies [30]. At the level of the leg, family 7 showed a differential pattern with relative preservation of muscles in the anterior compartment (tibialis anterior and posterior) as compared to the soleus and gastrocnemii, a finding which has been described in both *DNM2* and *RYR1*-related myopathies. However, in addition to such overlapping patterns there also seems to be a wide spectrum of involvement ranging from very mild atrophy of thigh muscle groups to extensive marked fatty infiltration, suggesting that the imaging of *CACNA1S*-related myopathy is heterogeneous.

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The finding of specific histological hallmarks and one of the muscle imaging pattern is of major diagnostic importance, as it can aid in directing the molecular diagnosis towards *CACNA1S* testing. Patients with centronuclear myopathies or cores myopathies without molecular diagnosis should be revaluated for specific hallmarks of *CACNA1S* congenital myopathy and offered *CACNA1S* testing if appropriate. Patient P10 was the only patient displaying dystrophic features; the characterization of additional families will confirm if *CACNA1S* defect lead to dystrophy under certain conditions.

. We believe the gene should be included both in NGS panels for congenital muscle disorders and in filtering pipelines for whole exome sequencing in such cases.

The association of *CACNA1S* with a congenital myopathy phenotype was recently suggested in one patient [12]. This patient displayed a similar phenotype as in our cohort, with congenital myopathy and ophthalmoplegia, and occasional internal nuclei and coarse myofibrillar architecture on muscle biopsy. He carried compound *CACNA1S* variants each inherited from a heterozygous parent, c.4947delA (p.Q1649Qfs*72) and c.3795G>T (p.Q1265H). In that report, no functional validation of the variants pathogenicity was provided, precluding a conclusion on the pathogenicity of the identified variants. Of note is that the predicted frameshift is very close to the frameshift mutation p.L1656Rfs*67 found in our recessive families 1 and 2 (Fig. 4), and we show here that the c.3795G>T change in fact leads to altered splicing in our patient P5 (Fig. 5a).

Dominant *CACNA1S* mutations have been associated with malignant hyperthermia or hypokalemic periodic paralysis [14,20,26]. Here we report for the first time dominant and recessive *CACNA1S* mutations associated with a myopathy. Most *CACNA1S* mutations linked to periodic paralysis are missense changes residing in the voltage-sensing domain (transmembrane segment S4 in each transmembrane domain), while most mutations in malignant hyperthermia are missense mutations in the loop regions (Fig. 4c). In contrast, the myopathy mutations uncovered in this study in congenital myopathy are evenly distributed in the sequence and structure of CACNA1S. Dominant mutations are either in the pore-forming domain (L1367) or in the II-III loop implicated in RYR1 coupling (P742), while recessive mutations primarily comprise truncations at different spots, or missenses in the pore-forming or voltage-sensing domains. There is thus no strict correlation between the type and location of mutations and the associated diseases, or their severity.

Noteworthy, recessive and dominant mutations in congenital myopathy lead to similar clinical and histological phenotypes, and our functional studies demonstrated that both mutations types comparably interfere with ECC, suggesting a similar overall alteration of CACNA1S function (Fig. 5). However, ophthalmoplegia is not a common sign even if observed in several patients, CK was elevated in only one family and episodes of periodic paralysis was observed in one patient. The discovery of additional CACNA1S patients with congenital myopathies and future functional work will specify the overall clinical phenotype and its potential correlation with the position and the transmission mode of the mutations.

The fact that all dominant mutations are missense changes while heterozygous carriers of recessive mutations are healthy excludes haploinsufficiency as the underlying pathomechanism in the dominant cases. Instead, the dominant mutations may act through a dominant-negative manner on the WT protein. Indeed, we noted that both recessive and dominant mutations correlated with a strong decrease in overall protein level (Fig. 4), suggesting that DHPR complexes containing a mutant CACNA1S subunit form unstable tetrads prone to degradation.

None of our patients presented with cardiac involvement. The heart-specific CACNA1C alpha 1 subunit is mutated in Brugada syndrome (MIM#611875) characterized by shortened QT interval and high incidence of sudden death, and in Timothy syndrome (MIM#61005) characterized by multi-organ dysfunction including lethal arrhythmias. These findings reflect the exquisite tissue-specific

expression of DHPR alpha 1 subunits, CACNA1C in heart and CACNA1S in skeletal muscle. More generally, this is also true for the other 10 different CACNA1 (alpha 1) subunits that are mutated in non-muscle diseases such as hemiplegic migraine, ataxias, bradycardia and deafness, night blindness, or aldosteronism, in relation with their tissue-specific expression [13].

Pathophysiological mechanism

While future experimental work will be needed to decipher the detailed pathological mechanism, the published and present data suggests a sequence of events leading from the *CACNA1S* mutations to the pathology and muscle disease. As both dominant and recessive mutations can lead to a rather homogeneous phenotype, we hypothesize they both lead to a decrease in overall DHPR function in skeletal muscle. We show here that several *CACNA1S* mutations strongly reduced Ca^{2+} release after depolarization, while basal cytosolic Ca^{2+} and SR store Ca^{2+} levels are not altered. Of note, a previous study showed that the artificial P742T mutation, affecting the same amino acid that is mutated in our dominant families F5 (p.P742Q), and F6 (p.P742S), strongly reduced ECC, highlighting the importance of the loop between DHPR domains II and III for RyR1 activation [16]. In order to activate directly RyR1 we used 4-CMC and observed a reduced Ca2+ release from the SR in patient 1 (Supplementary Fig. S3). We hypothesize that RyR1 function is impacted by the loss of CACNA1S through a feedback regulation or protein stabilization. The KCl-induced dose response curves are strikingly similar to those obtained in patients with RyR1-related central core disease (CCD) [6], although the resting calcium concentration were always higher in myotubes from RyR1 mutants compared to controls. This would then lead to the structural defects observed on histological analysis. In most patient biopsies analyzed, the T-tubules and the SR were frequently dilated or disrupted. Some of them presented an increased frequency of triads by sarcomere. It is unclear at present whether this is an indirect effect of abnormal Ca^{2+} signal or contraction (functional role), or due to the direct alteration in CACNA1S protein (structural role). These rather specific alterations were not found in RyR1-related myopathies nor in centronuclear myopathies. However, in our study, triads are still formed with visible « feet ». Indeed, mice lacking DHPR (muscular dysgenesis, mdg mouse) or RyR1 (dyspedic) form triadic junctions, with a similar architecture compared to wildtype [4,25,10]. These data in mice thus suggest that structural defects seen in patients are not linked to a loss of DHPR but rather to altered ECC. Abnormal nuclei positioning and focal zones of myofibrillar disorganisation are additional structural defects in patient muscles. The peculiar alveolar appearance of the intermyofibrillar network could be a result of the SR dilatation. Complete knockout of CACNA1S during development is lethal in the mdg mouse [4,23] and disease onset is antenatal or perinatal in our patients, pointing to an important role of DHPR in perinatal development. Moreover, the role of CACNA1S in adult muscle was studied by U7 mediated-skipping in adult mice, revealing that decreased CACNA1S protein level was linked to muscle atrophy [24]. These data in mice are in agreement with the observation that a low level of CACNA1S has to be present in the muscle and can lead to the muscle atrophy observed in our patients. While abnormal ECC can explain the severe muscle weakness in patients, it is possible that the concomitant structural defects contribute to the clinical phenotype.

Conclusions

Here we have characterized the CACNA1S congenital myopathy as a specific entity, at the interface between the structural congenital myopathies and the triadopathies defined by alteration of the EEC. The identification of *CACNA1S* mutations in a phenotype of early-onset myopathy should allow a better molecular diagnosis, genetic counselling and prognosis. It also points to CACNA1S and ECC as therapeutic targets for the development of treatments that may be facilitated by the already extensive prior knowledge accumulated on DHPR. In addition, treatment by acetazolamide showed positive results on the episodes of periodic paralysis of one patient. The results of this study also expand the clinical and genetic spectra of *CACNA1S* mutations and highlight that DHPR cellular roles are important for perinatal muscle function in human.

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Web resources

ExAC Browser / Exome Aggregation Consortium (URL: http://exac.broadinstitute.org/)
Exome Variant Server (URL: http://evs.gs.washington.edu/EVS/) [March, 2012])
1000 genomes (URL : http://www.1000genomes.org/)
Database of Single Nucleotide Polymorphisms (dbSNP Build ID: 134) (URL: http://www.ncbi.nlm.nih.gov/SNP/)
Online Mendelian Inheritance in Man (OMIM) (URL: http://www.omim.org/)

References

1. Al-Qusairi L, Laporte J (2011) T-tubule biogenesis and triad formation in skeletal muscle and implication in human diseases. Skelet Muscle 1:26. doi:2044-5040-1-26 [pii]

10.1186/2044-5040-1-26

2. Amburgey K, McNamara N, Bennett LR, McCormick ME, Acsadi G, Dowling JJ (2011) Prevalence of congenital myopathies in a representative pediatric united states population. Ann Neurol 70:662-665. doi:10.1002/ana.22510
3. Censier K, Urwyler A, Zorzato F, Treves S (1998) Intracellular calcium homeostasis in human primary muscle cells from malignant hyperthermia-susceptible and normal individuals. Effect Of overexpression of recombinant wild-type and Arg163Cys mutated ryanodine receptors. J Clin Invest 101:1233-1242. doi:10.1172/JCI993
4. Chaudhari N (1992) A single nucleotide deletion in the skeletal muscle-specific calcium channel transcript of muscular dysgenesis (mdg) mice. J Biol Chem 267:25636-25639
5. Dowling JJ, Lawlor MW, Dirksen RT (2014) Triadopathies: an emerging class of skeletal muscle diseases. Neurotherapeutics 11:773-785. doi:10.1007/s13311-014-0300-3
6. Ducreux S, Zorzato F, Muller C, Sewry C, Muntoni F, Quinlivan R, Restagno G, Girard T, Treves S (2004) Effect of ryanodine receptor mutations on interleukin-6 release and intracellular calcium homeostasis in human myotubes from malignant hyperthermia-susceptible individuals and patients affected by central core disease. J Biol Chem 279:43838-43846. doi:10.1074/jbc.M403612200
7. Dulhunty AF, Karunasekara Y, Curtis SM, Harvey PJ, Board PG, Casarotto MG (2005) Role of some unconserved residues in the "C" region of the skeletal DHPR II-III loop. Front Biosci 10:1368-1381
8. Eberl DF, Ren D, Feng G, Lorenz LJ, Van Vactor D, Hall LM (1998) Genetic and developmental characterization of Dmca1D, a calcium channel alpha1 subunit gene in Drosophila melanogaster. Genetics 148:1159-1169
9. Engel AG, Franzini-Armstrong C (2004) Myology, Basic and Clinical. McGraw-Hill, New York
10. Franzini-Armstrong C, Pincon-Raymond M, Rieger F (1991) Muscle fibers from dysgenic mouse in vivo lack a surface component of peripheral couplings. Dev Biol 146:364-376
11. Geoffroy V, Pizot C, Redin C, Piton A, Vasli N, Stoetzel C, Blavier A, Laporte J, Muller J (2015) VaRank: a simple and powerful tool for ranking genetic variants. PeerJ 3:e796. doi:10.7717/peerj.796

796 [pii]

12. Hunter JM, Ahearn ME, Balak CD, Liang WS, Kurdoglu A, Corneveaux JJ, Russell M, Huentelman MJ, Craig DW, Carpten J, Coons SW, DeMello DE, Hall JG, Bernes SM, Baumbach-Reardon L (2015) Novel pathogenic variants and genes for myopathies identified by whole exome sequencing. Mol Genet Genomic Med 3:283-301. doi:10.1002/mgg3.142
13. Jurkat-Rott K, Lehmann-Horn F (2004) The impact of splice isoforms on voltage-gated calcium channel alpha1 subunits. J Physiol 554:609-619. doi:10.1113/jphysiol.2003.052712
14. Jurkat-Rott K, Lehmann-Horn F, Elbaz A, Heine R, Gregg RG, Hogan K, Powers PA, Lapie P, Vale-Santos JE, Weissenbach J, et al. (1994) A calcium channel mutation causing hypokalemic periodic paralysis. Hum Mol Genet 3:1415-1419
15. Knudson CM, Chaudhari N, Sharp AH, Powell JA, Beam KG, Campbell KP (1989) Specific absence of the alpha 1 subunit of the dihydropyridine receptor in mice with muscular dysgenesis. J Biol Chem 264:1345-1348
16. Kugler G, Weiss RG, Flucher BE, Grabner M (2004) Structural requirements of the dihydropyridine receptor alpha1S II-III loop for skeletal-type excitation-contraction coupling. J Biol Chem 279:4721-4728. doi:10.1074/jbc.M307538200

17. Kung AW, Lau KS, Fong GC, Chan V (2004) Association of novel single nucleotide polymorphisms in the calcium channel alpha 1 subunit gene (Ca(v)1.1) and thyrotoxic periodic paralysis. J Clin Endocrinol Metab 89:1340-1345. doi:10.1210/jc.2003-030924
18. Lu X, Xu L, Meissner G (1994) Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor. J Biol Chem 269:6511-6516
19. Matthews E, Portaro S, Ke Q, Sud R, Haworth A, Davis MB, Griggs RC, Hanna MG (2011) Acetazolamide efficacy in hypokalemic periodic paralysis and the predictive role of genotype. Neurology 77:1960-1964. doi:10.1212/WNL.0b013e31823a0cb6
20. Monnier N, Procaccio V, Stieglitz P, Lunardi J (1997) Malignant-hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. Am J Hum Genet 60:1316-1325
21. Nance JR, Dowling JJ, Gibbs EM, Bonnemann CG (2012) Congenital myopathies: an update. Curr Neurol Neurosci Rep 12:165-174. doi:10.1007/s11910-012-0255-x
22. North KN, Wang CH, Clarke N, Jungbluth H, Vainzof M, Dowling JJ, Amburgey K, Quijano-Roy S, Beggs AH, Sewry C, Laing NG, Bonnemann CG, International Standard of Care Committee for Congenital M (2014) Approach to the diagnosis of congenital myopathies. Neuromuscul Disord 24:97-116. doi:10.1016/j.nmd.2013.11.003
23. Pai AC (1965) Developmental Genetics of a Lethal Mutation, Muscular Dysgenesis (Mdg), in the Mouse. Ii. Developmental Analysis. Dev Biol 11:93-109
24. Pietri-Rouxel F, Gentil C, Vassilopoulos S, Baas D, Mouisel E, Ferry A, Vignaud A, Hourde C, Marty I, Schaeffer L, Voit T, Garcia L (2010) DHPR alpha1S subunit controls skeletal muscle mass and morphogenesis. EMBO J 29:643-654. doi:10.1038/emboj.2009.366
25. Powell JA, Petherbridge L, Flucher BE (1996) Formation of triads without the dihydropyridine receptor alpha subunits in cell lines from dysgenic skeletal muscle. J Cell Biol 134:375-387
26. Ptacek LJ, Tawil R, Griggs RC, Engel AG, Layzer RB, Kwiecinski H, McManis PG, Santiago L, Moore M, Fouad G, et al. (1994) Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. Cell 77:863-868
27. Rebbeck RT, Karunasekara Y, Board PG, Beard NA, Casarotto MG, Dulhunty AF (2014) Skeletal muscle excitation-contraction coupling: who are the dancing partners? Int J Biochem Cell Biol 48:28-38. doi:10.1016/j.biocel.2013.12.001
28. Rezgui SS, Vassilopoulos S, Brocard J, Platel JC, Bouron A, Arnoult C, Oddoux S, Garcia L, De Waard M, Marty I (2005) Triadin (Trisk 95) overexpression blocks excitation-contraction coupling in rat skeletal myotubes. J Biol Chem 280:39302-39308. doi:10.1074/jbc.M506566200
29. Romero NB, Clarke NF (2013) Congenital myopathies. Handb Clin Neurol 113:1321-1336. doi:10.1016/B978-0-444-59565-2.00004-6

B978-0-444-59565-2.00004-6 [pii]
30. Susman RD, Quijano-Roy S, Yang N, Webster R, Clarke NF, Dowling J, Kennerson M, Nicholson G, Biancalana V, Ilkovski B, Flanigan KM, Arbuckle S, Malladi C, Robinson P, Vucic S, Mayer M, Romero NB, Urtizberea JA, Garcia-Bragado F, Guicheney P, Bitoun M, Carlier RY, North KN (2010) Expanding the clinical, pathological and MRI phenotype of DNM2-related centronuclear myopathy. Neuromuscul Disord 20:229-237. doi:10.1016/j.nmd.2010.02.016
31. Tang ZZ, Yarotskyy V, Wei L, Sobczak K, Nakamori M, Eichinger K, Moxley RT, Dirksen RT, Thornton CA (2012) Muscle weakness in myotonic dystrophy associated with misregulated splicing and altered gating of Ca(V)1.1 calcium channel. Hum Mol Genet 21:1312-1324. doi:10.1093/hmg/ddr568
32. Treves S, Pouliquin R, Moccagatta L, Zorzato F (2002) Functional properties of EGFP-tagged skeletal muscle calcium-release channel (ryanodine receptor) expressed in COS-7 cells: sensitivity to caffeine and 4-chloro-m-cresol. Cell Calcium 31:1-12
33. Wu J, Yan Z, Li Z, Yan C, Lu S, Dong M, Yan N (2015) Structure of the voltage-gated calcium channel Cav1.1 complex. Science 350:aad2395. doi:10.1126/science.aad2395